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Toxic and Teratogenic Effects of Sampa-sampalukan (*Phyllanthus niruri*) Leaves Extract Using *Danio rerio* Embryo Assay

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ABSTRACT

This paper established toxic and teratogenic effects of P. nururi leaves extract to the embryonic development of D. rerio. After 12 hours of exposure to various treatment concentrations, 100% coagulated embryos were observed in 1% and higher concentrations. Meanwhile, mortality in lower concentrations was found to be as time and dose-dependent. Coagulation was the most remarkable toxic effect of the plant leaves extract. On the other hand, heartbeat and hatchability rate of zebrafish embryo was affected in a dose-dependent manner. In teratogenicity testing, tail malformation was the most evident teratogenic effect of the plant leaves extract. Taken together, P. niruri leaves extract was embryo-toxic and teratogenic to D. rerio.

Key words: *Danio rerio, Embryo-toxicity, Phyllanthus niruri, Sampa-sampalukan, Teratogenicity, zebrafish*

INTRODUCTION

Phyllanthrus niruri, commonly known as *Sampa-sampalukan* belonging to the family *Phyllantaceae*, is commonly found in the tropical and sub-tropical region of the world. It grows as a weed in moist abandoned land. It has various applications in tradition and folk medicine for treatment of various diseases such as hepatitis B, HIV, microbial infections, plamodiasis, nematode infestation, hepatoxicity, cough, diuretic, menstruation problem and dysentery[1]. However, embryo-toxicity and teratogenicity of this invasive weed are still unexplored.

Toxicity can be defined as the degree to which a substance can harm organisms[2]. Teratogenicity, on the other hand, is the ability of any substances (teratogens) to cause malformations in developing organisms[3].

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However, Blagosklonny[4] stated that most of the anticancer drugs are teratogenic in nature and teratogens can be developed as anticancer drugs. Nowadays, zebrafish (*Danio rerio*) becomes a trending model when it comes to toxicity and teratogenicity assay due to its high fecundity, transparent embryos and larvae, no pain model, similarity to mammalian embryonic development[5] and physiological responses[6].

Herein, toxicity and teratogenicity of *P. niruri* leaves extract were evaluated using *D. rerio* embryo in return to the assessment of its bio-potentialities in drug development.

MATERIAL AND METHODS

Source of plant leaves specimen

The leaves of *P. niruri* were collected from Lias Marilao Bulacan, Philippines. The botanical specimen was brought to the Institute of Biology of the University of the Philippines, Diliman, Quezon City, Philippines for verification and authentication. The remaining leaves were air-dried for seven days, milled using a blender and prepared for hot water extraction.

Preparation for plant leaves extraction

The plant leaves extract was obtained after Eguchi *et al.*[7], with minor modifications. Thirty grams (30) of the pulverized plant sample were extracted in 300 mL hot water at 80-90^{o}C in the water bath for 2 hours. Afterward, the extract was filtered using Whatman filter paper No. 2. The filtrates were used for the preparation of different treatment concentrations by diluting to the embryo medium[8]. Eight treatment concentrations were formulated such as: 10%, 5%, 3%, 1%, 0.5%, 0.1%, 0.05% and the control (embryo medium).

Acclimatization and spawning of Zebrafish

Following the procedure established by Nagel[9], with minor modifications, 10 female and 20 male zebrafish were acclimatized in the aquarium with water saturated by oxygen for one (1) week. The zebrafish were fed using dry flakes twice a day and the water quality was maintained by removing excess feeds out of the aquarium after 1 hour (h). To initiate spawning, zebrafish were confined in the breeding tank with plastic mesh to avoid cannibalism of the released eggs. The breeding tank was enclosed using a black trash bag for 12 h. After 12 h, the trash bag was removed and allowed the eggs to be fertilized for another 12 h. Typically, fertilization occurs after 30 minutes of exposure to the light condition. At 12 hours post fertilization, embryos were siphoned out using a hose and then washed using distilled water thrice. The embryos were sorted out using a simple compound microscope. Fertilized eggs were used in the assay. Meanwhile, unfertilized and coagulated eggs were discarded.

Toxicity and teratogenicity assay

Two (2) mL of different treatment concentrations were doled out into each well of 24-well ELISA plate. Each treatment was triplicated. Four (4) embryos at segmentation period were exposed into each well of the plate. Afterward, the plate was maintained at room temperature (26 ± 1ºC)[10]. The mortality of zebrafish embryo was assessed at 12, 24, 36 and 48 hours post-treatment application (hpta). On the other hand, heartbeat was monitored at 36 hpta while hatchability was observed at 48 hpta. To evaluate the teratogenicity of the plant leaves extract, protocol of Nagel[9] was used: lethal (coagulation, no heartbeat, tail not detached and no somites), teratogenic (malformation of the head and tail, scoliosis, limited movement, stunted tail, light pigmentation and growth retardation or delayed growth) and normal. Malformations, if any, were captured using a mobile android phone with 8 megapixels.

