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Research Article

Toxicology for safe therapy

Embryotoxicity and Teratogenic Effect of Chloroform Extract of *Leucaena leucocephala (Lam.) de Wit* (Lam.) de Wit Leaf on Zebra fish (*Danio Rerio*)

Sumana Roy (Ghosh)*, Lopamudra Datta and Prerona Saha

Department of pharmacy. Guru Nanak Institute of Pharmaceutical Science and Technology, 157/ F Nilgunj Road. Sodepore. Kolkata -700114, India

Abstract: *Leucaena leucocephala* (Lam.) de Wit is a plant widely distributed in India and other Tropical countries widely as a source of nutrition to cattle and Humans. Literature review indicates varied medicinal property of *L. leucocephala* (Lam.) de Wit plant. However before further exploring its therapeutic potential, evaluation of in vitro embryotoxicity and teratogenic effect of the leaf extract of this plant are required to be performed in zebrafish model. The current study aimed to assess the toxicity of chloroform extract of *L. leucocephala* (Lam.) de Wit on Zebrafish (*Danio Rerio*). Successive soxhlation was carried out for extraction with solvent having variable polarity. Embryotoxicity and teratogenicity are studied by exposing twenty completely fertilized embryos to the extracts with varying concentrations. The zebrafish embryos were exposed to varying plant extract concentrations in a 0.1% DMSO solution, used as control. Toxicity , teratogenicity and some deformities in embryos was observed at a concentration of 600 µg/ml. Mortality was observed at higher concentration, whereas reduced hatching rate was observed with increasing concentration. Different physical deformities like kink tail, bent trunk, and enlarged yolk sac edema were attributed to the teratogenic effect of the extract observed at higher concentrations. Finally, LC50 value was determined to be significant with a p value < 0.0001. Teratogenic Index based on development of deformities was also significant (p value<0.0001) and claimed the nonteratogenic effect of the extract. The study divulges that plants with therapeutic efficacy could also develop some side effects when consumed at higher doses specially on the embryos. Detailed toxicity study should be carried out on medicinal plants to identify their safety and teratogenic effect on the embryos.

Keyword: Embryotoxicity, *Danio Rerio*, Teratogenicity, Hatching Rate, LC50, Zebrafish.

***Corresponding Author**

Sumana Roy (Ghosh) , Department of pharmacy. Guru Nanak Institute of Pharmaceutical Science and Technology, 157/ F Nilgunj Road. Sodepore. Kolkata -700114, India

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1. INTRODUCTION

Medicinal plants and phytoconstituents, reported for pharmacological effects, are widely applied to treat different disorders.[1] Although the medicinal plants offer various pharmacological activities, some phytoconstituents are responsible for developing different toxicity and teratogenicity. [1,2] An agent possessing the capacity to develop morphological abnormalities is known as teratogen.[2] Therefore, toxicity and teratogenicity study of medicinal plant extracts is pivotal. Different mammals like rats, mice, rabbits are common animal models used for toxicity studies.[3,4] Zebrafish (*Danio rerio*), is a freshwater and aquarium fish belonging to Cyprinidae family. [5] Zebrafish is a successful animal model for *in-vitro* analysis of drugs and for toxicological studies on embryonic and larval stage.[6] *Danio rerio* is an ideal model for replicating pathological condition in human. *Danio rerio* is found to be genetically complement to 70% of human gene responsible for development of disease.[1,7] The zebrafish embryo is a very popular and reliable tool as an *in vitro* model due to rapid development processes, transparency and low maintenance on a laboratory Scale, and its similarity in embryonic development to vertebrates of higher forms.[8] Due to increased reproduction and availability of transparent eggs, it is now become useful and cost-effective alternative to some mammalian models for drug discovery and toxicity study. [6,9] *Leucaena leucocephala* (Lam.) de Wit, locally known as Saw Babul is a topical tree from Fabaceae family. Different parts of this plant like leaves, flowers, young pods and seeds have high nutritional value. Additionally, it is one of the important medicinal plants from Mimosoideae's subfamily possessing anthelmintic, antimicrobial, antibacterial activity. [10,11] Natural chemical compounds with different pharmacological and therapeutic activity can be obtained from plants. Different parts of plants are widely used for the management of different significant and major illness.[12,13] Some of the components of the extracted compounds contain some toxic substance which can affect various organs. [14,15,16]. Although medicinal plants' toxicity on different human organs has been already reported, there are limited reports on the embryotoxicity and teratogenic effects of *L. leucocephala* (Lam.) de Wit leaf extract.[17,18] According to WHO (World Health Organization) healthcare medicine utilized by 80% of world population is from natural source and on traditional medicine.[16,17] Many of edible medicinal plants are safe, however list of survey shows some medicinal plants are toxic and even few of them shows teratogenicity as well.[19,20] Natural remedy from plant origin or medicinal herbs is considered as a safe alternative to synthetic medicine.[21,22] Different therapeutic and pharmacological properties of *L. leucocephala* (Lam.) de Wit have been reported; like antibacterial activity [23,24]. antioxidant and antidiabetic effects [25,26] stimulator for stimulates adipogenesis, lipolysis, and glucose uptake 26. It is also widely used for its nutritional activity and for the development of biofuel[27] Mimocine is an amino acid present in different parts of *Leucaena leucocephala*. Mimocine is a toxic amino acid present in higher amount in the leaves of the plant which reduce its activity as animal feed and reported to produce toxicity in animal and human.[28,29] Despite the widely reported [23,24,25,26] safe therapeutic and pharmaceutical activity of *L. Leucocephala (*Lam.) de Wit , a limited number of researches reported the in-vitro embryotoxicity and teratogenic effect of chloroform extract of leaf in zebrafish (*Danio rerio*) model are required before exploring further therapeutic potency. Therefore, in this study, the chloroform extract was examined for in-vitro toxicity developed in embryo and deformities in development using zebrafish embryos and larvae assay as a model.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The leaf of *Leucaena leucocephala* (Lam.) de Wit was collected from West Midnapore India and dried in shade. Identification and Authentication of *L. leucocephala* (Lam.) de Wit was done by K.KARTHIGEYAN Scientist -E, Central National Herbarium, Botanical Survey of India, Howrah-711103, with sample no GNIPST/LD/SR/002.

