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Embryo-toxic and Teratogenic Effects of Philippine Strain of *Lentinus tigrinus* (Tiger Sawgill Basidiomycetes) Extract on Zebrafish (*Danio rerio*) Embryos

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ABSTRACT

Lentinus tigrinus is a wild edible basidiomycete typically inhabiting fallen logs. With the aim to assess its biosafety and its potential as source of toxic compounds with multi-bioactivities, this paper highlighted the embryo-toxicity and teratogenic effects of this mushroom on zebrafish (Danio rerio) as animal model. Its functional components obtained through hot water extraction produced teratogenic and toxic effects in zebrafish embryos. L. tigrinus extract significantly reduced the hatchability of zebrafish eggs at 1% or higher concentrations of the water extract and the heartbeat rate at 5% or higher concentrations. Treated embryos at 0.5%-10% concentrations showed developmental delay prior to the various abnormalities. Tail malformations, pericardial edema and under-develop organs were identified as the three main growth-delay related endpoints. Delayed development resulted to coagulation of embryos at an earlier phase, whereas developmental abnormalities that impede embryonic activities caused the lack of heartbeat at the latter phase. This report provides benchmark data on the strong biological activity of L. tigrinus, thus, investigation on the other functionalities is deemed necessary in the future studies.

Keywords: bioactivity, embryo-toxicity, *Lentinus tigrinus*, Philippine wild mushrooms, teratogenicity, zebrafish embryo.

INTRODUCTION

Mushrooms have been recognized not only as source of food with high nutrient value but also a good source of medicinal compounds with functional properties. Several studies have been conducted and reported for the different biological activities of mushrooms worldwide. Mushrooms are reputed to exhibit medicinal benefits such as blood sugar lowering, cholesterol reducing, liver protective, antifibrotic, anti-inflammatory, antidiabetic, and antimicrobial activities [1]. They also possess antioxidant and antitumor properties [2]. With these various human health benefits of mushrooms, it is also noteworthy to regard their negative effects. Recently, we found out that *Ganoderma lucidum* hot water extract exhibited toxic and teratogenic effects on the developing zebrafish embryos. Coagulation of embryos was the most obvious lethal effect while tail malformation was the most marked morphological abnormality [3]. Moreover, ribosome inactivating protein, hypsin, from *Hypsizygus mamoreus* induces abnormal embryonic development in mouse embryos [4]. These teratogenic activities of mushrooms could indirectly equate to their potential as anticancer agent because most of the teratogens are anticancer drugs and vice versa [5].

Teratogenicity assay using zebrafish embryos as animal model is a very reliable and important tool in establishing whether certain compounds or food materials could cause deformity to the new being [6]. This is also used in

various toxicological research such environmental toxicity dealing with the harmful effects of chemical pollutants, safety assessment of new pharmaceutical products, and evaluation of new toxic compounds with potential anticancer properties. Blagosklonny [5] often compared embryo with cancer for two reasons; first, they both grow and invade. Secondly, certain embryonic pathways may reactivate by cancer cells, which are targets of teratogens.

Lentinus tigrinus is a newly domesticated Philippine wild edible mushroom. This basidiomycetous fungus is often seen growing on fallen logs in the forest from May to September. The optimum conditions for basidiospore germination, secondary mycelial growth and basidiocarp production were determined [7]. Its successful production technology was generated using rice straw – sawdust based formulation in an artificial cultivation. We also reported some bioactivities of this mushroom such as hypoglycemic and antibacterial activity [8, 9]. However, the toxic and teratogenic activity of *L. tigrinus* has not been investigated.

In order to assess the safety of *L. tigrinus* as food source and evaluate its potential as source of toxic compound for anticancer drug, the present work highlighted the toxic and teratogenic activity of *L. tigrinus* on developing embryos of zebrafish as animal model.

MATERIALS AND METHODS

Mushroom Sample

Air-dried fruiting bodies of *L. tigrinus* were obtained from the Center for Tropical Mushroom Research and Development (CTMRD). Sample was milled using food processor prior to the extraction of its functional components.

Extraction of functional components

Functional components of the *L. tigrinus* were obtained through hot water extraction following the procedure of Eguchi et al. [10]. The active components of the milled mushroom samples (20g) were extracted in 600 ml hot water at 80 - 90ºC in a water bath for 2 hrs. The milled mushrooms were separated from the extract by filtration using filter paper No. 2 then filter sterilized through 0.45µ filters. The filtrate was refrigerated until needed for used.

