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Optimal culture conditions and toxicity assessment of *Fomitopsis feei* (Fr.): a newly documented macro fungus from Philippines

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Abstract

Mushrooms are known to be utilized by ethnic communities and Paracelis, Mountain Province is one of the places in Philippines inhabited by several of these native groups. Many studies have been conducted on various macrofungi, however no studies have been reported about *Fomitopsis feei* in the Philippines particularly in Paracelis, Mountain Province. It is a brown-rot bracket fungus, belonging to the family Fomitopsidaceae, characterized by a sessile effuse-reflexed basidiomata, with its color ranging from white to pinkish or brown. This mushroom has been reported to have antimicrobial properties, hence optimization of its culture condition could lead to its mass production for its biopharmaceutical potential. In order to develop a mass cultivation protocol of this mushroom, this study was conducted to determine the optimum conditions for its mycelial growth. The effect of different culture media using local substrates and evaluating environmental factors such as pH, aeration, illumination, and temperature were assessed. Optimum conditions for the secondary mycelial growth of *F. feei* produced very thick and largest radial growth on coconut water gelatin (CWG) medium (83.57 mm) at pH 6.5 (83.13 mm), in sealed (85 mm), dark conditions (85.00 mm) at room temperature (28-32ºC) (81.96 mm). The most abundant mycelial growth was found in cracked corn as grain spawning material. This study also determined the teratogenic and cytotoxic activity of the ethanol extract of *F. feei* against the zebra fish (*Danio rerio*) embryos and brine shrimp (*Artemia salina*) nauplii. *Fomitopsis feei* exhibited teratogenic effects against the developing *D. rerio* embryos wherein growth retardation, malformation of tail, yolk deformity, pericardial edema, curved body, scoliosis and little pigmentation were the notable teratogenic effects of the ethanol extract to the developing embryos. Embryos treated with ≥1000 ppm recorded high mortality rate. Hatchability was most evident at lower concentrations ≤750 ppm. In terms of heartbeat, as the concentration of the extract increased, the heartbeat rate significantly decreased. For the cytotoxicity, 1250 ppm has the highest mortality rate with 73.33%. Using probit analysis, the LC_{50} is 534.676 ppm which is considered as mildly toxic. Thus, *F. feei* in higher concentrations exhibit toxic effect. These results indicate that *F. feei* has a pharmaceutical potential and could be harnessed for its bioactivities.

Key words – cultivation – cytotoxicity – grain spawn – local substrate media – teratogenicity

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Introduction

Mushrooms are sources of food and nutrients and have high potential as sources of bioactive compounds with pharmaceutical effects (Oyetayo 2011). Numerous studies have discovered how mushrooms can be useful in preventing and treating health conditions. Ware (2017) reported that, some species of mushrooms have medicinal properties such as antioxidant, antimicrobial, anticancer, cholesterol lowering and decrease the risk of obesity, diabetes and heart disease. Also, several studies have been conducted related to species belonging to the genus *Fomitopsis;* this include the study of Cheng et al. (2008) on the medicinal importance of *Fomitopsis pinicola* as anti-microbial, anti-inflammatory, anti-tumor, antioxidant, anti-angiogenic and immune-stimulating effect. The fomitopsin isolated from *Fomitopsis feei* exhibited activity against *Bacillus cereus* and herpes simplex virus (Isaka et al. 2017). Culture filtrates of *Fomitopsis feei* in ten days incubated rice bran extract showed good antibacterial activity on *Enterobacter aerogens*, *Bacillus subtilis*, *Microcococcus luteus* and *Proteus mirabilis* (Hima et al. 2011). Additionally, polypore macrofungi have been reported to be used as herbal medicine for intermittent fever, chronic diarrhea, nervous, headache and jaundice (Rogers 2011). Moreover, Chilkov (2017) confirmed that, mushrooms such as *Ganoderma reishi, Lentinula edodes, Trametes versicolor, Cordyceps sinensis, Grifola frondosa* and *Inonotus obliquus* are rich in polysaccharides and beta glucans, which are considered as the primary active immune–enhancing constituents. Kumar (2015) noted that, mushrooms have become a source of food and drugs, and nutraceuticals due to their antioxidant, antitumor and antimicrobial properties. Research findings in the past three to four decades had demonstrated that mushrooms are important sources of pharmacological bioactive compounds that can enhance human health (Bankole & Adekunle 2012). That is why determination of its bioactive constituents and its toxicity effect is crucial for the development of nutraceuticals. Raghunath & Perumal (2016) noted that zebrafish embryo has emerged as a potential tool to test teratogenicity. As declared by Finn (2007), the developing zebrafish in an *in vitro* system has a lot of potentials in meeting the need of a simple and inexpensive test and rapid assay to determine the teratogenicity of a specific compound or chemical. Moreover, Reneses et al. (2016) revealed that the teratogenic activities of mushroom are important as some teratogens can be used as anticancer drugs. For the cytotoxicity, Syahmi et al. (2010) stated that brine shrimp bioassay is considered as a quick preliminary screening for the presence or absence of bioactivity and is also used to determine cytotoxicity. Likewise, Golla et al. (2011) specified that, brine shrimp lethality assay is a technique that requires small number of samples and is easily measured, has low-cost and utilizes small amount of test material.

