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Der Pharmacia Lettre, 2016, 8 (7):163-168
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Moringa oleifera (Malunggay) Water Extracts Exhibit Embryo-toxic and Teratogenic Activity in Zebrafish (*Danio rerio*) Embryo Model

Camille Roxanne S. David[1], Angelica Angeles[1], Robert Cyrill Angoluan[1], John Paul E. Santos[1], Eden S. David[2] and Rich Milton R. Dulay[2]

[1]*University Science High School, Central Luzon State University, Science City of Munoz, Nueva Ecija 3120 Philippines*
[2]*Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija 3120 Philippines*

ABSTRACT

This paper demonstrated the teratogenicity and embryotoxicity of Moringa oleifera leaves and bark hot water extracts in Danio rerio embryos. Hatchability, heartbeat rate, morphological abnormalities and mortality rate of embryos exposed to M. oleifera extracts. M. oleifera leaves and bark hot water extracts showed teratogenic properties as indicated by no and low percentage hatchability, absence or low heartbeat rate, growth retardation and morphological abnormalities of the embryos including yolk deformity and stunted tail. Leaves and bark hot water extracts at 6000 ppm and 3000 ppm concentrations are highly toxic to D. rerio embryos as early as after 12 and 24 hours of exposure. However, lower concentrations of hot water extract showed high embryo-toxicity at 48 hours post treatment application. Inspite of the healthful benefits that can derive from M. oleifera, it is very interesting to note that extracts of this plant also exhibit embryo-toxic and teratogenic effects in the developing embryos of zebrafish.

Keywords: *Moringa oleifera*, malunggay, teratogen, *Danio rerio*.

INTRODUCTION

Moringa oleifera Lam (Moringaceae) is a highly valued plant, distributed in many countries of the tropics and subtropics. It has an impressive range of medicinal uses with high nutritional value. *M. oleifera* leaves have been reported to be a rich source of β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidants; and thus enhance the shelf-life of fat containing foods due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids [1]. A number of medicinal properties of this plant have been reported. The root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders [2]. In addition, the different parts act as cardiac and circulatory stimulants, possess anti-tumor, anti-pyretic, anti-epileptic, anti-inflammatory, anti-ulcer, anti-spasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia [3].

Despite the enormous medicinal properties of *M. oleifera*, its teratogenic and toxic effects are not yet well established. A teratogenic agent is a chemical, infectious agent, physical condition, or deficiency that can alter fetal morphology or subsequent function or congenital abnormalities. However, most of the teratogens are anticancer drugs, merely because they target vital cellular functions. Many teratogens may suppress cancer cells that reactivate embryonic pathways, while sparing most normal cells. Teratogens that interfere with morphogens (e.g., Hh pathways) may be preferentially toxic to such cancer cells, whereas normal cells lacking such pathways may be less affected [4]. Zebrafish embryo is now being used in the evaluation of the teratogenic effect of certain compounds or substances because of several advantages including high fecundity, very transparent, rapid developmental processes, no pain model and most importantly, it is similar to the human embryonic development.

This study was conducted to evaluate the teratogenic and embryotoxic effects of leaves and bark of *M. oleifera* hot water extracts in *D. rerio* embryos.

MATERIALS AND METHODS

2.1 Source and Extraction of *M. oleifera*:

The leaves and bark of *M. oleifera* were collected from Science City of Munoz, Nueva Ecija. The collected plant parts were air-dried then pulverized using blender. Functional components were obtained through hot water extraction following the procedure of Eguchi et al. [5] with modifications. Each powdered plant part (20 g) was extracted in 600 ml hot water at 80 - 90ºC in a water bath for 2 hrs. The extract separated through filtration using Whatman filter paper no. 2. The filtrate was placed in a flask and refrigerated until needed for evaluation. Ten ml of the different concentrations of extracts (300 ppm, 1500 ppm, 3000 ppm, and 6000 ppm) were prepared by dilution in embryo water [6].

2.2 Maintenance and Spawning of *D. rerio*:

The acclimatized adult *D. rerio* at 10 female and 20 male, confined in a plastic mesh were allowed to spawn and fertilize following the procedure of Dulay et al. [7]. After fertilization, embryos at segmentation phase (12 hour post fertilization) were collected, rinsed, and placed in a watch glass to check the phase uniformity of embryos. Unfertilized egg and coagulated embryos were discarded.

