



Mycochemical Composition, Antioxidant Properties, and Cytotoxic Activity of *Xylaria papulis* Lloyd (Xylariaceae) Mycelia Grown in Static and Shaking Indigenous Liquid Culture Conditions

Mark Kevin S. Lopez^{1*}, Sofronio P. Kalaw^{2,3}, Rich Milton R. Dulay^{2,3}, Angeles M. De Leon³ and Renato G. Reyes⁴

¹ Biology Department, College of Arts and Sciences, Don Mariano Marcos Memorial State University – North La Union Campus 2515 Sapilang, Bacnotan, La Union, Philippines

² Center for Tropical Mushroom Research and Development, Central Luzon State University, 3120 Science City of Muñoz, Nueva Ecija, Philippines

³ Biology Department, College of Science, Central Luzon State University, 3120 Science City of Muñoz, Nueva Ecija, Philippines

⁴ Office of the President, Aurora State College of Technology, 3200 Zabali, Baler, Aurora, Philippines

*Corresponding author: mlopez@dmmsu.edu.ph

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Abstract

The search for promising and sustainable sources of secondary metabolites with potential biofunctional activities is important for the possible sources of drugs to treat human diseases. Hence, this study qualitatively assessed the mycochemical composition and investigated the biofunctional activities (antioxidant activity and cytotoxic activity) of *Xylaria papulis* Lloyd mycelia grown in static and shaking indigenous liquid culture medium. Mycochemical screening revealed the presence of several classes of secondary metabolites, such as phenolics, terpenoids, fatty acid derivatives, alkaloids, and sugar derivatives, in mycelial mat (shaking) and in culture spent (static). Mycelial mat (static) and culture spent (shaking) also contain the same classes of secondary metabolites, with the former including essential oils. Mycelial extracts have greater antioxidant property ($\leq 43.19 \pm 1.63\%$ RSA) and total phenolics ($\leq 47.75 \pm 2.31$ mg/GAE) compared to the culture spent. Culture spent from shaking condition exhibited cytotoxicity ($LC_{50} = 94.39 \mu\text{g/mL}$) against brine shrimp larvae. These findings indicate that *X. papulis* (Lloyd) grown in liquid culture medium can be a sustainable source of diverse secondary metabolites with antioxidant properties and cytotoxic activity. Further, cytotoxicity studies against cancer cell lines can also be investigated for future studies.

Keywords: Antioxidant property, Cytotoxic activity, Mycelia, Mycochemicals, Total phenolics, *Xylaria papulis* Lloyd

1. Introduction

Drugs for the treatment of human ailments are most often discovered and developed from natural products. A viable strategy to combat the growing threat posed by drug-resistant forms of human diseases is the screening of naturally occurring bioactive chemicals from macrofungi. Among the many macrofungi in the Philippines, the genus *Xylaria* Hill ex Schrank (1786) is under the family Xylariaceae Tul. & C. Tul. (1861), which is one of the most diverse and largest families of Ascomycota and is primarily distributed throughout the world across various regions with broad biological diversity [1]. *Xylaria* is characterized mainly by more or less carbonaceous sessile or stipitate, erect stromata or fruiting structures with simple or branched cylindrical to clavate or globoid or irregular fertile parts [2]. Many species of this genus flourish in tropical and subtropical climates. Species belonging to this genus are saprophytic, some are pathogenic, and still others appear to exist in decayed wood, dung, and litter. Certain species are host-specific and are associated with bamboo, palm species, and even with teak trees [3]. Studies suggest that endophytic xylariaceous fungi are crucial in controlling the generation of secondary metabolites in their host plants, which may help increase the bioactive compounds they contain. For

example, the studies of Chien et al. [4] stated the increase in total alkaloids and polysaccharides in a host medicinal plant called *Dendrobium*.

Interestingly, *Xylaria* is valued for the various lignocellulosic enzymes it produces, which have a wide range of applications from pharmaceutical to various industries like textile, pulp, and paper industries [3]. Aside from that, the endophytic nature of this group of macrofungi with a wide range of host plants confers medicinal value and can be a source of structurally diverse bioactive compounds, which include alkaloids, exopolysaccharides, xylaramide, xylarin, and xyloketals [2,4]. Moreover, other natural products such as xylarenal, xyloallenolide, mellisol, griseofulvin, and xylactam showed promising biological activities like antibacterial, antifungal, antioxidant, and anti-inflammatory or anti-malarial activity [5,6]. Furthermore, the researchers have isolated isopimarane diterpene glycosides from the fruiting bodies of *Xylaria polymorpha*, which exhibited cytotoxicity against human cancer cell lines [6].