The discovery of various novel bioactive compounds from wild macrofungi has been increasing for the last decades. Several studies have proven the significance of mushroom cultivation for environmental management and as a source of medicinal products that are rich in phytochemicals, antioxidant, bioactive and phenolic compounds found within different mushroom varieties (Chang & Wasser 2012, Wasser 2010, 2014, Chang & Wasser 2017). However, climate change, deforestation, and over exploitation of the forest might cause the occurrence of wild mushrooms to decrease, thus, cultivation of mycological wild resources is essential as a source of cell lines towards the effective utilization and conservation of mushrooms (Dulay et al. 2012). Also, ethnic communities from Paracelis, Mountain Province have been known to utilize different species of mushrooms. Hence, this study was conducted to optimize culture conditions and evaluate the possible toxic substances of *Fomitopsis feei* which was collected from Paracelis, Mountain Province using zebrafish and brine shrimp as animal models.

Materials & methods

Source of macrofungi

Fomitopsis feei was collected from Paracelis, Mountain Province. The fruiting bodies were collected, wrapped in brown paper, labeled and were brought to the laboratory.

Preparation of culture media

Thirty-nine grams of Potato Dextrose Agar (PDA) was boiled in 1000 ml of distilled water, it was stirred thoroughly until the powder was dissolved. Homogenized culture medium was dispensed to glass bottles, plugged with cotton, covered with a clean paper, and sterilized at 121°C at 15 psi for 30 minutes. Then the medium was aseptically poured to sterile petri plates.

Surface disinfection of mushroom tissues

Fruiting body of *F. feei* was brushed to remove the dust. Then it was soaked with 10% sodium hypochlorite for 1 minute, then rinsed three times with distilled water.

Mushroom tissue culture

Fruiting body of *F. feei* was cut longitudinally in order to expose its inner portion. Using a sterile scalpel, a small portion was cut from the inner part of the mushroom tissue and then inoculated aseptically on the petri plates with the prepared PDA medium. Inoculated plates were incubated at room temperature until the mycelia has fully grown on the media surface.

Screening of appropriate indigenous culture media for the mycelial growth of *F. feei*

Various local substrates were used to prepare culture media in order to determine the most suitable media for *F. feei* growth. Each media was prepared with three replicates. The treatments used were the following: Potato Sucrose Gelatin (PSG), Coconut Water Gelatin (CWG), Rice Bran Decoction Sucrose Gelatin (RBDSG), Cracked Corn Decoction Sucrose Gelatin (CCDSG), and Taro Sucrose Gelatin (TSG). In the preparation of PSG and TSG, potato and taro were boiled separately in 1000 mL distilled water, filtered with cheese cloth, and the broths were adjusted to pH 6.0, mixed with 20 g shredded gelatin bars. For the preparation of CCDSG and RBDSG, media were boiled in a 1000 mL distilled water, filtered, adjusted to pH 6.0, and decoctions were mixed with 20 g gelatin bars. For CWG, one liter of coconut water was filtered, boiled for 20 minutes, adjusted to pH 6.0 and mixed with 20 g gelatin bars. All culture media were sterilized by autoclaving at 121°C (15 psi) for 15 minutes and pour plated. To assess the mycelial growth, 10 mm diameter mycelial plugs of *F. feei* was prepared using a cork borer and inoculated centrally on all culture media. All inoculated plates were incubated at room temperature and the mycelial growth diameter was measured and recorded daily. Mycelial density was also observed visually and rated as (+) very thin, (++) thin, (+++) thick and (++++) very thick. The best indigenous culture medium is defined as the medium that produced the most luxuriant mycelial growth in the shortest incubation period.

Influence of physical factors on the mycelial growth of *F. feei*

pH

As CWG was found to be the most suitable culture medium in terms of shortest incubation period with very thick mycelial density, its pH was adjusted in a range from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, pour plated and then inoculated with *F. feei* mycelia. Inoculated petri plates were then incubated at room temperature and the mycelial diameter was measured every 24 hours. Mycelial density and incubation period were also recorded.

Aeration

In the evaluation of the air requirement, the CWG medium was adjusted to the optimum pH 6.5. For sealed condition, layers of cling wrap were used while the other condition was left unsealed. Mycelial diameter and density were evaluated every 24 hours.

Illumination

To assess the influence of light in the mycelial growth of *F. feei*, 10 mm diameter mycelial plugs from cultures growing in CWG medium at pH 6.5 were utilized. The inoculated petri plates were subjected to full lighted, full dark, and alternating light and dark conditions at room

temperature. To facilitate the full lighted condition, a room with fluorescent light was used. In full dark, the petri plates were covered with clean black paper. While in alternating light and dark condition, inoculated plates were exposed to light for 12 hours with 12 hours of darkness. Each treatment was incubated at room temperature. Every 24 hours, mycelial diameter was measured and mycelial density was recorded.

Temperature

Mycelial plugs from CWG as the best culture medium, at pH 6.5, in sealed condition, incubated in total darkness were used to evaluate the temperature effect. Ten mm diameter of mycelial plugs were prepared using a sterile cork borer and incubated at the following conditions; room temperature, air-conditioned, and refrigerated. Mycelial diameter was measured every 24 hours and mycelial density were recorded.

Evaluation of different granular materials for mother spawn production

Various types of locally available granular materials (sorghum seeds, cracked corn, and palay seeds) for mother spawn production were evaluated for the mycelial growth of *F. feei*. The most suitable culture media and appropriate physical factors (pH, aeration, illumination and temperature) were used. Each treatment had three replicates. The treatment with the thickest mycelial density and with the shortest incubation period was identified as the best grain spawning material.

Treatment preparation

The procedure of Manasathien et al. (2012) with minor modification was used in this study. One ml of the mushroom extract was diluted in 9 ml of embryo water for teratogenicity and saltwater solution for brine shrimp lethality assay. Three ml of each solution was used and each dose was evaluated in triplicates.

Teratogenicity

Preparation of aquarium tank

In order to maintain the adult female and male zebrafish a 1:2 ratio was used. A glass aquarium of approximately 5-gallon capacity containing untreated and clean tap water with continuous flow of oxygen via aerator were used.

Zebrafish care, culture, and maintenance

Healthy and sexually matured zebrafish were acquired from the Wet Laboratory of the College of Fisheries, CLSU. They were acclimatized for a week at room temperature condition before using for spawning and fertilization. The zebrafish was fed daily with flakes and the leftover food was removed daily for maintenance of water cleanliness and quality.

Zebrafish spawning and egg production

Zebrafish have daily cycle of light and darkness that affects their behavior and physiological function. The matured zebrafish was placed in a coarse plastic mesh submerged into the aquarium water to protect the eggs from being devoured by the matured zebrafish once fertilized. The zebrafish was then exposed to dark condition to stimulate spawning by covering the aquarium with a black plastic bag. The black plastic bag was removed after 12 hours of dark condition and the light was turned on to stimulate fertilization. Fertilized eggs were seen at the bottom of the aquarium with the aid of artificial light.

Harvesting of fertilized eggs

After 12 hours post fertilization, the fertilized eggs were collected and tapped out of the aquarium using a hose tube and transferred to a beaker. Morphological assessment was done before placing them in the vials wherein cloudy and ruptured eggs was discarded.

Zebrafish (*Danio rerio*) teratogenicity and toxicity assay

Three milliliters of each treatment concentration were placed in each vial with four embryos at segmentation phase. Teratogenic activity was examined using a stereomicroscope after 12, 24, 36, and 48 hours of incubation. Morphological endpoint evaluation of zebrafish was based on the parameters established by Schulte & Nagel (1994): lethal (coagulation, tail not detached, no somites, and no heartbeat), teratogenic (malformation of head and tail, scoliosis, growth retardation, stunted tail, and limited movement) and normal. Hatchability and mortality rate were recorded and pictures were taken out and death was defined as coagulated embryos.

Cytotoxicity

Source of brine shrimp (*A. salina*) eggs

Artemia cysts INVE brine shrimp eggs were acquired from the Wet Laboratory of College of Fisheries, CLSU.

Hatching set-up

A 1.5 L plastic bottle was cut at its base. The cut plastic bottle served as the hatchery vessel and was inversely hang on the hatching rack. The set-up was provided with proper illumination using 18-watt fluorescent bulb and was positioned 12 inches above the hatchery vessel to maintain the temperature to 28-30ºC. Aquarium air pump with air stone was placed at the bottom of the hatchery vessel for proper aeration.

Hatching of brine shrimp eggs

The hatchery was filled with artificial seawater prepared by dissolving 38 g of sea salt or rock salt in 1 L of distilled water following the method of McLaughlin & Rogers (1998). One gram of brine shrimp eggs was hatched in the artificial seawater within 48 hours under illumination and with adequate aeration to keep the eggs in suspension until its 48 hours life. The hatchery was covered with thin gauze for protection against the entrance of undesirable insects or organisms that may be harmful to the developing eggs. The hatched eggs called nauplii were used for the assay.

Brine shrimp lethality assay

Using a hand lens, active 48-hour old nauplii were harvested and used for the assay. The larvae were transferred into each sample vials using a syringe. A drop of dry yeast suspension (3 mg yeast/5 mL saline solution) was added to each vial as food of the nauplii and 200 µL of different concentration was added. It was observed after 6, 12, 18, and 24 hours. The vials were maintained under illumination and dead nauplii were counted using a hand lens. The LC_{50} was evaluated according to the rating of Aldahi et al. (2015) where LC_{50} of <249 µg/mL as highly toxic, 250-499 µg/mL is moderately toxic and 500-1000 µg/mL is mildly toxic. Values above 1000 µg/mL are non-toxic according to the rating of McLaughlin & Rogers (1998).

Statistical analysis

All the treatments were arranged following the complete randomized design under laboratory conditions. One-way analysis of variance (ANOVA) was used to determine significant differences among treatments. Means were compared using least significant differences (LSD) at 5% level of significance. For the cytotoxicity, the median lethal concentration LC_{50} was computed using probit analysis. The SAS 9.1 program was used for the analyses.