2.3 *D. rerio* embryo-toxicity and teratogenicity assay:

Four embryos at segmentation phase were transferred into each well of ELISA plate containing the different treatments. These were incubated at 26°C ± 1°C. Teratogenic activity was examined using a compound microscope after 12, 24, 36, and 48 hours of incubation. Mortality, hatchability, heartbeat rate, malformations were recorded. Mortality was defined as coagulated embryos and as no visual heartbeat. Morphological endpoint evaluation of zebrafish was based on the parameters established by Nagel [8]: 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. The validity of the test was determined. Data were analyzed using analysis of variance (ANOVA) and compared using Duncan's Multiple Range Test (DMRT) at 5% level of significance. The Sirichai Statistics 6.07 program was used for analysis.

RESULTS AND DISCUSSION

The teratogenicity and embryo-toxicity of *M. oleifera* hot water extracts in *D. rerio* embryos were evaluated. The percentage hatchability, heartbeat rate, mortality rate and morphological abnormalities of the developing embryos were determined. The spawning and fertilization were found to be approximately 98% successful.

3.1 Hatchability of *D. rerio*

The hatchability of the embryos was evaluated after 48 hours post treatment application (hpta). As shown in Table 1, 300 ppm of bark extract had the highest percentage hatchability among the *M. oleifera* extracts with 25.00% but significantly lower compared to the control (embryo water). This was followed by 300 ppm of leaves extract and 1500 ppm of bark extract, both with 8.33% hatchability which is comparable with higher concentrations having 0% hatchability. Results revealed that *M. oleifera* hot water extracts of both leaves and bark exhibits teratogenic effects as showed by the inhibited or decreased hatchability rate. This effect could possibly explained by the active compounds present in *M. oleifera*. Extracts, secondary metabolites, essential oils and lectins (carbohydrate-binding and hemagglutinating proteins) of *M. oleifera* have been shown to exert deleterious effect in *Aedes aegypti*, delaying

development, impairing growth and digestive enzyme activities, reducing egg hatching and larval survival, as well as deterring feeding and oviposition activities [9]. Likewise, Santos et al. [10] reported that hatching was not observed to the stored eggs of *A. aegypti* treated with water-soluble *M. oleifera* lectin at 0.3 g/ml which indicates the embryos within the eggs were killed by lectin.

Table 1. Hatchability and heartbeat rate of *D. rerio* after 48 hours of exposure to varying concentrations of *M. oleifera* leaves and bark extracts

Extract	Concentration (ppm)	Hatchability (%)	Heartbeat rate (/min)
Leaves	6000	0.00^{c}	0.00^{c}
	3000	0.00^{c}	0.00^{c}
	1500	0.00^{c}	0.00^{c}
	300	8.33^{c}	32.33^{c}
Bark	6000	0.00^{c}	0.00^{c}
	3000	0.00^{c}	0.00^{c}
	1500	8.33^{c}	100.00^{b}
	300	25.00^{b}	97.67^{b}
Control	0.00	100.00^{a}	138.00^{a}

Treatment means having the same letter of superscript are not significantly different from each other at 5% level of significance using DMRT.

3.2 Heartbeat Rate of *D. rerio*

Heartbeat rate was monitored at the pharyngula stage of every embryo when the tail was distinctly pigmented to ensure that the heartbeat obtained was effect of the different extract concentrations and not by the delayed development. In zebrafish, the normal embryonic heart rate is much closer to that of humans, at 120–180 beats per minute [11]. The two lowest concentrations of bark extracts which are the 300 ppm and 1500 ppm recorded the highest heartbeat rates of 100 and 97.67, respectively. Statistical analysis revealed that these values are not significantly different from each other (Table 1). Nonetheless, their heartbeat rates were still significantly lower than the control embryos having 138 per minutes. Moreover, the heartbeat rate of embryos exposed at 300 ppm and 1500 ppm of bark extract were also lower than the control embryos which recorded within the normal rate. On the other hand, 300 ppm of leaves extract had a mean of 32.33 per minute, which is comparable with other treatments with 0 heartbeat rate. Similar with percentage hatchability, the heartbeat rate was observed only in lower concentrations such as 300 ppm and 1500 ppm. It only means that the heartbeat rate is also concentration dependent. The absence or decreased hatchability would indicate growth retardation or slow development hence, the normal development of the heart is also hampered thus, affecting the heartbeat rate. This could also be explained by high mortality rate or even 100% mortality of the embryos exposed in higher concentrations of *M. oleifera* hot water extracts (Table 2).

