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TERATOGENIC EFFECTS OF LYOPHILIZED WATER EXTRACTS OF LEAVES AND STEM-BARK OF *Artocarpus heterophyllus* IN ZEBRAFISH (*Danio rerio*) EMBRYO

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ABSTRACT

Artocarpus heterophyllus is a medicinal tropical fruit which belong to the family Moraceae. This study assessed the embryo-toxic and teratogenic effects lyophilized water extracts of *A. hetrophyllus* leaves and stem-bark against zebrafish (*Danio rerio*) embryos. Mortality, hatchability, heartbeat rate and morphological malformation were determined. Results revealed that the mortality was concentration and time dependent. Coagulation of embryos was the most lethal effect of both extracts. Embryos exposed to 0.05% and higher concentrations (0.1%, 0.5%, 1% and 5%) of leaves extract and to 0.1% and higher concentrations (0.5%, 1% and 5%) of bark extract significantly recorded 100% mortality at 48 hpta. Embryos at 0.01% of leaves extract had 16.67% hatchability while no hatched was noted at 0.05% and higher concentrations. In stem-bark extract, 0.01% exposed embryos had 41.67%, followed by 0.05% with 16.67% hatchability. Embryos at 0.05% to 1% leaves extract and 0.1 % and higher concentrations of stem-bark extract showed growth retardation, which was the most marked teratogenic effect. Tail malformations such as unformed tail, bent tail and hook tail were common morphological abnormalities observed at 0.01% of leaves extract and at 0.05% and lower concentrations of stem-bark extract. Both lyophilized water extracts of *A. heterophyllus* significantly affect the survival and embryonic development of *D. rerio*.

Keyword: *A. heterophyllus*, *D. rerio*, teratogen, lyophilized extract.

INTRODUCTION

Artocarpus heterophyllus Lam., also known as jackfruit, is a tropical fruit tree belongs to the family Moraceae which can be found throughout the Philippines. This plant is popular because of its tasty fruit which is green to greenish-yellow when ripe, oblong with pyramidal projections. Inside the fruit there are numerous seed covered with luscious custard yellow pulp. *A. heterophyllus* is used in folkloric remedy to treat various diseases. The leaves are useful in fever, boils, wounds and skin diseases. The fruits when young are acrid, astringent, and carminative while when ripe are sweet, cooling, laxative, aphrodisiac, and brain tonic. The wood is nervine, anti-diabetic, sedative, and is useful in convulsions **[1]**. Its seeds have a sufficient antibacterial activity against *Escherichia coli* and *Bacillus megaterium* **[2]**. Several studies showed that *A. heterophyllus* exhibits a wide range of biological and pharmacological activities including anti-diabetic, anti-fungal, anti-microbial and anti-inflammatory, anti-oxidant, and anti-cancer properties **[3, 4]**. However, despite of the considerable medicinal properties of this plant, it might still pose problems due to presence of toxic compounds. Therefore, evaluation of the embryo-toxic and teratogenic effects of *A. heterophyllus* leaves and stem bark is imperative in order to assess its safety as a therapeutic agent and screen its potential as a source of anti-cancer compound.

Teratogenicity assay using zebrafish embryos as animal model is a very reliable and important test in determining if certain compounds or substances from natural resource could cause morphological abnormalities. Importantly, this assay is also used in the evaluation of new toxic compounds with potential anticancer properties. According to Blagosklonny **[5]** most anticancer drugs are teratogens and vice versa, merely because they target vital cellular functions. In the present study, the teratogenic and toxic effects of *A. heterophyllus* lyophilized water extracts against *D. rerio* embryos were established.

MATERIALS AND METHOD

Collection of *A. heterophyllus* leaves and stem-bark

A. heterophyllus leaves and stem-bark were collected from Science City of Munoz, Nueva Ecija, Philippines. The collected samples were air-dried in room temperature and shaded condition. Ten grams of each dried *A. heterophyllus* part was cut into small pieces and pulverized using a blender.