Results

Culture media

Among the five evaluated culture media (PSG, CWG, RBDSG, CCDSG and TSG) CWG showed the largest mycelial growth diameter with 83.57 mm, which was significantly higher than

the other media after 12 days of incubation, while the smallest mycelial growth diameter was observed in PSG with 59.11 mm (Table 1). Moreover, CWG produced a very thick mycelial density, while CCDG have shown the thinnest mycelial density.

pH levels

No significant difference was found in the mycelial growth of *F. feei* in different pH levels (Table 1). However, pH 6.5 produced a thicker mycelial density compared to pH 6.0. The results revealed that the pH levels did not significantly affect the mycelial production of *F. feei* in the substrates. However, variation of growth of *F. feei* in mycelial density was evident at different pH levels. The results showed that pH 5.0 to 8.0 could support an efficient mycelial growth of *F. feei*, but the range of pH 6.0-6.5 produced the highest mean mycelial growth.

Aeration

From the most appropriate medium and suitable pH, the inoculated plates with *F. feei* were sealed and the other were left unsealed in order to evaluate aeration requirements. The unaerated condition significantly recorded the maximum diameter of 85.00 mm in comparison to aerated condition with 80.97 mm (Table 1). Furthermore, mycelial density under sealed condition produced a very thick and cottony growth as compared to unsealed plates which exhibited only thick mycelial density.

Table 1 Mycelial diameter and density of *F. feei* on various nutritional and physical requirements for mycelial growth.

	Mycelial Diameter (mm)	**Mycelial Density**
Culture Media		
PSG	59.11 ± 1.60^{c}	++
CWG	83.57 ± 2.48^{a}	++++
RBDSG	75.01 ± 3.19^{b}	++
CCDSG	73.46 ± 2.28^{b}	+
TSG	69.50 ± 2.08^{b}	+++
pH		
5	82.05 ± 1.72	++
5.5	79.62 ± 2.04	++
6	83.22 ± 2.04	+++
6.5	83.13 ± 2.94	++++
7	79.99 ± 2.41	++
7.5	81.01 ± 2.49	++
8	82.90 ± 3.64	+++
Aeration		
Sealed	85.00 ± 0.00^{a}	++++
Unsealed	80.97 ± 0.67^{b}	+++
Illumination		
Dark	85.00 ± 0.00^{a}	++++
Light	82.38 ± 1.16^{b}	+++
Light and Dark	81.90 ± 1.17^{b}	++
Temperature		
Room Temperature	81.96 ± 2.73^{a}	++++
Air conditioned	75.88 ± 1.14^{b}	+++
Refrigerated	10.00 ± 0.00^{c}	-

Values are means ± sd; for each variable means with the same letters are not significantly different at 5% level using Tukey's HSD as post hoc. Note: (+) very thin, (++) thin, (+++) thick, (++++) very thick, (-) no growth

Illumination

F. feei mycelial diameter in dark condition registered a full growth of 85.00 mm, analysis of variance showed that it is significantly higher than other treatments, while illuminated condition recorded a growth diameter of 82.38 mm, while the least mycelial growth diameter was observed in alternating light and dark which has a mean mycelial growth of 81.90 mm (Table 1). A very thick mycelial density was also observed in the dark condition after 14 days incubation period and the alternating light and dark resulted to a thin mycelial growth.

Temperature

F. feei produced a very thick and cottony growth at room temperature (28-33^{o}C) as presented in Table 1. There was a significant difference between the mean mycelial growth diameter in room temperature 81.96 mm and air conditioned temperature (18-24^{o}C) with a mean of 75.88 mm, while no growth was observed at refrigerated temperature (8-10^{o}C). At the same time, mycelial density at room temperature was recorded to be very thick, while mycelial density at air-conditioned temperature produced thick growth.

Spawning Materials

After 16 days of incubation, cracked corn showed the highest mycelial growth (Fig. 1), the sorghum seeds were colonized after 17 days, while there was a very thin growth in palay seeds.

Fig. 1 – Mycelial density of *F. feei* in different spawning materials after 16 days. A Sorghum seeds. B Cracked corn. C *palay* seeds.

Teratogenicity

Teratogenic activities of *F. feei* ethanolic extract were evaluated using *D. rerio* embryos. Zebrafish embryos at segmentation phase exposed to different treatment concentrations (1250 ppm, 1000 ppm, 750 ppm, 500 ppm, 250 ppm, 100 ppm and 0 ppm) were examined under different developmental stages such as segmentation (12 hours post treatment application [hpta]), pharyngula (24-36 hpta) and hatching stage (48-72 hpta).