2.2 Animal Treatment and Production of Fertilized Egg

The embryo and larva of zebrafish was maintained according to OECD fish embryo Acute Toxicity Test (FET) draft Guideline of 2006. Adult Danio Rio (Zebrafish) were collected from local supplies. The adult fish was maintained for four weeks for acclimatization. The developed fish were maintained in a 500 L aquarium with continuous air flow and dark and light cycle. Temperature was maintained at 29±2 degree with a constant light-dark cycle.[30] They were fed with brine shrimp. Before fertilization, female and male zebrafish were separated in different aquariums for dark and light periods for 10 hours and 14 hours, respectively. Before the toxicity testing on embryos, a standard method of breeding described in OECD, fish embryo acute toxicity was carried out.[30] Eggs were produced from the male and female spawning groups at a ratio of 2:3, respectively. After that, the fertilized embryos were kept at room temperature (29±2°C) and allowed to develop for 6 hours. Healthy eggs at the age of 6 hpf were collected and separated in different Petri plates with 0.1% DMSO Solution as per OECD guideline. [30,31]

2.3 Extraction preparation of L. Leucocephala

Shade dried leaves of *L. leucocephala* (Lam.) de Wit were homogenized to coarse powder and charged into Soxhlet apparatus. Successive extraction was carried out with n-hexane, Chloroform and Methanol.[11] Before extraction with solvent of higher polarity marc was dried properly. Each concentrated extract was collected and stored separately. Percentage yield of all the extracts were 15.01±0.02 %, 12.25± 0.08% and 17.90±0.05 % respectively.

2.4 Preparation and Dilution of test Extract

Chloroform extract was selected for toxicity study on zebrafish embryos and larvae. DMSO in a concentration of 0.1% used for dilution of extract for assay.[32,33] Stock solution of extract was prepared with 0.1% DMSO (1000 µg/ml). Different concentration from 100 µg/ml to 800 µg/ml of chloroform extract of *L. leucocephala* (Lam.) de Wit was prepared from stock solution with 0.1% DMSO Solution. [32,33]

2.5 Acute toxicity study on Zebrafish (Danio Rerio)

Zebrafish embryos and larvae were exposed to 0.1% DMSO in separate plates as per the protocol. [30] At 6 hour of post fertilization(6hpf), according to OECD guideline selected healthy embryos were washed and examined under microscope. Twenty fertilized eggs (n=20) at 12 hpf were

treated with the different concentration of extract. The experiment was repeated three times for a particular concentration of extract diluted in 0.1% DMSO. The control was exposed to 0.1% DMSO solution. All studies were repeated for three times.[37] Both the embryos and larvae were subsequently examined with the aid of different concentration (100 µg/ml to 800 µg/ml) of chloroform extract of *L. leucocephala* (Lam.) de Wit leaf for five day of exposure. The malformation of the body in each extract concentration was checked with an electron microscope. Five different concentrations 100 µg/ml to 800 µg/ml of *L. leucocephala* (Lam.) de Wit leaf extract was tested for toxicity developed in embryo and teratogenic effect on the development of zebrafish embryos and larvae, tail detachment, skeletal deformities, and somites were subsequently examined with microscope. Egg coagulation, hatching, heartbeat for toxic effect while somites, tail deformities, blood circulation, heartbeat, motility and skeletal mal-formation were evaluated for developmental deformities up to 120 hpf. (Table 1,2).

2.6 Evaluation of Hatching Rate

The Zebrafish embryos hatch rate was determined for 120 hpf at different concentrations of *L. leucocephala* (Lam.) de Wit (100 µg/ml to 800 µg/ml) extract. Hatching of embryos occurs due to chorion rupture for the release of larvae.

2.7 Evaluation of heart Rate

Heart rate counting throughout the study period of 120 hpf was performed through visual observation of the zebrafish larval cardiac ventricles using an optical microscope. Heart rate was measured per minute with the help of stopwatch.[35]

2.8 Evaluation of Embryotoxicity

Embryotoxicity was studied for different concentrations of *L. leucocephala* (Lam.) de Wit Extract up to 120 hpf and LD50 value was calculated for different concentration and duration of exposure.[35,37] (Figure 1)

2.9 Evaluation of Teratogenicity

Teratogenicity was determined on the basis of malformation percentage of larvae or embryo over total number of embryo alive at 24 hpf. Therefore, percentage of malformation can be used as an indication of teratogenicity.[35,37] (Figure 1) EC50 (Teratogenic effect) was calculated for different concentrations and duration of exposure. The ratio of LC50/EC50 was determined as Teratogenic Index, which is an indicator of teratogenicity development. Less the value, lesser is the teratogenic effect.

3. STATISTICAL ANALYSIS

Statistical significance (p) was calculated in all experiments using experiments using Graph Pad Prism version 5e (Graph Pad Software Inc., San Diego, CA, USA) and analyzed with one-way ANOVA followed by Dunnett's Post hoc test of significance where *$p < 0.05$ considered to be significant.

4. RESULT

4.1 Embryotoxicity and Teratogenicity Study

Deformation in morphology was evaluated to measure potency of toxicity of *L. leucocephala* (Lam.) de Wit extract on Zebrafish Larvae and Embryos. At 12 hpf (hour post fertilization), the embryos were incubated with *L. leucocephala* (Lam.) de Wit extract at various concentrations whereafter no embryotoxic effect and no hatching of embryo was observed. Even at 24 hpf no observable hatching and effect is visible. At 48 hpf, hatching of embryos was observed at 100, 200, 400 and 600 µg/ml concentration but no hatching was observed at 800 µg/ml. Observation at 48 hpf reported hatching at all applied concentration except 800 µg/ml. Change in Motility rate, normal blood circulation, measurable heartbeat, normal eye development was observed clearly. At 72 hpf, dead unhatched larvae were observed at higher concentration (800 µg/ml). Kink and bend tail were observed at 72 hpf at a concentration of 600 µg/ml. (Figure 1). At 96 hpf and 120 hpf larvae with several deformities were observed at 600 µg/ml concentration. Larvae at all concentrations were observed for any significant change in heart rate and hatched rate. Unhatched dead embryos were also discovered for all the concentration 100 µg/ml to 800 µg/ml at 24 hpf. At 800 µg/ml concentration, all the embryos are dead in unhatched condition. Depending on the number of larvae alive LC50 value was calculated with the help of probit model. The LC50 value was found to be gradually decreasing with increased exposure time (Figure 2) which is quite significant when compared with control. (p value < 0.0001). Gradual decrease in the value of EC50 (p value < 0.0001) (Figure 3) and the therapeutic value (TI) which is an indication for ranking the teratogenic effect (ratio of LC50/EC20) is less than 1 and is significant (p value < 0.0001) when compared with control. (Figure 4) is an indication of potency and reduced teratogenic effect of the compound.

4.2 Effect of L. leucocephala (Lam.) de Wit leaf extract on Embryo Hatch Rate

Hatching rate of Zebrafish embryos exposed to varying concentrations of *L. leucocephala* (Lam.) de Wit extract at higher concentration shows delayed hatching rate (93%) up to 72 hpf (600 µg/ml) which is statistically significant when compared with control (p value <0.0001) with no observed hatching at 800 µg/ml due to embryo mortality (Figure 1 and Figure 5). More than 80 % of embryos were hatched at 48 hpf for 400 and 600 µg/ml where more than 90 % embryos were hatched for 100, 200 µg/ml concentration and in the control group. (Figure 1 and Figure 5). More than 90% hatched rate were observed at 72 hpf for 400 and 600µg/ml as 100% hatched rate were observed for 100 and 200 µg/ml. Similar results were also observed for control. At 96 hpf and 120 hpf all eggs are hatched including those for the control as well. (Figure 5)

4.3 Effect of L. leucocephala (Lam.) de Wit Extract on the Heartbeat of Zebrafish Larvae

Heart rate of larvae at 24 hpf before hatching and at 48, 72, 96 and 120 hpf after hatching exposed to various concentrations (100 µg/ml to 600µg/ml) of *L. leucocephala* (Lam.) de Wit shows no significant difference in the mean heartbeat rate with respect to control in the concentration range of 100 µg/ ml to 600 µg/ml. No heart rate was measured or observed at higher concentration range due to embryos and larvae mortality (Figure 6).

Table 1: Morphological characteristics for the teratogenic potency of *L. leucocephala (Lam.) de Wit* (concentration range 100 µg/ml-800 µg/ml) at different point.

			Time point for observation of normal development.					
Life Stage	**Embryotoxicity**	**Developmental endpoints evaluated**	**6hpf**	**24hpf**	**48hpf**	**72hpf**	**96hpf**	**120hpf**
Zebrafish Egg	Egg Coagulation		_	+	+	+	+	+
		Somites	_	+	+	+	+	+
		Tail detachment	_	+	+	+	+	+
		Heartbeat	_	_	+	+	+	+
		Blood Circulation	_	_	+	+	+	+
		Eye	_	_	+	+	+	+
Hatching (Zebrafish larvae)	Larvae alive							
		Hatch rate	_	_	+	+	+	+
		Skeletal deformities	_	_	+	+	+	+
		Motility	-	-	+	+	+	+

+. Observation of normal development. -, No observation/ no development

TABLE 2: LC 50 (µg/ml) , EC 50 (µg/ml)(Teratogenic effect) and TI (Teratogenic index) value of *L. leucocephala (Lam.) de Wit* on Zebrafish embryo model.

