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**TOXIC AND TERATOGENIC EFFECTS OF *LENTINUS SAJOR-CAJU* AND
PLEUROTUS OSTREATUS ETHANOLIC EXTRACTS IN
DANIO RERIO EMBRYO MODEL**

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ABSTRACT

Mushrooms are popular nutraceutical resource worldwide. However, the toxic and teratogenic effects of mushroom extracts were recently realized. In the present work, we studied the toxic and teratogenic effects of *Lentinus sajor-caju* (LSE) and *Pleurotus ostreatus* (POE) ethanolic extracts in *Danio rerio* embryo model. Embryos exposed in 2.5% and 5% concentrations of POE significantly recorded 100% mortality after 12 hours whereas 5% LSE with initial mortality of 33.33% drastically raised to 83.33% after 24 hours up to the last observation period. Hatching of embryos treated with 2.5% or higher of LSE (75.00%) and 1% POE (83.33%) were significantly lower. High percentage of delayed growth and tail malformation were observed in embryos exposed to 2.5% or higher of LSE and 1% POE. Coagulation was the most marked toxic effect while delayed growth and tail malformations were the major teratogenic effects. These effects of the two mushroom extracts in *D. rerio* embryos were dependent on concentration and period of exposure.

Keywords: *L. sajor-caju*, *P. ostreatus*, Teratogenicity, Toxicity, *D. rerio* Embryo Model

INTRODUCTION

Mushrooms are popular healthy food source because of their organoleptic, high nutritional values and pharmacological characteristics. They richly contain carbohydrates, proteins, fiber, minerals but very low lipids, so, they can be considered as vital source of complete human diet. In addition, they are valued as natural sources

of novel bioactive metabolites that are accountable to their many beneficial properties. A number of biological activities of the different compounds isolated from edible and medicinal basidiomycetes have been studied these are summarized in the recent comprehensive review by De Silva *et al.* [1]. Some of these properties include anti-diabetic, anti-malarial, anti-microbial, anti-oxidant, antitumor, antiviral, anti-cholesterol, and anti-Alzheimer. Indeed, mushrooms could improve physiological processes, thus, excellent food for human well-being.

Many basidiomycetes still remain in the wild waiting to be harnessed, but some are being cultivated and used for food and pharmaceutical purposes. *Pleurotus ostreatus* is one of the most widely cultivated mushrooms because of its medicinal properties, culinary status, and good flavour. It is capable of colonizing a wide variety of natural lignocellulosic substrates and grows over a wide temperature range with high production efficiency. On the other hand, *Lentinus sajor-caju*, a leathery-textured wild edible mushroom, is commonly found growing on the fallen logs in the tropical forest. Apart from being source of food, these two mushrooms also improved physiological activities. For instance, *L. sajor-caju* exhibited antihypertensive effect in

spontaneously hypertensive rats [2] while *P. ostreatus* demonstrated hypolipidemic property in hypercholesterolemic rats [3]. Hence, these mushrooms hold tremendous potential in the nutraceutical industry.

Teratogens are agents that cause malformation in the developing embryos. Embryos of zebrafish (*D. rerio*) are now used as standard model in toxicity testing due to their similarity to higher forms of vertebrates [4]. Teratogenicity testing can be a desirable property because many anticancer drugs are teratogenic and teratogens can be used as anticancer drugs [5]. In the previous works, considering other edible mushroom, hot water extracts of *Ganoderma lucidum* and *Lentinus tigrinus* revealed toxic and teratogenic effects, for instance, tail malformation (bent tail and S-shaped tail) which was reported as the most marked morphological abnormality [6, 7].

In spite of the reported chemical or nutritional constituents and medicinal importance of these two mushrooms, their toxic and teratogenic effects, if there is any, were not studied yet. Therefore, we investigated the toxic and teratogenic activities of ethanol extracts of *L. sajor-caju* and *P. ostreatus* to generate alternative resource of teratogens that can potentially be used as anticancer drugs.

MATERIALS AND METHODS

Source of Mushroom Wild fruiting bodies of *L. sajor-caju* were collected from Mt. Makiling Forest Reserve, Los Banos, Laguna while *P. ostreatus* fruiting bodies were obtained from the Center for Tropical Mushroom Research and Development, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines. Mushrooms samples were brought to the Department of Biology Laboratory of De La Salle University, Manila and were air-dried and powdered using food processor prior to extraction.

