Current Research in Environmental & Applied Mycology (Journal of Fungal Biology)
10(1): 300–318 (2020) ISSN 2229-2225
www.creamjournal.org
Article
Doi 10.5943/cream/10/1/28

Mycochemical screening, antioxidant evaluation and assessment of bioactivities of *Xylaria papulis*: a newly reported macrofungi from Paracelis, Mountain Province, Philippines

De Leon AM[1,2*], Diego EO[1], Domingo LKF[1] and Kalaw SP[1,2]

[1]*Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines*
[2]*Center for Tropical Mushroom Research and Development, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines*

De Leon AM, Diego EO, Domingo LKF, Kalaw SP 2020 – Mycochemical screening, antioxidant evaluation and assessment of bioactivities of *Xylaria papulis*: a newly reported macrofungi from Paracelis, Mountain Province, Philippines. Current Research in Environmental & Applied Mycology (Journal of Fungal Biology) 10(1), 300–318, Doi 10.5943/cream/10/1/28

Abstract

Macrofungi is a diverse group of organisms that are known to be utilized by ancient people since earliest times for a number of their functions. In the Philippines, macrofungi have been part of the horticultural diversification of the people and were used by ethnic communities across the country. *Xylaria papulis* collected from Paracelis, Mountain Province have been assessed for its mycochemical, antioxidant, antibacterial, teratogenic and cytotoxic activities. Results showed that *X. papulis* exhibited secondary metabolites such as essential oil, triterpenes, coumarines, anthraquinones, tannins, flavonoids, anthrones, fatty acid, phenols, steroids and alkaloids. The recorded DPPH radical scavenging activity is 55.67%, however, it is lower than catechin with 71.13%. On the contrary, *X. papulis* exhibited high total phenolic content of 43.17 mg GAE/g. Moreover, the ethanolic extract of *X. papulis* showed a mean zone of inhibition ranging from 16-18 mm after 24 hours of incubation which is interpreted as an intermediate effect against *Escherichia coli* and *Staphylococcus aureus*. Furthermore, the teratogenic activity to the developing *Danio rerio* embryo showed that growth retardation, and head and tail malformations are the most notable effect. Mortality rates of the embryos were significantly high due to exposure to different concentrations of the *X. papulis* ethanol extract and the hatchability was completed after 48 hours for the concentrations ≤250 ppm. Abnormal heart beat rates, as well as pericardial edema causing weak and slow heart beats was also noted suggesting defective cardiac function and cardio-toxicity. Cytotoxicity was observed in all the treatments ranging from 40% to 90% mortality. The highest mean percentage mortality of 90% was recorded in 1250 ppm. Brine shrimp lethality assay showed an LC_{50} of 218.507 ppm, which indicates that the *X. papulis* ethanolic extract is highly toxic.

Key words – antibacterial – antioxidant – cytotoxicity – mycochemical – teratogenicity

Introduction

Macrofungi are one of the most diverse group of organisms inhabiting almost every type of ecosystem. Most popular and important of these fungi are the mushrooms that has been known for its functions since ancient times (Kinge et al. 2017). Apart from their biological and economical importance (Teke et al. 2018) and with its nutritional and medicinal purposes (Pala et al. 2013),

Submitted 23 April 2020, Accepted 21 July 2020, Published 19 August 2020
Corresponding Author: Angeles M. De Leon – e-mail – angelesdeleon@clsu.edu.ph

mushrooms have become part of the cultural diversification of ancient people as a center of mythological beliefs and as part of important rituals. Because of its availability in nature, mushroom started to be a source of food (Rahi & Malik 2016), then later on as a source of income (Teke et al. 2018). Mushrooms are considered as functional foods because they contain substances that might be used directly in the diet and promote healthiness. They are rich in protein, carbohydrates, minerals, vitamins, unsaturated fatty acids, phenolic compounds, antioxidants, tocopherols, ascorbic acid and carotenoids that fit the definition of food supplements (Reyes et al. 2016, De Leon et al. 2017, Adebola et al. 2016, Reis et al. 2011, Villares et al. 2012, Vieira et al. 2014, Dulay et al. 2015, Khatun et al. 2012). The medicinal benefits derived from mushrooms such as anti-fibrotic, anti-inflammatory, antidiabetic, antimicrobial, anti-tumor, anti-Alzheimer, anti-malarial, blood sugar lowering, cholesterol reducing, and liver protectant have been previously reported (Kalaw et al. 2016, Shikongo et al. 2013, Zhang et al. 2014, Wang et al. 2014, Ismail et al. 2015, Dulay et al. 2017, 2014). Due to the fact that mushrooms can be a rich resource of bioactive compound, people discovered their medicinal purposes and nutraceutical importance. In present times, mushrooms are involved in the development of drugs and other nutraceutical advancements because of its antioxidant, antitumor, and antimicrobial properties (Khatun et al. 2012).

Furthermore, mushrooms also exhibit toxic and teratogenic effects. Teratogenic activities of mushroom are important as some teratogens can be used as anticancer drugs and anticancer drugs are teratogenic as well (Reneses et al. 2016). Therefore, teratogenecity can be utilized to evaluate new toxic compounds with potential anticancer properties. Moreover, it can also be used in many toxicological researches including environmental toxicity in relation to the harmful effects brought by chemical pollutants, and safety appraisement of new pharmaceutical products (Dulay et al. 2014). Assessing the teratogenic activities of some drugs can be done with the use of zebrafish (*Danio rerio*) primarily because of its characteristics that are similar to other vertebrate animals including humans (Dulay et al. 2012). Therefore, teratogenicity assay with zebrafish as a model is an important tool in determining if certain compounds or substances could cause deformities to new individuals (Dulay et al. 2014). Moreover, another effect of mushroom is its cytotoxicity which can also be attributed to the anticancer and antitumor properties of mushroom. A cytotoxicity assay that has been popularly adopted in most researches is the brine shrimp lethality assay which is a simple, rapid, and inexpensive bioassay assessing bioactivities of plant extracts (Baravalia et al. 2012, Oany et al. 2016). This assay utilizes a model animal, brine shrimp (*Artemia salina*) as it is easy and practical to use in various lethality assays (Asaduzzaman et al. 2015).