Time of exposure(hpf)	LC5O	EC50	TI
24	428.706 ± 6.053	450.926 ± 4.461	0.951 ± 0.019
48	335.143 ± 6.834	358.04 ± 5.326	0.936 ± 0.028
72	253.403 ± 4.188	351.226 ± 1.665	0.721 ± 0.010
96	172.94 ± 6.998	285.393 ± 3.628	0.606 ± 0.032
120	105.086 ± 5.553	194.843 ± 0.927	0.539 ± 0.031

Values are Mean ± SEM (n= 3)

**** $p < 0.0001$ when compared with control.

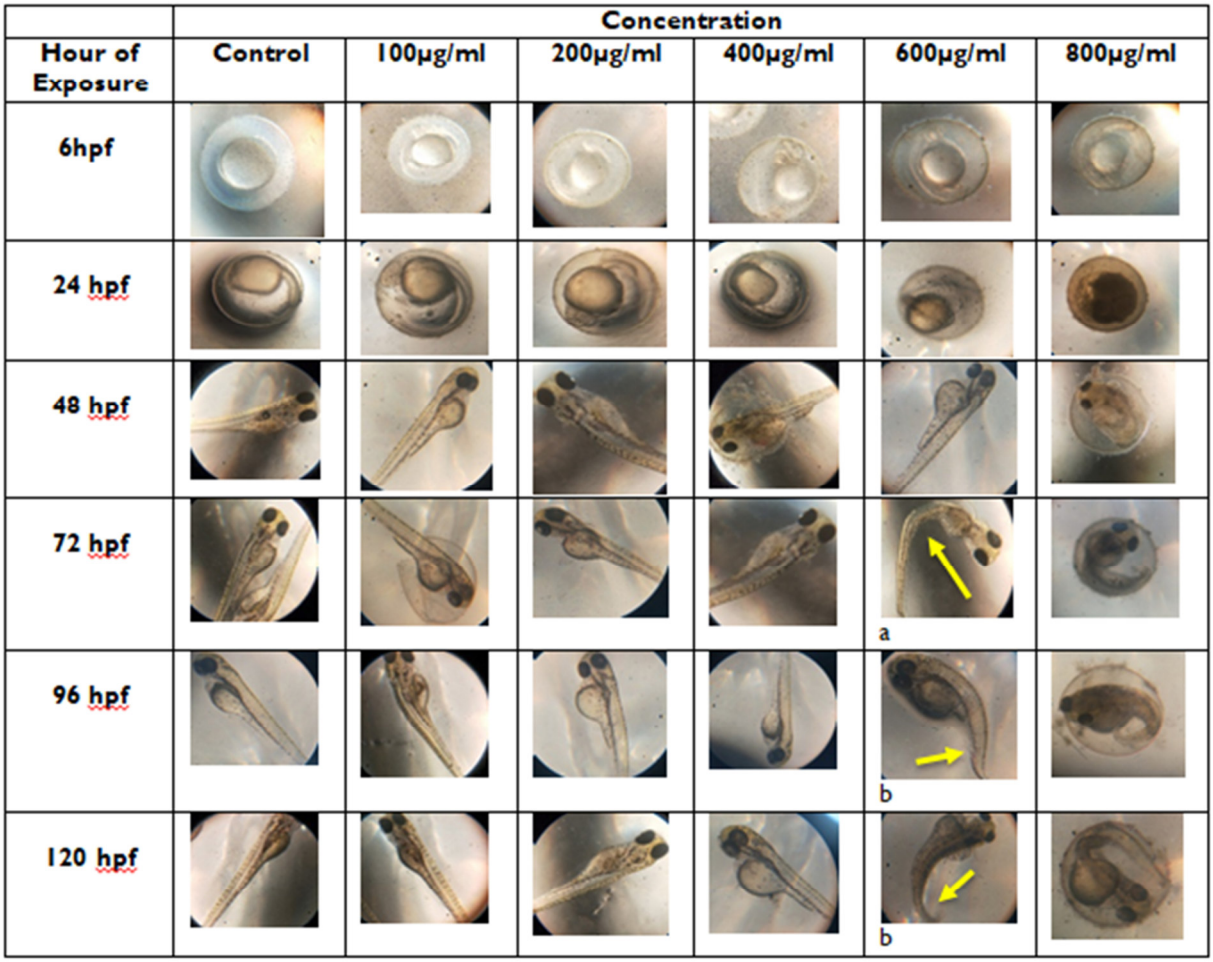

Fig 1: Morphological characteristics of zebra fish embryotoxicity and teratogenicity of L. Leucocephala extract at different time point. a-showing tail bending, b- kink formation