Statistical analyses

The collected data were analyzed using SPSS program (17.0 versions). Data were run in One-Way ANOVA followed by Duncan's Multiple Range Test, to compare the means at 5% level of significance.

RESULTS AND DISCUSSION

In this study, zebrafish embryo was used to determine the toxicity and teratogenicity of the *P. niruri* leaf extract. The assay was conducted from segmentation period up to hatching period of zebrafish.

Mortality of Zebrafish embryo

Mortality is defined as no visible heartbeat and coagulation. Herein, mortality of zebrafish embryo was observed after 12, 24, 36 and 48 hours of exposure to various treatment concentrations (see Table 1).

As early as 12 h of exposure in 1% and higher concentrations, 100% mortality was recorded. Meanwhile, embryos exposed in 0.5% had 58.33% mortality. However, 0% mortality was noted in 0.05% and 0.1%. At 24 hours post-treatment application (hpta), all embryos treated in 0.5% have died. After 36 h of exposure in 0.1%, 16.67% dead embryos were observed. At 48 hpta, an increase of mortality was recorded in 0.05% and 0.1% with 8.33% and 25%, respectively. Nevertheless, even the mortality of embryo in 0.05% increases, the result is still comparable to the control at 5% level of significance.

Table 1: Mortality rate of zebrafish embryo after exposure to different treatment concentrations

Treatment Concentrations	12 hpta	24 hpta	36 hpta	48 hpta
Control	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
0.05%	0.00^{a}	0.00^{a}	0.00^{a}	8.33^{a}
0.1%	0.00^{a}	0.00^{a}	16.67^{b}	25.00^{b}
0.5%	58.33^{b}	100.00^{b}	100.00^{c}	100.00^{c}
1.0%	100.00^{c}	100.00^{b}	100.00^{c}	100.00^{c}
3%	100.00^{c}	100.00^{b}	100.00^{c}	100.00^{c}
5%	100.00^{c}	100.00^{b}	100.00^{c}	100.00^{c}
10%	100.00^{c}	100.00^{b}	100.00^{c}	100.00^{c}

Means that do not share a superscript in a column are significantly different at 5% level of significance

In these results, it is clearly observed that the survivability of the embryo was affected as the amount of treatment concentration increases and as the time of exposure is prolonged. Coagulation was the most marked lethal or toxic effect of the plant leaves extract.

The toxic effects of this plant could be associated to its bioactive component. In the study of Paithankar *et al*.[11], alkaloid from aqueous extract of *P. niruri* shows an inhibition to Human Immunodeficiency Virus (HIV) on MT-4 cells culture. Similarly, the *Limonene*, a kind of terpenes, shows an inhibition of liver tumor[12] suggesting a potential anticancer property. On the other hand, even other plant extracts exhibit toxic effect to *D. rerio* embryo. For instance, the aqueous extract of *Artocarpus heterophyllus* stem-bark exhibited 100% mortality at 0.1% and higher concentrations meanwhile, the leaves extract exhibited 100% mortality at 0.5% and higher concentrations after 48 hours of exposure[13]. Likewise, fruit rind extract of *Annona muricata*, *Annona squamosa* and *Garcinia mangostana* exhibited a toxic effect to *D. rerio* embryo in time and dose-dependent manner[14]. These results strongly suggested that plants can be a source of bioactive constituents specifically, *P. nururi*. Hence, continuous identification of those active phytochemical components is indeed necessary for pharmacological purposes.

Cardio-toxicity of P. niruri leaves extract to D. rerio embryo

Heartbeat rate is one of the important parameters to determine the toxicity of natural compounds or substances. Many Philippine plant studies have shown that zebrafish was one of the suitable models to determine cardio-toxicity. Usually, the effect to the heartbeat of zebrafish embryo was monitored after 36 hours of exposure to plant extract due to transparency and the visibility of its heartbeat15. Mably & Childs16 reported that the heartbeat of zebrafish is closely the same to the heartbeat of human with 120-180 beats per minute (bpm). In the present study, the results on the effect of P. niruri leaves water extract to the heartbeat zebrafish embryos after 36 h of exposure were presented in Table 2.

Table 2: Heartbeat rate and hatchability rate of *P. niruri* to *D. rerio* embryo

Treatment Concentrations	Heartbeat
Control	152.00[a]
0.05%	147.17[a]
0.1%	150.33[a]
0.5%	Coagulated
1.0%	Coagulated
3%	Coagulated
5%	Coagulated
10%	Coagulated

Means that do not share a superscript in a column are significantly different at 5% level of significance

In 0.5% up to 10% treatment concentrations, no heartbeat was recorded due to coagulation at early developmental stages of zebrafish. However, embryos treated in the control obtained the highest heartbeat with 152 beats per minute (bpm) while those embryos exposed to 0.05% and 0.1% register 147.17 and 150.33 bpm, which shows comparability to the control at 5% level of significance.

Accordingly, cardiac function may have been affected due to underdeveloped heart and pericardium, which could induce an abnormal heartbeat and circulation failure and subsequently result in body growth retardation via insufficient nutrients[17]. Thus, this deficiency to zebrafish will eventually lead to death.