Macrofungi, being an ancient medicinal and nutraceutical resources have been known to be utilized by ethnic communities, especially in the Philippines. Mountain Province, which is a landlocked territory located in the north of Ifugao, south of Kalinga, east of Ilocos Sur, and west of Isabela, is a place where most of the indigenous communities in the country resides (PhilGIS 2019), particularly in the locality of Paracelis. Paracelis comprises several ethnic groups like the Gaddang, and Baliwon, with some migrants from Balangao, Madukayan, Kalinga, and Ifugao tribes including the Ilokanos (Igorotage 2016). It is a perfect place to search for mushroom utilized by these indigenous communities. Therefore, this study was conducted to evaluate the medicinal properties of *X. papulis* collected from Paracelis, Mountain Province.

Materials & Methods

Source of Mushroom

Xylaria papulis was collected from Paracelis, Mt. Province in January of 2019. It was photographed on site, harvested carefully, placed in a properly labeled paper bag and immediately brought to the laboratory for analysis.

Preparation of extracts

Wild fruiting body of the *X. papulis* was air dried at room temperature (27^{o}C) prior to extraction. This was cut into smaller pieces and powdered using a blender. Five grams of powdered

air dried fruiting body of *X. papulis* was extracted in a 500 ml of 95% ethanol for 48 hours. Extract were then filtered using Whatman filter No. 2 and concentrated to dryness using rotary evaporator. The functional component of the mushroom was obtained through ethanol extraction which was done and processed at Saint Mary's University, Bayombong, Nueva Vizcaya.

Mycochemical Screening of *Xylaria papulis*

To detect the secondary metabolites of the extract mycochemical screening was carried out. The extract was spotted on marked and labeled TLC (thin layer chromatography) 7 x 4 cm. This was developed in the acetate-methanol (7:3) mixture in the developing chamber. To check the separation of the different compounds, the spots for certain metabolites was visualized using TLC plates and it was exposed under the UV light and hot plate. To determine the presence of phenols, steroids, triterpenes and essential oils, vanillin-sulfuric acid reagents was utilized. For the visualization of secondary metabolites (anthraquinones, coumarins and anthrones) a metanolic potassium hydroxide was used. While the phenolic compounds and tannins was detected through the use of potassium ferricyanide-ferric chloride reagent. Finally, to detect the presence of flavonoids a Dragendorff's reagent was utilized.

DPPH Radical Scavenging Assay

The radical scavenging activity of the bioactive compounds of *X. papulis* extract was evaluated using DPPH assay. Concentrated extract of *X. papulis* was used as stock solution and aliquot was taken to make 1000 ppm dilution and 1000 ppm of Catechin served as control (1 mg/mL). Four milliliter of 0.1 mM DPPH solution was mixed with 1 ml of prepared stock solution in separate plastic cuvette. The reactions were done in triplicate. Prepared mixtures were incubated in the dark at 37°C for 30 minutes. At 517 nm using a UV VIS spectrophotometer the absorbance readings were monitored. Higher free radical scavenging activity was determined by the lower absorbance of the reaction mixture. The radical scavenging activities were compared to the activity of the control Catechin. The ability to scavenge the DPPH radical was calculated using the equation:

$$\%\ Radical\ Scavenging\ Effect = [\ (A_{control} - A_{sample})\ /\ A_{control}\ ]\ x\ 100$$

Where $A_{control}$ is the absorbance of the control which is the DPPH without the sample. A_{sample} is the absorbance of the test sample containing the DPPH sample. Catechin was used as the positive control.

Total Phenolic Content

Folin-Ciocalteu reagent was used to determine the amount of total phenolic in the extract of *X. papulis*. Gallic acid was used as a standard and the total phenolic was expressed as mg/g Gallic Acid Equivalents (GAE). Concentration of 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49 and 1 mg/ml of gallic acid was prepared in methanol. Concentration of 1 mg/ml of extract was prepared in methanol and 0.5 ml of each sample was introduced into test tubes and mixed with 2.5 ml of a 10-fold dilute Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. Test tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was read at 760 nm. This was done in triplicate. Follin-Ciocalteu is sensitive to reducing compounds including polyphenols, thereby producing a blue color upon reaction, this blue color is measured spectrophotometrically. Thus, total phenolic content was determined.

Disc Diffusion Assay

Prepared petri plates containing the Mueller Hinton Agar were inoculated with 10 µl of bacterial culture and was aseptically transferred at the center of the plates and was spread using sterile cotton swab. Even distribution of the culture was assured. The prepared 6 mm of Whattman filter paper discs were submerge to 100 µl of the ethanolic extracts, ethanol, streptomycin (positive

control) and distilled water (negative control) then this was allowed to dry for 30 minutes. After drying of the discs this were inoculated on the prepared media using sterile forceps. Four discs were inoculated in each petri plates consisting the pure *X. papulis* ethanolic extracts, ethanol, streptomycin as the positive control and distilled water as the negative control. All were replicated three times. This was incubated at 37°C in an inverted position. Zones of inhibition was measured after 12 and 24 hours of incubation using a digital vernier caliper.

Teratogenicity Assessment

Preparation of Aquarium Tank

A glass aquarium of approximately 5-gallon capacity which contained untreated and clean tap water with continuous flow of oxygen via aerator was used to maintain male and female zebrafish with a ratio of 2 males to 1 female.

Zebrafish care, culture, and maintenance

Healthy and sexually matured zebrafish were acquired from the Wet Laboratory of College of Fisheries, CLSU which were acclimatized for one week at 26±1ºC (room temperature) before using for spawning and fertilization. These were fed daily and the leftover food was also removed daily for the maintenance of water cleanliness and quality.

Zebrafish spawning and egg production

Zebrafish, being photoperiodic breeders, have daily cycle of light and darkness that affects the behavior and physiological function of the organism. Zebrafish were placed first in a coarse plastic mesh submerged into the aquarium water to protect the eggs from being devoured by the matured zebrafish once they were fertilized. For stimulation of spawning, the zebrafish were exposed in a dark condition by covering the aquarium with a black plastic mesh. After 12 hours in dark condition, the black plastic mesh was removed and the light was turned on. With the aid of the light, fertilized eggs were seen at the bottom of the aquarium.

Harvesting of fertilized eggs

Fertilized eggs were collected by siphoning them out of the aquarium using a hose tube after 12-hour post fertilization period, then these were transferred in a beaker. Morphological assessment of the fertilized eggs was done before placing them in the vials, wherein whitish and ruptured eggs were discarded. This would ensure the quality and uniformity of eggs prior to treatment suspension.