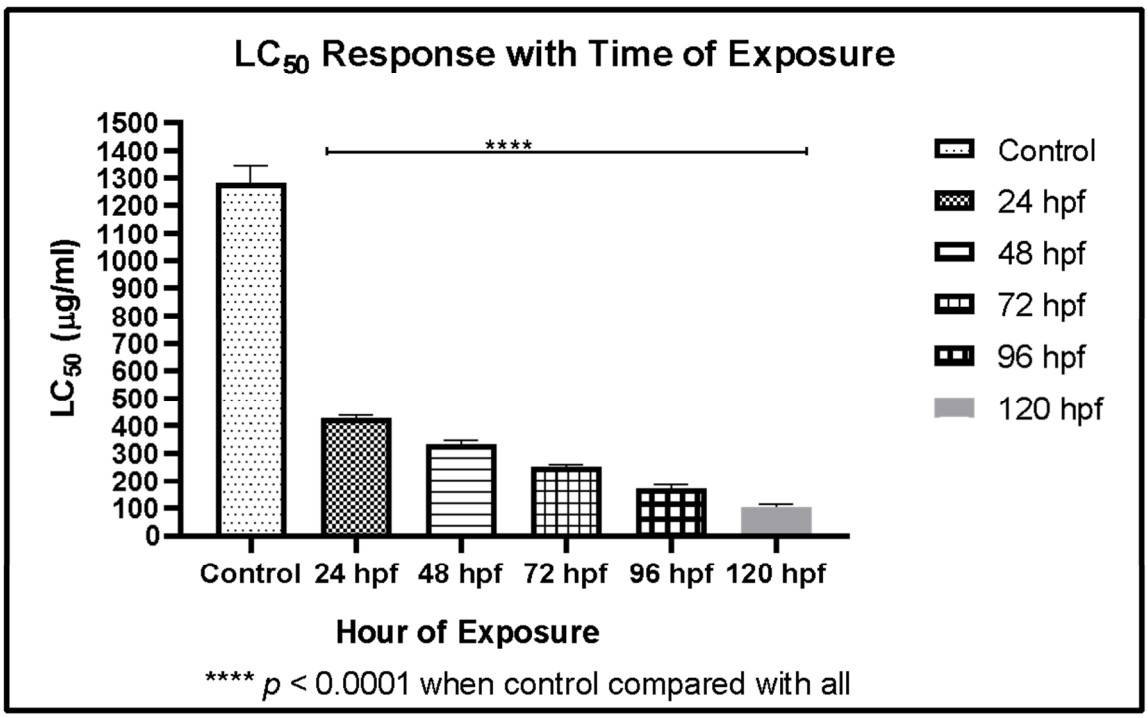

Fig 2: LC 50 value of L. leucocephala (Lam.) de Wit leaf extract at different time of exposer.

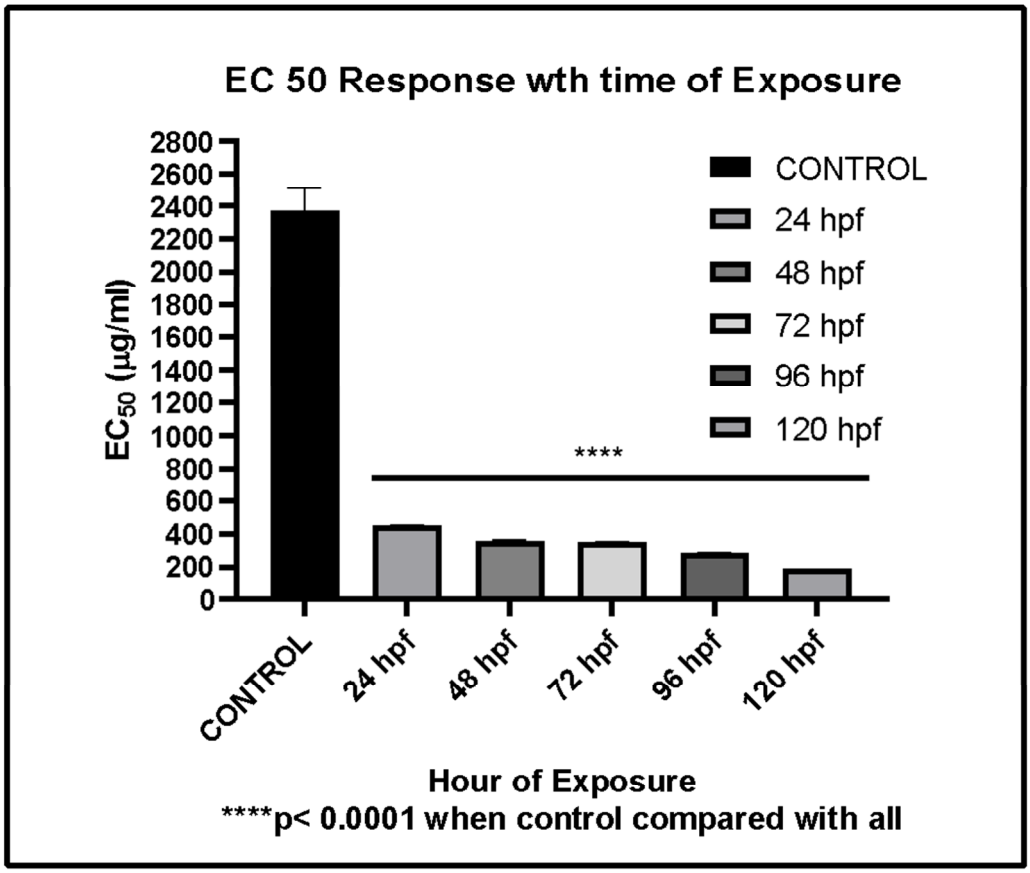

Fig 3: EC 50(Teratogenic effect) value of L. leucocephala (Lam.) de Wit leaf extract at different time of exposer.