Mortality

Mortality is one of the parameters examined to determine the toxicity of the ethanolic extract of *F. feei*. It is described as having no observable heartbeat and coagulation. The toxic effect of *F. feei* was recorded and the mean percentage mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure in varying concentrations are shown in Table 2. No mortality was observed at 12- and 24-hours post treatment application (hpta) in all the treatments. However, longer duration

of exposure of the embryos to the different concentrations of *F. feei* ethanol extract increased the percentage mortality and this was evident at 1250 ppm and 1000 ppm concentrations. Moreover, on 1250 ppm concentration, a mean percentage mortality of 41.67% was recorded at 36 hpta and increased to 100% at 48 hpta. The mean percentage mortality for 1250 ppm at 36 and 48 hpta was not significantly different with that of 1000 ppm with a mean percentage mortality of 33.33% observed at 36 hours and 100% on its 48 hours. However, the mean percentage mortality of 8.33% at 46 hpta on 750 ppm was significantly different from that of the mean percentage mortality of 1250 and 1000 ppm. The toxic effects of the mushroom extract were dependent on the concentration and time of exposure, as shown by the increased mortality of embryos with higher level of concentration and longer time of exposure. No heartbeat was the most notable lethal effect of the ethanol extract exposed to 1250 ppm and 1000 ppm from 36 hours until 48 hours.

Table 2 Mean percentage mortality of *Danio rerio* embryos after 36, and 48 hours of exposure to different concentrations of *Fomitopsis feei* ethanol extract.

Concentration (ppm)	Treatment Exposure	
	36 h	48 h
1250	41.67[a]	100.00[a]
1000	33.33[ab]	100.00[a]
750	0.00[b]	8.33[b]
500	0.00[b]	0.00[b]
250	0.00[b]	0.00[b]
100	0.00[b]	0.00[b]
0	0.00[b]	0.00[b]

Means having the same letter of superscript in the same column are not significantly different at $P \leq 0.05$ significance using LSD

Hatchability

Hatching is a significant key in successful embryonic developmental process. The percentage hatchability of embryos treated with different concentrations of lyophilized mushroom extract was observed (Table 3).

The 500 ppm had the lowest mean percentage hatchability of 25%. However, significant difference was observed between treatment concentrations ≥1000, ≤750 ppm and the control. Furthermore, no hatched embryo was recorded in 1250 ppm and 1000 ppm due to early death of embryos. It was observed that as the concentration of the ethanol extract decrease, the mean percentage hatchability of the embryos exposed on lower treatment concentrations increased. Thus, varying amount of the ethanol extract of *F. feei* affects the normal hatchability of the embryos exposed at 48 hours.

Table 3 Hatchability of embryos treated with different concentrations of *Fomitopsis feei* ethanol extract.

Concentration (ppm)	48 h
1250	0.00[b]
1000	0.00[b]
750	41.67[a]
500	25.00[a]
250	41.67[a]
100	50.00[a]
Control	41.67[a]

Means having the same letter of superscript in the same column are not significantly different at $P \leq 0.05$ significance using LSD

Heartbeat rate

The heartbeat rate of *D. rerio* was monitored microscopically at pharyngula stage (Table 4). The mean heartbeat rate of embryos exposed to *F. feei* ethanol extract was highest at 100 ppm with 202 beats per minute and was significantly different from that of the mean percentage heartbeat of 1250 ppm with only 154 beats per minute. It can be marked that the different concentrations of *F. feei* extract affects the heartbeat of zebrafish embryos.

Table 4 Heartbeat of *Danio rerio* after 36 hours of exposure to various concentrations of *Fomitopsis feei* ethanol extract.

Concentration (ppm)	Heartbeat
1250	154.00^{cd}
1000	128.00^{d}
750	182.00^{abc}
500	160.00^{bc}
250	186.00^{ab}
100	202.00^{a}
Control	188.00^{ab}

Means having the same letter of superscript in the same column are not significantly different at $P \leq 0.05$ significance using LSD

Morphological endpoints of treated *D. rerio* embryos

Parameters such as head and tail malformation, growth retardation, and little pigmentation were used as parameters in order to evaluate the teratogenic effects of the extract after 12, 24, 36, and 48 hours of exposure as presented on Table 5.

Growth retardation

The most prominent observed teratogenic effects of *F. feei* ethanol extract was growth retardation. It was distinguishable on embryos treated with 750 ppm.

Morphological abnormalities

Lethal and teratogenic effects exhibited by *F. feei* ethanol extract on developing embryos at various concentrations at different hours of exposure are presented in Figure 2. Ethanol extract of *F. feei* resulted to curved body which was notable in 750 ppm concentration at 48 hpta (Figure 2A). Yolk deformity of the embryo was also observed in 750 ppm concentration at 48 hpta (Figure 2B). The only observed tail malformation was bent tail (Figure 2B) in 750 ppm concentration at 48 hpta. Moreover, scoliosis can be seen prominent (Figure 2C) in 750 ppm concentration at 48 hpta. Furthermore, little pigmentation, no gut and pericardial edema were the other observed morphological abnormalities. Pericardial edema and little pigmentation (Figure 2D) was observed at 48 hpta in 500 ppm.

Table 5 Lethal and teratogenic effects of various concentrations of *F. feei* ethanol extract at 12, 24, 36, and 48 hours of exposure.

Toxicological Endpoints	Time of Exposure (h)	Concentration (ppm)						
		1250	1000	750	500	250	100	0
Lethal								
Coagulation	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-
No heartbeat	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	+	+	-	-	-	-	-

Table 5 Continued.