Treatment preparation

The extract was diluted using embryo water (Hank's solution) and the concentrations were prepared using the standard dilution formula, C1V1 = C2V2. Three mL of each solution was used.

Zebrafish (*Danio rerio*) teratogenicity and toxicity assay

Three milliliters of each treatment concentration were placed in each vial with four embryos at segmentation phase. The vials were incubated at 26±1ºC. Teratogenic activity was observed under a compound microscope with 40x magnification after 12, 24, 36, and 48 hours of incubation. Morphological endpoint evaluation such as teratogenic (malformation of head and tail, scoliosis, growth retardation, stunted tail, and limited movement), lethal (coagulation, tail not detached, no somites, and no heartbeat) and normal was examined. Hatchability and mortality rate were also recorded. Pictures were taken out and death was recognized as coagulated embryos.

Cytotoxicity Evaluation

Source of Brine Shrimp (*Artemia salina*) eggs

Artemia cysts INVE brine shrimp eggs were obtained from the Wet Laboratory of the College of Fisheries, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines.

Dilution of Extract

The extract was diluted modifying the procedure of Manasathien et al. (2012).

Hatching Set-up

A 1.5 L plastic bottle was cut at its base to serve as the hatchery vessel. The cut plastic bottle 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 supply temperature at 28-30ºC. For proper aeration, aquarium air pump with air stone was placed at the bottom of the hatchery vessel.

Hatching of Brine Shrimp Eggs

The hatchery was filled with artificial sea water following the procedure of McLaughlin & Rogers (1998), which was prepared by dissolving 38 g of sea salt or rock salt in 1 L of distilled water. 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-hour life. The hatchery was covered with a thin gauze to protect the eggs from undesirable organisms that may have a negative effect on their development. The hatched eggs produced the nauplii which was used for the assay.

Brine Shrimp Lethality Assay

Forty-eight-hour old nauplii were harvested using a hand lens and were used for the assay. The nauplii were transferred in each sample vials using a syringe. For the food of the larvae, a drop of yeast (3 mg yeast/5 mL saline solution) was added to each vial. Two hundred µL of different concentration was also added. It was observed after 6, 12, 18, and 24 hours. The vials were maintained under illumination. Survivors were counted using hand lens. The LC_{50} was evaluated according to the rating of Aldahi et al. (2015) stating that LC_{50} of <249 µg/mL is highly toxic, LC_{50} of 250-499 µg/mL is moderately toxic and LC_{50} of 500-1000 µg/mL is mildly toxic. Moreover, values above 1000 µg/mL are non-toxic according to the rating of McLaughlin & Rogers (1998).

Statistical Analysis

All the treatments were laid out in complete randomized design (CRD) under laboratory conditions. To analyze the data, one-way analysis of variance (ANOVA) was used to determine least significant differences (LSD) between treatments 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 all the analyses.

Results

Mycochemical Screening of the *Xylaria papulis*

Qualitative mycochemical composition of the 5 g powdered dried fruiting bodies of *X. papulis* collected from Paracelis, Mountain Province and extracted with 95% ethanol is presented in Table 1. The extract was evaluated for the presence of 11 secondary metabolites which includes essential oil, triterpenes, coumarines, anthraquinones, tannins, flavonoids, anthrones, fatty acid, phenols, steroids and alkaloids. All the aforementioned secondary metabolites evaluated were found present in *X. papulis*.

Antioxidant Property of *Xylaria papulis*

DPPH radical scavenging activity of the ethanolic extract of *X. papulis* was determined and the results are shown in Table 2. *X. papulis* exhibited high DPPH radical scavenging

activity (55.67%), however, it is lower than the control Catechin (71.13%). The phenolic content of ethanolic extract of *X. papulis* was evaluated and also presented in Table 2. Results revealed that *X. papulis* has a total phenolic content of 43.17 mg GAE/g.

Table 1 Mycochemical composition of *Xylaria papulis*

Mycochemicals	Results
Essential oil	+
Triterpenes	+
Coumarines	+
Anthraquinones	+
Tannins	+
Flavonoids	+
Anthrones	+
Fatty acid	+
Phenols	+
Steroids	+
Alkaloids	+

Table 2 Radical scavenging activity and total phenolic content of *Xylaria papulis*

Species	% Radical Scavenging Activity (RSA)	Total Phenolic Content (mg GAE/g)
Xylaria papulis	55.67	43.17
Catechin (control)	71.13	-

Antibacterial property against *E. coli* and *S. aureus*

The antibacterial activity using disc diffusion assay of the ethanolic extract of *X. papulis*, ethanol (negative check), streptomycin (positive control) and distilled water (negative control) against gram-negative bacteria *Escherichia coli* and gram positive *Staphylococcus aureus* were presented in Table 3 and Fig. 1. At 24 hours of incubation, the ethanolic extract of *X. papulis* exhibited a zone of inhibition with 16.87 mm and 18.87 mm against *E. coli* and *S. aureus*, respectively. Statistical analysis shows that at 5% level of significance there is significant difference among all the treatments after 24 hours of incubation. According to CLSI (2012) certain microbial species are considered susceptible to antimicrobial agent when its zone diameter (mm) is (≥20), intermediate (15-19) and resistant (≤14). Therefore, after 24 hours of incubation the ethanolic extract of *X. papulis* has an intermediate effect against *E. coli* and *S. aureus* which implies that the ethanolic extract of *X. papulis* has a broad spectrum antibacterial activity and can be a good source of naturally occurring metabolites against microbial infections.

Teratogenicity of *Xylaria papulis*

Percentage Mortality

Mortality of the embryos is said to be having no visible heartbeat and coagulation. The lethal effect of different concentrations of *X. papulis* ethanol extract to zebrafish embryos was observed and the mean percentage mortality of the embryos after 12, 24, 36, and 48 hour of exposure to varying concentrations was recorded and shown in Table 4.

At 12-hour post treatment application (hpta), mortality of 58.33% was observed in 1250 ppm, while 8.33% was noted in 1000 ppm. This increased at 24 hpta with 100% and 91.67%, respectively.