Several studies have also shown that many biological activities of the various compounds can be derived from *Xylaria* species. They possess antibacterial, antimalarial, antifungal, and cytotoxic activities. Nath et al. [7] reported that *Phomopsis* sp. and *Xylaria* sp. showed antibacterial properties against *Escherichia coli* MTCC730, *Enterococcus faecalis* MTCC2729, *Salmonella enterica* ser. *paratyphi* MTCC735, *Streptococcus pyogenes* MTCC1925, and the fungus *Candida albicans* MTCC183. Nandan Patel and Krishnappa [8] reported the antibacterial potential of *Xylaria carpophila* against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Pseudomonas syringae*, among others. Sawadstang et al. [9] reported the cytotoxic activity of *Xylaria cf. cubensis* PK108 against KB and 12 NCI-H187 cancer cell lines. In the same vein, Orachaipunlap et al. [5] reported the cytotoxic activity of a *Xylaria* sp. against the Jurkat cell line. Moreover, they also show potential activities such as bioherbicides, fungicides, and insecticides or biocontrol agents as applications in agriculture.

Due to the highly diverse bioactive compounds they contain, other researchers also showed that several members of the genus *Xylaria* exhibit biological activities that offer promising nutraceutical and pharmaceutical benefits. Peng et al. [10] reported that *X. nigripes* has anti-depressant and sleep-regulating effects and was used in treating depression and insomnia in clinical practice, especially in patients with epilepsy. In the study of Sawadstang et al. [9], the researchers extracted a compound from *Xylaria cubensis* (Mont.) Fr., which has anti-malarial activity. Further, they also extracted cytochalasin D, ergosterol, and ergosterol peroxide, which have high cytotoxic activity. Moreover, Rajendran et al. [11] isolated antibacterial eremophilane sesquiterpenoids from *Xylaria feejeensis*, which showed activity against *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus*.

In light of the discovery of many bioactive metabolites in many *Xylaria* species, there is a growing interest in mycelial production for nutraceutical or pharmacological applications. Mycelial biomass production using submerged culture conditions is more practical than cultivating fruiting bodies due to higher mycelial yields in a compact space and shorter cultivation times. Submerged culture conditions produce mycelial biomass and bioactive metabolites faster with fewer contamination risks [12,13]. Extensive studies have been made on bioactive metabolites of fungi using submerged culture conditions, which are influenced by several factors, including temperature, aeration, pH, and agitation, affecting secondary metabolite production. In this sense, various researchers used submerged culture conditions to produce a myriad of bioactive metabolites [14]. Zhou et al. [15] demonstrated the OSMAC (one strain many compounds) strategy, which involves alteration of cultivation conditions or addition of epigenetic modifiers to increase the diversity and yield of microorganism-derived natural products from a single strain.

Available research indicates that several functional studies have been made in different species of *Xylaria*. De Leon et al. [16] investigated the mycochemical composition, antioxidant activity, and bioactivities of the fruiting bodies of *Xylaria papulis* Lloyd. On the other hand, this study aimed to assess the secondary metabolites and investigate the biofunctional activities of the mycelial biomass of *Xylaria papulis* grown in static and shaking optimized indigenous liquid culture conditions based on the studies of Lopez et al. [13] for a potentially inexhaustible source of compounds with a great and wide range of benefits. This study could then open an avenue for natural product development from indigenous and locally grown mycelial biomass of *Xylaria papulis*.

2. Materials and methods

2.1 Mycelial Source of *X. papulis* Lloyd

The Tuklas Lunas Center (TLC) and the Center for Tropical Mushroom Research and Development (CTMRD) of the Department of Biological Sciences, College of Arts and Sciences of Central Luzon State University (CLSU), Science City of Muoz, Nueva Ecija, Philippines, provided the pure culture of *X. papulis* Lloyd (Sample_ID_4/GU300100.1). Potato dextrose agar (PDA) was used to subculture the aforementioned macrofungus.

2.2 Preparation of Mycelial Inoculants

Initially, one liter of potato dextrose agar (PDA) was prepared following the instructions indicated on the label of the commercial media (i.e., dissolving 39 g in 1 L of distilled H₂O). The mixture was placed in an Erlenmeyer flask sealed with a cotton plug and covered with paper secured by a rubber band. It was sterilized at 121°C and 15 psi for 30 min. The sterilized medium was then pour-plated.

