



Submerged Culture Optimization and Bioactivity Profiling of the Philippine Wild Hairy Sawgill Mushroom (*Panus neostrigosus* Drechsler-Santos & Wartchow)

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Abstract

Mushrooms are widely recognized for their nutritional value and diverse therapeutic properties, making them promising sources of bioactive compounds. This study aimed to optimize submerged culture conditions for *Panus neostrigosus*, a wild Philippine macrofungus, and to evaluate the bioactivities of its mycelial extract. Among the tested culture media, coconut water significantly enhanced mycelial biomass production, with optimal growth achieved at pH 6–7, 30 °C, and 150 rpm agitation. The ethanol extract of the mycelia was subjected to enzyme-based assays to assess its anti-inflammatory, antidiabetic, neuroprotective, and antioxidant potentials. Results showed that the ethanolic extract at 10 µg/mL exhibited inhibitory activity against α-glucosidase (5.84%), acetylcholinesterase (2.45%), and cyclooxygenase-1 (COX-1) (20.11%). No COX-2 inhibition was observed, suggesting selective anti-inflammatory potential. At 1000 µg/mL, the extract showed 29.77% antioxidant activity. Toxicity assessments revealed LC₅₀ values of 42.49 µg/mL in zebrafish (*Danio rerio*) embryo toxicity testing and 16.83 µg/mL in the brine shrimp (*Artemia salina*) lethality assay, indicating dose-dependent embryotoxic, teratogenic, and cytotoxic effects, particularly at higher extract concentrations. Overall, the results suggest that *P. neostrigosus* exhibits anti-inflammatory, antidiabetic, and neuroprotective activities, as well as cytotoxicity, highlighting its potential as a source of cytotoxic agents for future pharmacological investigations.

Keywords – Antioxidant – Antidiabetic – Acetylcholinesterase – Cytotoxic – Indigenous liquid media – Philippine mushrooms

Introduction

Mushrooms are increasingly recognized as a prolific source of bioactive compounds with significant therapeutic and nutritional value. Numerous studies have emphasized their antioxidant, antimicrobial, antitumor, anti-inflammatory, and immunomodulatory properties, positioning them as valuable candidates for applications in the nutraceutical and pharmaceutical industries (Kim et al.

2022, Mwangi et al. 2022, Xu et al. 2022, Mizuno & Minato 2024). These beneficial effects are primarily attributed to a diverse array of secondary metabolites, including polysaccharides, phenolics, terpenoids, and sterols (Valverde et al. 2015, Ma et al. 2018). Within this framework, the genus *Panus* represents a taxonomically and pharmacologically significant group of wood-rotting basidiomycetous fungi, which are widely distributed in tropical and temperate regions and known for their ecological importance as saprotrophic decomposers (Yue et al. 2024). Taxonomic revisions within the group have clarified species boundaries and nomenclature, including the recognition of *Panus neostrigosus* Drechsler-Santos & Wartchow as a distinct taxon formerly associated with *Lentinus strigosus* (Schwein) