OPTIMAL SUBMERGED CULTURE CONDITIONS AND BIOACTIVITIES OF MYCELIA OF WILD MUSHROOMS, *Cyathus striatus (*HUDSON) WILDENOW, AND *Xylaria hongkongensis* (A.M.C. TANG, R.Y.C. LAM and M.W.K. LEUNG)

Janice S. Aguilar[1, 2]*, Rich Milton R. Dulay[1], Sofronio P. Kalaw[1]and Renato G. Reyes[1]
[1]Center for Tropical Mushroom Research and Development, College of Science,
Central Luzon State University, Science City of Muñoz, Nueva Ecija, 3120, Philippines
[2]Science Education Institute, Department of Science and Technology,
Bicutan, Taguig City, Philippines
*Corresponding author: janiceaguilar919@clsu.edu.ph

(Received: November 25, 2022; Accepted: May 5, 2023)

ABSTRACT

The Philippines is a rich source of mushrooms with promising nutritional and pharmacological potentials. To date, a number of Philippine wild mushrooms have been investigated for their growth requirements and bioactivity profile. However, several mushrooms remain to be undiscovered and unutilized. Hence, the mycelial biomass production of *Cyathus striatus*, and *Xylaria hongkongensis* in submerged conditions was evaluated and the bioactivities of their ethanolic extracts were assessed. Potato sucrose broth at pH 6-8 favored the mycelial biomass production of both mushrooms, while coconut water and rice bran broth were also favorable for *X. hongkongensis*. The maximum yield of *C. striatus* mycelia was obtained when incubated at 28°C, lighted, and agitated submerged fermentation. *X. hongkongensis* recorded higher mycelial biomass yield at 28°C, dark, and agitated conditions. Mushroom extracts exhibited antibacterial activity against *Staphylococcus aureus*, but not against *Escherichia coli*. Both extracts showed toxic effects in brine shrimp with LC_{50} values of 134.90 µg/ml (*C. striatus*) and 141.25 µg/ml (*X. hongkongensis*). In zebrafish assay, 1000 µg/ml or higher concentrations of extracts showed 100% mortality of embryos after 48 hours of exposure, and no hatchability, indicating embryotoxicity and cytotoxicity. Extract-treated embryos also showed different morphological abnormalities, indicating teratogenic properties. The maximum yield of mycelial biomass of the two mushrooms can be obtained in optimized culture conditions, which can be sources of bioactive compounds with pharmacological properties.

Key words: mushrooms, antibacterial, cytotoxic, liquid media, mycelia

INTRODUCTION

Mushrooms are saprotrophic organisms that demonstrate remarkable nutritional and medicinal potential. They are highly considered one of the most valued crops due to the number of benefits they can offer to humanity. Edible fungi such as the group of oysters, enoki, shiitake, straw, and button mushrooms contain ample amounts of protein, vitamins, minerals, and carbohydrates (Nhi and Hung 2012; Eguchi et al. 2015). Apart from their nutritional attributes, mushrooms contain agents that can treat or prevent the emergence of serious health problems such as hypertension, cancer, diabetes, and diseases associated with microbial infection (Chaturvedi et al. 2018). Considering these numerous

capabilities of mushrooms, attention must be given to every species of mushroom to broaden the resources of excellent bioactive compounds.

Most of the wild mushrooms are observed when there is enough humidity in the environment. One of the wild mushrooms is *Cyathus striatus*, which is commonly known as a bird's nest (Zhao et al. 2008). It has tiny fruiting bodies with egg-like structures inside the nest-like fruiting body. This mushroom is usually observed in clusters attached to decaying wood or soil. It is capable of enhancing nerve growth factors and has anti-neuro-inflammatory activity since it contains neuroprotective agents (Bai et al. 2015; Yin et al. 2019). It has incredible anticancer potential as its low extract concentration inhibits cell proliferation within a short period of time (Sharvit et al. 2012). *Xylaria hongkongensis* is another species of mushroom with unique morphological characteristics wherein its pointed structure resembles a hardened coral. Mushrooms under the genus *Xylaria* contain a wide range of biologically active compounds with antioxidant, antimicrobial, and anti-cancer potentials (Changi 2015; Adnan et al. 2018). However, there is very little information regarding the optimized condition for the successful propagation of these mushrooms. Understanding the growth preferences will therefore lead to the establishment of efficient production technology.

Solid-state fermentation is the most common technology for the cultivation of mushroom fruiting bodies in the Philippines. However, this technique might not be suitable for all species of mushrooms. Some mushrooms take a longer incubation period in order to produce fruits. Submerged cultivation which involves the use of liquid media is a reliable technique that can be used to produce mycelial biomass. The fruiting body and mycelia can be good sources of bioactive compounds and can exhibit biological properties. Therefore, liquid cultivation could be used as an alternative technology that can provide a sufficient amount of mycelia.

Mycelia, as the vegetative part of mushrooms, require nutrients and appropriate environmental conditions such as temperature, agitation, and illumination. Some species of mushrooms can produce high biomass yield in a wide range of environmental conditions, others can tolerate extreme surroundings while other species have a very specific requirement. These factors clearly determine successful mycelial development, which varies depending on the species.

Many attempts have been made to culture the fruiting bodies of *C. striatus* and *X. hongkongensis*. However, the Center for Tropical Mushroom Research and Development at Central Luzon State University was not successful in culturing the fruiting bodies of these mushrooms. Since it is of utmost importance to establish the position of *C. striatus* and *X. hongkongensis* in the mushroom industry, submerged cultivation was utilized as the cultivation technique to mass produce the mycelial biomass for the screening of their biological properties. The optimal culture conditions for the maximum production of mycelia of the two mushrooms were established in this study.

MATERIALS AND METHODS

This study was conducted at the Center for Tropical Mushroom Research and Development (CTMRD) Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines, from January to March 2022.

Source of mycelial discs. Pure cultures of *C. striatus* and *X. hongkongensis* were obtained from the culture collection of the CTMRD. To prepare the source of inoculant, mycelial blocks from the pure culture were aseptically inoculated into potato dextrose agar plates and incubated for 7 days. Mycelial discs were prepared using a cork borer,

Evaluation of culture media and pH. The optimum liquid culture media for the mycelial biomass production of *C. striatus* and *X. hongkongensis* was determined using four indigenous liquid media namely corn grit broth (CGB), potato sucrose broth (PSB), rice bran broth (RBB), and coconut water

(CW) (Dulay et al. 2022). Each medium was prepared by boiling rice bran, corn grits, and potato in water separately with the ratio of 50g rice bran: 1L water, 50g corn grits: 1L water, and 250g diced potato: 1L water. After boiling, 10g of table sugar was added to the decoction. Prior to sterilization, the prepared broth was adjusted to pH 6.5. Thirty ml of the previously prepared liquid culture media was transferred into clean bottles and covered with plastic. The bottles were sterilized in an autoclave (KT-3065A, ALP Co., Ltd., Japan) for 30 minutes at 15 psi and 121°C. The bottles were allowed to cool after sterilization and a mycelial disc was aseptically inoculated into each bottled medium. All bottles were incubated at room temperature to allow mycelial growth. Mycelial biomasses were harvested after seven days of incubation, air-dried for three days, and weighed using an analytical balance.

PSB was then used to determine the optimum pH requirement of the mushrooms. Varying pH levels of PSB were prepared (4, 5, 6, 7, 8) using 1 M NaOH or 1 M HCl and adjusted using a pH meter (ST20, Ohaus, USA). Then, 30 ml of the medium was placed in clean bottles covered with plastic, and secured with a rubber band. The mycelia were harvested after seven days of incubation, air-dried, weighed, and recorded.

Effects of physical factors. After determining the optimum pH for the mycelial biomass production of the two mushrooms, the physical factors: temperature, illumination, and agitation were evaluated. To determine the optimum temperature, the culture bottles containing PSB adjusted to pH 7 were inoculated with mycelial discs and incubated at three temperature conditions; refrigerated (8°C), air-conditioned (21°C), and room temperature (28°C). After determining the temperature preference of the mushrooms, the influence of light was assessed by incubating the culture bottles under lighted and dark conditions. The culture bottles were covered with black paper for the dark condition while the other culture bottles were exposed to artificial light (137 lux). The culture bottles were incubated in agitated (100 rpm) and static conditions with the optimum illumination and temperature conditions to determine the influence of agitation on the mycelial biomass production of the mushrooms. Harvesting of mycelial biomasses was done after seven days followed by air-drying and weighing. The optimum temperature, illumination, and agitation were determined based on the mycelial biomass yield after seven days of incubation.

Mass production of mycelia. This was carried out by inoculating mycelia discs into culture bottles containing 30 ml of PSB at pH 7. The bottles were incubated under the optimum temperature, illumination, and agitation conditions. Fifty replicates of mycelial cultures for each mushroom were prepared. The mycelial biomasses were harvested and air-dried, after seven days of incubation.

Ethanolic extraction. The bioactive compounds of the mycelia were extracted following the method of Dulay et al. (2021) with some modifications. The dried mycelia were powdered using a blender, and then soaked in 95% ethanol for 48 hours. Filtration was done using Whatman filter No.2 and concentrated to dryness using a rotary evaporator (RV 10 D S99, IKA Germany).

Antibacterial assay. Two bacterial pathogens namely: *Staphylococcus aureus* (UPLB BIOTECH 1582) and *Escherichia coli* (UPLB BIOTECH 1634) in disc-diffusion method were used. Microbial cultures were prepared in a 9 ml nutrient broth medium and the turbidity was compared to 0.5 McFarland standard. Assay plates were swabbed with the bacterial inoculum and a 6-mm sterile paper disc previously soaked in mycelial extracts was placed on the surface of the medium. Streptomycin sulfate was used as the positive control and ethanol as the negative control. The zone of inhibition was measured using a Vernier caliper after 24 hours of incubation.

Brine shrimp lethality assay. The cytotoxicity of the extract was assessed using brine shrimp (*Artemia salina*). Larvae were prepared by hatching the eggs in artificial seawater (25 g salt in 1 L dH_2O). Varying concentrations of the extract were prepared (1, 10, 100, 1000, and 10,000 µg/ml) in vials, then ten larvae were placed in vials containing the treatments. Brine solution was used as control. The

number of dead nauplii was recorded after 24 hours and the percentage mortality was calculated. Lethal concentration (LC_{50}) was computed using Probit analysis and interpreted using the following: LC_{50} values > 1000 µg/ml (non-toxic), ≥ 500 ≤ 1000 µg/ml (weak toxic), and < 500 µg/ml (toxic) (Bastos et al. 2009).

Toxicity and teratogenicity assay. The method of toxicity and teratogenicity using zebrafish embryos used by Dulay et al. (2012) was followed. Ten ml of each treatment concentration of the extract was prepared using embryo water as a diluent (1 µg/ml, 10 µg/ml, 100 µg/ml, 1000 µg/ml, and 10000 µg/ml) and control (embryo water) and placed into each well of the 12-well ELISA plate. Three embryos at the segmentation phase were transferred into each well containing the different treatments. The plate was incubated at 26°C ± 1°C. The teratogenic effect was examined using a dissecting microscope after 48 hours of incubation. Morphological endpoint evaluation of zebrafish was based on the parameters established by Nagel (2002): Lethal (coagulation, tail not detached, no somite, and no heart-beat): teratogenic (malformation of head, tail, and heart, scoliosis, deformity of yolk, and growth retardation): and normal. Percentage hatchability and mortality were determined. A test is classified as valid if 100% of the embryos in the control group show normal conditions.

Statistical analysis. All treatments were laid out in a Complete Randomized Design (CRD). Data were analyzed using Analysis of Variance (ANOVA) and compared using Tukey's HSD at 5% level of significance. Treatment means in illumination and shaking conditions were compared using paired T-tests. The two mushrooms were not compared since they are of different species.

RESULTS AND DISCUSSION

Effect of liquid culture media and pH. The successful and luxurious growth of mushroom mycelia is dependent primarily on the nutritional composition of the substrate. The mean mycelial biomass yield of *C. striatus* and *X. hongkongensis* after seven days of incubation in the different liquid culture media is presented in Table 1. The highest mycelial biomass yields were recorded significantly in PSB with 73.60 mg/30 ml (*C. striatus*) and 136.33 mg/30 ml (*X. hongkongensis*). Aside from PSB, CW and RBB were also found favorable for the mycelial growth of *X. hongkongensis*. In contrast, CGB registered the lowest biomass yield in both mushrooms.