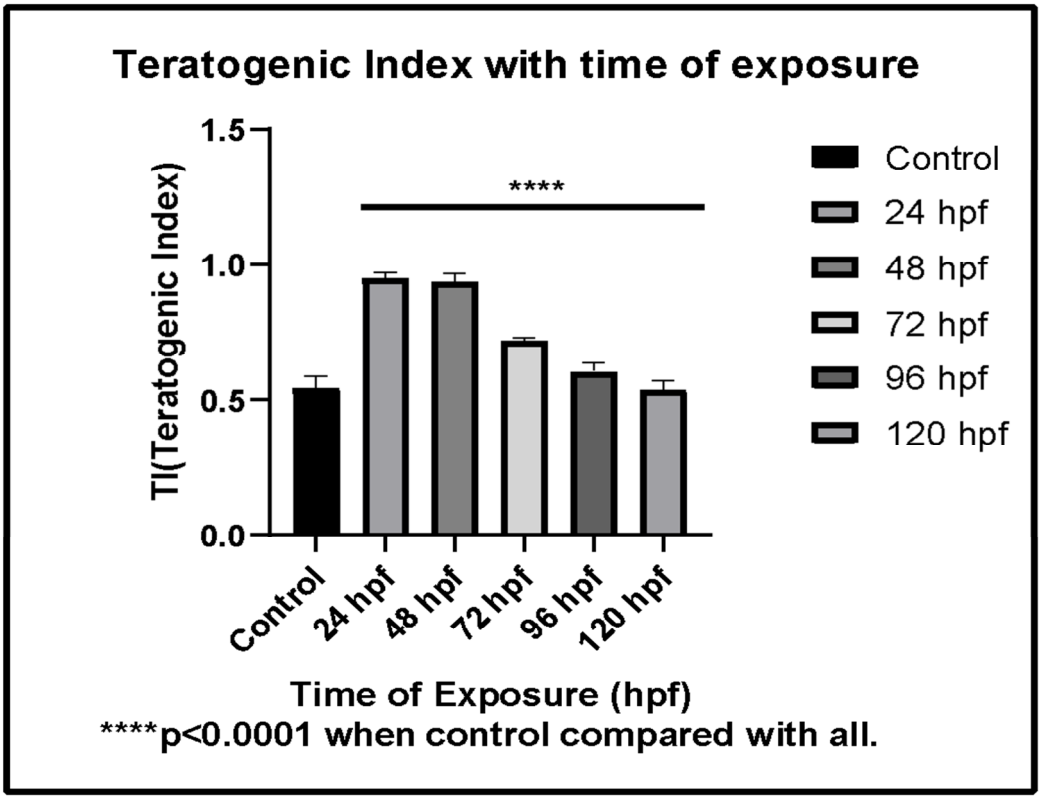

Fig 4: Teratogenic Index (TI) of L. leucocephala (Lam.) de Wit leaf extract at different time of exposure.

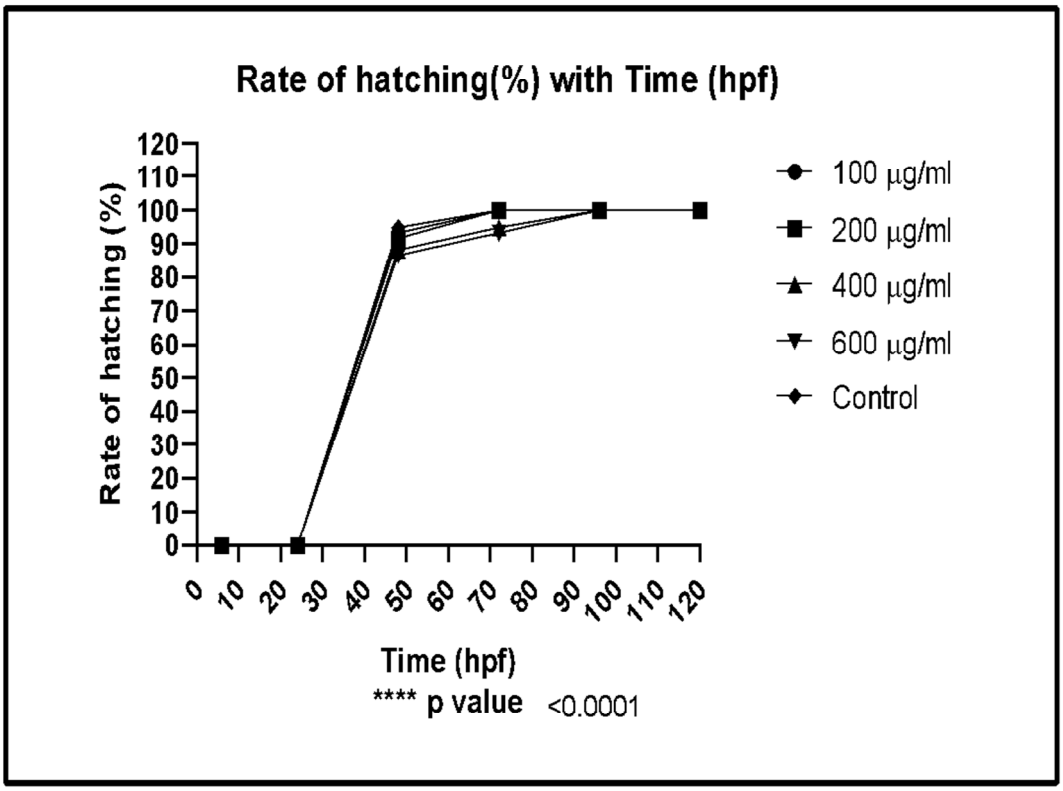

Fig 5: Hatching of Zebrafish Embryo on exposure to L. leucocephala (Lam.) de Wit Extract.