From the pure culture of *X. papulis*, agar blocks containing mycelia were aseptically inoculated into the prepared PDA plates. To promote mycelial growth, the culture plates were incubated at room temperature. Mycelial discs were harvested using a sterilized 10-mm cork borer after seven days of incubation. These mycelial discs served as the mycelial inoculants for the large-scale generation of mycelial biomass used in this study.

2.3 Mass Production of Mycelial Biomass

Mycelia were mass-produced following the optimized methods of Lopez et al. [13] for *X. papulis* to obtain adequate amounts of samples. It was done by inoculating the mycelial disc into 50 catsup bottles containing 100 mL of potato sucrose broth with a pH of 6.5. The inoculated bottles were incubated at room temperature (~28°C), static condition, and continued lighting condition (fluorescent light) for 10 days. Mycelia were also mass-produced in shaking conditions (70 rpm). The mycelial mats were harvested, air-dried, and subjected to analyses of their mycochemical composition, antioxidant properties, and cytotoxic activity.

2.4 Extraction of Bioactive Components

Ten grams of air-dried mycelial mats were cut into pieces and powdered using a food processor. One hundred mL of absolute ethanol was added to 10 grams of powdered mycelial mats, while a volume ratio of 1:1 of absolute ethanol and culture spent was mixed to extract bioactive compounds. Mixtures were filtered using filter paper (Whatman no. 1), and the ethyl alcohol-soluble portion was concentrated at 60°C for 48 hours under reduced pressure to produce the concentrated crude extract. The crude extracts were refrigerated (ca. 4°C) before mycochemical testing, antioxidant properties, and cytotoxic activity.

2.5 Determination of Antioxidant Activity

The mycelial mats and culture-spent harvested from shaking and static culture conditions were utilized to determine antioxidant activity. They were subjected to radical scavenging activity assay using DPPH, and the total phenolic contents were estimated using the Folin-Ciocalteu method. In the radical scavenging activity assay, the antioxidant activity of the ethanolic extracts of culture spent and mycelial mats was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (1, 1-Diphenyl -2-picrylhydrazyl) following the method of Kolak et al. [17] with modifications. A stock solution was created from the concentrated extract, and an aliquot was taken to create a 1,000 ppm dilution, and 1,000 ppm for catechin as the control. In a different plastic cuvette, 1 mL of the produced stock solution and 4 mL of 0.1 mM DPPH solution were combined. Reactions were done in triplicate. For 30 minutes, the produced combinations were incubated at 37°C in the dark. A UV-VIS spectrophotometer was used to track values of absorbance at 517 nm. Higher free radical scavenging activity is shown by a decreased absorbance of the reaction mixture. The activities of the Catechin, a controlled substance, were contrasted with those of the radical scavengers under study. The formula below was used to determine the capacity to scavenge the DPPH radical.

$$\% \text{Radical Scavenging Effect} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

A_{control} is the absorbance of the control, which is the DPPH without the test sample, and A_{sample} is the absorbance of the test sample containing the mixture of the DPPH and the sample. Catechin was used as a positive control.

2.6 Estimation of Phenolic Contents

Meanwhile, the amount of total phenolics present in the mycelial mat extracts and culture spent was estimated using the Folin-Ciocalteu method following the method of Sunita and Dhananjay [18]. Gallic acid was used as the standard, and the total phenolics were expressed as milligrams of gallic acid equivalents (GAEs) per gram of the sample. Several concentrations of gallic acid (31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49, and 1 mg/mL) were prepared in methanol. For the mushroom mycelial mat extract and culture spent, 1 mg/mL was also prepared in methanol, and 0.5 mL of each sample was introduced into the test tubes.

Each test tube was mixed with 2.5 mL of a 10-fold diluted Folin-Ciocalteu reagent (Folin: Methanol, 1:1, v/v) with 2 mL of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 min at room temperature before the absorbance was read at 760 nm using a spectrophotometer. All tests were performed in triplicate. The Folin-Ciocalteu reagent is sensitive to reducing compounds, including polyphenols, thereby producing a blue color upon reaction. This blue color was measured spectrophotometrically, thus the total phenolic content was determined.