Table 1. Mycelial biomass yield of *C. striatus* and X. *hongkongensis* grown in liquid culture media after seven days of incubation.

Culture media	Biomass yield (mg/30 ml)	
	C. striatus	***X. hongkongensis***
Potato sucrose broth	73.60±13.98[a]	136.33±12.61[a]
Corn grit broth	34.55±3.35[b]	62.93±5.51[b]
Coconut water	41.90±8.19[b]	132.20±22.00[a]
Rice bran broth	29.60±35.80[b]	132.73±9.15[a]

In each column, means with the same superscript are not significantly different from each other at 5% level of significance. Potato sucrose broth served as the control medium.

The suitability of PSB could be attributed to the nutritional component of potato that favors the efficient development of their mycelia. Potato contains high amounts of carbohydrates, proteins, vitamins, minerals, fructose, sucrose, and folic acid (Burlingame et al. 2009). Carbon and nitrogen are the primary nutrients needed by mushrooms, and the presence of other nutrients like potassium, phosphorus, manganese, iron, selenium, magnesium, copper, and molybdenum have positive effects on their growth (Chang and Miles, 2004). Mushrooms prefer the type of carbon, sugar, and other nutrients

that are present in potato sucrose broth than the other media used allowing their mycelia to grow luxuriantly. *Xylaria hypoxylon* and other *Xylaria* species also prefer potato dextrose agar (Ramesh et al. 2014; Ahmed and Jahan 2017) Meanwhile, *Xylaria nigripes* produced optimum biomass yield in media with fructose and yeast extract supplemented with magnesium sulfate heptahydrate, nitrogen, and some minerals (Chen et al. 2014). However, there were no published reports regarding the mycelial biomass production of *C. striatus* to compare with the results of this study. Other species of mushrooms that prefer potato as the carbon source include *Pleurotus floridanus* (Khan, 2017) and *V. volvacea* (Kalaw et al. 2016).

The mycelial biomass yield of the two mushrooms is influenced by pH (Table 2). Potato sucrose broth at pH 7 registered the highest yield of mycelial biomass with a mean value of 136.37 mg/30 ml and 140.43 mg/30 ml for *C. striatus* and *X. hongkongensis,* respectively. However, those biomass yields were found statistically comparable with those at pH 6 and 8 which also produced high yields. pH levels lower than pH 6 produced a low biomass yield of both mushrooms. The response of *X. hongkongensis* to illumination coincides with the findings for *Cyclocybe cylindracea, Pleurotus djamor,* and *Pleurotus salmoneostramineus,* which did not show sensitivity to light (Landingin et al. 2020; Jacob et al. 2015). The response of *X. hongkongensis* to illumination coincides with the findings for *Cyclocybe cylindracea, Pleurotus djamor,* and *Pleurotus salmoneostramineus,* which did not show sensitivity to light (Landingin et al. 2020; Jacob et al. 2015).

Table 2. Mycelial biomass yield of *C. striatus* and *X. hongkongensis* grown in PSB with varying pH levels after seven days of incubation.

pH	Biomass yield (mg/30 ml)	
	C. striatus	***X. hongkongensis***
4	90.70±4.61^{b}	50.77±8.24^{b}
5	99.10±26.10^{b}	86.37±16.27^{b}
6	102.70±8.60^{ab}	130.00±2.00^{a}
7	136.37±4.13^{a}	140.43±12.11^{a}
8	135.00±20.70^{a}	133.97±8.24^{a}

In each column, means with the same superscript are not significantly different from each other at 5% level of significance.

The differences in the yield among varying pH levels could be explained by the direct effect of the hydrogen ions in the media. pH affects the entry of sodium ions and necessary molecules present in the media to the individual cell (Elisashvili 2012). It can limit the absorption capacity of the cell membrane leading to poor mycelial growth since the necessary nutrients are not provided (Deacon 2006). These results are in consistent with earlier findings where pH 6-7 was found to be the optimum pH for most wild mushrooms collected in Central Luzon namely: *G. lucidum, L. tigrinus*, and *Coprinopsis cinerea* (Kalaw et al. 2016), Likewise, the mycelia of *Ganoderma applanatum* showed excellent growth in pH 6 – 9 (Jo et al. 2009). Previous reports on the pH preferences of mushrooms under the genus *Xylaria* obtained different results. These differences in pH preferences of mushrooms though under the same genus are possibly correlated to the location and type of substrate from where they are collected. *X. nigripes* in China preferred pH 5 (Chen et al. 2014): *X. hypoxylon* collected in Bangladesh was favored at pH 6 (Ahmed and Jahan 2017) while another strain of *Xylaria* species from India showed the highest biomass yield at pH 5.5 (Ramesh et al. 2014).

Effect of physical factors. The most suitable media with the best pH were used to evaluate the influence of physical factors such as temperature, illumination, and agitation on the mycelial biomass production of the two mushrooms. To efficiently exploit mushroom biomass, it is necessary to understand the relationship between these environmental factors and biomass production. Three different temperature conditions including 8°C, 21°C, and 28°C were used in this study in order to understand which of these conditions can support the better growth of the two mushrooms. Table 3 presents the mean biomass yield after 7 days of incubation to different physical factors. Cultures incubated at 28°C significantly recorded the highest mycelial biomass yield of both mushrooms, indication that the two mushrooms are tropical species. However, lower yield was obtained at 21 °C and no biomass growth was observed at 8 °C. Other species under the genus *Xylaria* showed optimum biomass production under 28°C -30°C such as *Xylaria* sp. (Ramesh et al. 2014) and *X. hypoxylon* (Ahmed and Jahan, 2017).

Table 3. Mycelial biomass yield of *C. striatus* and *X. hongkongensis* grown in PSB at pH 7 as affected by temperature, illumination, and agitation after seven days of incubation.

Physical factors	**Biomass yield (mg/30 ml)**	
	C. striatus	***X. hongkongensis***
Temperature		
8°C	0.00±0.00^{c}	0.00±0.00^{c}
21°C	106.63±11.65^{b}	145.90±44.64^{b}
28°C	205.80±19.00^{a}	183.80±1.20^{a}
Illumination		
Lighted	283.62±14.70^{a}	201.39±51.72^{b}
Dark	234.50±22.50^{b}	249.40±37.20^{a}
Agitation		
Agitated	407.62±65.91^{a}	266.11±12.91^{a}
Static	199.51±7.87^{b}	236.40±9.33^{b}

In each column, means with the same superscript are not significantly different from each other at 5% level of significance.

Illumination is crucial to the physiological processes of all organisms including mushrooms, some species rely on the availability of light for successful mycelial proliferation (Cheng et al. 2012). The culture bottles of *C. striatus* incubated at 28 °C under lighted conditions produced a higher yield (283.6 mg/30ml) compared to mycelial cultures incubated in the dark. The presence of light has a positive impact on the biomass production of *C. striatus*. Illumination helps greatly in regulating the fermentation process which in turn enhanced the mycelial biomass production of fungi (Cheng et al. 2012). This study supports the findings of other related studies (Smania et al. 1997; Kalaw et al. 2016). However, dark conditions favored biomass production of *X. hongkongensis* (249.4 mg/30ml). The response of *X. hongkongensis* to illumination coincides with the findings for *Cyclocybe cylindracea, Pleurotus djamor,* and *Pleurotus salmoneostramineus*, which did not show sensitivity to light (Landingin et al. 2020; Jacob et al. 2015).

Agitation (100 rpm) improved the yield of both mushrooms which produced 407.6 mg/30 ml (*C. striatus*) and 266. 1 mg/ 30 ml (*X. hongkongensis*). The mycelial biomass of *C. striatus* and *X. hongkongensis* in their optimized submerged culture conditions are shown in Fig. 1.

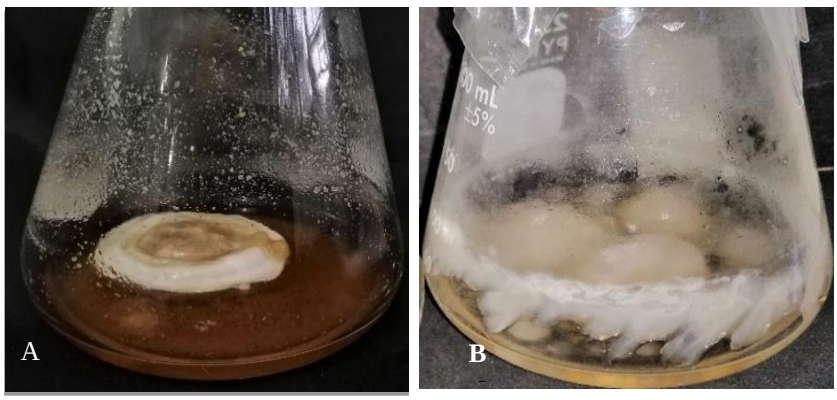

Fig. 1. Mycelial biomass of *C. striatus* (A) and *X. hongkongensis* (B) in an optimized submerged culture condition after seven days of incubation.

A significant increase in the biomass yield in agitated condition could be attributed to shaking of the media which resulted in the efficient distribution of nutrients and oxygen to the entire network of mycelia (Cui et al. 1997; Peng et al. 2000). There is a positive correlation between biomass growth and energy dissipation in liquid fermentation (Kelly et al. 2004). Similarly, the agitation has a positive effect on *Xylaria nigripes* which produced a high biomass yield in a shaker at 100 rpm (Chen et al. 2014). A considerable increase in biomass production, glucose consumption, and lipid biosynthesis was observed in different mushroom species including *P. ostreatus*, *G. lucidum*, *Auricularia auricula*, and *Lentinus edodes* in shaking conditions (Diamantopoulou et al. 2012).

Antibacterial activity of mycelial extracts. The ethanolic extracts of *C. striatus* and *X. hongkongensis* mycelia were assessed for their antibacterial potential against Gram-negative, E. *coli*, and Gram-positive *S. aureus* bacteria. The mean diameter zones of inhibitions after 24 hours of incubation are shown in Table 4. The two extracts showed inhibitory activity against *S. aureus* with a mean of 11.93 mm diameter zone of inhibition for *X. hongkongensis* and 11.27 mm diameter zone of inhibition for *C. striatus* while no inhibitory activity was observed against *E. coli* (Fig. 2).

Table 4. Antibacterial activities of *C. striatus* and *X. hongkongensis* mycelial extract against *E. coli* and *S. aureus.*

Treatments	Zone of inhibition (mm)	
	E. coli	***S. aureus***
C. striatus extract	6.00±0.00^{b}	11.27±0.55^{b}
Streptomycin sulfate	32.33±0.45^{a}	33.13±0.15^{a}
Ethanol	6.00±0.00^{b}	6.00±0.00^{c}
X. hongkongensis extract	6.00±0.10^{b}	11.93±0.15^{b}
Streptomycin sulfate	32.33±0.45^{a}	33.13±0.15^{a}
Ethanol	6.00±0.00^{b}	6.00±0.00^{c}

In each column, means with the same superscript are not significantly different from each other at 5% level of significance. Streptomycin sulfate served as positive control and ethanol served as negative control.

