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ASSESSMENT OF TOXIC EFFECTS OF *Lantana camara* L. EXTRACT USING *Danio rerio* EMBRYO ASSAY

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ABSTRACT

This paper reported the toxic and teratogenic effects of leaf and stem-bark of *Lantana camara* lyophilized extracts in *Danio rerio* embryos. Embryos at 1% and higher concentrations of both leaf and stem-bark extracts showed 100% mortality after 12 hours and those in the lower concentrations continuously increased after prolong exposure. This effect of both extracts was dependent on concentration and time of exposure, as evident by the increased mortality of embryos with higher extract dose and longer time of exposure. Coagulation was the most notable toxic effect of both extracts. Embryos at 0.01% leaf extract completed hatching while those at 0.01% of stem-bark extract had 50% hatchability. Tail malformation was the most marked morphological abnormalities of *L. camara* extract-treated embryos. Delayed growth was also obvious teratogenic effect of both extracts. Altogether, *L. camara* extracts exhibit toxic and teratogenic effects in developing embryos of *D. rerio*.

Keywords: *Lantana camara*, DarT assay, morphological abnormalities, zebrafish

INTRODUCTION

Medicinal plants have been used over the years as natural alternative to prevent and treat several diseases. However, some of these plants contain substances that may potentially toxic and teratogenic to a human system. Therefore, assessment of the toxicity and teratogenicity of certain medicinal plant, if any, is necessary. Teratogenicity assay is accomplished using different animal models and one of these is zebrafish (*Danio rerio*) embryo. This model is a very reliable and important tool in order to establish whether certain substance, food, and drink could cause malformations to new individual. *D. rerio* embryo assay is also used in the preliminary screening of anticancer properties. Embryo is compared with cancer by Blagosklonny **[1]** for two reasons; first, they both grow and invade, and secondly, certain embryonic pathways may reactivate by cancer cells, which are targets of teratogens.

Lantana camara (Verbenaceae), commonly known as "kantutay" or stink grass, is a fast-growing woody thicket-forming shrub. It is invasive weed species **[2]** and can aggressively grow in open forest lands, plantations, farmlands and wastelands. The different parts of this plant exhibit important bioactivities. The extracts are used to treat chicken pox, measles, asthma, ulcers, swellings, eczema, tumors, high blood pressure, bilious fevers, catarrhal infections, tetanus, rheumatism, malaria and atoxy of abdominal viscera **[3]**. Bioactive metabolites isolated from *L. camara* leaves showed significant antitumor, anti-inflammatory, antipyretic and antinociceptive activities **[4, 5]**.

This paper reported the effects of *L. camara* on developing embryos of zebrafish in our intention to establish the potential toxic and teratogenic activity of this plant, which could lead to the discovery of other promising bioactivities.

MATERIALS AND METHODS

Collection of *L. camara*

L. camara leaves and stem-bark were collected from Sto. Tomas, San Jose City, Nueva Ecija, Philippines. Samples were separately placed in plastic bags and brought to the laboratory for air-drying for 5 days. The air-dried samples were pulverized using a food processor and each powdered sample was prepared for the extraction process.

Extraction and Treatment Preparation

The extraction protocol of Eguchi *et al*. **[6]** was followed in this study. Twenty grams of each sample was weighed and separately placed in a 1000ml-capacity flask containing

600 ml of distilled water. These were maintained at 80 - 90ºC for 2 hours in a water bath. Extracts were filtered using Whatman filter paper No. 2 and the filtrates were freeze-dried up to dryness. Six extract concentrations (5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%) were prepared by dissolving the extract into embryo water **[7]**. Embryo water served as the control. Two ml of each concentration was dispensed into each well of the 12-well ELISA plate. Triplicate was done in each treatment.

Maintenance and Spawning

Mature female and male zebrafish at 1:2 ratios were acclimatized in a glass aquarium with water saturated with oxygen. They were fed two times a day with dry flakes and the quality of water was maintained. In spawning, the procedure described by Nagel **[8]** was followed. Fish were localized in a plastic mesh and the aquarium was covered with black plastic sheet for 12 hours. After spawning, eggs were exposed to lighted condition for another 12 hours. The eggs were fertilized after 30 minutes of exposure to light. Embryos were collected from the aquarium using a hose and examined for the uniformity of embryos using a microscope. Coagulated and unfertilized eggs were discarded and the normal embryos were used in the assay.

