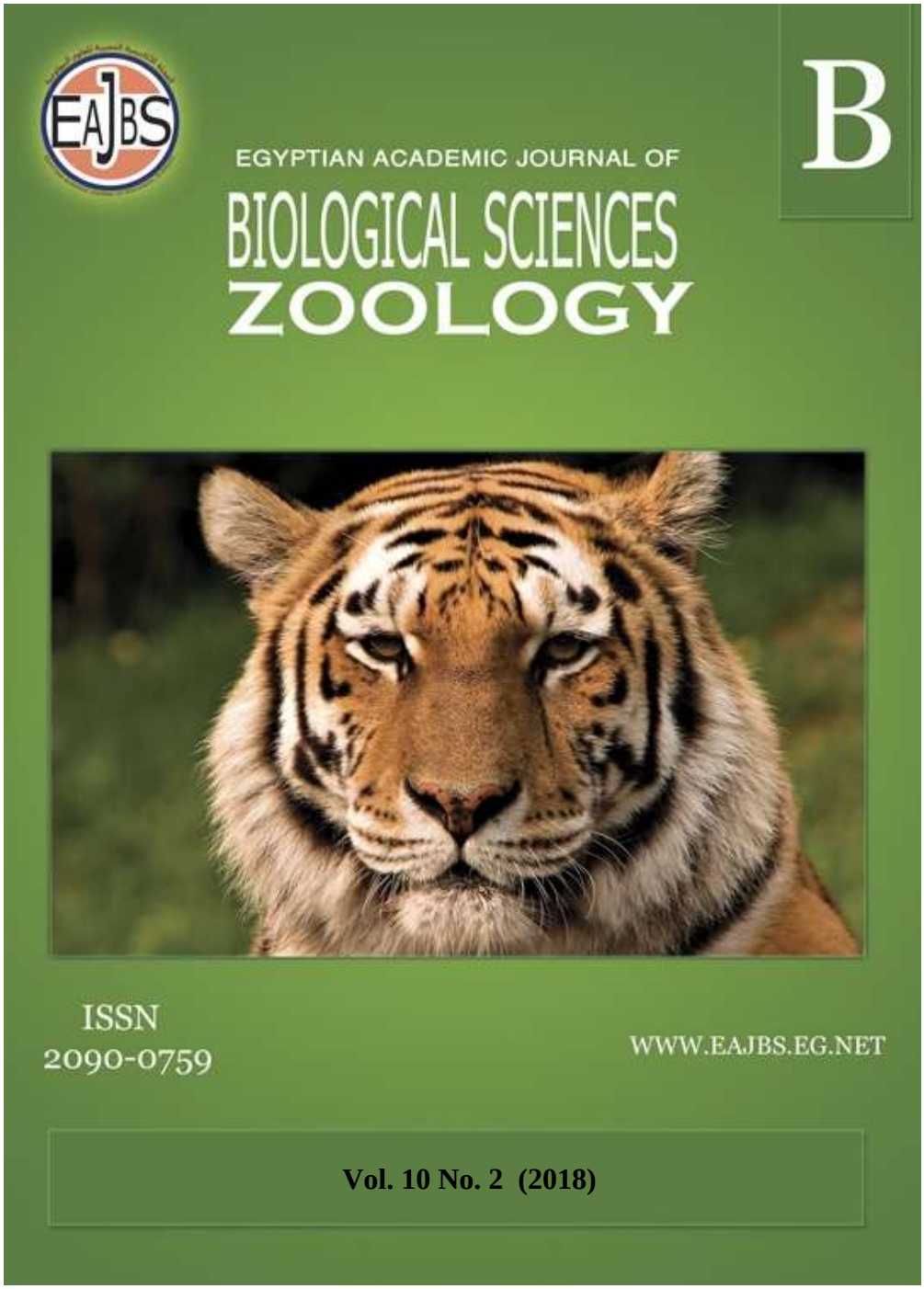

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Assessment of the Toxic and Teratogenic Effects of Kamagong (*Diospyros discolor* Willd.) Leaves Extract to the Developing Embryo of Zebrafish (*Danio rerio*)

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ABSTRACT

This work highlighted the embryo-toxic and teratogenic effects of *D. discolor* leaves extract to the embryo of *D. rerio*, a model. Apparently, 100% mortality of embryos was observed in 5% and 10% treatment concentration at 12 hpta. Meanwhile, mortality in lower concentrations increases as time of exposure is prolonged and as the amount of treatment concentration increases. Coagulation was the toxic effect of the plant water extract. On the other hand, heartbeat and hatchability rate decreases as the amount of treatment concentration increases. In terms of teratogenicity, tail malformation was the most marked teratogenic effect of the plant extract. Moreover, growth retardation, head malformation, yolk deformities and abdominal edema were observed. Altogether, *D. discolor* leaves water extract exhibit toxic and teratogenic effects against *D. rerio* embryo. Thus, this plant contains active phytochemical components that can be developed as anticancer drugs.

