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Der Pharmacia Lettre, 2016, 8 (5):237-242
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

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 Zebrafish (*Danio rerio*)

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ABSTRACT

This work presents the teratogenic activity of the termite mound mushroom Termitomyces clypeatus extract on Danio rerio embryos. Hatched embryos treated with 0.1% and higher concentration of the mushroom extract significantly yielded higher mortality rate and delayed growth than the control embryos. The hatchability rate of embryos treated with 0.05% and higher concentrations of T. cleaptus extract was found to be significantly lower than that of the control. Apparently, the effect of extract to embryos is dose-dependent. The different phenotypic endpoints of embryos observed include wavy somite embryo, unhatched embryo with twisted tail tip, delayed development of embryo (still at segmentation phase), tail malformed embryo and coagulated embryo. Our results indicate that T. clypeatus contains biologically-active compounds that could induce teratogenicity in zebrafish embryos.

Keywords: *Termitomyces clypeatus, Danio rerio,* teratogens, medicinal mushroom, termite mound

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INTRODUCTION

Mushrooms, a culinary favorite to millions of people, also possess many other functional biological properties proven to be medically important. While the nutritional value of mushrooms is undisputed, thereare other attributes that are being discovered and studied especially in the area of their pharmacological potential. The so called medicinal mushrooms may possess anti-microbial, hypoglycemic, anti-inflammatory, anti-cancer, immunomodulator, and other beneficial related activities **[1, 2]**. The anti-cancer property of mushrooms for example is well documented through its cytotoxic activity in various primary cultures and cancer cell lines **[3-5]**, eventually providing a platform for identifying potential anti-cancer compounds. The other extreme attribute of mushrooms is that they can also be poisonous. Many mushrooms identified, mostly growing in the wild contain very potent compounds that can cause vomiting, nausea, numbness, abdominal cramps and even death **[6]**. There are also documented studies that while some mushrooms are popularly edible they may also possess cytotoxic and teratogenic properties at the same time **[3-5]**.

Termitomyces clypeatus is a wild edible basidiomycete that is found growing on the termite mounds. It is considered an obligate symbiont of termites belonging to the subfamily Macrotermitinae. Its mycelia grow on the termite fecal pellets. The fruiting body has elongated stipe from the termite nest and conical pileus with grayish brown to dark brown color. This mushroom is a favorite ingredient of numerous native delicacies such as *diningding* because of

the high umami flavor. Ilocanos called this as *oong bunton*, which means mound mushroom. To the best of our knowledge, there are no comprehensive reports on the bioactivity of *T. clypeatus*, thus, the conduct of this present study is imperative.

Recent trend in medicine is to assess these various properties of mushrooms using "omics" technologies **[7]**. However there is still a need to standardize and validate these methods across different laboratories. In many of these laboratories especially those in developing countries, conventional methods still remain the best alternative for many of investigations. One of the more attractive and popular conventional method to study toxicity and teratogenicity is the use of fish embryos. One technology in particular, the zebra embryo toxicity (ZET) test is considered an attractive, cost-effective and scientifically sound model for assessing and understanding the teratogenic mechanisms of compounds from natural sources like mushrooms **[8]**. Zebrafish because of its small size and transparent embryos offers many advantages in laboratory studies **[9-13]**. In fact, its ease of use and reliability has prompted many laboratories form a consortium and do collaborative investigations to harmonize the zebrafish developmental toxicity assay **[14, 15]**. The versatility of zebrafish as a model species for most developmental and toxicity studies proves that it is indeed an intermediate between the high throughput *in vitro* screening and the classical expensive mammalian model **[16]**. This current study demonstrated the toxic and teratogenic activities of *Termitomyces clypeatus* on the developing embryos of zebrafish.

MATERIALS AND METHODS

Source of mushroom

The healthy fruiting bodies of *T. clypeatus* were collected from a termite mound in multistorey agroforestry farm situated at the foot of Mt. Makiling Forest Reserve, Los Baños, Laguna, Philippines. The collected mushrooms were brush to remove the dirt and dust and air-dried for 3-5 days.