Ethanol Extraction and Treatment Preparation

Bioactive components of the milled mushrooms were obtained using ethanol extraction. Twenty grams each of the powdered mushroom were extracted in 500 ml of ethanol for 24 hours and filtered using No. 2 Whatman filter paper. Extracts were concentrated in a rotary evaporator. Three millilitres of extract concentrations (0%, 1%, 2.5% and 5%) were prepared by diluting to embryo water [8]. Treatments were transferred into the 12-well ELISA plate.

Spawning of *Danio rerio*

The 7 day-acclimatized adult zebrafish were confined in a plastic mesh and the aquarium was covered with black plastic for 12 hours to induce spawning. After spawning, the eggs and sperms were exposed to light to

allow fertilization after 30 minutes from the time that the light was turned on. After 12 hours of light exposure, fertilized eggs were siphoned out of the aquarium using a hose and were rinsed three times with embryo water. Embryos were examined using a microscope to ensure the uniformity of developmental phase and normal condition of the embryos. Unfertilized and coagulated eggs were discarded.

Embryo-toxicity and teratogenicity assay

Four embryos at segmentation phase were transferred into each well containing the different treatments. Triplicate per treatment was done. The plates were incubated at $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$. 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 [9]: 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. Data were analyzed using analysis of variance and

treatment means were compared at 5% level of significance using LSD.

RESULTS AND DISCUSSION

Toxic effects of mushroom extracts

D. rerio embryo toxicity assay is a reliable method of testing consumable materials of important values that could potentially be used as source of toxic substance in cancer therapy. In the present work, mushrooms as source of embryo-toxic and teratogenic substances were investigated. The bioactive components of *L. sajour-caju* (LSE) and *P. ostreatus* (POE) were extracted and concentrated using ethanol as solvent and were bioassayed in segmentation-phase embryo. Coagulation and no visual heartbeat of embryos were the basis in determining toxic effects. The mortality of embryos after 12, 24, 36, and 48 hours of exposure in mushroom extracts is presented in **Table 1**. Apparently, toxic effects of the two mushroom extracts were dose and exposure period dependent. In all periods, no mortality was observed in embryos exposed at 1% of both extracts, except after 48 hours of exposure in POE with 16.67%. Embryos exposed in 2.5% LSE with 16.67% mortality from 12 hours to 36 hrs significantly increased to 33.33% after 48 hours of exposure whereas 5% LSE with initial mortality of 33.33% drastically raised to 83.33% after 24 hours up to the last observation period. On the other hand,

100% mortality was significantly recorded in embryos exposed in 2.5% and 5% concentrations of POE. Coagulation was the most marked toxic effect of the two extracts. No visual heartbeat was observed in developed embryos after 48 hours of exposure in 1% POE, and 48 and 24 hours treated with 2.5% and 5% LSE, respectively. These results obtained clearly indicate that *L. sajour-caju* and *P. ostreatus* ethanolic extracts at 2.5 and 5% are toxic to *D. rerio* embryos, thus, these mushrooms could be remarkable resource of toxic substances in pharmaceutical development. Likewise, high mortality of embryos was also demonstrated by other basidiomycetes. Hot water extract of *L. tigrinus* at 1% or higher concentrations and *Schizophyllum commune* at 4% or higher concentrations significantly increased the mortality of embryos after a prolonged exposure [7, 10]. These toxic effects of mushrooms could be accounted to their bioactive compositions with effective inhibitory activity against the proliferation of disease-causing agents like cancer cells. A wide variety of low-molecular-weight compounds from different mushrooms that exhibited toxic activity against cancer cells in several mechanisms were summarized in a review done by De Silva *et al.*, [11].

Teratogenic effects of mushroom extracts

To determine the teratogenic effects of the two mushroom extracts, the percentage hatchability, delayed growth, and tail malformation of the extract-treated embryos were compared with the control embryos.

Successful hatching process is anticipated in normal embryos. The hatchability of embryos treated with the different concentrations of mushroom extracts after 48 hours is presented in **Table 2**. Embryos treated with 2.5% LSE and 1% POE significantly registered lower hatchability, 75.00% and 83.33%, respectively, than the control embryos with 100%. However, no hatched embryo was recorded in 5% LSE and 2.5% or higher concentrations of POE. Hence, hatching of embryos was affected by the two extracts: as the extract concentration increased the percent hatchability decreased. The failure of hatching process could be explained by the morphological abnormalities which limit the ability of the embryos to break the chorion and hatch out [6].