All embryos at 1250 ppm was observed to be dead due to coagulation. On the other hand, percentage mortality in 750 ppm increased at 24 hpta by 50%. Moreover, there was no recorded

mortality in 500 ppm until 36 hpta with 66.67% which is significantly different to the percentage mortality of higher concentrations of the same hour. However, by 48 hpta, there was no significant difference recorded among all the concentrations ≥500 ppm. Furthermore, there was no mortality observed on the lower concentrations of 250 ppm, 100 ppm, and the control throughout the observation.

In view of the aforementioned, the mortality of the embryos was directly proportional to the varying concentrations of *X. papulis* ethanol extract and the time of exposure of the embryos. As the concentration and the time of exposure increased, percentage mortality increased, as well. This result implies that *X. papulis* ethanol extract is toxic to the embryos. The most notable toxic effect of the *X. papulis* ethanol extract is coagulation of the embryos exposed to ≥750 ppm starting from 36 hpta as shown in Fig. 2.

Table 3 Mean Zone of Inhibition of *Xylaria papulis* against *Escherichia coli* and *Staphylococcus aureus*

Treatments	Zone of Inhibition (mm)	
	E. coli	***S. aureus***
X. papulis Extract	16.87 ± 0.81^{a}	18.87 ± 1.60^{a}
Streptomycin	26.73 ± 0.06^{b}	26.53 ± 0.55^{b}
Distilled Water	6.00 ± 0.00^{c}	6.00 ± 0.00^{c}
Ethanol	6.00 ± 0.00^{c}	6.00 ± 0.00^{c}

*Mean ± SD. Means having the same letters are not significantly different at 5% level using Turkey's HSD test

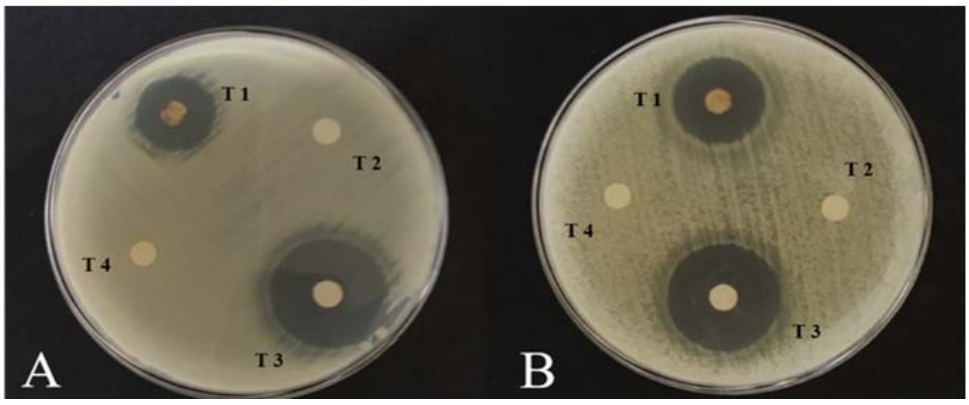

Fig. 1 – Effect of ethanolic extract of *X. papulis* against *E. coli* and *S. aureus* at 24 hours of incubation A = *E. coli*. B = *S. aureus*. T1 = *X. papulis* extract. T2 = distilled water. T3 = streptomycin sulfate. T4 = ethanol.

Table 4 Mean percentage mortality of *D. rerio* embryos after 12, 24, 36 and 48 hours of exposure to different concentrations of *X. papulis* ethanol extract.

Concentration (ppm)	Mortality (H)			
	12	**24**	**36**	**48**
1250	58.33^{a}	100.00^{a}	100.00^{a}	100.00^{a}
1000	8.33^{b}	91.67^{a}	100.00^{a}	100.00^{a}
750	0.00^{b}	50.00^{b}	100.00^{a}	100.00^{a}
500	0.00^{b}	0.00^{c}	66.67^{b}	91.67^{a}
250	0.00^{b}	0.00^{c}	0.00^{c}	0.00^{b}
100	0.00^{b}	0.00^{c}	0.00^{c}	0.00^{b}
0	0.00^{b}	0.00^{c}	0.00^{c}	0.00^{b}

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

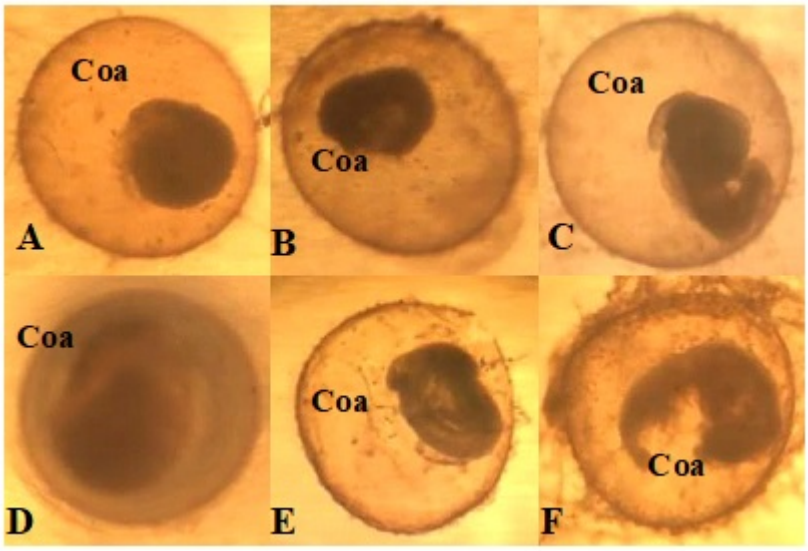

Fig. 2 – Toxic effects of various concentrations *X. papulis* ethanol extract at different hours of exposure. A coagulation (Coa) at 36 hpta in 1250 ppm concentration. B coagulation (Coa) at 36 hpta in 1000 ppm concentration. C coagulation (Coa) at 36 hpta in 750 ppm concentration. D coagulation (Coa) at 48 hpta in 1250 ppm concentration. E coagulation (Coa) at 48 hpta in 1000 ppm concentration. F coagulation at 48 hpta in 750 ppm concentration.

Heartbeat Rate

The heartbeat rate was measured under the microscope at pharyngula stage of the embryo when pigmentation in the zebrafish is prominent. The mean heartbeat rate for each concentrations of *X. papulis* ethanol extract is shown in Table 5. The highest mean heartbeat rate was recorded in the control with 188 beats per minute, whereas the lowest was noted in 500 ppm with 24 beats per minute. Moreover, heartbeat was not observed in concentrations ≥500 ppm due to early death of the embryos. It can be deduced that as extract concentration increased, heartbeat rate significantly decreased. Furthermore, there was an observed abnormal heartbeat rate on 500 ppm due to slow and weak heartbeat implying pericardial edema.

Table 5 Mean heartbeat of embryos on various concentrations of *X. papulis* ethanol extract.

Concentration (ppm)	Heartbeat
1250	0.00^{c}
1000	0.00^{c}
750	0.00^{c}
500	24.00^{c}
250	148.00^{b}
100	182.00^{ab}
0	188.00^{a}

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

Percent Hatchability

The mean percentage hatchability of the embryos exposed to different concentrations of the *X. papulis* ethanol extract is presented in Table 6. The hatchability of zebrafish embryos was observed after 48 hpta. There was no observed hatchability in concentrations ≥500 ppm due to coagulation or halted growth before even reaching the hatching phase. Percent hatchability was only observed in 250 ppm with 8.33%, 100 ppm with 33.33%, and the control with 100% having significant difference between the latter two. The hatching rate was greatly affected by the varying concentrations of *X. papulis* ethanol extract. As the concentration increased, the hatching rate

decreased. Likewise, prolonged exposure of the embryos to different concentrations of the extract causing malformation could lead to the observed hatching process.

Table 6 Mean percentage hatchability of *D. rerio* embryos after 48 hours of exposure to different concentrations of *X. papulis* ethanol extract.

Concentration (ppm)	Hatchability
1250	0.00^{c}
1000	0.00^{c}
750	0.00^{c}
500	0.00^{c}
250	8.33^{bc}
100	33.33^{b}
0	100.00^{a}

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

Morphological Endpoints of Treated *D. rerio* Embryos

Different parameters such as head and tail malformation, growth retardation, little pigmentation, and limited movement were used as morphological endpoints to assess the teratogenic effects of the *X. papulis* ethanol extract to embryos after 12, 24, 36, and 48 hours of exposure as presented in Table 7. It was observable that the most prominent teratogenic effect of the macrofungi ethanol extract was growth retardation. This was observed in embryos exposed to ≥ 750 ppm during 12 up to 48 hours, as well as those exposed at 500 ppm at 36 and 48 hours. As shown in Figure 3. As early as 12 hours, delayed development and eventually, arrested growth were already noticeable at 1250 ppm of the *Xylaria papulis* extract.

Other malformation and morphological abnormalities

Other teratogenic effects of *X. papulis* ethanol extract on developing zebrafish embryos are presented on Fig. 4. The only observed head malformation was microcephaly at 12-hours exposure in 1250 ppm (Fig. 4A), 24-hours (Fig. 4B) and 36-hours (Fig. 4C) exposure at 1000 ppm. Moreover, the tail malformation observed was curved tail at 36-hours exposure in 1000 ppm (Fig. 4C). Little pigmentation was also observed at 12-hours exposure to 1250 ppm (Fig. 4A), and 36-hours exposure to 1000 ppm (Fig. 4C). On the other hand, other morphological abnormalities present but were not included in the toxicological endpoints in Table 7 were abdominal edema, yolk deformity, no gut, and pericardial edema.

Table 7 Lethal and teratogenic effects of various concentrations of *X. papulis* ethanol extract at 12, 24, 36, and 48 hours of exposure.

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

Table 7 Continued.

Toxicological Endpoints	Time of Exposure (Hour)	Concentration (ppm)						
		0	100	250	500	750	1000	1250
Tail not detached	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-
Teratogenic								
Malformation of head	12	-	-	-	-	-	-	+
	24	-	-	-	-	-	+	-
	36	-	-	-	-	-	+	-
	48	-	-	-	-	+	-	-
Malformation of tail	12	-	-	-	-	-	-	+
	24	-	-	-	-	-	+	-
	36	-	-	-	-	-	+	-
	48	-	-	-	-	-	-	-
Growth retardation	12	-	-	-	-	+	+	+
	24	-	-	-	-	+	+	+
	36	-	-	-	+	+	+	+
	48	-	-	-	+	+	+	+
Limited movement	12	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-
	48	-	-	+	+	-	-	-
Little pigmentation	12	-	-	-	-	-	-	+
	24	-	-	-	-	-	+	-
	36	-	-	-	-	-	-	-
	48	-	-	-	+	+	-	-

Cytotoxicity

The cytotoxic activity of *X. papulis* ethanol extract was determined by performing brine shrimp lethality assay. The mean percentage mortality of *A. salina* 48-hour old nauplii exposed to varying concentrations of the extract during the assay is shown in Table 8.

The highest mortality rate was noted at 1250 ppm with 90% while the lowest was recorded at 100 ppm with 40% mortality. Analysis of variance (ANOVA) showed that there is significant difference among all the treatments except for 750 ppm and 500 ppm which has no significant difference with treatments 750 ppm and 250 ppm, respectively.

Median lethal concentration (LC_{50}) of the extract was determined using probit analysis based on the data presented in Table 8. Confidence limits of lethality concentration of brine shrimp lethality assay showed that the lower bound is set up at 104.866 ppm and the upper bound is 455.297 ppm. As presented in Fig. 5, the LC_{50} of *X. papulis* ethanol extract was 218.507 ppm which is considered to be highly toxic according to the ratings of Aldahi et al. (2015) wherein LC_{50} of <249 µg/mL is highly toxic.