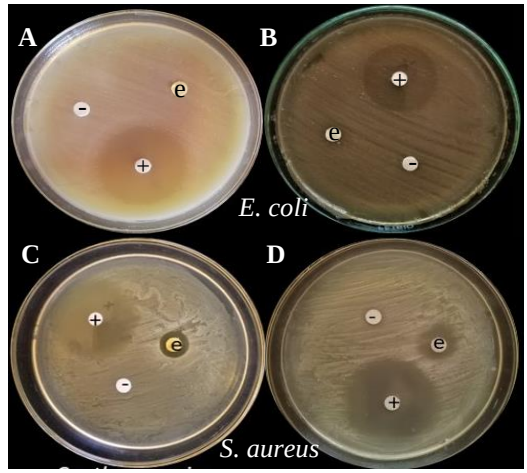

Fig. 2. Antibacterial assay plates showing the zone of inhibitions exhibited by *C. striatus* (A, C) and *X. hongkongensis* (B, D) mycelial extract against *E. coli* and *S. aureus.*

C. striatus and *X. hongkongensis* are capable of suppressing the growth of pathogenic bacteria. Previous studies demonstrated that mushrooms naturally produce some novel secondary metabolites with known antibacterial properties such as triterpenes, alkaloids, and anthrones (Chudzik et al. 2015). Related studies on *Xylaria* species revealed the antibacterial potential of these mushrooms. For example, *X. papulis* demonstrated an intermediate effect against *S. aureus* and *E. coli* (De Leon et al. 2020). Another *Xylaria* extract inhibited the growth of *Pseudomonas aeruginosa* and *S. aureus* (Ramesh et al. 2014). Ten *Xylaria* isolates inhibited the growth of *S. aureus,* Bacillus *subtilis, Pseudomonas aeruginosa,* and *Escherichia coli* (Orachaipunlap et al. 2015). Other mushrooms under the genus *Cyathus* including *C. intermedius, C. colensoi,* and *C. pallidus* possess antibacterial activity against *E. coli*, *Mycobacterium tuberculosis, Staphylococcus albus, Streptococcus hemolyticus α, Streptococcus hemolyticus β* and *Streptococcus pneumonia* (Liu and Zhan 2004; Shakouri et al. 2014).

Cytotoxic activity of mycelial extracts. Mushrooms are good resources for bioactive compounds with promising anti-cancer activity. Brine shrimp lethality assay, a preliminary step to a more advanced study of cell toxicity, can be used to reveal anticancer activity. Both extracts were toxic to brine shrimp larvae with LC_{50} values of 134.90 µg/ml for *C. striatus* and 141.25 µg/mm for *X. hongkongensis*. Although the mushroom extracts demonstrated toxicity, their efficacy is incomparable to cyclophosphamide, an anticancer drug, which is cytotoxic at a concentration of 16.30 µg/ml (Moshi et al. 2010). Both extracts were more toxic than *X. hypoxylon* and *X. papulis* extracts (Ahmed and Jahan 2017; De Leon et al. 2020). In addition to this, *Cyathus subglobisporus* and *C. striatus* showed great potential in inhibiting the proliferation of cancer cells (Nitthithanasilp et al. 2018; Sharvit et al. 2017). *Xylaria curta* and other *Xylaria* species also exert antitumor activities against lung cancer cells (Orachaipunlap et al. 2015; Ramesh et al. 2015). The cytotoxic activities of these mushrooms can be linked to the secondary metabolites they contain such as terpenoids, steroids, flavonoids, and phenolic compounds (Ramesh et al. 2015). Indolic compounds, striatins C and cyathins from *C. striatus* demonstrated strong activity against human breast and pancreatic cancer cells (Sharvit et al. 2021; Fares et al. 2022). Although indole derivatives exhibited strong cell growth inhibition ability, these can be used in anticancer drug development since they are not toxic to normal cells (Fares et al. 2010). *X. allantoidea* was found to contain several secondary metabolites such as allantoside which demonstrated antiproliferative activity (McCloskey et al. 2017). However, the compounds responsible for the cytotoxic activity of *C. striatus* and *X. hongkongensis* used in this study are still unknown. Further studies need to be conducted in order to investigate the mycochemical constituents and their mechanisms of action.

Teratogenic and toxic effects of *C. striatus* and *X. hongkongensis* extract. The use of zebrafish embryos is an efficient way to assess the teratogenic activity of certain compounds in humans. The morphological abnormalities can be readily observed due to the transparency and visibility of the internal structures; aside from that, its genetic composition is almost similar to the human genome (Howe et al. 2013). Some compounds can negatively affect the processes during embryonic development which in turn alters the morphological characteristics of the organism and is obviously shown as it matures (Elefant et al. 2020).

In this study, the hatching rate was found to be dependent on the concentration of the extracts (Table 5). Control embryos hatched normally while a lower hatching rate was observed in groups directly exposed to the different concentrations of the extracts after 72 hours. The highest number of embryos hatched at 1 µg/ml, with 88.91 % (*C. striatus*) and 77.85 % (*X. hongkongensis*) being not significantly different from the control group. However, embryos exposed to 10 µg/ml concentration of the extracts showed 44.4% and 33.3% hatching rates. A much lower number of embryos hatched at 100 µg/ml extract concentration (22.2 % for *C. striatus* and 11.1 % for *X. hongkongensis*) however, no embryos hatched at 1000 and 10,000 µg/ml suggesting that the extracts have a dose-dependent effect on the hatchability of zebrafish embryos.

Table 5. Hatchability rate of zebrafish embryos at different concentrations of the mushroom extracts after 72 hours.

Mushroom Extract (µg/ml)	% Hatchability	
	C. striatus	***X. hongkongensis***
Embryo water	100.00±0.00^{a}	100.00±0.00^{a}
1	88.91±19.27^{a}	77.85±38.59^{ab}
10	44.41±19.28^{b}	33.34±0.00^{bc}
100	22.28±19.23^{bc}	11.17±19.27^{c}
1,000	0.00±0.00^{c}	0.00±0.00^{c}
10,000	0.00±0.00^{c}	0.00±0.00^{c}

In each column, means with the same superscript are not significantly different from each other at 5% level of significance. Embryo water served as the control.

Growth retardation was observed among the embryos as a result of direct exposure to the mushroom extracts (Fig. 3B-E). It is possible that the mushroom extracts inhibit enzymes responsible for hatching (David and Pancharatna 2009). Aside from growth retardation, embryos with bent tail were observed in 100 µg/ml concentration of *C. striatus* extract (Fig. 4F). These morphological abnormalities are considered teratogenic effects of the extracts (Nagel, 2002). Tail malformations (perverted tail, hook-like tail, twisted tail) wavy somite, pericardial edema, and head deformation are the other reported morphological abnormalities caused by other mushroom extracts (Dulay et al. 2012; Dulay et al. 2014; De Castro et al. 2016; Sogan et al. 2018). Natural teratogenic compounds from mushrooms trigger irregular circulation of blood, limiting the cells with the necessary nutrients it needs to function properly, similar to known teratogens like hydroxyurea, retinoic acid, and valproic acid. It also affects the expression of genes responsible for the production of glucose, triglycerides, and cholesterol (Miao et al. 2022). Thus, these results demonstrated that exposure of zebrafish embryos to *C. striatus* and *X. hongkongensis* mycelia extracts can lead to undesirable morphological conditions.

