RESEARCH PAPER

Toxic and teratogenic effects of mycelia and fruiting body extracts of *Lentinus strigosus* (BIL 1324) in zebra fish (*Danio rerio*) embryo

Rich Milton R. Dulay[*], Danielle G. Pamiloza, Rolen L. Ramirez

Bioassay Laboratory, Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, 3120 Philippines

Abstract

We reported in the previous works the successful rescue, domestication, optimization, enriched cultivation, compositions, and functional activities of *Lentinus strigosus*, a naturally occurring, wood-rotting edible mushroom. Herein, the embryo-toxic and teratogenic effects of water extracts of mycelia and fruiting body of this mushroom in zebra fish (*Danio rerio*) embryos were investigated. The toxic effects of both extracts were dependent on concentration and time of exposure. Embryos at 1000 µg/ml (after 24 hrs) and 100 µg/ml (after 48 hrs) and lower concentrations of mycelial extract survived while 100% mortality was recorded to those exposed at 10000 µg/ml (after 24 hrs) and 1000 µg/ml (after 48 hrs) concentration of fruiting body extract. Both extracts significantly reduced the hatchability and heartbeat rate of zebra fish. Delayed development was the sole teratogenic effect and this was obvious to embryos exposed at 100 µg/ml of both mycelia and fruiting body extracts. Therefore, *L. strigosus* holds promising potential as source of teratogens, which are also known as anticancer agents.

* Corresponding Author: Rich Milton R. Dulay ✉ richmiltondulay@clsu.edu.ph

Introduction

Lentinus strigosus is a hairy sawgill mushroom that is commonly found growing in the wild. Recently, the growth conditions for both mycelia and fruiting bodies were successfully optimized (Dulay *et al.*, 2017; Dulay and Gracia, 2017).

This mushroom is considered as a natural source of functional food. Both wild and domesticated fruiting bodies of *L. strigosus* contain carbohydrates, protein, dietary fiber, reducing sugar, soluble polysaccharide, crude fat, minerals (potassium, phosphorous, magnesium, calcium, iron, and zinc), and mycochemicals such as saponins alkaloids, flavonoids, anthraquinones, anthrones, phenols, steroids, coumarins, fatty acids, phenolic and exhibit radical scavenging activity and antibacterial properties (Dulay and Pamiloza, 2018).

Moreover, it is also reported that hypnophilin and panepoxydone, terpenoids isolated from *L. strigosus* have significant inhibitory activity against *Trypanosoma cruzi* trypanothione reductase that causes Chagas disease and leishmaniasis (Souza-Fagundes *et al.*, 2010). Despite of the promising nutraceutical potential, it is necessary to understand the embryo-toxic and teratogenic effects, if any, of *L. strigosus*.

Teratogenicity can be a desirable property because teratogens can be used as anticancer drugs, and many anticancer drugs are teratogenic (Blagosklonny, 2005) and zebra fish (*Danio rerio*) embryo is used to determine this activity. Zebra fish embryos as model for testing teratogens combine the advantages of embryo culture systems with cell culture in which the essential developmental processes of embryos can be studied (Busquet *et al.*, 2008). Previous works reported that mushroom extracts exhibit toxic and teratogenic activities in zebra fish embryos. Some of these mushrooms include *Ganoderma lucidum*, *Lentinus tigrinus*, *Lentinus sajor-caju*, *Pleurotus ostreatus*, *Panaeolus antillarium*, *Panaeolus cyanescens*, and *Termitomyces clypeatus* (Dulay *et al.*, 2012; Dulay *et al.*, 2014; De Castro and Dulay,

2015; Bustillos *et al.*, 2016; De Castro and Dulay, 2016).

Since previous works have focused on the health benefits and functional activities of *L. strigosus*, this present study evaluated the toxic and teratogenic effects of mycelia and fruiting body extracts of *L. strigosus* in the developing embryos of zebra fish as model organism.

Materials and methods

Mushroom source and culture

The pure culture of *L. strigosus* was obtained from the Bioassay Laboratory, Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines. The mycelia were sub-cultured onto potato dextrose agar plates and were incubated for 5 days to allow mycelial growth. Mycelial discs were prepared using a flame sterile 10 mm-diameter cork borer and were aseptically inoculated into glass bottles containing potato dextrose broth. Mycelial cultures were incubated for 15 days at room temperature. The mycelia were harvested and air-dried. On the other hand, the mass production of fruiting bodies of *L. strigosus* was the same as our recent report (Dulay and Garcia, 2017).

Extraction of bioactive components

The bioactive components of mycelia and fruiting bodies were extracted using hot water. The air-dried mycelia were pulverized using a blender and 20 g of the powdered mushroom was placed in 1L-capacity flask containing 600 ml distilled water. This was subjected in a water bath at 80-90°C for 2hrs. The extract was filtered using a Whatman filter paper No.2 and refrigerated until needed for the assay.

Maintenance and spawning of zebra fish

A non-treated stock of tap water in a glass aquarium with oxygen saturation was used for the spawning of zebra fish. Eight mature females and 16 males were maintained. They were fed two times a day and excess food was removed to maintain high water quality. To induce spawning, zebra fish were localized in a plastic

mesh and subjected in dark condition by covering the aquarium with black plastic for 12 hours. After incubation in the dark, eggs were exposed in the light condition for another 12 hours. Fertilization occurs after 30 min from the light is turned on. Embryos at 12 hour post fertilization (hpf) were siphoned out of the aquarium using a hose into a beaker. They were rinsed three times, placed in watch glass with embryo water medium and observed under the compound microscope to examine their uniformity and normal condition. Coagulated embryos and unferlitized eggs were not considered and discarded.

Teratogenicity test
The different concentrations of the extracts were prepared by diluting it to embryo water (Thomas, 2000). Three ml of each concentration was dispensed into each vial. Triplicate test was done. Four embryos were exposed to each replicate vial and placed at room temperature. The percentage mortality was recorded after 24 and 48 hours of exposure. The hatchability and heartbeat rate were also determined. Morphological endpoint evaluation of the zebra fish embryo was based on the parameters established by Nagel (2002) where: lethal (coagulation, tail not detached, no somites, and no heart beat); teratogenic

(malformation of head, tail and heart, scoliosis, deformity of yolk, and delayed development); and normal.

Statistical analysis
The data were analyzed using Analysis of Variance (ANOVA) in one way classification analysis in SAS Statistics program. Treatment means were compared using Duncan Multiple Range Test (DMRT) at 5% level of significance

Results and discussion
Embryo-toxic effects of L. strigosus mycelia and fruiting bodies
Zebra fish is an ideal animal model and reliable tool in investigating the embyo-toxic and teratogenic effects of certain compounds, extracts, and/or food materials. With the aim to assess the biosafety and potential of *L. strigosus* as source of toxic compounds, this study evaluated the embryo-toxic and teratogenic effects of extracts of mycelia and fruiting body in developing embryos of zebra fish.

The mean percentage mortality of embryos after 24 and 48 hrs of exposure in the different concentrations of both extracts are presented in Table 1.

Table 1. Mortality of embryos of zebra fish after 24 and 48 hours of exposure in the different concentrations of mycelia and fruiting body extracts of *L. strigosus*.

Extract Concentration (µg/ml)	Mortality (%)			
	Mycelia		Fruiting body	
	24 hours	48 hours	24 hours	48 hours
10000	100.00[a]	100.00[a]	100.00[a]	100.00[a]
1000	0.00[b]	100.00[a]	16.67[c]	100.00[a]
100	0.00[b]	0.00[b]	0.00[c]	8.33[b]
10	0.00[b]	0.00[b]	0.00[c]	0.00[b]
1	0.00[b]	0.00[b]	0.00[c]	0.00[b]
0	0.00[b]	0.00[b]	0.00[c]	0.00[b]

In each column, treatment means having the same letter of superscripts are not significantly different from each other at 5% level of significance using DMRT.

In both periods of observation, it can be seen that the toxic effects of both extracts of *L. strigosus* were dependent on concentration and time of exposure. All embryos exposed to 1000 µg/ml (after 24 hrs) and

100 µg/ml (after 48 hrs) and lower concentrations of mycelial extract were survived. However, in fruiting body extract, 100% mortality was recorded to those exposed to 10000 µg/ml (after 24 hrs) and 1000

µg/ml (after 48 hrs). Although some embryos exposed to 1000 µg/ml (16.67% mortality) and 100 µg/ml (8.33% mortality) died after 24 and 48 hrs, respectively, these results were not found to be statistically significant from those exposed to lower concentrations and to the control (p>0.05). It is also interesting to note that the percentage mortality of embryos treated with both extracts at 1000 µg/ml significantly increased as the exposure to the extract was prolonged. The most marked toxic effect of both extracts was coagulation of embryo (Figure 1A & 1B and Figure 2A & 2B).

Table 2. Hatchability and heartbeat rate of zebrafish after 48 hours exposure to different concentrations of mycelia and fruiting body extracts of *L. strigosus*.