3.3 Morphological characteristics and teratogenic effects

Normal development was observed in control embryos while growth retardation or slow development was evident in all extract concentrations both in leaves and bark (Figure 1). At 12 hpta, coagulated embryos were already observed in 1500, 3000 and 6000 ppm of leaves extract. On the other hand, coagulation was seen in 3000 and 6000 ppm of bark extract. After 24 hours, the normal development was also observed in 1500 and 300 ppm of bark extract as well as 300 ppm leaves extract. However, growth retardation or slow development became evident even in 1500 ppm bark and 300 ppm leaves extracts after 36 hpta. The remaining concentration with normal development during this time was the 300 ppm bark. However, after 48 hpta, slow development and delayed hatching process happened in most embryos in 300 ppm of bark extract.

Among the observed survived embryos exposed in hot water extracts of *M. oleifera*, the morphological abnormalities observed were deformed yolk and constricted tail which were common in 1500 ppm and 300 ppm leaves extract at 24, 36 and 48 hpta (Figure 2). On the other hand, the most common teratogenic effect of this plant was growth retardation while coagulation was its lethal or toxic effect. In the study of Nath et al. [12], aqueous or 90% ethanol extracts of the plants of interest were studied in rats orally dosed for 10 days after insemination with special reference to effects on fetal development. Results revealed that leaf extracts of *Moringa oleifera* and *Adhatoda vasica* were 100% abortive at doses equivalent to 175 mg/kg of starting dry material. This shows the teratologic potential of the plants at the doses tested.

As a whole, the low or absence of percentage hatchability, without or not within the normal range of heartbeat, growth retardation and morphological abnormalities of the developing embryos indicated strong teratogenic effects of *M. oleifera*. These mean that precautionary measures must be done in consuming large amount of this plant by the

pregnant women. Nonetheless, such teratogenic activity could denote the strong anti-cancer potential of *M. oleifera* leaf and bark. Teratogenic effect signified antiproliferative activity, and thus potential antineoplastic activity. Anti-neoplastic or chemotherapeutic agents are highly teratogenic as these agents inhibit rapidly dividing cells [13].