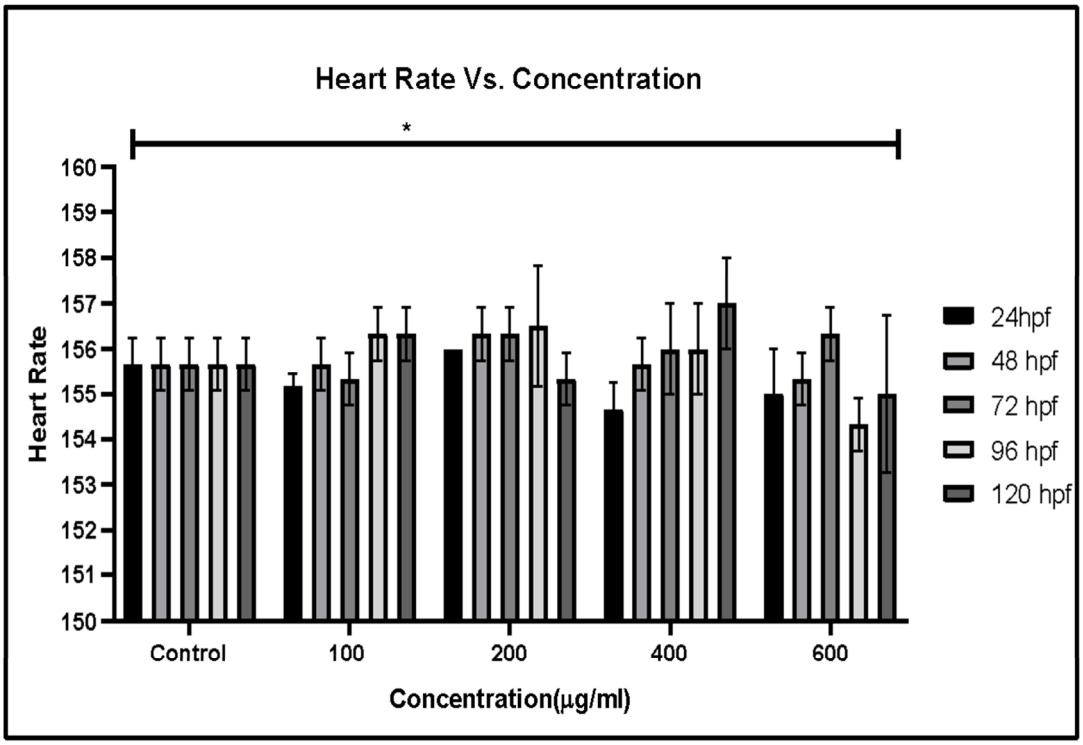

Fig 6: Effect of L. leucocephala (Lam.) de Wit Extract on Heart rate of Zebra fish embryo.

5. DISCUSSION

Development of a fetus is an organized process in which different changes are sequentially, and the changes at the cellular and molecular levels are amalgamated for a phenotype. In recent times, herbal plant-derived products, claiming for their pharmacological and therapeutic potentials, are gaining massive popularity. Therefore, it is becoming crucial to study their toxicological profile. Assessment of embryotoxicity of therapeutic plants during development of the fetus is, therefore, very important. Hatching of embryos was observed at 48 hpf at 100, 200, 400 and 600 µg/ml concentration of the plant extract but no hatching was observed at 800 µg/ml. Observation at 48 hpf reported hatching at all applied concentration except 800 µg/ml. Motility, blood circulation, heartbeat, eye development was observed clearly after 48 hpf for all exposed concentration. At 72 hpf, dead unhatched larvae were observed at higher