Extract Concentration (µg/ml)	Hatchability (%)		Heartbeat (per min)	
	Mycelia	Fruiting body	Mycelia	Fruiting body
10000	0.00[d]	0.00[c]	NHB	NHB
1000	0.00[d]	0.00[c]	NHB	NHB
100	58.33[c]	0.00[c]	135.00[c]	NHB
10	83.33[b]	66.67[b]	146.33[b]	152.67[b]
1	100.00[a]	100.00[a]	144.13[b]	148.34[b]
0	100.00[a]	100.00[a]	160.00[a]	160.00[a]

In each column, treatment means having the same letter of superscripts are not significantly different from each other at 5% level of significance using DMRT. NHB, no heartbeat due to coagulation and/or delayed development.

Hatchability and heartbeat rate of treated zebra fish

In order to determine the successful development of embryos, the hatchability of embryos after 48 hrs of treatment exposure was also recorded in this study (Table 2). In mycelial extract, hatching was observed to embryos at 100 µg/ml and lower concentrations. However, significantly lower percentage hatchability was noted to those at 10 µg/ml (58.33%) and 100 µg/ml (83.33%) when compared to embryos at 1 µg/ml and control both having 100% hatchability. In contrast, no hatched was registered in 1000 µg/ml and higher concentration of the extract. On the other hand, embryos at 100 µg/ml and higher concentrations of fruiting body extract did not hatch while those embryos at 1 µg/ml and control showed 100% hatchability. Although some embryos hatched at 10 µg/ml, the percentage hatchability of 66.67% was significantly lower than those at 1 µg/ml and control. These results strongly suggest that the hatchability of embryos is concentration dependent. The failure of hatching and significant lower hatchability could be attributed to the delayed development of embryos as one of the teratogenic fingerprints of both extracts.

Heartbeat rate was monitored at the pharyngula stage of every embryo when the head and tail were distinctly pigmented to ensure that the heartbeat obtained was effect of the different extract concentrations and not by the delayed development. Apparently, normal embryos significantly recorded the highest heartbeat rate of 160 per min (Table 2). Embryos exposed to lower concentrations of both extracts showed lower heartbeat rate ranging from 135.00 to 146.33 per min (for mycelia extract) and from 148.34 to 152.67 per min (for fruiting body extract). However, no heartbeat was observed to those at 1000 µg/ml and 100 µg/ml and/or higher concentrations of mycelia and fruiting body extracts, respectively. This could be attributed to the coagulation of embryos at early phase of development and to the delayed development of embryos.

Morphological endpoints of treated embryos

Delayed development of zebra fish embryo could cause different morphological abnormalities. In the present work, it was found out that delayed development is a teratogenic fingerprint of *L. strigosus*.
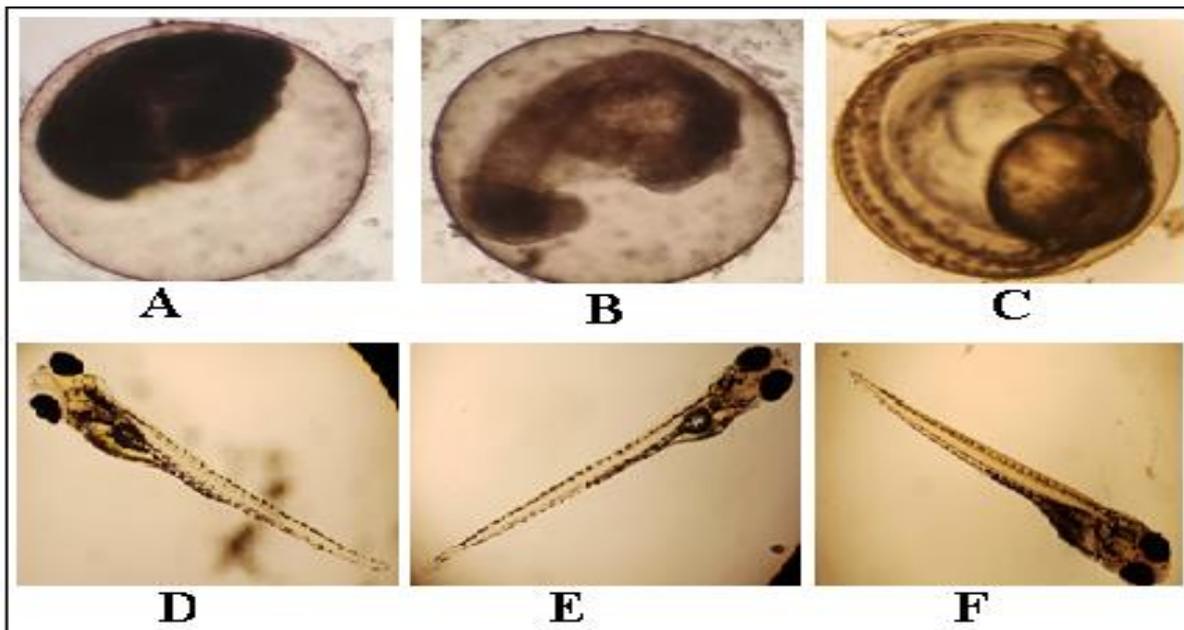
Fig. 1. Morphological endpoints of zebra fish after 48 hours of exposure to the different concentrations of mycelial extract of *L. strigosus*; (A) 10000 μg/ml, (B) 1000 μg/ml, (C) 100 μg/ml, (D) 10 μg/ml, (E) 1 μg/ml, and (F) embryo water (0 μg/ml).