2.7 Mycochemical Composition Tests

The qualitative screening of secondary metabolites in mycelial mat and culture spent broth was determined using the methods of Guevara et al. [19], with modifications that involved the use of fungal extracts. The mycelial mat extracts and culture spent were screened for phenols, sterols, triterpenes, essential oils, fatty acids, sugars, anthraquinones, coumarins, anthrones, alkaloids, flavonoids, and tannins. The test was done in triplicate, and the results were compared with distilled water as the control. The results were interpreted as (+) if mycochemical was present, and (-) if mycochemical was absent.

Each sample underwent mycochemical screening to identify any secondary metabolites that might be present. Each sample was spotted on a 7 x 4 cm TLC (thin layer chromatography) sheet that was tagged and labeled, and it was then developed in an ethyl acetate-methanol solution with a 7:3 volume ratio within the developing chamber. To verify the separation of the various components, the spots for a specific metabolite were seen on the TLC plates and exposed to UV light and a hot plate.

Typically, vanillin-sulfuric acid reagent was used to visualize secondary metabolites. The presence of sterols, triterpenes, and essential oils can be detected using this solution. Anthraquinones, coumarins, and anthrones were tested using methanolic potassium hydroxide, whereas phenolic compounds and tannins were tested using the potassium ferricyanide-ferrous chloride reagent. Alkaloids were detected using Dragendorff's reagent, and flavonoids were detected using Antimony (III) chloride.

2.8 Brine Shrimp Cytotoxicity Assay

A preliminary cytotoxicity assay of the mycelial mat extracts and culture spent was performed in brine shrimp (*Artemia salina*) larvae – nauplii. When the shrimp larvae hatched after 48 h, 10 mL of seawater containing nauplii was placed on a Petri dish. From that, 10 nauplii were placed in each vial containing 4 mL of each concentration of mycelial extracts and culture spent. The experiment used five replicates. Thus, there were 50 nauplii in every corresponding concentration of mycelial extracts and culture spent. The extracts were tested at concentrations of 10, 100, 1,000, and 10,000 µg/mL. The volume of each vial was adjusted with artificial seawater up to 5 mL per vial. The vials were left uncovered under an incandescent lamp (for constant illumination and temperature maintenance) and were supplemented with a drop of yeast suspension (3 mg of yeast in 5 mL of saline solution) to serve as food for the nauplii.

A negative control was assessed without the crude extract. After 24 hours, the number of dead nauplii was tallied. Lethality concentration (LC₅₀) was evaluated using probit analysis. The percentage mortality (%M) was calculated by dividing the number of dead nauplii by the total number of nauplii, multiplying by 100. The LC₅₀ was assessed following the rating of toxicity by Aldahi et al. [20] and McLaughlin and Rogers [21] specifying that LC₅₀ value of <249 µg/mL is highly toxic, LC₅₀ value of 250-499 µg/mL is moderately toxic, and LC₅₀ value of 500-1000 µg/mL is fairly toxic while LC₅₀ value of >1000 µg/mL is non-toxic.

2.9 Statistical Analyses

For statistical analyses of the data, Minitab ver. 16 statistical software was utilized. Analysis of variance was employed in the treatments involving %RSA and total phenolic contents. Utilizing Tukey's comparison of means, treatments identified as significant by analysis of variance (ANOVA) were further compared. Likewise, the Probit analysis was employed to determine the LC₅₀ (the lethal concentration that can kill 50% of the population of nauplii) in the brine shrimp cytotoxicity assay.

3. Results

3.1 Mycochemical Composition

Mycochemical screening provides basic information about the medicinal importance of mushroom extracts. Both cultivated and wild mushrooms have adequate levels of proteins, carbs, minerals, fibers, and vitamins. Literature shows that mushrooms contain mycochemical substances with potent functional properties [4]. The mycochemical composition of *X. papulis* Lloyd was analyzed to determine its potential as a source of possible

novel bioactive metabolites. Twelve secondary metabolites were screened, such as phenols, steroids, triterpenes, essential oils, anthraquinones, coumarins, anthrones, alkaloids, flavonoids, fatty acids, sugars, and tannins. Table 1 shows the different mycochemicals in the mycelial mats and culture spent produced from static and shaking liquid culture conditions.

The ethanolic extracts of the mycelial mat in static condition contain essential oils, phenols, triterpenes, steroids, fatty acids, sugars, anthraquinones, coumarines, anthrones, tannins, flavonoids, and alkaloids. In contrast, mycelial mat in shaking condition contains the same secondary metabolites except essential oils. Meanwhile, the culture spent in the shaking condition contains all the secondary metabolites tested except sugars. In contrast, the culture spent in the static condition lacks essential oils and sugars; the rest are present. The absence of essential oils in the mycelial mat (shaking) and its presence in the culture spent (shaking) suggest that the shaking condition could affect the presence of essential oils in the extracts.