Preparation of lyophilized water extract

The water extracts of the two plant parts were prepared following the protocol of Eguchi et al. **[6]**. A 300 mL of water was added to 10 grams of each pulverized plant part and the mixtures were subjected into double boiler water bath for two hours at 80-90^{o}C. Then, hot water extracts were filtered using a Whatman filter paper no. 2. The filtrate was subjected to freeze drying for 24 hours to remove the water from the plant extract. Extracts were diluted using embryo water to obtain the different concentrations (5%, 1%, 0.5%, 0.1%, 0.05%, and 0.01%) as treatments. Ten ml of each treatment and embryo water as control were prepared.

***D. rerio* culture, spawning and fertilization**

The 15 female and 30 male mature zebrafish were kept on a glass aquarium filled with tap water and with oxygen saturation. The mating condition was set at room temperature which is 26±1^{0}C at 12 hours day/light. The zebrafish were incubated in dark condition by wrapping the aquarium with black plastic bag for 12 hours to induce spawning and then, incubated to light condition for another 12 hours to allow fertilization within 30 minutes **[7]**. Twelve hours post fertilized embryos were siphoned out of the aquarium using a hose and placed in a petri plate. Staging of embryo was performed according to Kimmel **[8]**. Embryos were rinsed three times and observed under the compound microscope to examine uniformity and normal condition. Unfertilized eggs and coagulated embryos were discarded.

***D. rerio* embryo-toxic and teratogenic assay**

The protocol on the toxicity and teratogenicity using zebrafish embryos established by Dulay et al. **[9]** was followed. Four embryos at segmentation phase were transferred into each well containing the different treatments. The plate was incubated at 26°C ± 1°C. Teratogenic activity was examined using a dissecting microscope after 12, 24, 36, and 48h of incubation. Morphological endpoint evaluation of zebra fish was based on the parameters established by Nagel [7]: 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. Percentage hatchability, heartbeat rate, and mortality were determined. A test was classified as valid, if 100% of the embryos in the control (embryo water) show normal conditions. Data were analyzed using Analysis of Variance (ANOVA) and Duncan Multiple

Range Test (DMRT) was used to compare the means at 5% level of significance.

RESULTS AND DISCUSSION

Embryo-toxic effect of *A. heterophyllus*

Lethal effect of lyophilized water extracts of *A. heterophyllus* leaves and stem-bark in zebrafish embryos was recorded. Mortality was defined as coagulated and no visual of heartbeat. The mean percentage of mortality of embryos after 12, 24, 36 and 48 hrs of exposure at different extract concentrations of *A. heterophyllus* leaves and bark extract is shown in Table 1. The lethal effects of both extracts were found dependent on dose and time of exposure. At 12 hpta, no mortality was observed at 0.01% concentration of leaves extract which was comparable to the control. On the other hand, 100% mortality was already recorded at 5% of leaves extract. After 24 hrs, the mortality was increased in embryos exposed to 0.05% and higher concentrations of leaves extract. After 36 hours, aside from the 5%, both the 0.5% and 1% leaves extract had 100% mortality which was significantly higher than the other treatments. The 0.01 % extract had the lowest mortality of 8.33% and still comparable with the control. At 48 hpta, all the concentrations of leaves extract (0.05%, 0.10%, 0.50%, 1% & 5%) except for 0.01% recorded 100% mortality.

On the other hand, stem-bark extract registered the highest mortality of 50% at 5% extract which was statistically comparable to the 33.33% mortality of embryos at 1% extract after 12 hours of exposure. An increased in mortality was evident after 24 hours wherein the 5% extract had a 100% mortality. No mortality was recorded at 0.01% extract. At 36 hpta, 0.5% and higher concentrations obtained 100% mortality, followed by 0.10%, 0.05% and 0.01 % with 58.33%, 25% and 8.33% mortality, respectively. After 48 hours, the 0.10 % and higher concentrations of stem-bark extract recorded 100% mortality. However, the 0.01% extract recorded the lowest mortality of 8.33%. Coagulation was the most marked toxic effect of the two extracts. The results of the present study clearly indicate that *A. heterphyllus* leaves extract is more toxic than the stem-bark extract.