Toxicological Endpoints	Time of Exposure (h)	Concentration (ppm)						
		1250	1000	750	500	250	100	0
	48	+	+	+	-	-	-	-
Teratogenic								
Malformation of head	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-
Malformation of tail	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	+	-	-	-	-
Scoliosis	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	+	-	-	-	-
Growth retardation	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	+	-	-	-	-
Limited movement	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-
Little pigmentation	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	+	+	-	-	-

+ indicates presence of lethal or teratogenic effects to developing embryos at various observation hours
- indicates absence of lethal or teratogenic effects to developing embryos at various observation hours

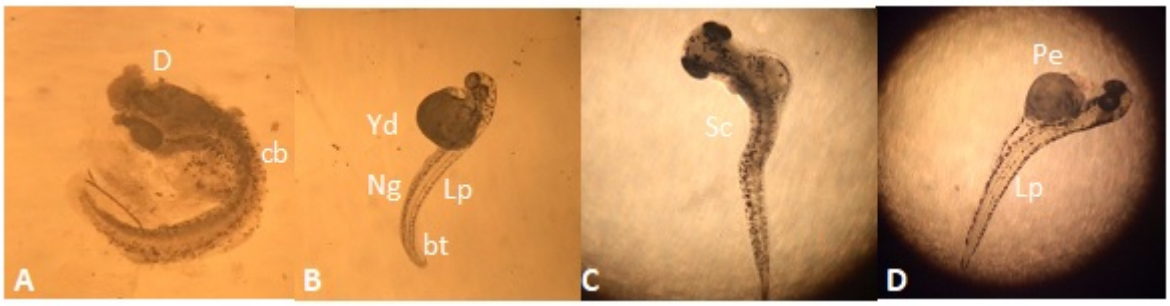

Fig. 2 – Lethal and teratogenic effects of various concentrations of *F. feei* ethanol extract. A dead embryo (D) and curved body (cb) at 48hpta in 750 ppm. B yolk deformity (Yd), no gut (Ng), little pigmentation (Lp) and bent tail (bt) at 48 hpta in 750 ppm. C scoliosis (Sc) at 48 hpta in 750 ppm. D pericardial edema (Pe) and little pigmentation (Lp) at 48 hpta in 500 ppm.

Cytotoxicity

Brine shrimp lethality was used in order to determine the cytotoxic activity of *F. feei.* Table 6 shows the mean percentage mortality of *A. salina* nauplii after 24 hours of exposure to different concentrations of *F. feei* ethanol extract.

It can be seen that 1250 ppm recorded the highest mortality rate with 73.33% while 100 ppm registered the lowest mortality rate with 26.67%. Analysis of variance (ANOVA) shows that after 24 hours of exposure to the extract, there was no significant difference among treatment concentrations ≥1000 ppm. However, significant difference was observed between 1250 ppm and concentrations ≤750 ppm and the control.

Using probit analysis, Fig. 3 shows the median lethal concentration (LC_{50}) of the *F. feei* ethanol extract. It was estimated that the LC_{50} of *F. feei* ethanol extract is 534.676 ppm which is considered to be mildly toxic based on the ratings of Aldahi et al. (2015).

Table 6 Mean percentage mortality of *A. salina* nauplii after 24 hours of exposure to different concentrations of *F. feei* ethanol extract.

Concentration (ppm)	Mortality (%)
1250.00	73.33[a]
1000.00	66.67[a]
750.00	50.00[b]
500.00	43.33[b]
250.00	26.67[c]
100.00	26.67[c]
0.00	0.00[d]

Means having the same letter of superscript in the same column are not significantly different at P ≤ 0.05 significance using LSD

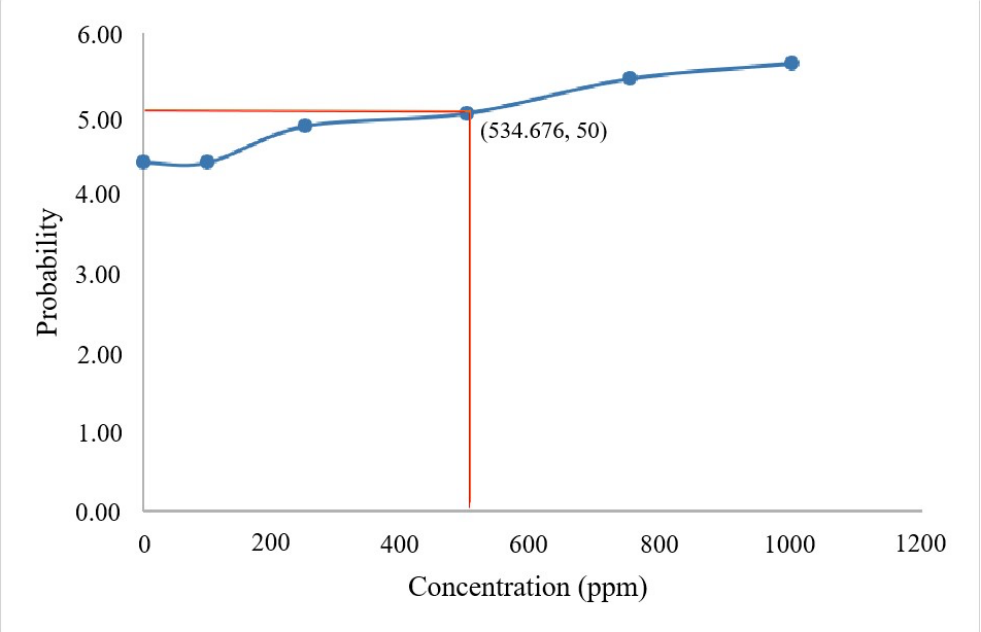

Fig. 3 – Point estimate of LC50 value of *Fomitopsis feei* ethanol extract after 24 hours of exposure.