Extraction of bioactive components

The active components of the milled mushroom sample (20 g) were extracted in 600 mL hot water at 80–90°C in a water bath for 2 hours. The milled mushrooms were separated from the extract by filtration using filter paper No. 2 then filter sterilized through 0.45 µ filters. The filtrate was freeze-dried for 3 days to obtain the lyophilized form of extract. The yield of the extract was 7.9% (1.58 g)on a dry weight basis. The extract was used for embryo toxicity and teratogenic assay.

Spawning of *Danio rerio*

The protocol of this study was patterned after Nagel [10]. Adult zebrafish at 1 male: 2 female ratio were localized in a plastic mesh and the aquarium was covered with black plastic to induce spawning. After 12 hours incubation in the dark, eggs were exposed to lighted condition for another 12 hours. Fertilization occurs within 30 minutes after light is turned on. Embryos at segmentation phase, the fertilized eggs were siphoned out of the aquarium using a hose. Embryos were rinsed three times, placed in a watch glass with embryo water and observed under the compound microscope to examine uniformity and normal condition. Unfertilized oocytes and damaged embryos were discarded.

Embryo-toxicity and teratogenicity assay

The established procedure on embryo-toxicity and teratogenicity was patterned after Dulay et al. **[17]**. Three ml of each treatment concentration of *T.clypeatus* lyophilized extract prepared using embryo water as diluent (5%, 1%, 0.5%, 0.1%, 0.05% and 0.01%) and control (embryo water) were placed into each well of the 12-well ELISA plate. Four embryos were transferred into each well containing the different treatments. Triplicate per treatment concentration was done. The plates were incubated at 26°C ± 1°C. Teratogenic activity was examined after 12, 24, 36 and 48h of incubation using a compound microscope at 40X magnification. Morphological endpoint evaluation of zebra fish was based on the following parameters: lethal (coagulation, tail not detached, no somites, and no heart-beat), teratogenic(malformation of head, tail and heart, scoliosis, deformity of yolk, and delayed growth), and normal. Hatchability, delayed growth, malformation and mortality rates were recorded, and death was defined as coagulated embryos and as no visual heartbeat. All tests were carried out three times.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA). Treatment 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

Toxic and teratogenic effects of mushroom extract in *D. rerio* embryos

The toxic effect of lyophilized extract of *T. clypeatus* was assessed using *D. rerio* embryos at segmentation phase (12 hours post fertilization). The mortality rates of embryos after 48 hrs of exposure in different extract concentrations are shown in Fig 1. It can be seen that toxic effect of the extract was dose-dependent. All embryos in the control group and those treated with 0.01% of mushroom extract survived at 48-hours post treatment application (hpta). Although some embryos exposed to 0.05% of mushroom extract died, the result was statistically comparable with those in the control ($p>0.05$). However, those embryos treated with 0.1% or higher concentration of mushroom extract significantly registered higher mortality rate than the control embryos. Coagulated embryo as shown in Figure 5Fpresent images of some of the most toxic effects of the mushroom extracts used. These same extract concentrations (0.1% and higher), also significantly affected the survival of developing embryos of *D. rerio*. The effect of different concentrations of mushroom extract were clearly seen in the developing zebrafish embryos used since they grow very fast (only 48 hours) and react immediately to any toxins upon which they are exposed. This finding confirmed the potential of zebrafish as a powerful toxicity model.