The toxic effect of *A. heterophyllus* could be due to the presence of its bioactive components such as alkaloids, tannins, phenolic compounds, flavonoids, and saponins. As cited by Soobrattee et al. **[10]**, *A. heterophyllus* contains phytonutrients: lignans, isoflavones, and saponins. There is a significant correlation between saponins content and cytotoxic effect **[11]**. On the other hand, cytotoxic effect of artcarpin

isolated from wood of *A. heterophyllus* caused a reduction of cell viability in a concentration dependent manner and an alteration of cell and nuclear morphology **[12]**. This toxic effect observed in the present work is the same with other previous studies. For instance, zebrafish embryos at 5% and higher concentrations of *Tinospora cordifolia* extract significantly showed 100% mortality **[13]**. Hence, the toxic effects of medicinal plants could be attributed to their important bioactive components.

Hatching rate of treated zebrafish embryos

Hatching is an indicative of successful developmental processes of the embryos. It takes place between 48-72 h depending on the thickness of the chorion. The percentage hatchability of embryos treated with different concentrations of leaves and stem-bark extract of *A. heterophyllus* after 48-72 hpta is depicted in Table 2. After 48 hours, hatching of embryos the the control were completed. Hatching of embryos at 0.01 % leaves extract was 16.67% after 72 hpta while no hatched at the other higher concentrations of the leaves extract (0.05%, 0.1%, 0.5%, 1% and 5%). In the case of stem-bark extract, the 0.01% concentration had 41.67% hatching rate which was followed by 0.05% extract with 16.67%. No hatched was observed at 0.10% and higher concentrations. Evidently, low concentrations of stem-bark extract revealed higher percentage of hatchability than those embryos treated with various concentrations of leaves extract. The varying concentrations of *A. heterophyllus* lyophilized water extracts affected the hatchability of embryos: as the extract concentration increased the percent hatchability decreased. The delayed hatching of embryos is due to morphological abnormalities which limit the ability to break the chorion and hatch out **[14]**. In like manner, this can also be correlated with the embryotoxicity and teratogenic effects of *A. heterophyllus* wherein results showed high mortality rate and delayed development is evident on both extracts. Therefore, hatchability was affected by these factors.

Heartbeat rate of treated embryos

Heartbeat rate is another important parameter in determining physiological effects of certain substances being tested. Heartbeat rate was monitored at the pharyngula stage of embryo when the tail is distinctly pigmented. The mean heartbeat rates of the embryos at different concentrations of leaves and stem-bark lyophilized water extracts are presented in Table 3. The embryos exposed to 0.01% leaves extract showed no significant difference with the control having a mean of 127.67 beats per minute. However, no

heartbeat was recorded in embryos treated with 0.05% and higher concentrations of leaves extract since 100% mortality was observed in these treatments. On the other hand, embryos treated with 0.01 % stem-bark extract had 109.33 beats per minute which was significantly lower than that of the control. This was followed by 0.05 % stem-bark extract with 95.67 beats per minute. No heartbeat was noted in embryos at 0.10% extract. Thus, the absence of visual heartbeat on embryos incubated to 0.05% and higher concentrations of leaves extract and 0.10% and higher concentrations stem-bark extract conform with the toxic effect of both extracts. Indeed, *A. heterophyllus* lyophilized water extracts have cardiotoxic activity.