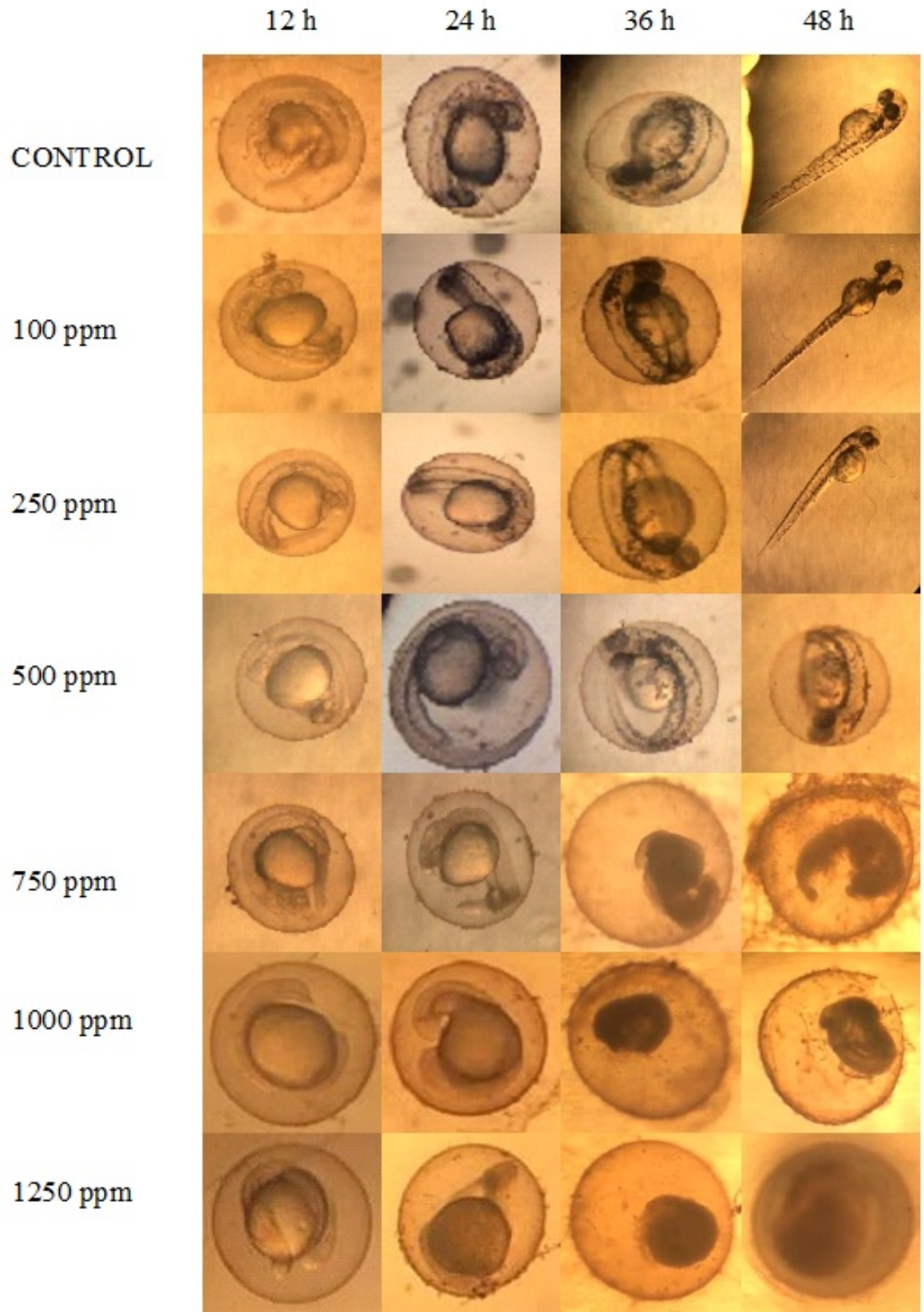

Fig. 3 – Morphological development of embryos exposed to different concentrations of *X. papulis* ethanol extract.

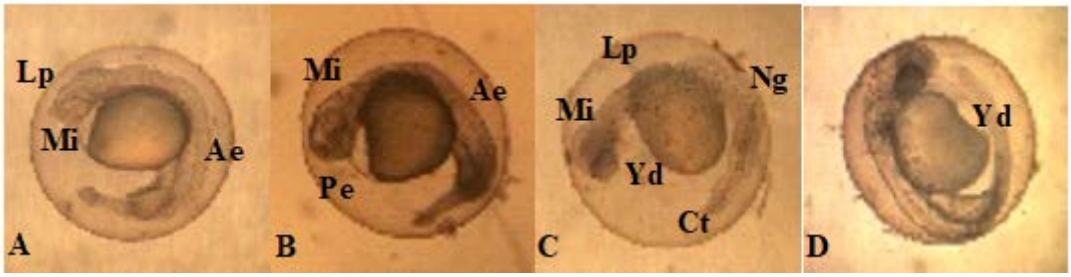

Fig. 4 – Toxic and teratogenic effects of various concentrations of *X. papulis* ethanol extract. A little pigmentation (Lp), microcephaly (Mi), and abdominal edema (Ae) at 12 hpta in 1250 ppm concentration. B microcephaly (Mi), pericardial edema (Pe), and abdominal edema (Ae) at 24 hpta in 1000 ppm concentration. C limited pigmentation (Lp), microcephaly (Mi), yolk deformity (Yd), curved tail (Ct), and no gut (Ng) at 36 hpta in 1000 ppm concentration. D yolk deformity (Yd) at 48 hpta in 750 ppm concentration.

Table 8 Mean percentage mortality of *A. salina* nauplii after 24 hours of exposure to different concentrations of *X. papulis* ethanol extract.

Concentration (PPM)	Mortality (%)
1250.00	90.00[a]
1000.00	66.67[b]
750.00	63.33[bc]
500.00	60.00[bc]
250.00	53.33[c]
100.00	40.00[d]
0.00	0.00[e]

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

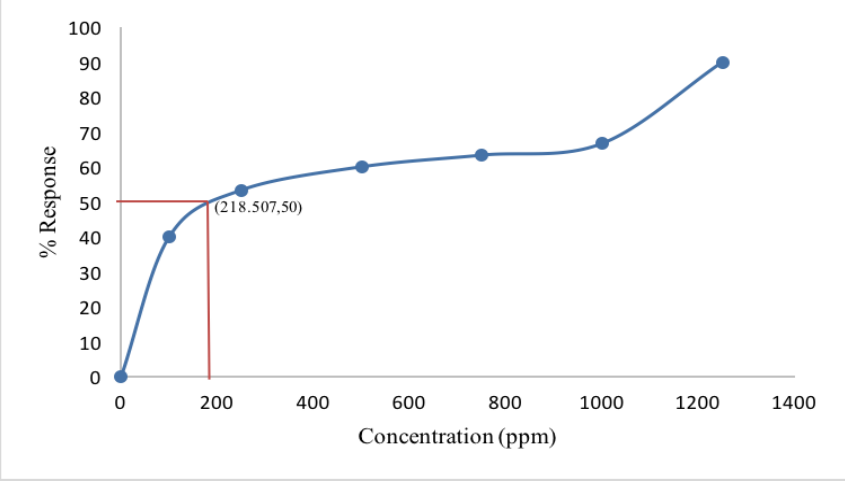

Fig. 5 – Point estimate of LC_{50} value of *X. papulis* ethanol extract after 24 hours of exposure