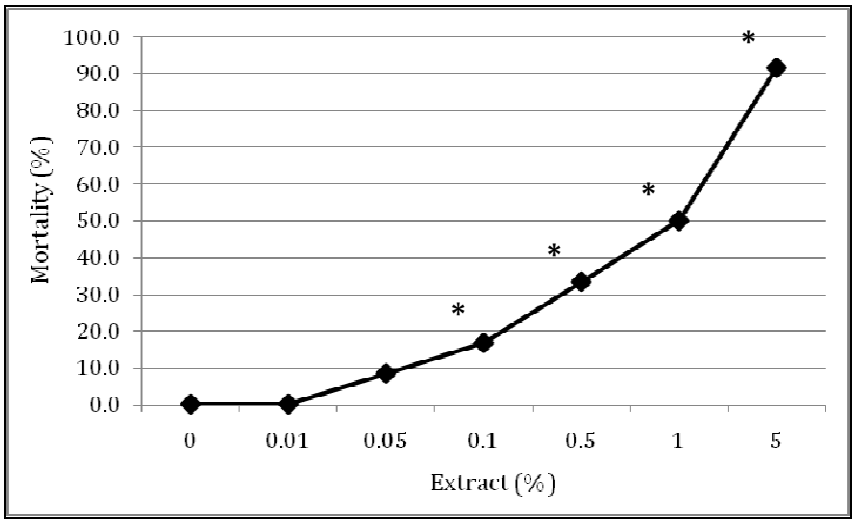

Fig. 1 Mortality rates of 48-hpta *D. rerio* embryos exposed to the different concentrations of *T. clypeatus* lyophilized extract. The death of an embryo was defined as coagulation and no visual heartbeat. The mortality rates of embryos treated with 0.1% or higher of mushroom-extract was significantly higher than that of the control embryos. *Represents significantly different from the control

Hatching is the process of breaking out of the chorion by the embryos that dictates a successful embryonic development. In this paper, the hatchability rates of embryos exposed at varying concentrations of mushroom extracts were studied and the results are presented in Fig 2. The control and 0.01%-treated embryos developed normally in embryo water medium and completed the hatching after 48 hpta. The hatchability rate of embryos treated with 0.05% or higher was significantly lower than that of the control. Some embryos at 0.05% and 0.1% completed the hatching process at 72 hpta due to the delayed development. Since delayed growth significantly affects the normal embryonic development, we also determined the effect of the extract on the growth retardation of embryos. The rate of delayed growth of embryos treated with the varying level of the extract at 24 hpta is shown in Fig 3. Embryos at 0.1% or higher concentrations of mushroom extract significantly demonstrated higher rate of delayed growth than that of the control embryos. Apparently, delayed growth in embryos is dose-dependent in this animal model.

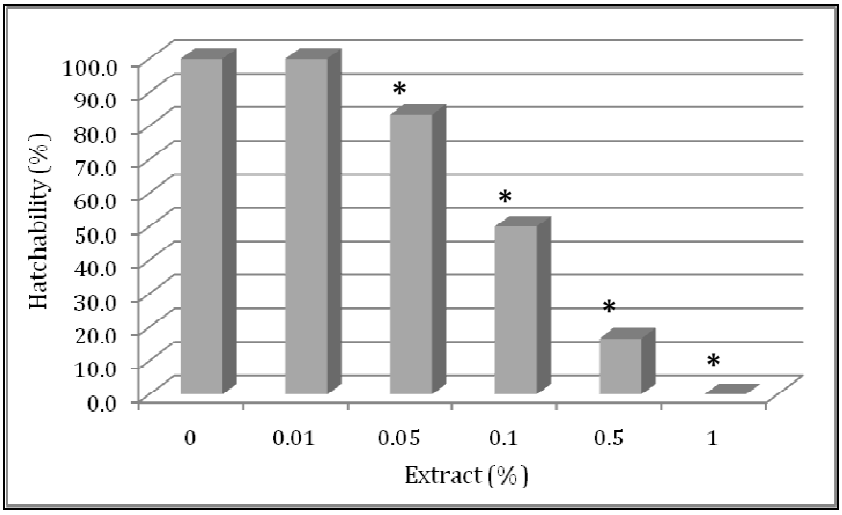

Fig. 2 Hatchability rates of 48-hpta *D. rerio* embryos exposed to the different concentrations of *T. clypeatus* lyophilized extract. The hatchability rates of embryos exposed at 0.05% or higher mushroom-extract were significantly lower than that of the control embryos. No hatched embryo was noted at 1% of extract. *Represents significantly different from the control

