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Embryo-toxic and teratogenic effects of *Tinospora cordifolia* leaves and bark extracts in Zebrafish (*Danio rerio*) embryos

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ABSTRACT

In the present work, the toxic and teratogenic activities of the Tinospora cordifolia leaves and bark extracts were examined in zebrafish embryo model. Embryos at segmentation phase were exposed to the varying concentrations of extracts and the mortality, hatchability, heartbeat rate, and teratogenic effects were determined. Results revealed that the embryo-toxic effects of the extracts were found dependent on dose and time of exposure. Among the two extracts, 5% and 10% of leaves extract recorded the highest mortality of 100% while bark extract showed mortality of 11.11% and 33.33% at 5% and 10% concentrations, respectively. Hatching was completed at 48 hours post treatment exposure in control embryos, 0.01% of leaves extract-treated embryos, and 0.1% or lower concentrations of bark extract-treated embryos. Control embryos significantly recorded the highest heartbeat rate of 167.00 per minute while treated embryos showed significant decreased in heartbeat rate in the increasing concentration of both extracts. The different teratogenic effects of T. cordifolia in zebrafish embryo include head and tail malformations, delayed growth, limited movement, scoliosis/flexure, and stunted tail and these are dose- and plant parts-dependent. Despite of the various bioactivities of T. cordifolia, extracts of this plant also exhibit embryo-toxic and teratogenic effects in the developing embryos of zebrafish.

Keywords: *T. cordifolia*, teratogenic, medicinal plants, *D. rerio*, toxicity.

INTRODUCTION

Plants have been utilized as medicinal remedy over centuries and been part of the human healing practices and cultures. In the Philippine, a number of plant species have been considered as herbal medicine being used to treat several diseases. One of these medicinal plants is *Tinospora cordifolia*, commonly known as *makabuhay* (to give life) for Filipinos, is a climbing shrub with creamy white to grey and deeply left spirally stem that contains rosette like lenticels while the leaves are membranous and cordate in shape. This plant is widely used as antibacterial, analgesic, antipyretic and also for the treatment of jaundice, skin diseases, anemia, and among others. The stem is used in dyspepsia, fever and urinary diseases [1]. The root is a powerful emetic and used for visceral obstructions; its water extract is used in leprosy and showed antidiabetic effect. The extracts of stem, leaves, barks and roots show strong antioxidant activities [2]. In Ayurvedic medicine, the extract of *T. cordifolia* is used to treat fever, jaundice, chronic diarrhea, cancer, dysentery, bone fracture, pain, asthuma, skin disease, poisonous insect, snake bite, eye disorders [3]. In a review of published scientific research conducted by Upadhyay et al. [4], the potential medicinal properties *T. cordifolia* include anti-diabetic, antipyretic, antispasmodic, anti-inflammatory, anti-arthritic, antioxidant, anti-allergic, anti-stress, anti-leprotic, antimalarial, hepato-protective, immuno-modulatory and anti-neoplastic activities.

Despite of the profound therapeutic advantages possess by medicinal plants, some of their chemical constituents have been shown to be potentially toxic, mutagenic, carcinogenic and teratogenic [5]. Teratogens are any substances responsible for the formation of anatomical abnormalities or defects of embryos. Teratogenicity assay is evaluated using zebrafish (*Danio rerio*) embryos. Zebrafish embryo as animal model is a very reliable and important tool due to its very rapid developmental processes, high fecundity, transparency, easy maintenance in the laboratory, accessible to experimental manipulation and similarity to the embryonic development of higher forms of vertebrates. On the other side, a number of teratogens are considered as anti-cancer [6]. Some reported Philippine plants with teratogenic effects include *Ficus odorata, Baccaurea tetrandra, Carica papaya, Anona squamousa, Sarcandra glabra, Hibiscus rosa-sinensis, Goniothalamus amuyan,* and *Alstonia macrophylla* [7][8][9][10].