Toxicity and Teratogenicity Assay

The protocol on the toxicity and teratogenicity using zebrafish embryos was adopted from Dulay *et al*. **[9]**. Four embryos at segmentation phase were exposed to the different concentrations of the extract. Mortality was determined after 12, 24, 36, and 48 hours of extract exposure. The hatching rate was also recorded. The morphological abnormalities of the treated embryos were based on the parameters established by Nagel **[8]**. The validity of the results was also noted. Analysis of Variance (ANOVA) was used to analyze the data and Least Significant Difference (LSD) was used to compare the means at 5% level of significance.

RESULTS AND DISCUSSION

Mortality of *D. rerio* Embryo

Mortality is described as having no visible heartbeat and coagulation. The toxic effect of *L. camara* leaf and stem-bark lyophilized extracts was recorded and the mean percentage mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure in varying concentrations are shown in Table 1. As early as 12 hours, 100% of embryos were observed in 1% and higher concentrations of both *L. camara* leaf and stem-bark extracts. At 0.5% concentration of leaf extract, 66.67% was recorded at 12 hpta and

increased to 100% after 24 hours of exposure. Meanwhile, 0.5% of stem-bark extract at 12 hpta had 41.67% then became 83.33% at 24 hpta and 100% at 36 hpta. No mortality was observed in embryos at 0.1% or lower concentrations of leaf extract while 33.33% mortality was noted in 0.1% of stem-bark extract after 12 hours. After further exposure for 36 hours, increased mortality was observed in embryos exposed at 0.05% and 0.1% concentration of leaf extract while remain the same in those exposed to stem-bark sample. In leaf extract, the mortality increased after 48 hours of exposure in 0.1%, 0.05% and 0.01% concentrations with 83.33%, 66.67% and 33.33%, respectively. However, the embryos exposed to stem-bark extract at 48 hpta, 0.1% had 50% and 0.05% had 33.33%. All embryos exposed to 0.01% of stem-bark extract survived at 48 hpta. It can be noticed that the embryo-toxic effects of *L. camara* leaf and stem-bark extracts were dependent on concentration and time of exposure, as evident by the increased mortality of embryos with higher extract dose and longer time of exposure. Coagulation was the most notable toxic effect of both extracts. In addition, non-detachment of tail was also observed as a lethal effect on embryos exposed to 0.01% concentration of leaf extract at 60 hpta and to 0.05% of stem-bark extract at 48 hpta.

Table 1: Mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure at the different concentrations of leaf and stem-bark extracts of *L. camara*

Extract	Concentration (%)	Mortality (%)			
		12 hours	24 hours	36 hours	48 hours
Leaf	5.0	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	1.0	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	0.5	66.67^{b}	100.00^{a}	100.00^{a}	100.00^{a}
	0.1	0.00^{c}	0.00^{b}	66.67^{b}	83.33^{a}
	0.05	0.00^{c}	0.00^{b}	50.00^{c}	66.67^{b}
	0.01	0.00^{c}	0.00^{b}	0.00^{d}	33.33^{c}
Control	0.00	0.00^{c}	0.00^{b}	0.00^{d}	0.00^{d}
Stem-bark	5.0	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	1.0	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	0.5	41.67^{b}	83.33^{a}	100.00^{a}	100.00^{a}
	0.1	33.33^{b}	41.67^{b}	41.67^{b}	50.00^{b}
	0.05	25.00^{c}	33.33^{b}	33.33^{b}	33.33^{b}
	0.01	0.00^{d}	0.00^{c}	0.00^{c}	0.00^{c}
Control	0.00	0.00^{d}	0.00^{c}	0.00^{c}	0.00^{c}

Values are treatment means. Means with the same letter of superscript are not significantly different from each other at 5% level of significance using DMRT.

The embryo-toxic effect of *L. camara* leaf and stem-bark extracts could be attributed to the compounds which are lantanilic acid, camaric acid and oleanolic acid from the aerial parts of *L. camara*. These compounds exhibited significant mortality against root-knot nematode *Meloidogyne incognita* at 0.5% concentration **[10]**. Similarly, active fractions from extracts of leaves, twigs, stems and roots of *L. camara* yielded lantadene A, oleanonic acid 8 and oleanolic acid which were very toxic to brine shrimp larvae in a brine-shrimp lethality test **[11]**.