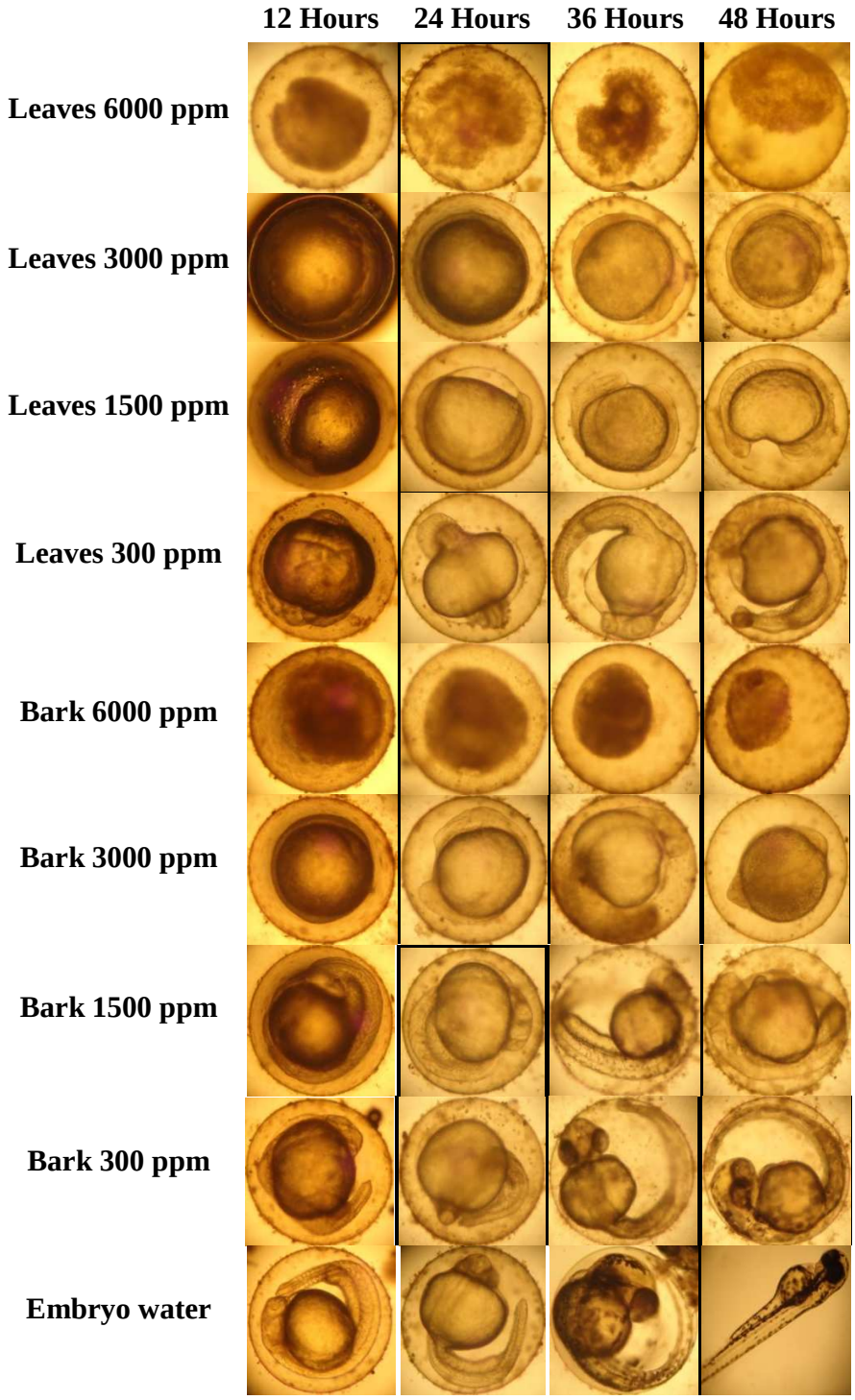

Figure 1. Effects of *M. oleifera* extracts on zebrafish embryos. Growth retardation or slow development was evident in all concentrations of extracts. Hatching was completed after 48 hours of treatment exposure in embryo medium

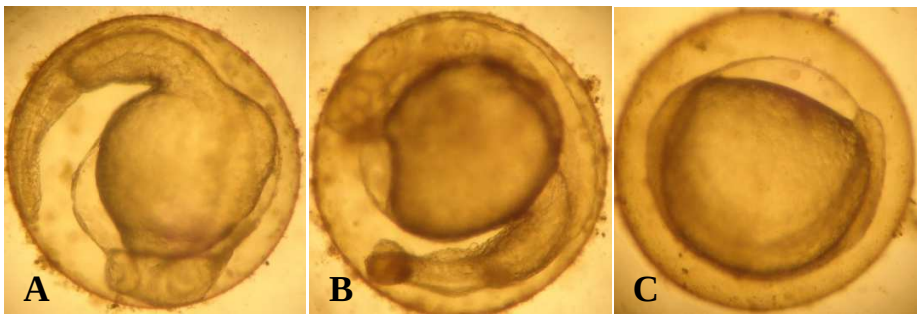

Figure 1. Morphological abnormalities of *D. rerio* embryos exposed in *M. oleifera* hot water extracts. A & C Yolk deformities in 300 and 1500 ppm of leaves extract after 24, 36 and 48 hpta. B Stunted tail in 1% leaf extract after 48 hpta