Hatchability of zebrafish embryos treated in different treatments

The hatchability of zebrafish embryo defines its normal and successful development. Usually, it takes place between 48-72 hours post-fertilization. In this study, the hatchability rate was evaluated after 48 hours of exposure to different treatment concentrations (see Table 3).

Apparently, no hatched embryo treated in 0.5% and higher concentration 10% was observed due to early coagulation. On the other hand, 41.67% hatched embryos were recorded in 0.1% treatment concentration. However, 83.33% hatched embryos was noted in 0.05%, which shows no significant difference to the control at 5% level of significance.

Table 3: Hatchability rate of *P. niruri* to *D. rerio* embryo

Treatment Concentration	Hatchability
Control	100.00[a]
0.05%	83.33[a]
0.1%	41.67[b]
0.5%	Coagulated
1.0%	Coagulated
3%	Coagulated
5%	Coagulated
10%	Coagulated

Means that do not share a superscript in a column are significantly different at 5% level of significance

Teratogenicity of *P. niruri* leaves extract to *D. rerio* embryo

Teratogenicity assay is a desirable property because many anticancer drugs are teratogenic in nature and teratogens can be developed as anticancer drugs[4]. Herein, the morphological endpoints of the plant leaves extract were based on the parameters established by Nagel[9]. The teratogenic effect of *P. niruri* leaves extract was observed at 72-84 hpta. Tail

malformation was the most marked teratogenic effect of the plant extract (Figure 1).

In early developmental observation (12-48 hpta), most of the embryos were coagulated. However, those embryos treated in lower concentrations exhibited teratogenic effects at 72-84 hpta. Particularly, embryos exposed in 0.05%, head malformation, yolk deformities, bent body tail and the loop-like tail were observed. On the other hand, those embryos treated in 0.1% showed scoliosis, hook-like tail, malformed head, yolk deformities and bent tail. These results suggested that plant extract contains important teratogenic component/s that can be developed as an anticancer drug.

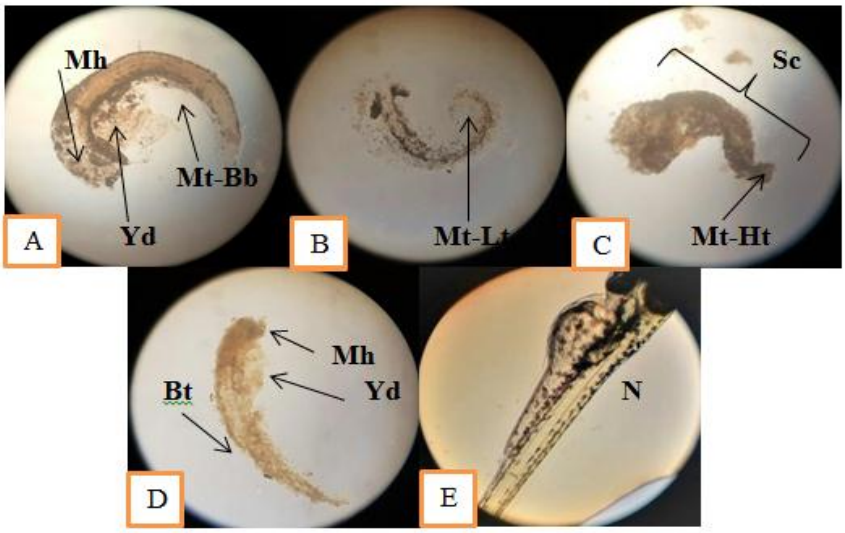

Fig. 1: Teratogenic effects of plant extract to *D. rerio* at 72-84 hpta. **(A).** Larva with head malformation, yolk deformities and bent body tail (observed in 0.05%) **(B)**. Larva with loop-like tail (observed in 0.05%) **(C)**. Larva with scoliosis and hook-like tail (observed in 0.1%) **(D)**. Larva with malformed head, yolk deformities and bent tail (observed in 0.1%). **(E)**. Normal hatched embryo (control)

These observed teratogenic effects were similar to the effect observed in other Philippine medicinal plants. In the study of Jose *et al*.[18], tail malformation was evident particularly in 0.01% leaf extract of *Garcinia mangostana* (Mangosteen). Also, this plant can cause head malformation to zebrafish embryo. Similarly, the result of this study conforms to the outcome obtained in Trinidad *et al*.[19], wherein the extract of *Lantana camara* (Stink grass), a weed, exhibited tail malformation (hook and bent tail), scoliosis and head malformation. On the other hand, yolk deformities were also observed in the extract of *Moringa oleifera* (Malunggay)[5].

CONCLUSION

Based on the collected findings, *P. niruri* leaves extract was toxic and teratogenic to *D. rerio* embryo. Thus, plant leaves contain phytochemicals that can be developed as anticancer drugs since, many anticancer drugs are teratogenic and teratogens can be developed as anticancer drugs. Identification of the specific phytochemical component(s) is highly recommended for future studies.

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