Spawning of *Danio rerio*

The protocol of this study was based after Nagel [11]. A non-treated stock of tap water in a glass aquarium with oxygen saturation was used for spawning of zebrafish where mature females and males were presented at 1:2 ratio. Adult zebra fish were localized in a plastic mesh and the aquarium was covered with black plastic for 12 hours to induce spawning. After incubation in the dark, eggs were exposed to lighted condition for another 12 hours. Fertilization occurs within 30 minutes after light is turned on [11]. Twelve hours after fertilization, the fertilized eggs were siphoned out of the aquarium using a hose. Embryos were rinsed three times, placed in a watch glass with embryo medium and observed under the compound microscope to examine uniformity and normal condition.

Embryo-toxicity and teratogenicity assay

Three ml of each treatment concentration of *L. tigrinus* extract prepared using embryo water as diluent (20%, 10%, 5%, 1%, 0.5%, 0.1% and 0.05%) and control (embryo water) were placed into each well of the 12-well ELISA plate. Six embryos at segmentation phase were transferred into each well containing the different treatments. The plates were incubated at 26°C ± 1°C. Teratogenic activity was examined under 40X magnification using a compound microscope after 12, 24 and 48h of incubation. Morphological endpoint evaluation of zebra fish was based on the parameters established by Nagel [11]: 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. Hatchability, malformation and mortality rates were recorded, and death was defined as coagulated embryos and as no visual heartbeat. All tests were repeated three times and conducted in accord with national guidelines for animal welfare.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in one way classification analysis. Means were compared using Duncan Multiple Range Test (DMRT) at 5% level of significance. The Sirichai Statistics 6.07 program was used for analysis.

RESULTS

Teratogenic effects of *L. tigrinus* extract

The control group of embryos developed normally in embryo water medium. Hatching was completed after 48 hours post treatment application (hpta) at lower concentrations of 0.05% and 0.1% of extract and embryo water (control)

and after 72 hpta at 0.5% and 1%, and 96 hpta at higher concentrations of the extract. The percent hatchability of embryos treated with 1% or higher was significantly lower than that of the control (Fig. 1).

Heartbeat rate was significantly affected by the different concentrations of extract (Fig. 2). Embryos incubated in embryo water had the highest number of heartbeat with a mean of 128 per min, which was not significantly different from those exposed to the lower concentrations of extract (0.05% to 0.5% except for 0.1%). However, heartbeat rates of embryos treated with 5% or higher were lower as compared to embryos in the control. Similar with the percent hatchability, heartbeat rate was also showed to be concentration-dependent.

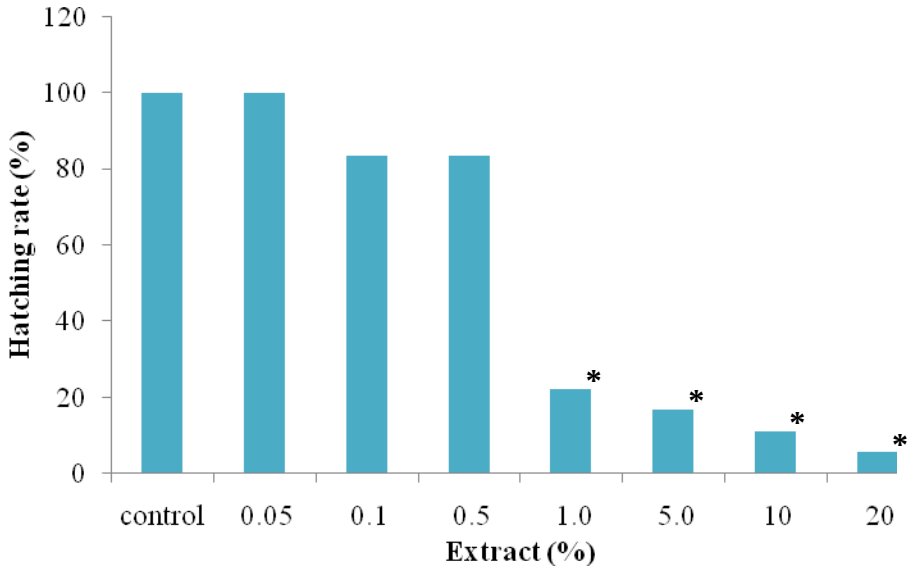

Fig. 1 Hatchability rate after 48hpta of embryos at different extract concentrations. The hatchability of embryos treated with 1% or higher was significantly lower than that of the control. *Represents significantly different from the control.