The findings of the present study are in conformity with the study conducted by Dixit et al., **[12]**. The petroleum ether and methanol extracts of the aerial part of *L. camara* have been reported to be toxic to *Callosobruchus chinensis*. The extracts showed 10-43% mortality at 5% concentrations, with fecundity loss at higher doses. Tokarnia *et al*. **[13]** diagnosed an outbreak of poisoning by *L. camara var. aculeata* in cattle in Quatis County, state of Rio of Janeiro. The results showed that the plant caused lethal poisoning when given as a single dose of 40 g/kg; 20 g/kg caused severe poisoning, 10 g/kg slight or no poisoning and 5 g/kg failed to provoke symptoms. In addition, other plants also exhibited toxic effects. According to the study conducted by De Castro et al. **[14]**, embryos exposed at 10% of young seed extract and 3% of mature seed extract of *Carica papaya* significantly recorded 100% mortality after 48 hours of treatment exposure. The decoction of *L. operculata* administered to female mice during the implantation of embryos caused a reduction in birth rate **[15]**.

Hatchability of *D. rerio*

Hatching is a significant indicator of normal and successful embryonic developmental processes. The percentage hatchability of embryos treated with different concentrations of *L. camara* at 48 hours of exposure is depicted in Table 2. Hatching was completed after 48 hours of exposure in control embryos and 0.01% of stem-bark extract-treated embryos while those embryos at 0.01% leaf extract had 50% hatchability. Some embryos exposed to 0.05% and 0.1% concentrations of both leaf and stem-bark extracts were hatched but significantly lower when compared to the hatchability of control embryos. No hatched embryo was recorded in 0.5% or higher concentrations. The results of the study clearly indicate that hatchability of embryos was affected by the increasing concentrations of *L. camara* leaf and stem-bark extract. Hence, *L. camara* extracts could impede the activity of chorionase resulting into delay hatching.

Table 2: Hatchability of *D. rerio* embryos after 48 hours of exposure at the different concentrations of leaf and stem-bark extracts of *L. camara*

Concentration (%)	Hatchability (%)	
	Leaf Extract	Stem-bark Extract
5.0	0.00^{d}	0.00^{c}
1.0	0.00^{d}	0.00^{c}
0.5	0.00^{d}	0.00^{c}
0.1	8.33^{cd}	16.67^{b}
0.05	25.00^{c}	25.00^{b}
0.01	50.00^{b}	100.00^{a}
0.00	100.00^{a}	100.00^{a}

Values are treatment means. Means with the same letter of superscript are not significantly different from each other at 5% level of significance using DMRT.

Teratogenic Activity of *L. camara* Extracts

The different teratogenic parameters such as growth retardation, malformation of head and tail, scoliosis/flexure, limited movement were observed in the extract treated embryos. The different teratogenic effects of *L. camara* leaf and stem-bark extracts are shown in Figure 1 and Figure 2. Embryos exposed to lower concentrations of leaf extract resulted to larvae with different morphological abnormalities including wavy tail, bent tail tip and micro and twisted head, stunted tail, C-shaped ventral body, C-shaped dorsal body, hook like tail, and C-shaped lateral body. However, embryos at lower concentrations developed to larvae with non-pigmented retina, C-shaped ventral body with bent tail tip, and elongated tail with bent tail tip. Based on the results, tail malformation was the most marked morphological abnormalities of *L. camara* extract-treated embryos. Aside from tail malformation, delayed growth was also obvious teratogenic effect of both extracts. Similar observation was reported by Morya et al. **[16]** on the developmental cycle of *C. cephalonica* treated with powdered leaves of *L. camara*. There was delayed development in the pupal-adult moult after treatment of *L. camara*. In case of *L. camara*, only 0.05, 0.1, and 0.15 g treated insects could complete the life cycle, whereas with the remaining concentrations, larval to pupal conversion is delayed or they were dead after certain period of exposure. Interestingly, the silken cocoon produced for pupation by the treated larvae were less rigid and were absent in some. More than 50% of such pupae produced abnormal adults with distorted wings. However, the adults formed were unable to survive and reproduce. Jaipal *et al*. **[17]** reported a juvenile hormone-like activity of *L. camara* on *D. koenigii* when they observed morphological aberrations in the insect body followed by developmental delay and infertility. Moreover, Prasad and Purohit **[18]** showed the morphological abnormalities in the 4th instar larva of *Helicoverpa*

armigera (Hub.) treated with leaf extract of *L. camara* including abnormal cephalothorax, reduced abdominal region, sometimes unproportionate pupa body and general darkening of pupa body.

In conclusion, the lyophilized extracts of leaf and stem bark of *L. camara* exhibited embryo-toxic and teratogenic effects in zebrafish embryos.