INTRODUCTION

Aside from food, shelter and fiber, human have used plants as medicine (Santos *et al*., 2017) due to its richness in phytochemicals that can be used in drug development and synthesis (Hassan, 2012). They are also considered as an alternative medicine due to their minor side effects and less toxicity but very strong bioactivities (Dulay & De Castro, 2017). However, many plants are now vulnerable but their biological activities are not completely known. One of these plants is the *Diospyros discolor* Willd. (Pobar, 2013).

D. discolor, from the family of Ebanaceae, is also known as *Diospyros blancoi* and *Diospyros philippinensis* (Ragasa *et al*., 2009). It is an endemic and vulnerable plant species in the Philippines with a high quality of *Kamagong* timber (Pineda *et al*., 2018). This tree produces a fruit called *Mabolo* which is known as a good source of calcium, vitamin B, iron and proteins and composed of phenolic compounds that provide powerful antioxidants. It is nutritious but is neglected due to its unpleasant

odor similar to rotten cheese or cat feces and is covered with fine, velvet fur which is irritating to sensitive skin (Pobar, 2013). Similarly, plant leaves has antibacterial (Vijaya & Chandra, 2015), astringent, antidiarrheal, anti-inflammatory, antioxidant, analgesic, anti-tumor, anti-asthma and vasorelaxant properties (Stuart, 2018). Despite of its medicinal properties, it is imperative to test its toxic and teratogenic effects to determine its biosafety and other promising bioactivities.

Toxicity is defined as the ability of substances to harm an individual (Kasper *et al*., 2015). Teratogenicity, on the other hand, is characterized by the malformation or abnormal development of the shapes and forms on the body of the developing embryo caused by teratogens (De Vera *et al*., 2016). However, many anticancer drugs are teratogenic in nature, and teratogens can be developed as anticancer drugs (Blagosklonny, 2005).

Today, embryonic and larval form of *Danio rerio* (zebrafish) is increasingly used as a toxicological and teratological model due its transparent larvae and embryo, rapid developmental processes, high fecundity, easy to maintain in the laboratory (Romagosa *et al*., 2016) and high genetic homology to mammals (Caballero & Candiracci, 2018).

In the present study, toxicity and teratogenicity of *D. discolor* leaves extract were evaluated using *D. rerio* embryo in able to assess its biosafety and bio-potentialities in discovering its other bioactivities.

MATERIALS AND METHODS

Collection, Identification and Preparation of *D. discolor* Leaves:

The plant leaves specimen was collected from Brgy. Lias Marilao, Bulacan, Philippines. Then, it was brought to the Institute of Biology, University of the Philippines, Diliman, Quezon City, Philippines for verification and authentication. After which, the remaining leaves were washed using tap water. It was then air dried, milled and prepared for aqueous extraction.

Plant Water Extraction and Preparation of Treatment Concentrations:

Following the protocol of Eguchi *et al*. (1999), with minor modifications, thirty grams (30 g) of pulverized sample was placed in Erlenmeyer flask with 300 mL of double distilled water. Then it was sealed and placed in water bath for 2 hours (h) at 80-90^{o}C. After 2 h, it was cooled and filtered using Whatman Filter Paper no. 2. The different treatment concentrations were prepared by diluting the extract in embryo medium (Thomas, 2000). These concentrations were: 10%, 5%, 3%, 1%, 0.5%, 0.1%, 0.05% and the control.

Acclimatization and Spawning of *D. rerio*:

Twenty male (20) and 10 female zebrafish were acclimatized in the aquarium with tap water that continuously aerated. They were fed using dry flakes twice a day. In spawning, the aquarium was covered using black trash bag for 12 h. After 12 h, the aquarium was uncovered and the eggs were exposed to light condition for another 12 for fertilization. Typically, fertilization occurs 30 minutes after exposure to light. At 12 hours post fertilization, the embryos were siphoned out using a hose, rinsed thrice using distilled water and were selected using a simple compound microscope. All fertilized embryos were used in the assay while those unfertilized eggs were discarded (Jose *et al*., 2016).

Toxicity and Teratogenicity Assay of *D. discolor:*

Two mL (2 mL) of the different treatment concentrations were dispensed into each well of 24-well ELISA plate. Three replicates of treatment concentrations were done. After dispensing the treatments, four zebrafish embryos at segmentation period were doled out into each well of the plate. The plate was incubated at 26 ± 1^{O}C (Cabuhat *et al*., 2018). Mortality of embryos was observed at 12, 24, 36 and 48 hours post treatment application (hpta). The heartbeat and hatchability of embryos were observed after 36 and 48 hours of exposure to different treatment concentrations, respectively. Teratogenic effects were recorded as it is manifested during the observation period. Morphological endpoint evaluation of zebrafish was based on the parameters established by Nagel (2002): Lethal (coagulation, tail not detached, no somites, and no heart-beat), teratogenic (malformation of head, tail and heart, scoliosis, deformity of yolk, and growth retardation), and normal.

Data Analyses:

Assay was classified as valid, if 100% of the embryos in the control show normal condition. Analysis of Variance (ANOVA) was used to analyze data and Duncan's Multiple Range Test was used to compare the means at 5% level of significance (Romagosa *et al*., 2016).

RESULTS AND DISCUSSION

Zebrafish embryo is now gaining popularity in toxicology and teratology. Thus, the importance of this organism has been recognized in drug discovery (Reneses *et al*., 2016). In this paper, the toxicity and teratogenicity of *D. discolor* leaves has been tested using the embryo of *D. rerio*. Mortality is defined as no visible heartbeat and coagulation. Herein, the mortality of zebrafish embryo after exposure to various treatment concentrations was presented in Table1.

As early as 12 hpta, 100% mortality or coagulated embryo was observed in 5% and 10%. Meanwhile, 91.67% was recorded in 3%. However, 1% obtained 8.33% mortality, which shows no significant difference to the control and lower concentrations (0.05%-0.5%) at 5% level of significance. At 24 hpta, an increase of mortality was observed in 1% with 66.67% while all embryos exposed in 3% were completely died. After 36 hours of exposure to 0.1% and 0.5%, 8.33% was noted but still, result was comparable to the control. At 48 hpta, 100% mortality was recorded in 1% whilst 0.05%, 0.1% and 0.5% treatment concentrations obtained 25% mortality of embryos which shows a significant difference to the control. Herein, it can be observed that as the amount of treatment concentration increases and as the time of exposure is prolonged, mortality rate increases. Coagulation was the toxic effect of the plant leaves extract.

This finding can be attributed by its active phytochemicals. In methanolic extraction of *D. discolor* leaves, several phytochemicals were identified such as: tannis, flavonoids, alkaloids, phytosterols, diterpenes, proteins, amino acids and sugars (Vijaya & Chandra, 2015). The result of the present study showed a similar effect using ethanolic extract of *D. blancoi* in brine shrimp lethality assay wherein an increase of mortality was observed in time and dose dependent manner (Howlder *et al*., 2012). Similarly, the crude methanolic and crude ethyl acetate extract of the seed and peels of *D. blancoi* showed cytotoxic activity in brine shrimp lethality assay (Setu *et al*., 2017). On the other hand, even other plants exhibit toxic effect to the embryo of *D. rerio*. Specifically, the 3% up to 10% extract of *Lagerstroemia speciosa* leaves recorded 100% mortality as early as 12 hpta (Cabuhat *et al*., 2018). Also, the leaves

extract of *Garcinia mangostana* (Jose *et al*., 2016), *Persea americana, Syzigium cumini* (Dulay & De Castro, 2017) *and Tinospora cordifolia* (Romagosa *et al*., 2016) exhibit toxic effect in zebrafish embryo in time and dose dependent manner.

Table 1. Mortality rate of zebrafish embryo after exposure to *D. discolor* leaves water extract

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]	25.00[b]
0.1%	0.00[a]	0.00[a]	8.33[a]	25.00[b]
0.5%	0.00[a]	0.00[a]	8.33[a]	25.00[b]
1.0%	8.33[a]	66.67[b]	83.33[b]	100.00[c]
3%	91.67[b]	100.00[c]	100.00[b]	100.00[c]
5%	100.00[b]	100.00[c]	100.00[b]	100.00[c]
10%	100.00[b]	100.00[c]	100.00[b]	100.00[c]

Means that do not share a letter in a column are significantly different to the control at 5% level of significant

Cardio-toxicity of *D. discolor* Leaves Extract :

The heartbeat of zebrafish is similar to human ranging 120-180 bpm (Mably & Childs, 2010). Herein, the heartbeat of zebrafish embryo was observed at 36 hpta when the body is distinctly pigmented. The heartbeat rate mean of the embryo is presented in Table (2).