Herein, the embryo-toxic and teratogenic effects of *T. cordifolia* in developing embryos of zebrafish were established. The mortality, hatchability, heartbeat rate and different morphological abnormalities of embryos exposed to the varying concentrations of extracts of leaves and bark of *T. cordifolia* were examined.

MATERIALS AND METHODS

2.1 Source and Extraction of Plant Material:

The leaves and bark of *T. cordifolia* were collected from Science City of Munoz, Nueva Ecija. Samples were placed in separate plastic bags, air-dried, and milled. The bioactive chemical attributes of each milled plant sample (10g) were extracted in 300 ml distilled water at 80-90°C in water bath for 2 hours. The extracts were filtered using Whatman filter paper No. 2, Ten ml of the different treatment concentrations of extracts were prepared by dilution in embryo water [11].

2.2 Maintenance and Spawning of *D. rerio*:

The acclimatized adult *D. rerio* at 1:2 ratio of female and male, confined in a plastic mesh were allowed to spawn and fertilize following the procedure of Dulay et al. [12]. 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 Embryo-toxicity and teratogenicity assay:

The assay established by Dulay et al., [12] was followed in this study. Embryos were exposed to the different concentrations (10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%) of each extract, and embryo water served as the control in the 12-well ELISA plate. Four embryos per treatment were assayed and each treatment was replicated three times. The plates were incubated at 26°C ± 1°C. Toxic and teratogenic effects were examined using a compound microscope every after 12 hours of treatment exposure. Mortality, hatchability, heartbeat rate, malformations were recorded. Death was defined as coagulated embryos and as no visual heartbeat. Morphological endpoint evaluation of zebrafish was based on the parameters established by Nagel [13]: 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

Zebrafish embryo, a new vertebrate model, was used as experimental animal in the toxicity and teratogenicity assay. In this study, the spawning and fertilization were successful with approximately 95% rate of fertilization. The successful embryonic development was observed in three distinct periods, namely segmentation phase (12 hours post fertilization), pharyngula period (24-36 hours post fertilization) and hatching period (48-72 hours post fertilization). The effects of the different concentrations of *T. cordifolia* leaves and bark extracts on the developmental processes of the embryos were evaluated.

3.1 Embryo-toxic Effect of *T. cordifolia*:

Mortality was defined as coagulation and no visual heartbeat of embryos. The percentage mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure in varying concentrations of *T. cordifolia* leaves and bark extracts are shown in Table 1. The embryo-toxic effects of the extracts were found dependent on dose and time of exposure. At 12 hpta, no mortality was observed in embryos exposed at 0.1% or lower concentrations of leaves

extract and at 1% or lower concentrations of bark extract. Among the two extracts, 5% and 10% of the leaves extract recorded the highest mortality of 100% while bark extract showed mortality of 11.11% and 33.33% at 5% and 10% concentrations, respectively. After further exposure for 24 hours, increased mortality was observed in embryos exposed to 0.5% and 1% of leaves extract while remain the same in those exposed to bark extract. In both extracts, the mortality did not increase after 36 and 48 hours of exposure. However, no mortality was noted in embryos exposed at lower concentrations up to 48 hours. The results of the present study indicate that *T. cordifolia* leaves extract is more toxic than the bark extract.

The toxic effect of *T. cordifolia* extract could be due to its myriad of biologically active compounds. Its aqueous extract contain alkaloids, di-terpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds or polysaccharides which have been reported for their cytotoxic actions in rat model [14]. This strong cytotoxic activity is significantly considered in the evaluation of its anti-cancer, anti-tumor and apoptotic properties. Hexane fraction of *T. cordifolia* could block the G1 phase in mice and cause apoptosis by the formation of apoptotic bodies, nuclear condensation, activation of caspase-3, decreased cell number and ascites volume, increased expression of pro-apoptotic gene, *Bax*, and decreased expression of anti-apoptotic gene, *Bcl-2* [15]. Moreover, extract of this plant could also induce a reduction of papillomas, tumor yield, tumor burden, and tumor weight while increase phase II detoxifying enzymes in skin carcinoma animal models [16]. On the other hand, *T. cordifolia* extract could exhibit anti-toxic effects. For instance, stem and leaves extracts have shown hepato-protective effect in Swiss albino male mice against lead nitrate induced toxicity [17]. In addition, it also showed protection against aflatoxin-induced nephrotoxicity and the active compounds involved in this property includes choline, tinosporin, isocolumbin, palmatine, tetrahydropalmatine, and magnoflorine [18].