Discussion

Mycochemicals are bioactive compounds in mushrooms that plays an important role in reducing the risk of diseases (Rusenova & Parvanov 2009) such as cancer, microbial infection, diabetes, inflammation and stroke (Thakur et al. 2015). The presence of essential oil, triterpenes, coumarines, anthraquinones, tannins, flavonoids, anthrones, fatty acid, phenols, steroids and alkaloids in *X. papulis* correlates to the findings of the study conducted by Adebola et al. (2016) that certain species of mushrooms are rich in mycochemicals. Kalaw

& Albinto (2014) reported that *Coprinus comatus* has alkaloids, cardiac glycosides, flavonoids, saponins, steroids and terpenoids were present in this mushroom while *Pleurotus cystidiosus* only possessed alkaloids, flavonoids, saponins and terpenoids. These mycochemicals are responsible for many biological activities and pharmacological properties (Figueiredo et al. 2008). Essential oils acts as phytoprotective agents which are distillates of the volatile compounds of a plant's secondary metabolism (Rusenova & Parvanov 2009). They been used as an active ingredients or constituents of drugs, soaps, shampoos, perfumes and cosmetics (Nerio et al. 2010). They also been used as an alternative remedy for treatment of many infectious diseases**.** Certain studies on different species of mushrooms have also been reported to contain alkaloids, tannins and saponins such as *Panaeolus cyanescens* (Bustillos et al. 2014), *Cantharelle cibarius, Laccaria amethysta, Clitocybe odora, Lepista nuda, Macrolepiota procera, Lepista saeva, Lactarius deliciousus, Laccaria laccata, Pleurotus ostreatus, Hericium erinaceus* (Egwim et al. 2011), *Rigidoporus microporus* (Sw) (Falade et al. 2017) *Daedelea quercina, Lentinus squarrosulus, Lepiota procera, Rigidosporous lignosus* (Ilondu 2013) and *Pleurotus roseus* (Adebola et al. 2016).

Moreover, antioxidant or molecules with radical scavenging capacity are thought to exert a potential protective effect against free radical damage (Ferreira et al. 2007). The radical scavenging activity acts by inhibiting the oxidation process that produces free radicals. These free radicals are unstable atoms that causes damage to cells that leads to several diseases (Keles et al. 2011). Studies have been reported that certain species of mushroom have an ability to act as an antioxidant (Hung & Nhi 2012). The 55.67% DPPH radical scavenging activity of *X. papulis* is grater that the percentage radical scavenging activity from those in *Lentinus edodes* (53.90%) (Boonsong et al. 2016), *Tricholoma portentosum* (Fr.) (30.40%) (Ferreira et al. 2007), *Auricularia polytricha* (21.10%) (Hung & Nhi 2012), and *Polyporus squamosus* (43.30%) (Keles et al. 2011). On the other hand, it is lower than those in *Pleurotus porrigens* (85%) (Wong & Chye 2009), *Morchella conica* (78.66%) (Gursoy et al. 2009), *Agaricus bisporus* (67%) (Keles et al. 2011) and *Volvariela volvacea* (82.9%) (Hung & Nhi 2012).

Studies have proven that mushrooms contain a wide variety of secondary metabolites, including phenolic compounds. Phenolic compounds play an important role in the antioxidant activity of certain mushroom species (Edeoga et al. 2005). They function by decreasing oxidative damage in the human body. Since oxidative stress resulting from the imbalance between the level of the free radicals and the body's defense system causes serious diseases, including cancer, heart diseases, and stroke (Keles et al. 2011). Numerous studies have proven that polyphenol-rich foods and beverages consumed in human diet reduced the risk of having major diseases associated with the cardiovascular system (Ferreira et al. 2007). *X. papulis* has a total phenolic content of 43.17 mg GAE/g. The result obtained herein is greater than those of *Lentinus deliciosus* (17.25 mg GAE/g) (Ferreira et al. 2007), *Morchella elata* (15.36 mg GAE/g), *Schizophyllum commune* (25.03 mg GAE/g) (Wong & Chye 2009), *Lentinus edodes* (24.25), *Volvariella volvacea* (27.89), *Pleurotus eous* (14.03), *Pleurotus sajor-caju* (12.34) and *Auricularia auricula* (2.75) which were all determined by Boonsong et al. (2016).

Current problem of microbial drug resistance and increased concern on opportunistic infections makes the alternative drugs especially those originating from plants and Basidiomycetes to be prospective (Chelela et al. 2014). Thus, evaluation of the antibacterial property of *X. papulis* is of utmost importance. Result showed that ethanolic extract of *X. papulis* has intermediate effect against *E. coli* and *S. aureus*. These findings correlate to certain studies that mushrooms such as *Ganoderma lucidum* (Shikongo et al. 2013), *Trametes gibbosa, Trametes elegans, Schizophyllum commune* (Appiah et al. 2017) and *Geastrum triplex* (Chittaragi et al. 2013) has an antibacterial property against these two bacterial pathogens. Moreover, the antibacterial activity obtain herein is supported by the findings based on the presence of mycochemicals in *X. papulis* (Table 1). According to Rusenova &

Parvanov (2009) certain secondary metabolites plays an important role in reducing the risk of microbial infection. Such as tannins, which has been already utilized by pharmaceutical companies in order to treat diseases like microbial infection (Khanbabaee & Van Ree 2001). These results also affirmed the claims of Mehta et al. (2012) that the type of extractive solvent used affects the effectiveness of the extracts against microbial pathogens. Additionally, the results also favorably agreed to the study conducted by Gebreyohannes et al. (2019) that ethanol and hot water extracts of mushrooms contained higher bioactive compounds compared to water extracts. This explains why this mushroom possessed potent secondary metabolites which is responsible for its antibacterial activity.