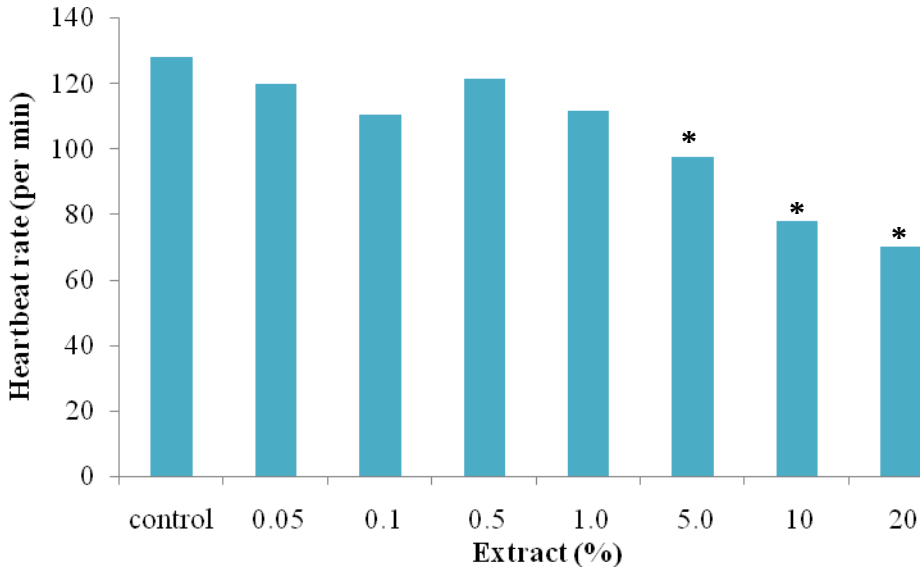

Fig. 2 Heartbeat rate at pharyngula stage (tail pigmentation) of embryos at different extract concentrations. The highest rate with a mean of 128 per min was recorded in control embryos. Heartbeat rates of embryos treated with 5% or higher were lower as compared to embryos in the control. *Represents significantly different from the control.

Morphological abnormalities in embryos caused by *L. tigrinus* extract included under-developed head, unformed head and tail, perverted tail, serious pericardial edema and hook-like tail starting with embryos exposed to 0.5% of the extract (Fig. 3 B-F). Embryos exposed to 1% extract after 24 hours of treatment application showed underdeveloped head region while the tail was fully detached, but still had a heartbeat. However, in the same observation period, a more serious developmental inhibition of embryos (no head and tail formed) was found when incubated at higher concentration of extract (5%). Tail malformations were noted in 24-hpta embryo (perverted tail)

at 10% and in 48-hpta embryo (hook-like tail) at 1% extract concentrations. In 48-hpta, serious edema in the pericardial sac was observed in sublethal embryo at 0.5% of extract, which was not induced in higher concentrations with very low heartbeat rate.