Discussion

Several studies have been conducted about *Fomitopsis feei* in India but none in the Philippines. This mushroom was reported to exhibit different biological activities such as antibacterial, antifungal, antioxidant and 5-lipoxygenase, alpha glucosidase and tyrosinase enzyme inhibitory activities using exopolysaccharide (Bindu & Charya 2017a). This mushroom contains essential amino acids such as arginine which play an important role in the activity of macrophages and considerable amount of linolenic acid which are essential for human health (Bindu & Charya 2017b). Furthermore, this mushroom also exhibited lignolytic enzyme activities, which can be used in biobleaching and dye reduction industries (Bindu et al. 2011). Since *F. feei* can decolorize several synthetic dyes belonging to triphenylmethane group efficiently (Lyra et al. 2009), it can be

used in biological treatment to control pollution generated by textile and dye-stuff industries (Bindu et al. 2013). With these significant functionalities of *F. feei* reported in other countries, it is of utmost importance that the culture conditions of this mushroom be optimized under Philippine condition to harness its potential in the nutraceutical industry.

Nutritional and environmental factors affecting mycelial growth of *F. feei*

In mushroom production, mushroom tissue culture is highly important for it is the source of mycelia and mushroom cell lines. Hence, the nutritional composition of the culture medium is one of the factors to be considered in mycelial production (De Leon et al. 2013). Nutrients present in the culture medium and environmental conditions are one of the major factors in the growth of mushrooms (Thongklang et al. 2010). The growth of *F. feei* was recorded to be abundant and very thick in the CWG medium. The abundance of the growth in CWG can be attributed to its nutritional content such as sugars, vitamins, minerals, and amino acids, with nitrogen (0.62%), phosphorus (0.36%), potassium (2.36%), sulfur (1.64%), sodium (0.86%), calcium (0.1%), magnesium (0.48%), zinc (20.6%), copper (17.4%), iron (93.2%), manganese (84%) (Umesha & Naranayaswamy 2016). The presence of sugar in CWG medium accords with the report of Bindu & Charya (2017c) that glucose as carbon source (6.88 g/L, 6.04 g/L) yielded the best mycelial biomass of *F. feei* both in 7 and 14 days respectively. In congruence with the study of De Leon et al. (2017) wherein *Lentinus sajor-caju* was reported to have the highest mycelial growth diameter in CWG with 88.75 mm. Same is true with the study of Kalaw et al. (2016) in which CWG significantly showed the largest mycelial growth diameter with ten mushroom species assessed. Furthermore, similar studies by Magday et al. (2014) have proven that CWG was the most suitable culture medium for several mushroom species like *Ganoderma lucidum*. On the other hand, growth pattern of *F. feei* mycelia in commercial solid media was found to be good in malt extract agar (MEA) (Bindu & Charya 2017b).

Furthermore, various environmental conditions were found to affect the mycelial growth including pH, aeration, light, and temperature as previously recorded (Shah & Modi 2018, Stott & Mohammed 2004). Results showing that the range of pH 6.0-6.5 produced the highest mean mycelial growth for *F. feei* is in accordance to the study of Bindu & Charya (2017c) that pH 6.0 is the most suitable pH level for the exopolysaccharide production from mycelial production of *F. feei* and *F. pinicola* (Dubok et al. 2007). Similar findings were reported by Magday et al. (2014) that a slightly acidic pH of 6.0 and 6.5 could support a rapid and thick mycelial growth of *G. lucidum*, similarly, low pH levels like 5.0 supports the growth of *Stropharia rugosoannulata* and *Pleurotus ostreatus* (Furlan et al. 1997). The luxuriant growth in unaerated condition is congruent to the observartion of Chang & Miles (2004) that oxygen and carbon dioxide are the components of air that are significant to the growth of most fungi. Similar to what this present study has revealed, *Lentinus squarrosulus* incubated at sealed conditions also produced larger mycelial growth (De Leon et al. 2017), possibly, due to high concentration of CO_2, the rate of the mycelial growth is triggered. In accordance with the study of Magday et al. (2014), wherein rich biomass production was also observed with *G. lucidum* in sealed condition. Moreover, assessment of the effect of illumination is necessary to determine the most suitable environmental condition for mycelial growth (Chang & Miles 2004). Results showing that total darkness influenced the mycelial growth of *F. feei* as it showed thickest mycelial density and rapid incubation period is similar with the study of Kalaw et al. (2016) wherein dark conditions proved maximum growth for *G. lucidum* strain B, *L. tigrinus* CLSU strain, *G. lucidum* strain A, *L. tigrinus* CLSU strain, and *Coprinopsis cinerea* Sto. Domingo strain. This also conforms to the study of Reyes et al. (2009), which shows that light is not essential for the growth of *Coprinus comatus,* as well as in the growth of *Paneolus cyanescens* and *Paneolus antillarium* (Bustillos et al. 2014). Better growth of mushroom in darkness was also observed in *Laricifomes officinalis* (Zhu Na et al. 2011) and *L. sajor-caju* (Cuevas et al. 2009). According to Chang & Miles (2004), light might hinder the ramification or even cause the death of mycelia in strong light conditions. Another ecological factor that affects mushroom growth is temperature (Bellettini et al. 2019). The result of this study is supported by the