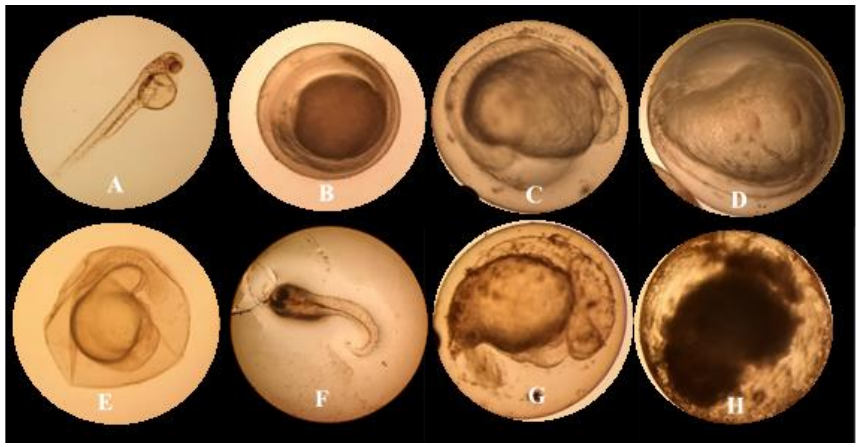

Fig. 3. Teratogenic and toxic effects of *C. striatus*. and *X. hongkongensis* extracts on zebrafish embryos after 72 hours. Normal hatched embryo (A), growth retardation at 1 µg/ml to 1000 µg/ml extract concentration (B-E), Bent tail at 100 µg/ml extract concentration of *C. striatus* (F), coagulated embryo (G, H)

Embryo mortality (100 %) was observed at 10, 000 µg/ml concentration of both extracts after 12 hours (Table 6). Increasing mortality was observed in 1000 µg/ml as a result of prolonged exposure to the extracts. However, dead embryo was not observed in 1 to 100 µg/ml concentrations of both extracts in all observation periods except for 100 µg/ml concentration of *X. hongkongensis* which caused 11.11 % mortality after 48 hours. These results indicate that high concentrations and prolonged exposure of zebrafish embryos to *C. striatus* and *X. hongkongensis* extracts have lethal or toxic effects (Nagel et al, 2002).

Table 6. Mortality of zebrafish embryos exposed to the different concentrations of mushroom extracts after 12, 24, and 48 hours.

Mushroom Extract (µg/ml)	**% Mortality**		
	12H	**24H**	**48H**
C. striatus			
Embryo water	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
1	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
10	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
100	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
1000	11.11±19.20^{b}	88.90±19.20^{a}	100.00±0.00^{a}
10000	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}
X. hongkongensis			
Embryo water	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}
1	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}
10	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{b}
100	0.00±0.00^{c}	0.00±0.00^{b}	11.11±19.20^{b}
1,000	33.33±0.00^{b}	88.99±19.20^{a}	100.00±0.00^{a}
10,000	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}

In each column, means with the same superscript are not significantly different from each other at 5% level of significance. Embryo water served as the negative control

The introduction of various chemicals to the embryonic system can cause obstruction in blood flow leading to the malfunctioning of the different organs (Wang and Zhong 2020). Coagulation observed at high concentrations and longer exposure time could be due to the activity of toxic compounds present in the extracts. Some active compounds require a longer time of accumulation in the cell before taking effect (Abdel-Tawab 2021). Results obtained in this study validate the findings for *X. papulis* ethanol extract which caused heart problems in zebrafish embryos (De Leon et al. 2020). These mushrooms are natural sources of triterpenes, flavonoids, and alkaloids, all of which have cytotoxic properties (Mendoza et al. 2020). Given these findings on the cytotoxic ability of *C. striatus* and *X. hongkongensis*, further testing against various cancer cell lines to determine their antiproliferative property needs to be done. Furthermore, the identification of the active constituents responsible for this biological activity should be elucidated.

CONCLUSION

The productivity of mushrooms in submerged fermentation varies depending on the species. In this study, both species prefer potato sucrose broth with pH 6-8 as their optimum nutrient source. *X. hongkongensis* can be effectively grown in PSB, CW, and RBB. Incubation at room temperature and agitating conditions increased mycelial biomass production. The presence of light can improve the mycelial growth of *C. striatus* while it has no direct effect on the growth of *X. hongkongensis*. Both mushrooms have antibacterial properties against *S. aureus*. The ethanol extracts of the mushroom mycelia have potential cytotoxic and teratogenic properties. Therefore, these mushrooms can be used as sources of novel compounds for the development of effective antibacterial and cytotoxic drugs.

ACKNOWLEDGMENT

This study was funded by the Academic Research Council of Central Luzon State University.

REFERENCES CITED

Abdel-Tawab, M. 2021. Considerations to be taken when carrying out medicinal plant research-what we learn from an insight into the ic50 values, bioavailability, and clinical efficacy of exemplary anti-inflammatory herbal components. J. Pharm. 14(5): 437.

Adnan, M., M. Patel, M.N. Reddy, and E. Alshammari. 2018. Formulation, evaluation and bioactive potential of *Xylaria primorskensis* terpenoid nanoparticles from its major compound xylaranic acid. Sci. Rep. 8(1): 1740.

Ahmed, F.A, and N. Jahan. 2017. Optimization of culture condition, phytoconstituents, and cytotoxicity of *Xylaria hypoxylon* (L.) Grev. Jahangirnagar. J. Biol. Sci. 6(2): 1-9.

Bai, R., C. Zhang, X. Yin, J. Wei, and J. Gao. 2015. Striatoids A–F, cyathane diterpenoids with neurotrophic activity from cultures of the fungus *Cyathus striatus*. J. Nat. Prod. 78(4): 783–788.