concentrations. Kink and bend tail were observed at a concentration of 600 µg/ml. (Figure 1). At 96 hpf and 120 hpf, larvae with several deformities were observed at 600 µg/ml concentration. All Concentrations were observed for heart rate and hatched rate. Depending on no larvae alive LC50 value was calculated with the help of Microsoft Excel 2019 and probit table. The LC50 value is gradually decreasing with increased exposure time (Figure 2) which is quite significant (p value< 0.001) The therapeutic value (TI), which is an indication for ranking the teratogenic effect (ratio of LC50/EC20), is less than 1 and significant (p<0.0001). Gradual decreasing in TI value is an indication of reduced teratogenic effect of the extract. *L. leucocephala (Lam.) de Wit* extract reported delayed hatching at higher concentration (600 µg/ml) with no observed hatching at 800 µg/ml due to embryo mortality (Fig 2). More than 80 % of embryos were hatched at 48 hpf for all the concentration. (Fig 3). 90% hatched rate were observed at 72 hpf for 400 and 600µg/ml as 100% hatched rate were observed for 100 and 200 µg/ml. Similar results were observed for control also. At 96 hpf and 120 hpf all the eggs are hatched including control also. (Fig 3) Heart rate of hatched larvae exposed to various concentrations of *L. Leucocephala*. No significant difference in the mean heartbeat rate was observed with respect to control in the concentration range of 100 µg/ ml to 600 µg/ml. No heart rate was measured or observed at higher concentration range due to embryos and larvae mortality. Embryotoxicity and teratogenic toxicity study of therapeutically potential plants are gaining the popularity in recent research. This finding was mainly focused on determination of embryotoxicity and teratogenicity of chloroform extract of *L. leucocephala (Lam.) de Wit* on Zebrafish (*Danio Rerio*) embryo and larvae. Fertilized embryos (at 6hpf) were exposed to different concentrations of chloroform extract of *L. leucocephala (Lam.) de Wit* leaf ranging from 100µg/ml to 800µg/ml. The toxicological study of chloroform extract for *L. leucocephala (Lam.) de Wit* divulge embryotoxic effect on zebrafish embryo at higher concentration of 800 µg/ml (Figure 1). Development of deformities like kink formation and tail bending were observed at 72 hpf, 96 hpf and 120 hpf for 600 µg/ml concentration. At 24 hpf hatching was not found and no observable teratogenic effect was identified on embryos for any concentration (100 µg/ml to 600 µg/ml) when compared with control (Figure 5). No hatching was observed for 800 µg/ml with increasing exposure of time (for 24hpf -120hpf). This indicates possible embryotoxic effect at higher concentration (800 µg/ml) for chloroform extract of *L. Leucocephala*.[34] At 600 µg/ml concentration, teratogenic effects in developmental stage was found which includes structural deformities like tail bending, kink formation at 72 hpf, 96 hpf and 120 hpf, when compared with control. (Figure 1) No malformation was observed (Figure 1) on zebrafish larvae development at lower concentration (100 µg/ml to 400 µg/ml) confirming the safety of chloroform extract of *L. leucocephala (Lam.) de Wit* at low concentration. [35] The LC50 value for different time of exposure 24 hpf to 120 hpf was found to be significant (p value < 0.0001) when compared with control (Figure 2). Change in the value of LC50 (Figure 2) is probably due to an increased accumulation of the extract in the embryo. Until when it reaches a concentration that can induce toxicity both in embryos and larvae with increased exposure to concentration 100 µg/ml to 800 µg/ml of extract as well as days of exposure. Because of increased exposure of the extract this accumulation becomes so high that it leads to the toxicity both in embryos and larvae (at 120 hpf LC50 was 105.086 $\pm$ 5.553). Gradual decrease in LC50 is probably due to the presence of a Chorion layer acting as a protective layer in embryo. This early protection of developing larvae eroded with age thus toxicity of the extract is more with increasing age.[36]Teratogenic effect as determined in terms of EC50 (malformation) was significant (p value <0.0001) when compared with control. (Figure 3) The ratio of LC50/EC50 produces the Teratogenic index (TI) value for each day of treatment. The TI values are used as an indication of teratogenic effect of any toxic compound. The higher the TI value greater the teratogenic potential of a compound.[37] Hence in this study, the gradually decreasing TI values, for the 5-day (120hpf) treatment suggest reduced teratogenic potency of the chloroform extract.

6. CONCLUSION

The present study has focused on the toxic effect of chloroform extract of *L. leucocephala* (Lam.) de Wit leaf at different concentrations on embryos and development of abnormality on larvae especially at higher concentration (600 µg/ml). Toxicity developed was found to be dependent upon concentration and hence may be toxic to human at higher dose. Plant is widely used as a source of nutrition to cattle and humans. Instead of its therapeutic benefit it contains one toxic amino acid mimocine. A detailed toxicity assessment should be carried out for safe and effective consumption of the extract. Through this study safety of the extract at a concentration lower than 105.086 $\pm$ 5.553 µg/ml on embryo was established (LC5O value at 120 hpf was 105.086 $\pm$ 5.553 µg/ml). Overall, the study revealed that although this plant is known to have important medicinal activity, the chloroform extract of *Leucaena leucocephala* (Lam.) de Wit produces some adverse effect at higher concentration (>600µg/ml) which indicates that further pharmacological activity can be carried out at a concentration lower than 600µg/ml. Hence based on both LC50 and EC50 (194.843 $\pm$ 0.927) values at highest time of exposure (120 hpf) it can be concluded that lower than 105.086$\pm$5.553 µg/ml concentration is safe for further *in vitro* pharmacological studies. Gradual decrease in TI (Teratogenic Index) value was an indication of reduced teratogenic effect and safety of the extract with time of exposure. Further research is required in order to explain specific effects regarding human risk assessment through *in vivo* model.

Abbreviations:

DMSO: Dimethyl sulfoxide
OECD: Organization for Economic Cooperation and Development
hpf: Hour of post fertilization.
FET: fish embryo Acute Toxicity Test

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8. AUTHORS CONTRIBUTION STATEMENT

All the authors contributed equally for successful completion of the work and designing of manuscript.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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