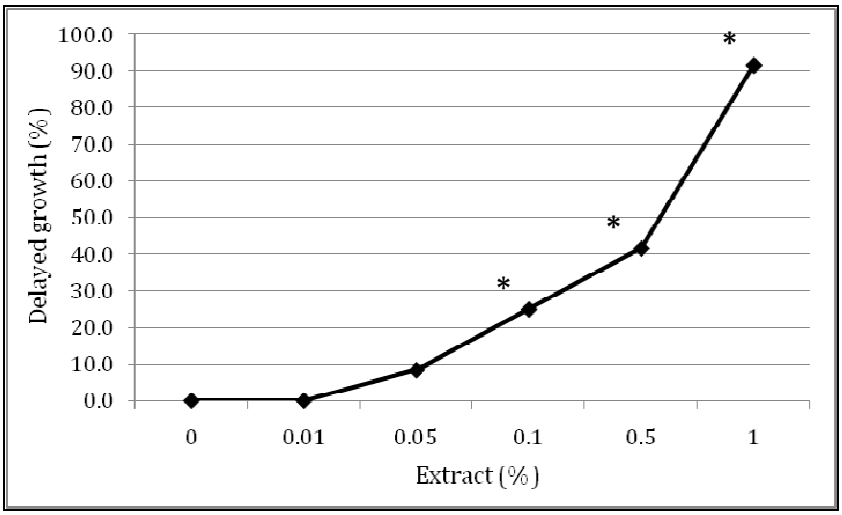

Fig. 3 Delayed growth rates of 24-hpta *D. rerio* embryos exposed to the different concentrations of *T. clypeatus* lyophilized extract. Delayed growthwas observed in embryos at 0.05% or higher concentrations of mushroom-extract. *Represents significantly different from the control

A variety of developmental abnormalities can be seen in *D. rerio* embryos after exposure to certain substances or compounds. One of the most distinct abnormalities observed in this present work is tail malformation. Fig 4 shows the rate of tail malformation of embryos treated with mushroom lyophilized extract at 72 hpta. Tail malformation was observed in embryos at 0.1% or higher concentrations and is shown to be dose-dependent. No tail malformation was observed at 0.05% or lower concentrations. The different phenotypic endpoints of embryos are depicted in Fig 5. These include wavy somite embryo, unhatched embryo with twisted tail tip, delayed development of embryo (still at segmentation phase), tail malformed embryo and coagulated embryo. It can be noted that these morphological endpoints of embryos are growth-delay related malformations.

Recently, toxic and teratogenic effects of many basidiomycetes captured interest and are now being subjected to various studies. In one study, *Lentinus tigrinus* water extract was proven to significantly reduced the hatchability of zebrafish eggs at 1% or higher concentrations, the heartbeat rate at 5% or higher concentrations, showed delayed development at 0.5%-10% concentrations and revealed different dysmorphologies such as tail malformation, pericardial edema and under-develop organs which were noted as growth-delay related endpoints **[18]**. In a related work, zebrafish embryos exposed to 2.5% and 5% concentrations of *Pleurotus ostreatus* ethanol extract (POE) significantly recorded 100% mortality after 12 hours whereas 5% *Lentinus sajor-caju* ethanol extract (LSE) with initial mortality of 33.33% was drastically raised to 83.33% after 24 hours to the last observation period **[19]**. 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. In *Ganoderma lucidum* treated embryos, however, the lethal effect of extract was both time and dose-dependent where the mortality rates of embryos treated with 5% (44.44%) or higher concentrations was significantly higher ($p > 0.05$) than that of the control embryos at 72 hpta. Tail malformation was obviously caused by 1% extract (55.56% tail malformation) and was observed in all embryos exposed to 5% of extract. Growth retardation was evident in embryos exposed to 5%, 10%, and 20% **[20]**. These observation was made possible due to the transparency of zebrafish embryo bodies which allow the direct observations of the tissue development *in vivo* **[15]**.