Table 1. Mortality of embryos treated with the different concentrations of lyophilized water extracts of *A. heterophyllus* leaves and stem-bark after 12, 24, 36 and 48 of exposure

EXTRACT	CONCENTRATION (%)	MORTALITY (%)			
		12 hpta	24 hpta	36 hpta	48 hpta
Leaves	0.00	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	0.01	0.00^{a}	0.00^{a}	8.333^{a}	8.33^{a}
	0.05	25.00^{b}	33.33^{b}	66.67^{b}	100.00^{b}
	0.10	33.33^{b}	33.33^{b}	58.33^{b}	100.00^{b}
	0.50	13.33^{b}	33.33^{b}	100.00^{c}	100.00^{b}
	1.00	41.67^{c}	75.0^{c}	100.00^{c}	100.00^{b}
	5.00	100.00^{d}	100.00^{d}	100.00^{c}	100.00^{b}
Stem-bark	0.00	0.00^{a}	00.00^{a}	00.00^{a}	0.00^{a}
	0.01	0.00^{a}	0.00^{a}	8.33^{ab}	8.33^{a}
	0.05	8.33^{ab}	8.33^{a}	25.00^{b}	25.00^{b}
	0.10	16.67^{ab}	33.33^{b}	58.33^{c}	100.00^{c}
	0.50	25.00^{ab}	33.33^{b}	100.00^{d}	100.00^{c}
	1.00	33.33^{bc}	75.00^{c}	100.00^{d}	100.00^{c}
	5.00	50.00^{c}	100.00^{d}	100.00^{d}	100.00^{c}

Values are expressed as mean of three replicates. In each column, means with the same letter superscript are not significantly different at P<0.05 using DMRT

Table 2. Hatchability of *D. rerio* treated with the different concentrations of lyophilized water extract of *A. heterophyllus* leaves and stem-bark extract after 72 hpta

Extract Concentration (%)	Hatchability (%)	
	Leaves	Stem-bark
0.00	100.00^{a}	100.00^{a}
0.01	16.67^{b}	41.67^{b}
0.05	0.00^{b}	16.67^{c}
0.10	0.00^{b}	0.00^{c}
0.50	00.00^{b}	0.00^{c}
1.00	0.00^{b}	0.00^{c}
5.00	0.00^{b}	0.00^{c}

Values are expressed as mean of three replicates. In each column, means with the same letter superscript are not significantly different at P<0.05 using DMRT.

Table 3. Heartbeat rate of embryos treated with the different concentrations of lyophilized water extracts of *A. heterophyllus* leaves and stem-bark		
Extract Concentration (%)	**Heartbeat Rate (per min)**	
	Leaves	**Stem-bark**
0.00	**135.67[a]**	**136.00[a]**
0.01	**127.67[a]**	**109.33[b]**
0.05	**NH**	**95.67[c]**
0.10	**NH**	**NH**
0.50	**NH**	**NH**
1.00	**NH**	**NH**
5.00	**NH**	**NH**
Values are expressed as mean of three replicates. In each column, means with the same letter superscript are not significantly different at P<0.05 using DMRT. NH means no heartbeat		

Teratogenic effect of *A. heterophyllus* extracts

A variety of abnormalities can be perceived in zebrafish embryos as affected by different concentrations of *A. heterophyllus* leaves and stem-bark extracts after 12, 24, 36 and 48 hpta. The morphological endpoints of embryos exposed to leaves and stem-bark extracts of *A. heterophyllus* are shown in Table 4 and Figure 1. Embryos treated with *A. heteropyhllus* leaves extract showed delayed development at 0.5% to 1% concentrations. Growth retardation was evident even at the lowest concentration of 0.01%. Moreover, several morphological abnormalities were observed including head malformation, tail malformations (bent tail, unformed tail & hook tail) that caused very limited movement. These abnormalities were likewise observed in embryos treated with stem-bark extract. In stem-bark treated embryos, delayed growth was evident to those embryos exposed to 0.1% and higher concentrations. Head malformation was also observed at 1% extract. In addition, deformities of tail were obvious at 0.01% to 0.05% and 1% of the extract. Growth retardation and tail malformation were the most marked teratogenic effects of both leaves and stem-bark lyophilized water extracts of *A. heterophyllus*. The observation suggests that the different abnormalities are growth-delay related malformations which characterized the morphological endpoints of zebrafish embryos exposed to the different teratogens **[15]**. Weigt et al. **[16]** reported that tail malformation was a fingerprint morphological endpoint of warfarin, an anticoagulant coumarin derivative. This might also be attributed to the inhibition or disturbance of essential substances for growth and developmental processes of embryos **[14]**. Accordingly, growth-delay related malformations were also observed in the toxicity tests performed with two human proteratogens, the ethanol and cyclophosphamide **[17]**. The results in related with the findings conducted by De