Apparently, no heartbeat was observed in 3% and higher concentrations because of early coagulation. However, the 0.05%, 0.1% and 0.5% registered 145.61 beats per minute (bpm), 144.42 bpm and 141 bpm, respectively. On the other hand, the 1% obtained the lowest heartbeat rate mean with 54 bpm which shows a significant difference to the control at 5% level of significance. In this result, it can be observed that the cardio-toxicity of *D. discolor* was found to be as dose dependent. Meaning, as the amount of treatment concentrations increases, heartbeat rate decreases.

In Diaz (2011) as cited in Jose *et al*. (2016), cardiac glycosides are specific type of toxic glycosides that can cause fatal toxicosis by affecting the cardiac muscle. It increases the contraction force of the heart by inhibiting the myocardial Na-K ATP-ase, which can lead to cardiac arrest (Poindexter *et al*., 2007)

Table 2. Heartbeat of zebrafish embryo at 36 hpta.

Treatment Concentrations	Heartbeat
Control	171.00[a]
0.05%	145.61[a]
0.1%	144.42[a]
0.5%	141.00[a]
1.0%	54.00[b]
3%	0.00[b]
5%	0.00[b]
10%	0.00[b]

Means that do not share a letter in a column are significantly different to the control at 5% level of significance

Hatchability of Zebrafish Embryo:

Hatching indicates the successful embryonic processes in the development of zebrafish. Usually, it takes place between 48-72 hpf depending on the thickness of the chorion and enzymes (chorionase) it releases. In the present study, hatchability of zebrafish embryos exposed to various treatment concentrations were observed after 48 hpta (Figure 1).

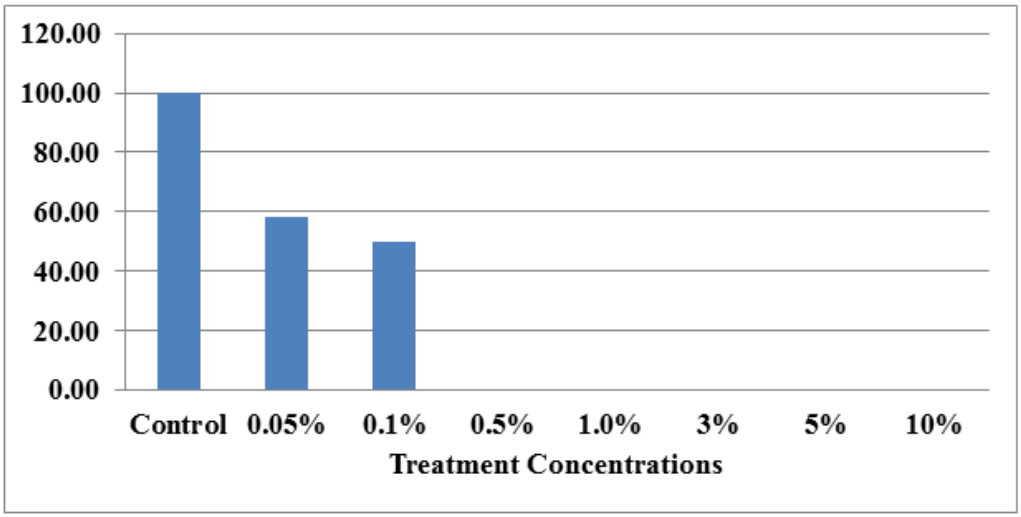

Fig. 1. Hatchability of *D. rerio* after exposure to the different treatment concentrations

Apparently, 0% hatchability was observed in 0.5% and higher concentrations due to early arrest of embryos. Meanwhile, 0.05% and 0.1% obtained 58.33% and 50%, separately which are significantly different to the control with 100% hatched embryos. Based on the results, the hatchability is affected depending on the amount of treatment concentration. The hatchability decreases as the amount of treatment concentration increases. This effect could be explained by the developmental abnormalities in the growth of zebrafish which eventually results to incapability to break the chorion (Weigt *et al*., 2011).

Teratogenic effects of *D. discolor* in *D. rerio* Embryo:

Teratogenic effect is accounted as sublethal effect of any substances called teratogens. In this study, manifold defects were observed in zebrafish embryo after exposure to different treatment concentrations. The teratogenic effects of plant leaves extract were presented in Figure (2).