study of Bindu & Charya (2017a) wherein, the abundance of mycelial growth and exopolysaccharide production of *F. feei* was found at 30ºC, while *F. pinicola* was observed to have maximum mycelial growth at 25ºC (Dubok et al. 2007). This also conforms with the study of Jacob et al. (2015) wherein three species of mushroom; *Pleurotus citrinopileatus, Pleurotus djamor* and *Pleurotus salmoneostramineus*, grew best at room temperature. Similarly, in terms of the growth at refrigerated condition, the present study is congruent to the report of Lee et al. (2008). Thus, temperature greatly affects growth of mycelia (Kalaw et al. 2016). Additionally, the quality of spawning material contributes the most in the production of macrofungi (Awi–Waadu & Stanley 2010). In terms of the spawn evaluation, the luxuriant and fast mycelial growth in corn grits is similar to the results obtained by Magday et al. (2014), in which *G. lucidum* exhibited a robust growth in corn grits, fully colonizing the substrate within five-days incubation period. These results suggest that the protein, fatty acid, and high carbohydrate content of cracked corn enhances the growth of *F. feei* mycelia.

Teratogenicity

The result of the study wherein the toxic effects of the *F. feei* ethanolic extract was dependent on the concentration and time of exposure is in conformity with the findings of Bustillos et al. (2016), who reported that prolonged period at higher concentration of *P. antillarium* and *P. cyanescens* extracts increased the mortality of zebrafish embryos. The study of Reneses et al. (2016) also suggested that *L. sajor-caju* exhibited toxic and teratogenic effects to zebrafish embryo as a 100% mortality was observed at 3% and 1% extract concentrations and at 0.5% extract concentration after 36 hours and 48 hours, respectively. Moreover, Dulay et al. (2017), disclosed no mortality to embryos at 0.10% and lower concentrations of *V. volvacea*. In terms of hatchability it was observed that as the concentration of the ethanol extract decrease, the mean percentage hatchability of the embryos exposed on lower treatment concentrations increased. Thus, varying amount of the ethanol extract of *F. feei* affects the normal hatchability of the embryos exposed at 48 hours. In line with this, De Castro et al. (2016), found out that the effect of the extract is dose dependent. The treated embryos with higher concentrations of *T. clepeatus* ethanol extract was said to be significantly different from that of the control. De Castro & Dulay (2015), also reported that the effect of ethanol extracts from *L. sajor-caju* and *P. ostreatus* in *D. rerio* embryos were dependent on concentration and period of exposure. Based on the results obtained, several factors such as antioxidants could probably affect the hatching time of zebrafish embryo. Thus, the success of hatching rate of the *F. feei* ethanol extract may be attributed to some of its functional components. Moreover, the normal embryonic heartbeat rate in zebrafish is much closer to that of humans, at 120-180 beats per minute (Mabley & Childs 2010). Evaluation of heartbeat shows that as the concentration of the *F. feei* ethanolic extract increased, the heartbeat rate significantly decreased. These results are correlated with the findings of Dulay et al. (2014), wherein the extract of *Lentinus tigrinus* induce a significant decreased in heartbeat rate at 5% or higher concentrations. Furthermore, growth retardation is the most prominent teratogenic effect of *F. feei* ethanolic extract. Similarly, the major teratogenic effects of *L. sajor-caju* and *P. ostreatus* ethanol extract is delayed growth (De Castro & Dulay 2015). This is also correlated with the study of Dulay et al. (2012), wherein growth retardation was evident among embryos exposed to 5%, 10% and 20%.

Cytotoxicity

The brine shrimp lethality assay showed that *F. feei* in higher concentrations exhibited toxic effect. This is an interesting finding since some anti-cancer drugs are known to be cytotoxic. These results could be correlated to the study of Shnyreva et al. (2018), wherein *F. pinicola* exhibited cytotoxicity against Jukart leukemia cell line. Additionally, Wu et al. (2014) showed that *F. pinicola* exerted anti-cancer effect against cancer cell lines since its ethanol extract induces cell apoptosis to exert significant anti-tumor activity. Furthermore, the study of Younis et al. (2014) showed that *Lentinula edodes* extract exhibited high toxicity in human liver carcinoma cell line and concluded that mushrooms could be a source of antitumor compounds. Similarly, Badshah et al.

(2015) reported that *Astraeus hygrometricus* and *Calvatia gigantea* have high phenolic content and total antioxidant activity as well as brine shrimp cytotoxicity. Therefore, the bioactive compounds present in *F. feei* could be harnessed for its pharmaceutical potential.

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