Table 1. Mortality of *D. rerio* embryos after 12, 24, 36 and 48 hours of exposure to varying concentrations of *T. cordifolia* leaves and bark extracts

Extract	Concentration (%)	Mortality (%)			
		12 hours	24 hours	36 hours	48 hours
Leaves	10.00	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	5.00	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	1.00	44.44^{b}	55.56^{b}	55.56^{b}	55.56^{b}
	0.50	11.11^{c}	22.22^{c}	22.22^{c}	22.22^{c}
	0.10	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
	0.05	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
	0.01	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
Bark	10.00	33.33^{b}	33.33^{b}	33.33^{b}	33.33^{b}
	5.00	11.11^{c}	11.11^{c}	11.11^{c}	11.11^{c}
	1.00	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
	0.50	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
	0.10	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
	0.05	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
	0.01	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
Control	0.00	0.00^{c}	0.00^{c}	0.00^{c}	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.

3.2 Hatchability of Zebrafish Embryo:

Hatching is an indicative of the successful developmental processes of the embryos. The percentage hatchability of embryos treated with the different concentrations of *T. cordifolia* extracts at 48 hpta is depicted in Table 2. Hatching was completed at 48 hours post treatment exposure in control embryos, 0.01% of leaves extract-treated embryos, and 0.1% or lower concentrations of bark extract-treated embryos. However, hatching of some embryos at 0.05% to 1% of leaves extract and at 0.5% to 5% of bark extract was also observed. No hatched embryo was recorded at 5% or higher concentrations for leaves extract and at 10% of bark extract. Apparently, hatching of embryos was affected by the varying concentrations of the different extracts: as the extract concentration increased the percent hatchability decreased. This delayed hatching process strongly dictates growth retardation and possibly explained by the morphological abnormalities observed in embryos that limit hatching.

3.3 Heartbeat Rate:

Heartbeat rate is another important parameter in determining the physiological effects of the extract. The heartbeat rate was monitored at the pharyngula stage of embryo, when the distinct pigmentation was observed, and the results are presented in Table 2. Heartbeat rate was significantly affected by the different concentrations of the extracts. Embryos incubated in embryo water significantly recorded the highest heartbeat rate of 167.00 per minute. It can be

noticed that as the extract concentration increased the heartbeat rate decreased. However, no heartbeat rate was noted in embryos treated with 5% and 10% of leaves extract due to the early arrested growth. Similar with the percent hatchability, heartbeat rate was also showed to be concentration-dependent. These results suggest that the *T. cordifolia* extracts could induce a significant decreased in heartbeat rate. The lower heartbeat rate in treated embryos than in normal embryos indicates cadiotoxicity. Cardiac glycoside in plant material is one of the most commonly associated compounds with cardiac functions. Among the large number of chemicals that have been isolated from *T. cordifolia*, one of the active components is cardiac glycoside [4]. In some cardiac glycoside-containing plants like *Nerium oleander*, which contains two cardenolides (oleandroside and neriin) showed potent cardiotoxic effect and the thevetin isolated from *Thevetia peruviana* and *Thevetia thevetioides* is a potent toxic cardenolide that is widespread throughout the plant, but particularly concentrated in the fruits [19]. Moreover, the cardiotoxic bufadienolides from *Drimia sanguinea* and *Bowiea volubilis* plants have also been implicated in human poisoning [20].