Bastos, M.L., M.R. Lima, L.M. Conserva, V.S. Andrade, E.M. Rocha, and R.P. Lemos. 2009. Studies on the antimicrobial activity and brine shrimp toxicity of *Zeyheria tuberculosa* (Vell.) Bur. (Bignoniaceae) extracts and their main constituents. Ann. Clin. Microbiol. Antimicrob. 8(1): 1-6.

Burlingame, B., B. Mouille, and R. Charrondiere. 2009. Nutrients, bioactive non-nutrients and anti-nutrients in potatoes. J. Food Compos. Anal. 22(6): 494-502.

Chang, S.T., and P.G. Miles. 2004. Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact. CRC press.

Changi, W. 2015. Biosorption of copper by *Nepenthes Ampullaria*-associated-endophytic fungi. Doctoral dissertation, Swinburne Univ. of Technology, Hawthorn, Australia.

Chen, J.Z., H.C Lo, F.Y. Lin, S.L. Chang, C.W. Hsieh, Z.C. Liang, W.J. Ho, T.H. Hsu. 2014. Effects of medium components and culture conditions on mycelial biomass and the production of bioactive ingredients in submerged culture of *Xylaria nigripes* (Ascomycetes): a Chinese medicinal fungus. Int. J. Med. Mushrooms 16(5).

Cheng, C.W., C.K. Chen, C.J. Chang, L.Y. and Chen. 2012. Effect of color LEDs on mycelia growth of *Aspergillus ficuum* and phytase production in photo-fermentations. J. Photochem. Photobiol. B: Biology 106, 81-6.

Chudzik, M, I. Korzonek-Szlacheta and W. Krol. 2015. Triterpenes as potentially cytotoxic compounds. Molecules 20, 1610-25.

Cui, Y.Q., R.G. Van der Lans, and K.C. Luyben. 1997. Effect of agitation intensities on fungal morphology of submerged fermentation. Biotechnol. Bioeng. 55(5): 715-26.

David, A., and K. Pancharatna. 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 27(3): 390-5.

De Castro, M.E., R.M. Dulay, and M.L. Enriquez. 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 zebrafish (*Danio rerio*). Der Pharm. Lett. 8(5): 237.

De Leon, A.M., E.O. Diego, L. Domingo, and S.P. Kalaw. 2020. Mycochemical screening, antioxidant evaluation and assessment of bioactivities of *Xylaria papulis*: a newly reported macrofungi from Paracelis, Mountain Province, Philippines. Curr. Res. Environ. Appl. Mycol 10(1): 300-318.

Deacon, J. W. 2006 – Fungal biology. (4th edn.): Blackwell Publishing Ltd, Oxford., John Wiley & Sons, Inc. 1-380.

Diamantopoulou, P., S. Papanikolaou, M. Kapoti, M. Komaitis, G. Aggelis, and A. Philippoussis. 2012. Mushroom polysaccharides and lipids synthesized in liquid agitated and static cultures. Part I: Screening various mushroom species. Appl. Biochem. Biotechnol. 167(3): 536-51.

Dulay, R.M., B.C. Valdez, Y. Li, S. Chakrabarti, B. Dhillon, S.P. Kalaw, R.G. Reyes, and E.C. Cabrera. 2021. Cytotoxicity of *Gymnopilus purpureosquamulosus* extracts on hematologic malignant cells through activation of the SAPK/JNK signaling pathway. PloS one 16(5): e0252541.

Dulay, R.M., S.P. Kalaw, R.G. Reyes and E.C. Cabrera. 2014. Embryo-toxic and teratogenic effects of Philippine strain of *Lentinus tigrinus* (tiger sawgill basidiomycetes) extract on zebrafish (*Danio rerio*) embryos. Ann. Biol. Res. 5(6): 9-14.

Dulay, R.M., S.P. Kalaw, R.G. Reyes, N.F. Alfonso, F. Eguchi. 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. Int. J. Med. Mushrooms 14(5): 507-512.

Eguchi, F., S.P. Kalaw, R.M. Dulay, N. Miyasawa, H. Yoshimoto, T. Seyama, and R.G. Reyes. 2015. Nutrient composition and functional activity of different stages in the fruiting body development of Philippine paddy straw mushroom, *Volvariella volvacea* (Bull.: Fr.) Sing. Adv. Environ. Biol. 9(22): 54-66.

Elefant, E., C. Hanin, and D. Cohen. 2020. Pregnant women, prescription, and fetal risk. Handbook of Clinical Neurology 173, 377-89.

Elisashvili, V. 2012 – Submerged cultivation of medicinal mushrooms: bioprocesses and products (Review). Adv. Environ. Biol. 14(3): 211–239.

Fares, F, Azzam N, Appel B, Fares B, Stein A. 2010. The potential efficacy of 3, 3′-diindolylmethane in prevention of prostate cancer development. Eur. J. Cancer Prev. 19(3): 199-203.

Fares, F., S. Khatib, J. Vaya, L. Sharvit, E. Eizenberg and S. Wasser. 2022. Striatal isolated from *Cyathus striatus* extracts induces apoptosis in human pancreatic cancer cells. Molecules 27(9): 2746.

Howe, K., M.D. Clark, C.F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J.E. Collins, S. Humphray, K. McLaren, L. Matthews, S. McLaren. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496(7446): 498-503.

Jacob, J.K., S.P. Kalaw, and R.G. Reyes. 2015. Mycelial growth performance of three species of *Pleurotus* on coconut water gelatin. Curr. Res. Environ. Appl. 5(3): 263-8.

Jo, W.S., Y.J. Cho, D.H. Cho, S.D. Park, Y.B. Yoo, and S.J. Seok. 2009. Culture conditions for the mycelial growth of *Ganoderma applanatum*. Mycobiology 37(2): 94-102.

Kalaw, S.P., D.O. Alfonso, R.M. Dulay, A.M. De Leon, J.Q. Undan, J.R. Undan, R.G. Reyes. 2016. Optimization of culture conditions for secondary mycelial growth of wild macrofungi from selected areas in Central Luzon, Philippines. Curr. Res. Environ. Appl. Mycol. 6(4): 277-87.

Kelly, S., L.H. Grimm, J. Hengstler, E. Schultheis, R. Krull, D.C. Hempel. 2004. Agitation effects on submerged growth and product formation of *Aspergillus niger*. Bioprocess Biosyst Eng 26(5): 315-23.