No teratogenic effect was observed in 1% and higher concentrations due to coagulation at early developmental stages. However, tail malformation was the most marked teratogenic effect of the plant leaves extract. Some of the embryos exposed at 0.05% exhibit bent body, stunted tail, yolk deformities and bent tail with C-shaped dorsal body. The *D. rerio* embryo exposed to 0.1% concentrations exhibited bent tail with C-shaped ventral body, C-shaped ventral body with hook-like tail, yolk deformities and head malformations. Even 0.5% treatment caused bent tail in the developing embryo. Tail malformation was the most marked teratogenic effect of the plant leaves extract.

Tail malformation was similar to the outcome obtained by De Vera *et al*. (2016), Dulay and De Castro *et al*. (2017) and Trinidad *et al*. (2017) as zebrafish embryo tested in *Bixa orellana* & *Piper betle*, *Persea americana* & *Syzigium cumini* and *Lantana camara*, respectively.

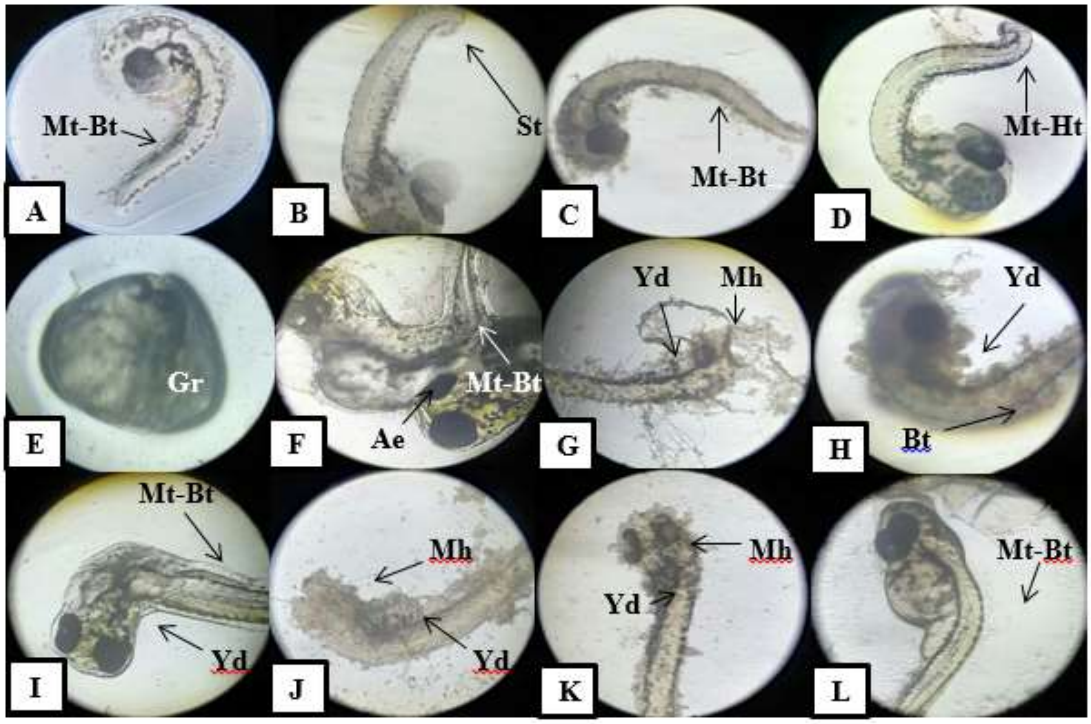

Fig. 2. Morphological malformations of zebrafish embryos exposed to the different treatment concentrations of *D. discolor* leaves extract. **(A)** Bent tail with c-shaped ventral body (0.1% at 36 hpta), **(B)** Bent body, stunted tail, yolk deformities (0.05% at 48 hpta), **(C)** Bent tail-Bent body and yolk deformities (0.05% at 48 hpta), **(D)** C-shaped ventral body with hook-like tail and deformed yolk (0.1% at 48 hpta), **(E)** Growth retarded embryo (0.1% at 48 hpta), **(F)** Bent tail with C—shaped dorsal body (0.05% at 72 hpta), **(G)** Yolk deformities and head malformation (0.05% at 72 hpta) **(H-I)** Yolk malformations and bent tail- with C-shaped lateral body (0.05% at 72 hpta), **(J-K)** Head malformation and yolk deformities (0.1% at 72 hpta) and **(L)** Bent tail (0.5% at 72 hpta).

CONCLUSION

Taken together, *D. discolor* leaves extract exhibit toxic and teratogenic effects to *D. rerio* embryo. Thus, this plant contains important bioactive component/s that can be developed as anticancer drugs since, many anticancer drugs are teratogenic in nature and teratogens can be developed as anticancer drugs. Due to its medicinal advantages, conservation of this species is imperative since it is now considered as endangered.

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