Growth retardation or delayed growth is another important parameter in determining teratogenic effects of the extracts tested. It can be seen that in the increasing concentration of the extracts, embryos with delayed growth were significantly increased (**Table 2**). The obvious delayed growth of treated embryos is illustrated in **Figure 1**. Embryos exposed in 2.5% or higher

concentration of LSE and 1% of POE significantly showed high percentage of delayed growth with 91.67% and 83.33%, respectively. The same with hatchability, delayed growth was also showed concentration-dependent. These results suggest that these mushroom extracts at 1% or higher could induce growth retardation that eventually resulted to morphological abnormalities.

One of the most common morphological abnormalities in teratogenic evaluation is tail malformation. In this study, tail malformations such as bent tail and hook-like tail were the most obvious teratogenic effect of the two mushrooms extracts. The percentage tail malformation of embryos after 72 hours exposure in the different concentrations of extracts is depicted in **Table 2**. Apparently, tail malformation was observed in embryos at 2.5% or higher concentrations of LSE and 1% of POE which again indicate concentration dependent. However, tail malformation was not observed at 2.5% or higher concentration of POE due to early arrested growth of embryos.

Evidently, ethanolic extracts of *L. sajor-caju* and *P. ostreatus* revealed significant teratogenic effects in *D. rerio* embryos, indicating the presence of bioactive substances which are necessary to elucidate and characterize for further studies. In some

other basidiomycetes, bioactive organogenesis of cultured mouse embryos compositions exhibited teratogenic effects [12] and lyophyllin from *Lyophyllum* in other animal model. The hypsin from *shimeji* at a concentration as low as 50 $\mu\text{g/ml}$ can induce embryonic abnormalities *Hypsizigus mamoreus* induces abnormal embryonic development during in mouse [13].

Extract (%)	Mortality (%)			
	12 h	36 h	24 h	48 h
<i>L. sajor-caju</i> (LSE)				
0.0	0.00 ^c	0.00 ^c	0.00 ^c	0.00 ^c
1.0	0.00 ^c	0.00 ^c	0.00 ^c	0.00 ^c
2.5	16.67 ^b	16.67 ^b	16.67 ^b	33.33 ^b
5.0	33.33 ^a	83.33 ^a	83.33 ^a	83.33 ^a
<i>P. ostreatus</i> (POE)				
0.0	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^c
1.0	0.00 ^b	0.00 ^b	0.00 ^b	16.67 ^b
2.5	100.00 ^a	100.00 ^a	100.00 ^a	100.00 ^a
5.0	100.00 ^a	100.00 ^a	100.00 ^a	100.00 ^a

In each extract, treatment means with the same letter of superscript are not significantly different from each other at 5% level of significance using LSD.

Extract (%)	<i>L. sajor-caju</i>	<i>P. ostreatus</i>
Hatchability (%)		
0.0	100.00 ^a	100.00 ^a
1.0	100.00 ^a	83.33 ^b
2.5	75.00 ^b	0.00 ^c
5.0	0.00 ^c	0.00 ^c
Delayed growth (%)		
0.0	0.00 ^b	0.00 ^b
1.0	8.33 ^b	83.33 ^a
2.5	91.67 ^a	NA
5.0	100.00 ^a	NA
Tail malformation (%)		
0.0	0.00 ^c	0.00 ^b
1.0	0.00 ^c	66.67 ^a
2.5	58.33 ^b	NA
5.0	83.33 ^a	NA

In each parameter, treatment means with the same letter of superscript are not significantly different from each other at 5% level of significance using LSD; NA, not applicable due to the early arrested growth of embryos