Table 1 The mycochemicals screened in *X. papulis* Llyod's mycelial mat extracts and culture spent.

Mycochemicals	Mycelial Mat		Culture Spent		Distilled Water
	Static	Shaking	Static	Shaking	
Phenols	+	+	+	+	-
Triterpenes	+	+	+	+	-
Steroids	+	+	+	+	-
Fatty acids	+	+	+	+	-
Sugars	+	+	-	-	-
Anthraquinones	+	+	+	+	-
Coumarines	+	+	+	+	-
Anthrones	+	+	+	+	-
Tannins	+	+	+	+	-
Alkaloids	+	+	+	+	-
Flavonoids	+	+	+	+	-
Essential oils	+	-	-	+	-

(+) present, (-) absent

3.2 Antioxidant property

Natural products are bioactive metabolites and byproducts that are naturally produced by many organisms, such as fungi and plants. These products with functional activities have been exploited for years for medicinal purposes, such as the possible antioxidant potential of putative sources [1]. Chemicals known as antioxidants or free radical scavengers interact with free radicals to neutralize and stop them from harming living things [22]. Accordingly, free radicals are the main cause of aging, coronary heart disease, inflammation, cancer, and other chronic and degenerative disorders [23]. Searching for potential and inexhaustible sources of bioactive metabolites with promising antioxidant activity is then essential. The present study evaluated the antioxidant activity of the mycelial mat and culture spent from both shaking and static conditions using the DPPH assay. Likewise, total phenolics were estimated using the Folin-Ciocalteu method. Table 2 shows the percent radical scavenging activity and total phenolics of mycelial mat and culture spent produced from static and shaking culture conditions. Among the extracts, the mycelial mat (static) recorded the highest radical scavenging activity (43.19 ± 1.63 %), while the culture spent (shaking) exhibited the lowest radical scavenging activity (19.17 ± 1.38 %). However, the radical scavenging activity obtained in the present study is lower than that of catechin. In terms of total phenolic contents, the mycelial mat (shaking) has the highest total phenolics (47.75 ± 2.31 mg/GAE), while the culture spent (shaking) has the lowest total phenolics (20.67 ± 1.07 mg/GAE).

Table 2 Percent Radical Scavenging Activity and Total Phenolics of mycelial mat and culture spent from both static and shaking conditions.

Sample	% Radical Scavenging Activity	Total Phenolics (mg/GAE)
Mycelial Mat-Static	43.19 ± 1.63^b	44.42 ± 1.56^A
Mycelial Mat-Shaking	33.75 ± 1.51^c	47.75 ± 2.31^A
Culture Spent-Static	22.36 ± 1.31^d	30.67 ± 3.04^B
Culture Spent-Shaking	19.17 ± 1.38^d	20.67 ± 1.07^C
Catechin (Control)	79.17 ± 1.68^a	

%Radical Scavenging Effect of Catechin is based on 1,000 ppm concentration; n = 3, Means that do not share a letter are significantly different ($p < 0.05$)

GAE = Gallic Acid Equivalent based on 1,000 ppm concentration of extracts; n = 3, Means that do not share a letter are significantly different ($p < 0.05$)

3.3 Cytotoxic activity

Since cancer is one of the primary causes of death, ongoing research efforts in this area are being made worldwide. These investigations frequently examine how biologically active chemicals affect various test organisms or cancer cells directly [24]. For centuries, the natural world has provided medical substances, and an astounding number of contemporary medications have been identified from these sources [25]. Thus, there is a necessity to look into dependable and endless sources of natural compounds. In the present study, the cytotoxic activities of mycelial mat extracts and culture spent of *X. papulis* Lloyd from both static and shaking conditions were examined against brine shrimp larvae. Table 3 shows the LC₅₀ value and the interpretation of toxicity for the samples evaluated. Culture spent from the shaking condition exhibited the lowest LC₅₀ of 94.39 µg/mL, while the mycelial mat from the shaking condition produced the highest LC₅₀ value of 1983.15 µg/mL.

Table 3 Cytotoxic activity of *X. papulis* mycelial mat and culture spent against brine shrimp (*Artemia salina*) larvae.