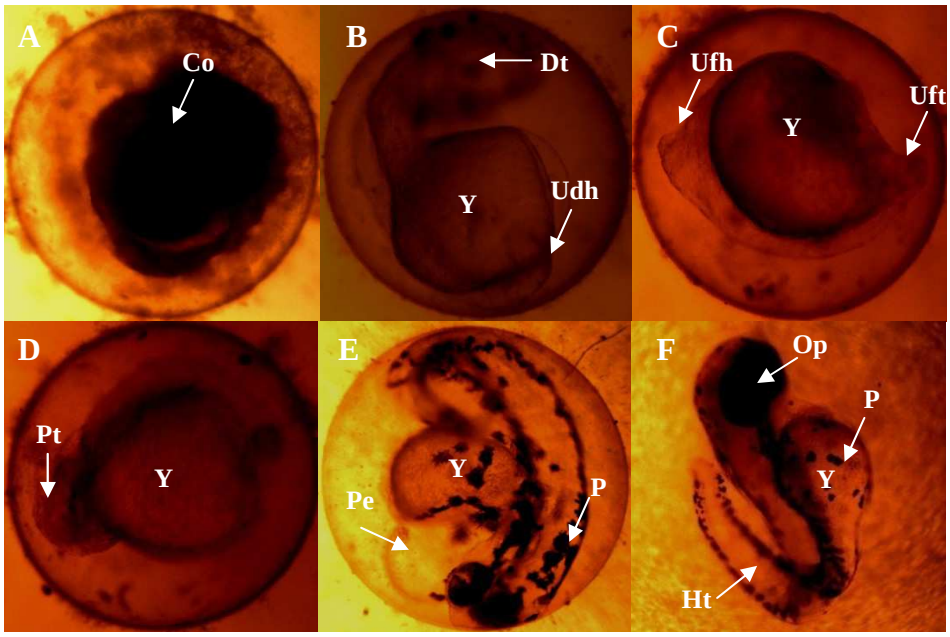

Fig. 3 Lethal and sublethal effects of *L. tigrinus* extract on zebrafish embryos. (A) A coagulated embryo at 20% concentration after 12 hpta (hours post treatment application). (B) Embryo with underdeveloped head region but with fully detached tail at 1% concentration after 24 hpta. (C) Embryo with no head and tail formed at 5% concentration after 24 hpta. (D) A 24-hpta embryo with perverted tail at 10%. (E) A 48-hpta embryo with serious pericardial edema incubated in 0.5%. (F) The 48-hpta larva with hook-like tail hatched from 1% of extract. Co,coagulated; Dt, detached tail; Udh, under developed head region; Ufh, unformed head; Uft, unformed tail; Pt, perverted tail; Pe, pericardial edema; P, pigmentation; Ht, hook-like tail; Op, optic; Y, yolk.

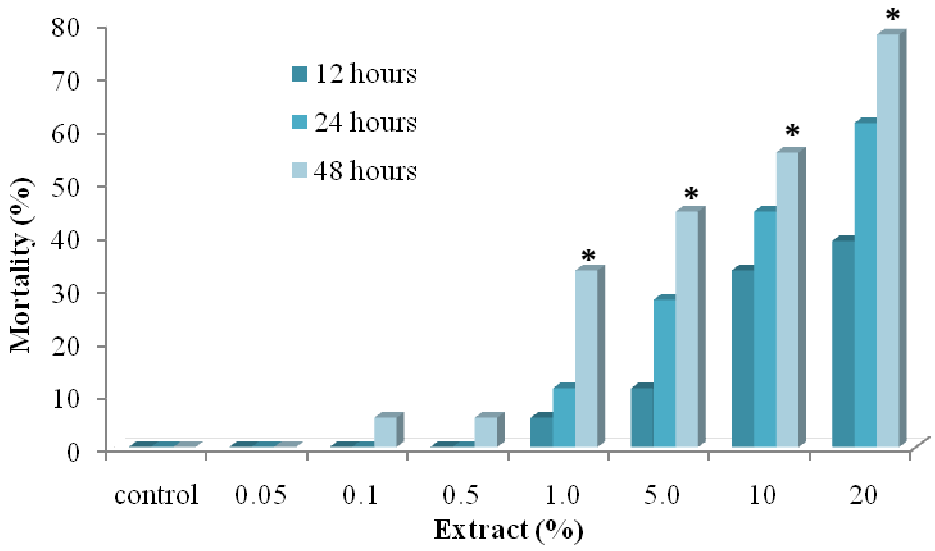

Fig. 4 Mortality rates of zebrafish embryos after 12h, 24h and 48h of extract exposure. The death of an embryo was defined as coagulation and no visual heartbeat. The mortality rates of embryos treated with 5% or higher of extract was significantly higher than that of the control embryos. *Represents significantly different from the control at 48hpta.

Lethal effect of *L. tigrinus* on development of embryos

Lethal effect of *L. tigrinus* extract on zebrafish embryos was recorded and mortality was defined as coagulation and no visual heartbeat of embryos. The mean percentage mortality of embryos after 12, 24 and 48 hrs of exposure in different extract concentrations are shown in Fig. 4. In all periods of observation, it can be seen that lethal effect of extract of *L. tigrinus* was dependent on dose and time of exposure. All embryos exposed to 0.05%, 0.1% and 0.5% extract survived at 12-hpta, 24-hpta and 48-hpta. Although some embryos exposed to 0.1% and 0.5% died 48 hpta,

these results were not found to be statistically significant from those exposed to the control and 0.05% concentration ($p>0.05$). On the other hand, percentage mortality of embryos treated with extract with concentration 1% or higher significantly increased as extract exposure prolonged. Coagulated embryo (Fig. 3 A) was the most marked lethal effect 12hpta, while the absence of visual heartbeat in embryos 24 and 48hpta confirmed the lethal effect of the extract. It is noted that extracts with concentrations of 1% or higher also significantly affected the hatchability and heartbeat rate of the embryos.

DISCUSSION

The zebrafish (*D. rerio*) embryo is an ideal animal model in investigating embyo-toxic and teratogenic compounds or food materials of potential value. The embryonic development of zebrafish is very similar with the higher vertebrates, including human [12]. Therefore, toxic effects of certain chemical to zebrafish embryos could be similar to other vertebrate embryos. Studies on mushrooms as source of embryo-toxic and teratogenic substances are very limited. However, there are several studies revealing important bioactivities of mushrooms particularly the famous medicinal *G. lucidum*, which exhibits antitumor and anticancer properties [13, 14, 15]. These significant properties could be associated to its toxic and teratogenic activities in zebrafish embryos, which we previously studied [3].