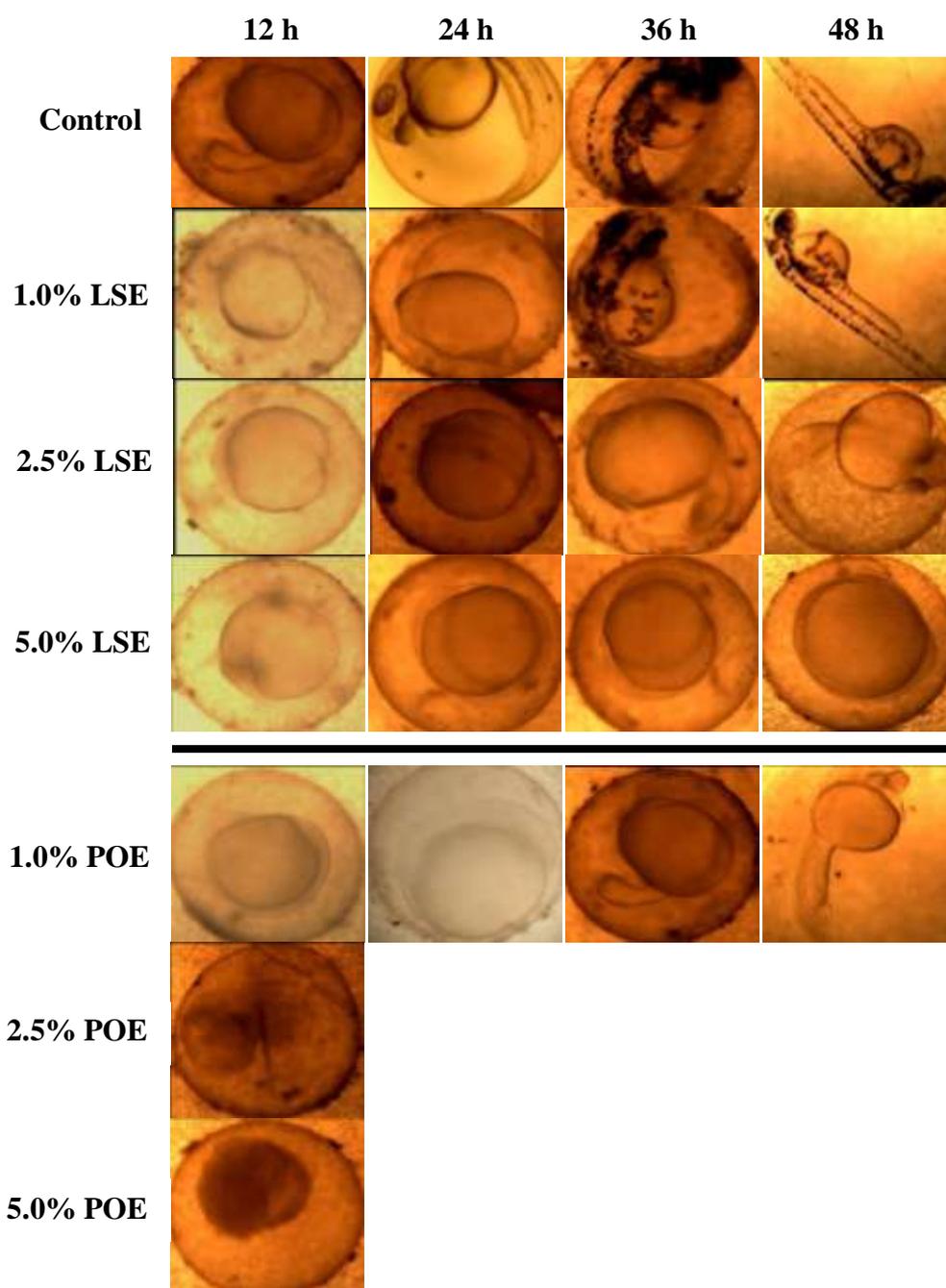


Figure 1: Morphological development of zebrafish embryo as affected by the three concentrations of *L. sajour-caju* (LSE) and *P. ostreatus* (POE) extracts every after 12 hours of treatment exposure. The obvious delayed growth of treated embryos was observed at 2.5% or higher concentrations of LSE and 1% of POE

CONCLUSION

Based on the results obtained in this study, it can be concluded that ethanol extracts of *L. sajour-caju* and *P. ostreatus* exhibited

toxic and teratogenic effects in the developing embryos of *D. rerio* as manifested by high percentage mortality, growth retardation, tail malformations, and

low success of hatchability, which strongly indicate that these two mushrooms could be important sources of toxic and teratogenic compounds that could ultimately be used in the development of anticancer drugs.

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