3.4 Embryo-toxic effect of *M. oleifera* extracts

The embryotoxicity of *M. oleifera* hot water extracts was measured by determining the mortality rate. Mortality was defined as coagulation and no visual heartbeat of embryos. Table 3 shows the percent mortality of *D. rerio* embryos at different concentration of *M. oleifera* extracts after 12, 24, 36 and 48 hours of exposure. Both 6000 ppm of leaves and bark extracts recorded 41.67% mortality rate at 12 hpta, which is the highest among the extracts and not significantly different from the 33.33% mortality of 3000 ppm of leaves extract. On the other hand, the 3000 ppm of bark extract had 8.33% mortality which is comparable with the other treatments with 0% mortality rate. At 24 hpta, the 6000 ppm of leaves extract registered the highest mortality of 83.33% which was followed by 3000 ppm of leaves extract and 6000 ppm of bark extract with 66.67% and 58.33%, respectively. These three treatment concentrations were not significantly different from each other. The 1500 ppm of leaves extract and 3000 ppm of bark extract have 25% which are not significantly different from the rest of the treatment including the embryo water. A 100 % mortality was already observed in 6000 ppm of leaves extract at 36 hpta, but still comparable with 3000 ppm of leaves extract and 6000 ppm of bark extract with both 83.33% mortality. The lowest mortality rate was observed in 300 ppm of both leaves and bark extract with 16.67% which is comparable with the control. At 48 hpta, four treatments recorded 100 % mortality including 6000, 3000, and 1500 ppm of leaves extract as well as 6000 ppm of bark extract. This was followed by 300 ppm of leaves extract and 3000 ppm of bark extract with both 75% mortality, and 1500 ppm of bark extract with 66.67%, which are not significantly different from the 100% mortality. These clearly indicate that the high concentrations of leaves and bark extracts are toxic to *D. rerio* embryos as early as 12 hpta. All of the treatments had high mortality rate at 48 hpta including those at lower concentrations (300 and 1500 ppm).

Results obtained in the present work are congruent with the study of Rocha-Filho et al. [14], who reported the effect of an aqueous extract from *Moringa oleifera* Lam. flowers in *Biomphalaria glabrata* embryos and adults and in *Schistosoma mansoni* adult worms. The extract contains tannins, saponins, flavones, flavonols, xanthones, and trypsin inhibitor activity. The toxicity of the extract on *Artemia salina* larvae was also investigated to determine the safety of its use for schistosomiasis control. After incubation for 24 h, the flower extract significantly ($p < 0.05$) delayed the development of *B. glabrata* embryos and promoted mortality of adult snails (LC50: 2.37 ± 0.5 mg/ml). Furthermore, treatment with the extract disrupted the development of embryos generated by snails, with most of them remaining in the blastula stage while control embryos were already in the gastrula stage. In like manner, in the study of Zade and Dabhadkar [15], the oral administration of *M. oleifera* stem bark extract (aqueous, alcohol, benzene and diethyl ether) at the doses of 25, 50 and 100 mg/kg body weight resulted in 10 % to 100 % abortion.

Table 2. Mortality of *D. rerio* embryos after 12, 24, 36 and 48 hours of exposure to varying concentrations of *M. oleifera* leaves and bark extracts

Extract	Concentration (ppm)	Mortality (%)			
		12 hours	24 hours	36 hours	48 hours
Leaves	6000	41.67^{a}	83.33^{a}	100.00^{a}	100.00^{a}
	3000	33.33^{a}	66.67^{a}	83.33^{ab}	100.00^{a}
	1500	8.33^{b}	25.00^{b}	33.33^{cd}	100.00^{a}
	300	0.00^{b}	8.33^{b}	16.67^{cd}	75.00^{ab}
Bark	6000	41.07^{a}	58.33^{a}	83.33^{ab}	100.00^{a}
	3000	8.33^{b}	25.00^{b}	50.00^{bc}	75.00^{ab}
	1500	0.00^{b}	8.33^{b}	33.33^{cd}	66.67^{ab}
	300	0.00^{b}	0.00^{b}	16.67^{cd}	58.33^{c}
Control	0.00	0.00^{b}	0.00^{b}	0.00^{d}	0.00^{c}

Treatment means having the same letter of superscript are not significantly different from each other at 5% level of significance using DMRT.

Altogether, all of the *M. oleifera* leaves and bark hot water extracts showed teratogenic properties as indicated in the low or no percentage hatchability, low or absence of heartbeat, growth retardation and morphological abnormalities of the embryos. The common abnormalities found are yolk deformity and stunted tail. The high concentration of leaves and bark hot water extracts such as 6000 and 3000 ppm are highly toxic to *D. rerio* embryos as early as 12 hpta and 24 hpta. All of the treatments including the low concentrations of hot water extract showed high embryo-toxicity at 48 hpta. These important findings strongly dictate that *M. oleifera* could be a valuable resource of bioactive compounds that can be used in the chemotherapy against aggressive cancer and tumor cells.

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