In this paper we investigated the developmental toxicity and teratogenicity of *L. tigrinus* extract in zebrafish embryo model. The varying concentrations of extract of *L. tigrinus* affected the hatchability of embryos: as the extract concentration increased the percent hatchability decreased. The low hatchability could be attributed to the delayed development of embryos as one of the important sublethal effects of the extract. It is also worth mentioning that the different developmental abnormalities could interrupt the hatching processes. For instance, malformation in the tail impedes the breaking of the chorion which allows the larvae to hatch out. Similar disturbance in the hatching process was observed when embryos are exposed to *G. lucidum* extract [3] and other compounds such as coumarin and warfarin [16].

Heartbeat rate is another important sublethal parameter used. 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. The results of the present study suggested that extract of *L. tigrinus* at 5% or higher concentrations induce a significant decreased in heartbeat rate. In contrast, Tiedeken et al. [17] found that the developmental heartbeat rates of control and domoic acid-treated zebrafish embryos were not significantly differed from each other. In this study, it can also be noticed that the developmental heartbeat rate was not affected by the nominal concentrations. Similar observation was found in 2-day post-fertilization zebrafish embryos exposed at low concentrations of diclofenac (1 to 2000 μg/L) [18]. Indeed, this mushroom plays important functions in the cardiovascular system. Thus, it is necessary to evaluate its cardio-toxicity in the future studies.

A variety of developmental abnormalities can be perceived in zebrafish embryos after exposure to a certain chemical compound. In this study, malformations such as perverted and hook-like tail were the most common teratogenic effect of *L. tigrinus* extract. Dulay et al. [3] found the same effect in embryos exposed to *G. lucidum* extract which showed tail malformation (bent tail and S-shaped tail) as the most marked morphological abnormality at 72 hpta. Furthermore, Weigt et al. [16] identified tail malformation as a fingerprint morphological endpoint of warfarin, an anticoagulant coumarin derivative. Severe flexure of the entire posterior-most tail region was observed in CH_3HgCl/L (20 μg and 30 μg) - treated embryos [19]. The failure of organogenesis leading to the underdevelopment of the head and tail morphology was also noticed in the treated embryos. This might be attributed to the inhibition or disturbance of essential substances for growth and developmental processes of embryos. Abnormal embryos also showed pericardial edema with weak and slow heartbeat, which confirmed the potential cardio-toxicity of the extract. Pericardial edema and tail malformations were frequently found endpoints of zebrafish embryos after exposure to the known embryo-toxic and teratogen compounds such as valproic acid, retinoic acid, hydroxyurea, methoxyacetic acid, and boric acid [20].

Exposure of zebrafish embryos to teratogen also resulted to delayed development. In the present study, we found that embryos treated with *L. tigrinus* extract at 0.5%-10% concentrations showed delayed development prior to severe malformations. This observation suggests that the different abnormalities are growth-delay related malformations. Teixido et al. [20] characterized the morphological endpoints related to developmental delay of zebrafish embryos exposed to the different teratogens. They found that valproic acid and methoxyacetic acid showed developmental delay at non-teratogenic concentrations but boric acid caused delayed development with teratogenic effect and caffeine performed as teratogen with no delayed growth at nominal concentrations.

However, embryo-toxicity assay confirmed the lethal effect of *L. tigrinus* extract on zebrafish embryos. Developmental delay has been a reason for coagulation at earlier phase whereas developmental abnormalities that impede embryonic activities have been the cause for the lack of heartbeat at the latter phase. This toxic effect of *L. tigrinus* can be compared with other basidiomycetes with strong bioactive compound. For instance, 1170 μg/kg of ostreolysin, a cytolytic protein from *Pleurotus ostreatus*, is lethal and cardiorespiratory toxic in rodents [21]. Lectin from toxic mushroom, *Inocybe umbrinella*, inhibited the proliferation of tumor cells including hepatoma HepG2 cells and breast cancer MCF7 cells with an IC_{50} of 3.5 ± 0.2 μM and 7.4 ± 0.3 μM, respectively, and HIV-1 reverse transcriptase with an IC_{50} of 4.7 ± 0.2 μM [22].

This present report on the toxic and teratogenic effects of *L. tigrinus* extract on the embryonic development of zebrafish provides data on the biological activity of this wild edible mushroom. Teratogenicity and embryo-toxicity to other form of mammals, molecular mechanism of its toxic and teratogenic effect and other multifunctional activities should be further investigated in the future studies.

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