This was obvious to embryos exposed at 100 μg/ml of both mycelia and fruiting body extracts. However, comparing the two extracts at this concentration, more developed embryos were observed in mycelial extract which are at pharyngula phase (Figure 1C) than those in fruiting body extract which are still at late segmentation phase (Figure 2C) after 48 hrs of treatment exposure. Apparently, delayed development is dependent on the concentration of extract and the type of extract. This delayed development observed in the present study might be due to the inhibition or disturbance of essential substances for the normal growth and developmental processes of embryos.
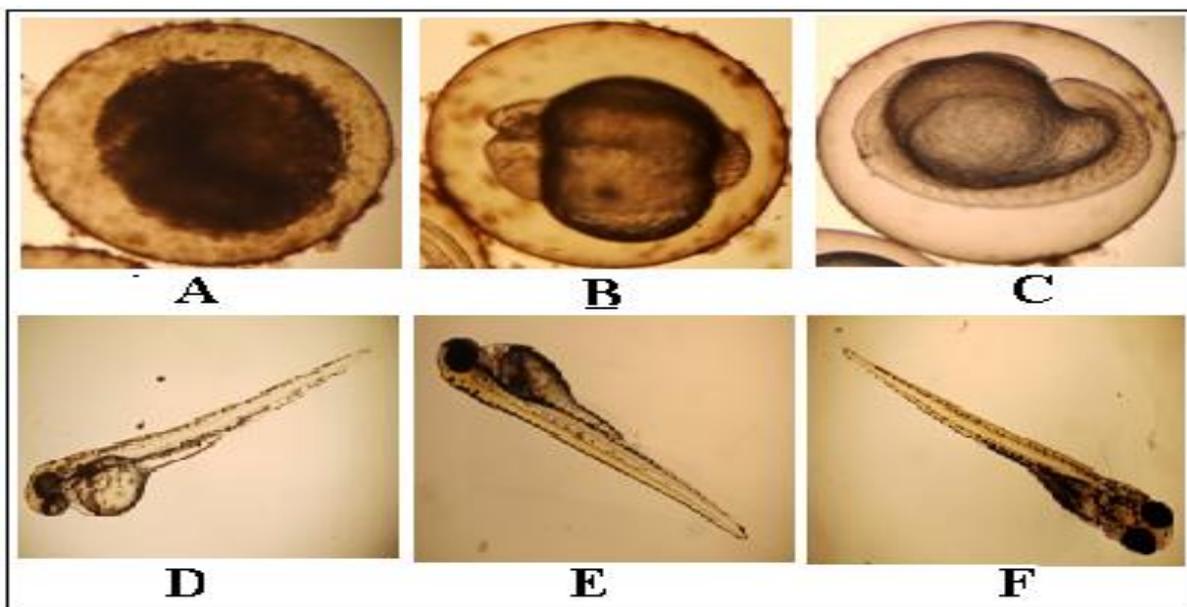

Fig. 2. Morphological endpoints of zebra fish after 48 hours of exposure to the different concentrations of fruiting bodies extract of *L. strigosus*; (A) 10000 μg/ml, (B) 1000 μg/ml, (C) 100 μg/ml, (D) 10 μg/ml, (E) 1 μg/ml, and (F) embryo water (0 μg/ml).