Castro et al. **[18]** that the activity of *C. papaya* is equivalent to the activity of these two proteratogens. Likewise, teratogenic compound are also present in other species of plants such as *R. vomitoria*, *M. tinoiflora*, *Anona squamousa*, *Sarcandra glabra* and *Hibiscus rosa-sinensis* **[19]**.

Table 4. Teratogenic effects of the different concentrations of lyophilized water extracts of *A. heterophyllus* leaves and stem-bark in *D. rerio* embryos.

Extract	Concentration (%)	Teratogenic effects					
		HM	TM	GR	SC	LM	ST
Leaves	0.00	-	-	-	-	-	-
	0.01	+	+	-	-	-	-
	0.05	-	+	+	-	+	-
	0.10	-	+	+	-	+	-
	0.50	-	-	+	-	-	-
	1.00	-	-	-	-	-	-
	5.00	-	-	-	-	-	-
Stem-bark	0.00	-	-	-	-	-	-
	0.01		+	-	-	-	-
	0.05	-	+	-	-	-	-
	0.10	-	-	+	-	+	-
	0.50	-	-	+	-	-	-
	1.00	+	+	+	-	-	-
	5.00	-	-	+	-	-	-

+ teratogenic effect is present, - no teratogenic effect, HM head malformation, TM tail malformation, GR growth retardation, SC scoliosis, LM limited movement, ST stunted tail.

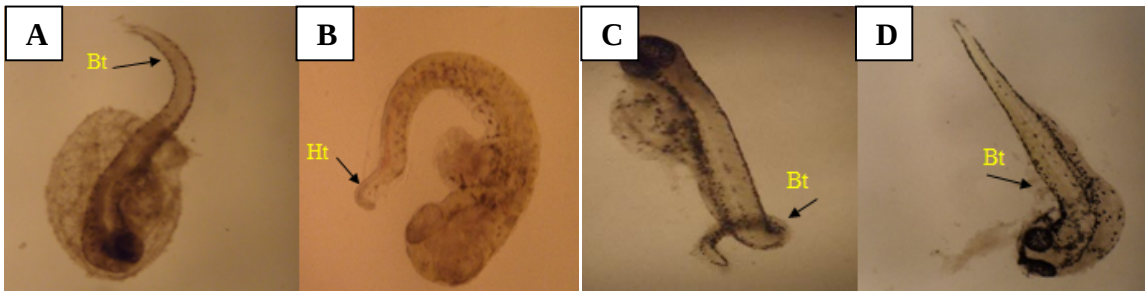

Figure 1. Teratogenic effects of the different concentrations of the *A. heterophyllus* leaves water extract: (A) 48-hpta larva with bent tail malformation from 0.01% of extract, (B) 72 hpta larva with hook tail, and *A. heterophyllus* stem-bark extract: (C) 72-hpta larva with bent tail malformation from 0.05% of extract, (D) 72 hpta larva with bent tail from 0.01%.

CONCLUSION

The lyophilized water extracts of *A. heterophyllus* leaves and stem-bark are toxic and exhibit teratogenic activities against zebrafish embryos. The 0.05% and higher concentrations of leaves extract and 0.10% and higher concentrations of stem-bark extract are both toxic to embryos. However, leaves extract is more toxic than the stem-bark extract. Hatchability and heartbeat rate

of zebrafish embryos were significantly affected on concentration-dependent manner. Growth retardation and tail malformation were the most marked teratogenic effects of *A. heterophyllus* extracts. Thus, this medicinal plant can be valuable sources of teratogens which are in turn could be potential anticancer agents.

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