Furthermore, the mortality of *D. rerio* embryos was found to be directly proportional to the varying concentrations of *X. papulis* ethanol extract and the time of exposure of the embryos. As the concentration and the time of exposure increased, percentage mortality increased, as well. This result implies that *X. papulis* ethanol extract is toxic to the embryos. The most notable toxic effect of the *X. papulis* ethanol extract is coagulation of the embryos exposed to ≥750 ppm starting from 36 hpta as shown in Fig. 2. In accordance with the study of De Castro & Dulay (2015) using ethanol extracts of *Lentinus sajor-caju* and *Pleurotus ostreatus*, the toxic effects of the mushroom extracts were dose dependent and coagulation was the most notable toxic effect. Moreover, according to these studies, the toxic effects could be accredited to the bioactive compounds present in the extract. Similar observation was reported by Sogan et al. (2018) with *Trichaleurina celebica*, wherein the embryo-toxicity and teratogenicity of *T. celebica* could be due to its secondary metabolites, namely coumarins, anthrones, alkaloids, phenolics, flavonoids, steroids, and anthraquinones. Accordingly, *Xylaria* species are quite diverse in terms of their chemical constituents, as well as bioactive compounds which includes terpenoids, xanthones, cytochalasins, cyclopeptides, polyketides, xyloketals, antifungal metabolites multiplolides A and B20, NPY Y5 receptor antagonists xyarenals A and B10, polypropionates like xylaranic acids A and B22 (Adnan et al. 2018). Furthermore, in the study of Adnan et al. (2018), xylaranic acid from *Xylaria primorskensis* revealed several medicinal importance, including its antibacterial, anti-oxidant, and anticancer properties.

Heartbeat is described as the action of the heart as it pumps blood. This is another notable parameter to assess the sub-lethal effect of certain substances, particularly for cardio-vascular assessment. Zebrafish was used as a model to measure heartbeat rate because its normal embryonic heartbeat rate is much closer to that of humans which is 120-180 beats per minute (Baker et al. 1996). According to Sarmah & Marrs (2016), heart rate, as well as pericardial edema has been used as endpoints for cardio-toxicity studies as pericardial edema provide general information for cardio-toxicity, whereas abnormal heart rate indicates defective cardiac function. Findings in this study that as extract concentration increased, heartbeat rate significantly decreased is similar with the result of Reneses et al. (2016) with *L. sajor-caju* wherein absence of heartbeat was observed in higher concentrations such as 0.05%, 1% and 3% due to coagulation at earlier phase and the highest heartbeat was recorded at the control group of embryos with a mean percentage of 106.67%.

Hatchability is an important indication for a successful developmental process. Thus, interrupted hatching process could indicate distinguishing developmental abnormalities. In the similar study of Dulay et al. (2012) using *Ganoderma lucidum*, morphological abnormalities limit the ability of the embryo to break the chorion and hatch out. Accordingly, hatchability is influenced by the embryo's ability to rupture the protective chorion (Sogan et al. 2018). The chorion has been weakened or damaged as a result of the changes in the profile of the chorion protein that might have caused an increase in the opening or widening of the chorion pore channel permitting greater influx of external solute (Alafiatayo et al. 2019).

In the same study of Reneses et al. (2016) using *Lentinus sajor-caju* hot water extract, delayed growth was the most manifested teratogenic effect which caused mortality on the zebrafish embryos at higher concentrations. This conforms with the study of Dulay et al. (2018) using *Lentinus strigosus*, wherein delayed development was evident to embryos exposed at 100 µg/ml of both mycelia and fruiting body extracts.

The cytotoxicity observed in the recent study conforms with some studies of anticancer activity of several wild mushroom species using brine shrimp assay, as well as *in vitro* cytotoxicity against human cancer cell lines (Chelela et al. 2014, Bézivin et al. 2002). In the study of Chelela et al. (2014) using brine shrimp, crude extracts of *Amanita muscaria*, exhibited high cytotoxicity of 11 µg/mL and 13.72 µg/mL for ethanol and petroleum ether extracts, respectively. Moreover, *Lactarius denigricans* possessed high cytotoxicity with LC_{50} of 12.77 µg/mL for ethanol extract, and 18.96 µg/mL for petroleum ether extract. On the other hand, similar study of Bézivin et al. (2002) with 2 murine cell lines (L1210 and 3LL) and 4 human cancer cell lines (K-562, DU145, MCF7 and U251) revealed that *Lepista inversa* showed high cytotoxicity of 20 µg/mL. Several *Xylaria* species also exhibited cytotoxicity against human cancer cell lines, in which secondary metabolites and chemical constituents isolated from the macrofungi were responsible for their cytotoxic activity (Dagne et al. 1994, Wang et al. 2005, Ramesh et al. 2015). Based on the study of Wang et al. (2005), penochalasin B from *Xylaria euglossa* exhibited potent cytotoxicity against cultured P388 cells. Moreover, cytochalasins from *Xylaria obovata* were reported to be lethal to brine shrimp and cytotoxic to HL-60 cells (Dagne et al. 1994). Ramesh et al. (2015) also obtained the same results with extracted and fractionized *Xylaria curta*, wherein fraction D showed significant cytotoxic activity against A-549 human lung cancer cells at a concentration of 60 µg/mL.

In summary, the results of this study showed that *X. papulis* consists of several secondary metabolites such as essential oil, triterpenes, coumarines, anthraquinones, tannins, flavonoids, anthrones, fatty acid, phenols, steroids and alkaloids. These mycochemicals are known to be responsible for many biological activities and pharmacological properties of mushrooms. Which also explains why *X. papulis* possessed an antioxidant activity. Moreover, the exhibited antibacterial activity of *X. papulis* against human pathogens *E. coli* and *S. aureus* is also attributed to certain secondary metabolites which plays an important role in reducing the risk of microbial infection, e.g. are tannins, which has been already utilized by pharmaceutical companies in order to treat diseases like microbial infection. Furthermore, *X. papulis* ethanol extract also demonstrated teratogenic effects on zebrafish embryos at different stages of their development and the brine shrimp lethality assay of the extract showed that it is highly toxic. The toxic effects of the mushroom extract could be accredited to the bioactive compounds present in the extract, wherein, the embryo-toxicity and teratogenicity could be due to its secondary metabolites, namely coumarins, anthrones, alkaloids, phenolics, flavonoids, steroids, and anthraquinones. However, teratogenicity and cytotocicity testing can be a desirable property for mushrooms because many anticancer drugs are teratogenic and teratogens can be used as anticancer drugs.

Acknowledgements

Our heartfelt gratitude to the people who helped to make this paper came into existence: Agana Reyn Dulay, Alicia Leecel Villanueva, Bryan Panto, Mariane Ann Pagaduan, Larny Martin and Jessa Mae Domingo. We also thank the people of Paracelis, Mountain Province, for letting us collect samples in the area.

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