Table 2. Hatchability and heartbeat rate of *D. rerio* after 48 hours of exposure to varying concentrations of *T. cordifolia* leaves and bark extracts

Extract	Concentration (%)	Hatchability (%)	Heartbeat rate (/min)
Leaves	10.00	0.00^{d}	N/A
	5.00	0.00^{d}	N/A
	1.00	44.44^{c}	65.33^{d}
	0.50	55.56^{bc}	93.00^{c}
	0.10	55.56^{bc}	106.67^{c}
	0.05	77.78^{a}	121.33^{bc}
	0.01	100.00^{a}	130.33^{b}
Bark	10.00	0.00^{c}	80.00^{b}
	5.00	44.44^{b}	115.00^{c}
	1.00	77.78^{a}	133.67^{b}
	0.50	88.89^{a}	135.67^{b}
	0.10	100.00^{a}	137.33^{b}
	0.05	100.00^{a}	140.67^{b}
	0.01	100.00^{a}	144.67^{b}
Control	0.00	100.00^{a}	167.00^{a}

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

3.4 Teratogenic activity of *T. cordifolia*:

Examination of embryos as affected by the different concentrations of the *T. cordifolia* extracts was done after 12, 24, 36, and 48 hours of exposure. The teratogenic effects of *T. cordifolia* extracts are presented in Table 3. Treated embryos after 12 hours of exposure significantly showed delayed growth, limited movement, slightly detached tail from the yolk, and underdeveloped eyes were observed in delayed embryos. However, spontaneous movement and very active embryos due to the complete detached tail were observed at 0.5% or lower concentrations of both extract which apparently comparable to the control embryos.

Table 3. Teratogenic effects of varying concentrations of *T. cordifolia* leaves and bark extracts in *D. rerio* embryos

Extract	Concentration (%)	Teratogenic Effect					
		Head malformation	Tail malformation	Delayed Growth	Scoliosis/Flexure	Limited movement	Stunted tail
Leaves	10.00	-	-	-	-	-	-
	5.00	-	-	-	-	-	-
	1.00	●	●	●	●	●	●
	0.50	●	●	●	-	●	●
	0.10	●	●	●	-	●	-
	0.05	-	●	●	-	●	-
	0.01	-	-	●	-	-	-
Bark	10.00	-	-	-	-	-	-
	5.00	-	-	●	●	●	-
	1.00	-	-	●	●	●	●
	0.50	●	●	●	-	-	-
	0.10	●	●	●	-	-	-
	0.05	●	●	●	-	-	-
	0.01	-	-	●	-	-	-
Control	0.00	-	-	-	-	-	-

● *indicate the presence of teratogenic effect; - indicate that teratogenic effect was not observed.*

At 24 hpta embryos treated with the different concentrations of the two extracts further conform the first observation regarding delayed growth of embryos. Control embryos significantly established very distinct pigmentation on both head and tail region, pigmented retina of the eyes, narrowing of the yolk and strong circulation characterized by visible heartbeat. In contrast, embryos treated with 0.5% for leaves extract and 1% for bark extract showed complete detached tail with some degree of motility and eyes become well developed but no pigmentation was observed in head and tail regions. However, no further development (growth arrested) was observed at 5% and 10% concentrations of leaves.

Obvious delayed growth was also noted at 48 hours post treatment exposure and found to be the most mark teratogenic effect of both extracts. This was followed by the malformations in head and tail. It can be noticed that head and tail malformations were more serious in embryos at 0.05% to 1% of leaves extract and at 0.05% to 0.5% of bark extract. Scoliosis or flexure and stunted tail were also observed at 0.5% to 1% for leaves and 1% to 5% for bark extracts. Based on the results of evaluation, it is safe to believe that the teratogenic effects of *T. cordifolia* are dependent on the extract concentration and plant parts. The same is true with the teratogenic properties of *Rauwolfia vomitoria* in mice, which the leaves extract is less teratogenic than its root extract [21].

Altogether, it can be concluded that *T. cordifolia* has toxic and teratogenic activities against zebrafish embryo model. This activity is dependent on the extract concentration and plant parts. It was revealed that leaves extract is more potent than its bark extract. These important findings strongly dictate that *T. cordifolia* 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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