Sample	LC ₅₀ (µg/mL)	Equation (R ²)	Toxicity*
Mycelial Mat-Static	149.31	y = 0.307x + 4.332 (0.96)	highly toxic
Mycelial Mat-Shaking	1983.15	y = 0.727x + 2.602 (0.98)	non-toxic
Culture Spent-Static	974.88	y = 0.716x + 2.859 (0.95)	fairly toxic
Culture Spent-Shaking	94.39	y = 0.955x + 3.114 (0.99)	highly toxic

LC₅₀ = lethal concentration (concentration to kill 50% of test organisms)

*The LC₅₀ was assessed following the rating of toxicity by Aldahi et al. [20] and McLaughlin and Rogers [21] specifying that LC₅₀ value of <249 µg/mL is highly toxic, LC₅₀ value of 250-499 µg/mL is moderately toxic, and LC₅₀ value of 500-1000 µg/mL is fairly toxic while LC₅₀ value of >1000 µg/mL is non-toxic.

4. Discussion

4.1 Mycochemical composition of *Xylaria papulis* Lloyd

The search for bioactive substances, especially novel compounds, has strongly shown that nature offers a promising reservoir for novel bioactive compounds. Among these sources, fungi are incredibly valuable sources with a wide range of chemical properties, making them an increasingly recognized resource for novel drug discovery [6]. Accordingly, many genera in ascomycetous fungi *Xylaria* Hill ex Schrank (1786) have received attention for their presence of enormous secondary metabolites over the past 25 years [8]. With this, the results of the present study in mycochemical screening of *X. papulis* coincide with previous reported findings.

Nakashima et al. [26] isolated a new chromone derivative and a known isocoumarine mullein from *Xylaria* sp. ECN-008. They also mentioned the presence of various bioactive metabolites, including triterpene glycosides, steroids, sesquiterpenoids, diterpenoids, diterpene glycosides, N-containing compounds, aromatic compounds (ketone derivatives), pyrone derivatives, and polyketides in a *Xylaria* species. Nadan Patel and Krishnappa [8] reported the presence of alkaloids, tannins, flavonoids, sterols, glycosides, terpenoids, and phenols in the ascomycetous fungus *Xylaria carpophila*. Moreover, Adnan et al. [27] reported the presence of terpenoids and xylaric acid in *Xylaria primorskensis*, while Wang et al. [28] isolated a new coumarin glycoside, a new phenylethanol glycoside, and five known steroids in *Xylaria* sp. cfcc 87468. Furthermore, Jang et al. [29] isolated linoleic acid, linoleic acid methyl ester, ergosterol, and other known valuable compounds from *Xylaria polymorpha*. Interestingly, the present study noted the presence of anthrones and anthraquinones in mycelial mats and culture spent of *X. papulis*. Anthrones and anthraquinones are anthranoid derivatives that are widely used laxatives of natural origin [30]. Moreover, anthraquinones are a class of aromatic compounds that possess a broad spectrum of bioactivities, which include cathartic, anticancer, vasorelaxing, and anti-inflammatory activities [4].

Several classes of secondary metabolites, such as alkaloids, triterpenoids, steroids, tannins, and flavonoids, were also present in the extracts. Alkaloids are reported to act as powerful pain relievers and topical anesthetics in ophthalmology. One of the most important biological properties of alkaloids is their toxicity against cells of foreign organisms [8]. Triterpenoids and other compounds from *X. papulis* have demonstrated immunomodulatory, anti-inflammatory, hepatoprotective, antitumor, and neuroprotective activities, as well as the prevention of spatial memory impairment and antidepressant effects [9]. Tannins have applications as an astringent, as a diuretic, against stomach and duodenal tumors, and anti-inflammatory, antiseptic, and hemostatic properties [31]. Flavonoids are among the most ubiquitous groups of phenolic compounds that can act as antioxidants, anti-inflammatories, antiallergic, antithrombotic, anticarcinogenic, and metal chelators [32].

4.2 Antioxidant activity and phenolic content

It is noteworthy that the mycelial mat extracts have higher antioxidant activity compared to the culture spent. Nevertheless, with these results, it can be deduced that the various samples have promising antioxidant activities.