Khan, M.B. 2017. Evaluation of mycelial growth of oyster mushroom (*Pleurotus floridanus* Singer) on different media and cereal grains. J. Biodivers. Environ. Sci. 11(3): 67-72.

Landingin, H.R., B.E. Francisco, R.M. Dulay, S.P. Kalaw, and R.G. Reyes. 2020. Optimization of culture conditions for mycelial growth and basidiocarp production of *Cyclocybe cylindracea* (Maire). CLSU Int. J. Sci. Environ. Technol. 4(1): 1-7.

Liu, Y.J., and K.Q. Zhan. 2004. Antimicrobial activities of selected *Cyathus* species. Mycopathologia 157(2): 185-9.

McCloskey, S., S. Noppawan, W. Mongkolthanaruk, N. Suwannasai, T. Senawong, and U. Prawat. 2017. A new cerebroside and the cytotoxic constituents isolated from *Xylaria allantoidea* SWUF76. Nat. Prod. Res. 31(12): 1422-30.

Mendoza, W.C., Dulay R.M., Valentino M. J., Reyes, R. G. 2020 - Mycelial biomass and biological activities of Philippine mushroom *Pycnoporus sanguineus* in timecourse submerged culture. J. Appl. Biol. Biotechnol. 8(05), 88-93.

Miao, W., L. He, T. Zhang, and C. Li. 2022. Lentinan impairs the early development of zebrafish embryos, possibly by disrupting glucose and lipid metabolism. Processes 10(1): 120.

Moshi, M.J., E. Innocent, J.J. Magadula, D.F. Otieno, A. Weisheit, and P.K. Mbabazi. 2010. Brine shrimp toxicity of some plants used as traditional medicines in Kagera region, North Western Tanzania. Tanzan. J. Health Res. 12, 63-7.

Nagel, R. 2002. Dart: the embryo test with zebrafish *Danio rerio*- a general method in eco-toxicology and toxicology. Altex 19(1/02): 38-48.

Nhi, N.N.Y. and P.V. Hung. 2012. Nutritional composition and antioxidant capacity of several edible mushrooms grown in the Southern Vietnam. Int. Food Res. J. 19(2): 611.

Nitthithanasilp, S., C. Intaraudom, N. Boonyuen, R. Suvannakad, and P. Pittayakhajonwut. 2018. Antimicrobial activity of cyathane derivatives from *Cyathus subglobisporus* BCC44381. Tetrahedron 74(48): 6907-6916.

Orachaipunlap, K., N. Suwannasai, A.J. Whalley, S. Roengsumran, P. Sihanonth. 2015. Antibacterial and anticancer activity of stromatic *Xylaria* spp. from tropical forest Thailand. Curr. Appl. Sci. Technol. 15(1): 1-9.

Peng, J., C. Lee, and T. Y. Tsai. 2000. Effect of rice bran on the production of different king oyster mushroom strains during bottle cultivation. J. Agric. Res. China 49(3): 60-7.

Ramesh, V., C. Karunakaran, and A. Rajendran. 2014. Optimization of submerged culture conditions for mycelial biomass production with enhanced antibacterial activity of the medicinal macro fungus *Xylaria* sp. strain R006 against drug resistant bacterial pathogens. Curr. Res. Environ. Appl. Mycol. 4(1): 88-98.

Ramesh, V., K. Santosh, M. Pavunraj, C. Karunakaran, and A. Rajendran. 2015. In-vitro antifungal and anticancer potential of *Xylaria curt*a fruiting body fractions against human fungal pathogen and cancer cell lines. Curr. Res. Environ. Appl. Mycol. 5(1): 20-6.

Shakouri, A., F. Nematpour, N. Adibpour, and A. Ameri. 2014. The investigation of antibacterial activity of *H. leucospilota* sea cucumber extracts (Body wall and white strings) at Chabahar bay in Oman Sea. Environmental Studies of Persian Gulf 1(2): 135-140.

Sharvit, L., R. Bar-Shalom, N. Azzam, Y. Yechiel, S. Wasser, and F. Fares. 2021. *Cyathus striatus* extract induces apoptosis in human pancreatic cancer cells and inhibits xenograft tumor growth *in vivo*. Cancers. 13(9): 2017.

Sharvit, L., R. Bar-Shalom, N. Azzam, Y. Yechiel, Wasser S, F. Fares. 2017. *Cyathus striatus* extract induces apoptosis in human pancreatic cancer cells and inhibits xenograft tumor growth *in vivo*. Cancers 13(9).

Sharvit, L.E., S.P. Wasser, and F. 2012. The effect of culture liquid ethyl acetate mycelium extracts of medicinal mushrooms on the viability of human pancreatic cancer cells. Int. J. Med. Mushrooms 14(2).

Smania, E.F., A. Smania Jr., C. Loguercio-Leite, and M.L. Gil. 1997. Optimal parameters for cinnabarin synthesis by *Pycnoporus sanguineus*. J. Chem. Technol.: International Research in Process, Environmental and Clean Technology 70(1): 57-9.

Sogan, M.M., J.A. Maslang, and R.M.R. Dulay. 2018. Myco-chemicals and teratogenic activity of wild mushroom *Trichaleurina celebica* from Mt. Palali, Quezon, Nueva Vizcaya, Luzon Island, Philippines. CLSU Int J Environ Sci Technol 3(2): 17-23.

Wang, S.J. and J.J. Zhong. 2020. Bioreactor engineering. In bioprocessing for value-added products from renewable resources. Elsevier, 131-161.

Yin, X., J. Wei, W. Wang, Y. Gao, M. Stadler, R. Kou and J. Gao. 2019. New cyathane diterpenoids with neurotrophic and anti-neuroinflammatory activity from the bird's nest fungus *Cyathus africanus*. Fitoterapia 134, 201–209

Zhao, R.L., D.E. Desjardin, K. Soytong, and K.D. Hyde. 2008. A new species of bird's nest fungi: characterization of *Cyathus subglobisporus* sp. nov. based on morphological and molecular data. Pers.: Mol. Phylogeny Evol. Fungi 21(1): 71-6.