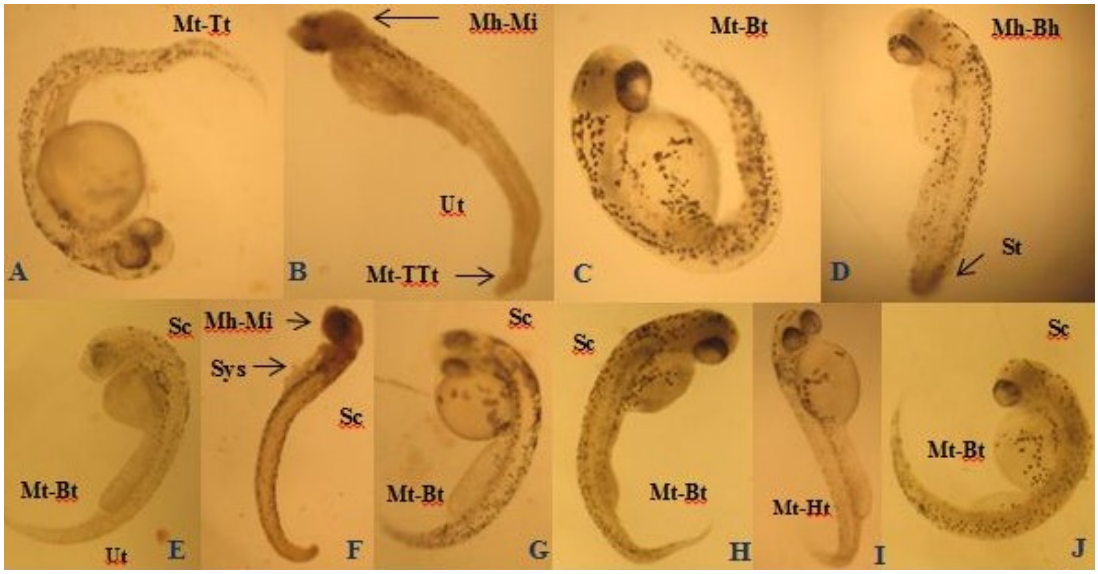

Figure 1: Malformations of *D. rerio* larvaetreated with the different concentrations of *L.camara* leaf extract after 60 and 72 hours of exposure; (A) wavy tail, (B) bent tail tip and micro and twisted head, (C) wavy tail, (D) stunted tail, (E) C-shaped ventralbody, (F) C-shaped dorsal body, (G) C-shaped lateral body, (H) bent tail tip, (I) hook like tail, and (J) C-shaped lateral body

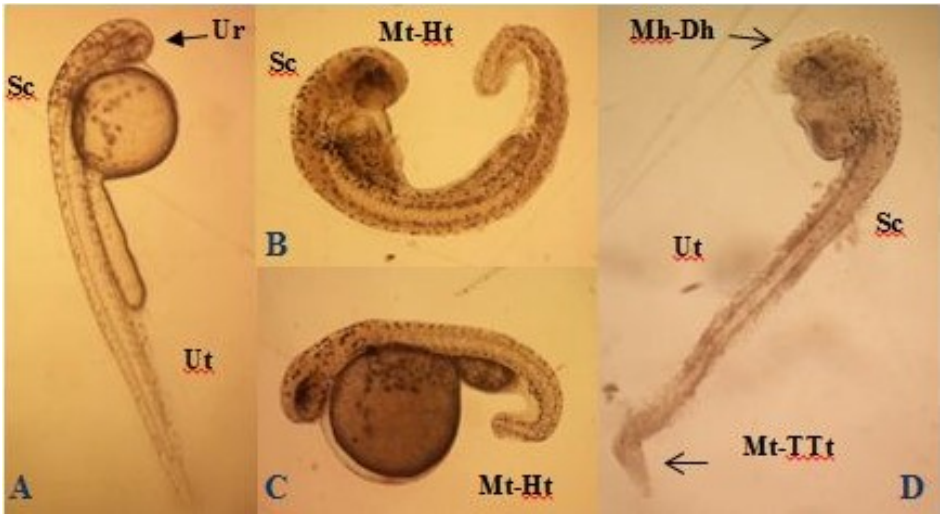

Figure 2: Malformations of *D. rerio* larvae treated with the different concentrations of *L. camara* leaf extract after 60 and 72 hours of exposure; (A) non-pigmented retina, (B) C-shaped ventral body with bent tail tip, (C) bent tail tip, and (D) elongated tail with bent tail tip

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