These results are parallel to the findings of several studies about the antioxidant activities of different *Xylaria* species. Pan et al. [33] reported that in the 53 fungal endophytes they have isolated (where a *Xylaria* sp. is included), the DPPH antioxidant values ranged widely from 6.88% to 107.32% DPPH radical inhibition, which coincides with the present study having a range of percent DPPH radical scavenging activity of 22.36% to 43.19%. Meanwhile, De Leon et al. [16] reported that the fruiting bodies of *X. papulis* exhibited a DPPH radical scavenging activity (RSA) of 55.67% and total phenolics of 43.17 mg/GAE. The present study evaluated the mycelia of *X. papulis* grown in liquid culture and showed higher total phenolics (47.75 mg/GAE) but with relatively lower %RSA (43.19%) compared to fruiting bodies reported by De Leon et al. [16]. However, the present study employed a submerged culture condition of mycelia production, a more practical and more sustainable approach compared to fruiting body cultivation. Adnan et al. [27] reported that xylaranic acid from *Xylaria* species and synthetic xylaranic acid AgNPs had effective DPPH radical scavenging abilities. Nath et al. [7] asserted that the DPPH activities in all the ethanolic extracts were dose-dependent, and among the endophytes studied, *Xylaria* sp. showed the highest antioxidant activity and total phenolic levels. According to Divate et al. [34], ethanolic extracts of *Xylaria nigripes*, compared to water extracts, showed greater antioxidant activity by scavenging DPPH radicals. Moreover, they also mentioned its neuroprotective effects, which accounted for the high phenolic contents and antioxidant activity of their extracts. Aside from that, Ko et al. [23] also reported the antioxidant activity of *Xylaria nigripes* (Wu Ling Shen), a well-known medicinal fungus used in traditional Chinese medicine. They also noted the higher antioxidant activity of *Xylaria nigripes* mycelial extracts compared to the existing commercial *Xylaria nigripes* product.

Fungal endophytes are prolific producers of antioxidant chemicals, and among these bioactive compounds are phenols, flavonoids, and saponins [32]. De Leon et al. [35] mentioned that phenols or phenolic compounds are important in their hydroxyl groups' propensity to act as scavengers, which play crucial roles in the scavenging of excessive free radicals and contribute directly to antioxidative action. The main free radical scavenging molecules in many therapeutic plants have been identified as phenolic acids, tannins, and other substances. Additionally, secondary metabolites from fungal endophytes with antioxidative activity have been discovered to contain phenolic compounds as their principal antioxidant components [33]. Free radical-mediated reactions or excessive reactive oxygen species (ROS) may harm biomolecules through oxidative stress, which eventually speeds up aging and causes diseases, including Alzheimer's, diabetes, atherosclerosis, cancer, and other neurological disorders [16]. Consumption of foods high in antioxidants, vitamins, and phenolic substances can help prevent disease associated with excessive free radicals by preventing lipid peroxidation and lowering oxidative stress in biological systems [23].

4.3 Cytotoxic potential and correlation with previous studies

Researchers have reported the anti-cancer or cytotoxic activity of different *Xylaria* species. In more advanced studies on the cytotoxic activities of various *Xylaria* species, Orachaiunlap et al. [5] reported the efficient cytotoxic activity of two *Xylaria* spp. against the Jurkat cancer line and HEK293 cell line with IC₅₀ values of 2.63 µg/mL and 2.94 µg/mL, respectively. In the findings of Ramesh et al. [6], *Xylaria curta* extracts demonstrated considerable cytotoxic activity of 58.5% against the A-549 human cancer cell line at a concentration of 60 µg/mL. Moreover, several studies reported the cytotoxic activities of many compounds isolated from *Xylaria allantoidea* SWUF76 against cancer cell lines such as HeLa, HT29, HCT116, and MCF-7.

In the present study, *X. papulis* culture spent from shaking culture conditions showed toxicity against brine shrimp nauplii with an LC₅₀ value of 94.39 µg/mL. Asaduzzaman et al. [36] stated that brine shrimp toxicity is correlated with 9KB (human nasopharyngeal carcinoma) cytotoxicity, in which its ED₅₀ values are generally about one-tenth the LC₅₀ values found in the brine shrimp toxicity test. The brine shrimp cytotoxicity assay is a quick, low-cost, and effective bioassay for determining an extract's lethality, which typically corresponds fairly well with cytotoxic and anti-tumor capabilities of the extracts [36,37]. Aside from that, the brine shrimp lethality assay has been proven to be convenient, rapid, inexpensive, easily mastered, effective, and requires small amounts of test material for initial monitoring of the cytotoxic activities of natural products [37,38]. The cytotoxic activity of natural products can be attributed to the various secondary metabolites present. Asaduzzaman et al. [36] associated the cytotoxicity of their extracts with the phenolics and flavonoid bioactive compounds. De Leon et al. [35] mentioned that one of the most important properties of alkaloids is their toxicity against foreign bodies. Moreover, Sawadsitang et al. [9] reported cytotoxic activity of the ergosterol and ergosterol peroxide isolated from *Xylaria cf. cubensis* PK108 against cancer cell lines.