Some mushrooms have been studied for their toxic and teratogenic properties using zebra fish embryos. For instance, hot water extract of *Ganoderma lucidum* showed toxic (coagulation) and teratogenic effects in both time and dose-dependent manner. Tail malformation and delayed development were the most common teratogenic fingerprints of the extract (Dulay *et al.*, 2012). However, *Lentinus tigrinus* water extract significantly decreased the hatchability, heartbeat rate, and induced delayed development at certain concentrations. Morphological abnormalities such as tail malformation, pericardial edema and under-develop organs were noted as growth-delay related endpoints (Dulay *et al.*, 2014). Moreover, *Termitomyces clypeatus* extract induced the toxicity and teratogenicity in zebra fish embryos including wavy somite embryo, unhatched embryo with twisted tail tip, delayed development of embryo (still at segmentation phase), tail malformed embryo and coagulated embryo (De Castro and Dulay, 2016). These previous works and the results obtained in the present study prove that mushrooms contain bioactive compounds with promising functional activities particularly as anticancer, since most of the teratogens are anticancer agents or vice versa (Blagosklonny, 2005).

Conclusion

In conclusion, extracts of both mycelia and fruiting body of *L. strigosus* exhibits embryo-toxic and teratogenic effects (delayed development) on the developing embryos of zebra fish. However, these activities are greatly influenced by the type of mushroom biomass; fruiting body extract is more toxic than mycelia extract. The anticancer activity of both extracts must be studied in the future.

References

Blagosklonny M. 2005. Teratogens as anticancer drugs. Cell Cycle **4(11),** 1518–21.

Busquet F, Nagel R, Landenberg F, Mueller S, Huebler N, Broschard T. 2008. Development of a new screening assay to identify proteratogenic substances using zebra fish Danio rerio embryo combined with an exogenous mammalian metabolic activation system (mDarT). Toxicological Sciences **104(1)**, 177–188.

Bustillos RG, Paguio ZKG, Hermosa DP, Dulay RMR. 2016. Philippine coprophilous mushrooms (Panaeolus antillarium and Panaeolus cyanescens) exhibit toxic and teratogenic effects in zebra fish (Danio rerio) embryo model. Advances in Environmental Biology **10(3),** 75-80.

De Castro MEG, Dulay RMR, Enriquez MLD. 2016. Toxic and Teratogenic Effects of Medicinal and Culinary Mushroom, Termitomyces clypeatus, Collected from the Termite Mound in Mt. Makiling Forest Reserve, Los Baños, Laguna, Philippines on Developing Embryos of Zebra fish (Danio rerio). Der Pharmacia Lettre **8(5),** 237-242.

De Castro MEG, Dulay RMR. 2015. Toxic and teratogenic effects of Lentinus sajor-caju and Pleurotus ostreatus ethanolic extracts in Danio rerio embryo model. International Journal of Biology, Pharmacy and Allied Sciences **4(4),** 2261-2269.

Dulay RMR, Garcia EJB. 2017. Optimization and enriched cultivation of Philippine (CLSU) strain of Lentinus strigosus (BIL1324). Biocatalysis and Agricultural Biotechnology **12,** 323-328.

Dulay RMR, Kalaw SP, Reyes RG, Alfonso NF, Eguchi F. 2012. Teratogenic and toxic effects of lingzhi or reishi medicinal mushroom, Ganoderma lucidum (W.Curt.:Fr.) P. Karst. (Higher basidiomycetes), on zebrafish embryo as model. International Journal of Medicinal Mushrooms **14(5),** 507–512.

Dulay RMR, Kalaw SP, Reyes RG, Cabrera EC. 2014. Embryo-toxic and teratogenic effects of Philippine strain of Lentinus tigrinus (tiger sawgill basidiomycetes) extract on zebra fish (Danio rerio) embryos. Annals of Biological Research **5(6),** 9–14.

Dulay RMR, Pamiloza DG. 2018. Proximate composition and bioactivities of hairy sawgill mushroom, Lentinus strigosus (BIL 1324) from the Philippines. International Journal of Biology, Pharmacy and Allied Sciences **7(3),** 361-369.

Dulay RMR, Rivera AGC, Garcia EJB. 2017. Mycelial growth and basidiocarp production of wild hairy sawgill Lentinus strigosus, a new record of naturally occurring mushroom in the Philippines. Biocatalysis and Agricultural Biotechnology **10,** 242-246.

Nagel R. 2002. DarT: The embryo test with the Zebra fish Danio rerio--a general model in ecotoxicology and toxicology. ALTEX **19(1),** 38-48.

Souza-Fagundes EM, Cota BB, Rosa LH, Romanha AJ, Correa-Oliveira R, Rosa CA, Zani CL, Teixeira-Carvalho A, Martins-Filho OA. 2010. In vitro activity of hypnophilin from Lentinus strigosus: a potential prototype for Chagas disease and leishmaniasis chemotherapy. Brazillian Journal of Medical and Biological Research **43,** 1054-1061.

Thomas J. 2000. The Zebra fish Book 4th edition, University of Oregon.