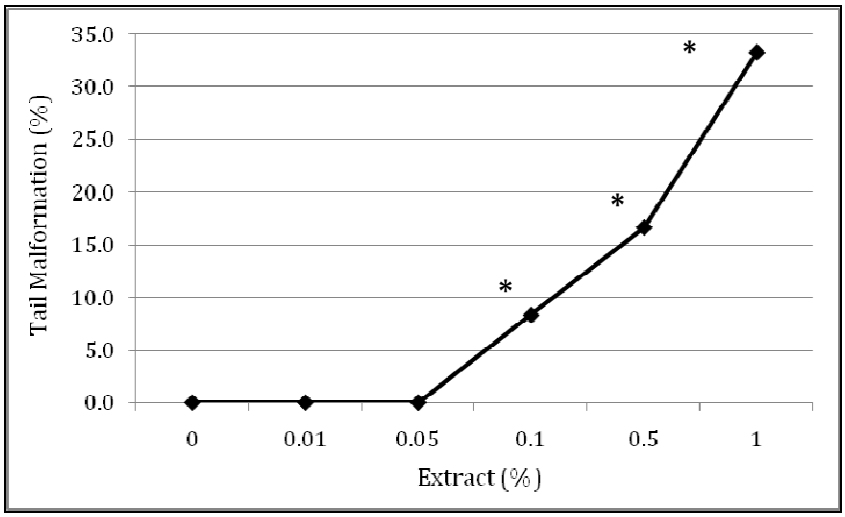

Fig. 4 Tail malformation rates of 72-hpta *D. rerio* embryos exposed to the different concentrations of *T. clypeatus* lyophilized extract. Bent tail was the most marked dysmorphology, was observed in embryos at 0.1% or higher concentrations of mushroom-extract. *Represents significantly different from the control

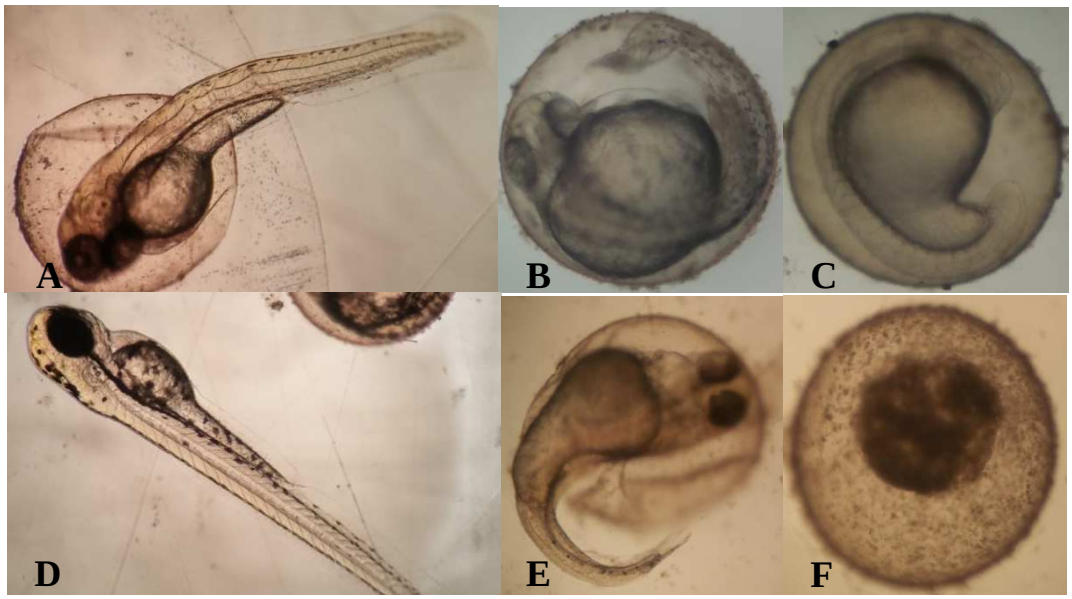

Fig. 5 Phenotypic endpoints of 48 hpta-embryos exposed to different concentrations of *T. clypeatus* extract. (A) wavy somite embryo at 0.5% extract, (B) unhatched embryo with twisted tail tip at 1% extract, (C) delayed development of embryo at 1% extract (still at segmentation phase), (D) normal control embryo, (E) tail malformed embryo at 1% extract and (F) coagulated embryo at 5% extract

Taken together, the results of this study demonstrated that *T. clypeatus* lyophilized extract exhibited toxic and teratogenic effects in developing *D. rerio* embryos as shown by the following parameters: mortality rates, delayed growth, tail malformations and other embryological abnormalities. Our data provide interesting results because edible mushrooms like *T. clypeatus* also possesses toxic and teratogenic properties. It is best to investigate further if the nutritive compounds in this mushroom are different from those that elicit toxic or teratogenic activities. More so, the potential heavy metal content of mushroom species must first be screened especially if they were collected in the forested areas adjacent to a mining site, small-scale or large-scale. Aside from this conventional method (ZET test), a complete phytochemical profile of the mushroom be established and together with the use of the "omics"

technology, particularly genomics and proteomics, a more comprehensive characterization of *T. clypeatus* can be achieved.

Conflict of Interests

The author(s) declare that there is no conflict of interest regarding the publication of this paper.

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