Extensive research efforts have been made to derive potent anticancer agents from natural products. Many classes of bioactive compounds from natural resources have shown promising potential for cytotoxic, anti-tumor, or anticancer capabilities. Zhao et al. [39] reviewed more than 1000 triterpenoids with cytotoxicity, including recent advancements in their synthesis and molecular simulation for structure-activity relationship analyses. Phenolic compounds, including flavonoids, showed major protective and therapeutic benefits against many types of human cancers, attributed to their functional groups that exert several anti-cancer mechanisms, including

induction of apoptosis, autophagy, and cell cycle arrest. These compounds also exhibit anti-angiogenic properties that inhibit invasion [40,41]. Coumarins and natural as well as synthetic steroidal derivatives have also been documented for antiproliferative and cytotoxic activities, while some fatty acids are associated with reduced cancer risk or anti-tumor effects [42,43]. Many anthraquinones were also studied and shown to target key cellular proteins with apoptosis-inducing activities [44]. In the many classes of bioactive metabolites, alkaloids have been comprehensively studied and have shown promising anticancer activities, often used in drug development [45]. All of these classes of bioactive metabolites are present in the mycelial mat and culture spent extracts of *X. papulis* grown under submerged culture conditions of the present study, which highlights a promising and sustainable source of potential anticancer bioactive compounds.

4.4 Biological implications and future perspectives

The findings of this study highlight *Xylaria papulis* as a potential natural resource of a wide variety of secondary metabolites with significant biological activities. The detection of several classes of secondary metabolites, such as alkaloids, triterpenoids, steroids, tannins, flavonoids, phenols, anthraquinones, and anthrones reveals a complex bioactive metabolite profile associated with biofunctional activities, including antioxidant and cytotoxic properties. The significant antioxidant activity of *X. papulis* extracts, particularly from the mycelial mats, underscores the role of phenolic compounds in scavenging free radicals and preventing oxidative stress, which is a major contributing factor to aging and many chronic diseases. These findings align with numerous previous studies on other *Xylaria* species, underscoring the potential of this genus as a valuable source of antioxidant compounds that contribute to maintaining cellular redox balance and preventing disease.

Moreover, the cytotoxic activity observed in the brine shrimp lethality assay of the present study provides preliminary evidence of the anticancer potential of *X. papulis* grown under submerged culture conditions. Also, the presence of many classes of bioactive metabolites known for their anticancer activities underscores their potential pharmacological significance. Moving forward, future studies may focus on bioassay-guided fractionation and purification as well as molecular characterization of the bioactive compounds. It is also important to consider the optimization of submerged culture conditions for metabolite production to increase the amounts of target active substances. These future studies will not only deepen the understanding of the biological roles of these metabolites but also offer an avenue for the development of *X. papulis*-based natural products as novel therapeutic agents.

5. Conclusion

All the tested mycochemicals were detected in the mycelial mat under static conditions, whereas the mycelial mat grown under shaking conditions contained the same compounds except for essential oils. All the tested mycochemicals were detected in the culture spent under shaking conditions, except for sugars. In contrast, the culture spent under static conditions lacked both essential oils and sugars, while all other compounds were present. Mycelial mat and culture spent exhibit antioxidant properties in DPPH radicals (as high as 43.19 ± 1.63 %RSA) and appreciable amounts of total phenolic contents (as high as 47.75 ± 2.31 mg/GAE). In cytotoxic activity, culture spent (shaking) exhibits high cytotoxicity ($LC_{50} = 94.39$ μ g/mL) against brine shrimp larvae. The researchers are interested in the future direction of this study, which is to further test the cytotoxicity of the culture spent (shaking conditions) and the mycelial mat (static conditions) to human cancer cell lines.

6. Author Contributions

Lopez, M.K.S.: Conceptualization, Methodology, Data curation, Writing –original draft; Kalaw, S.P.: Conceptualization, Resources, Writing – review & editing, Supervision; Dulay, R.M.R.: Conceptualization, Resources, Writing – review & editing; De Leon, A.M.: Conceptualization, Writing – review & editing; Reyes, R.G.: Conceptualization, Resources, Writing –review & editing, Supervision

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8. Conflict of Interests

The authors have no conflict of interest.

9. References

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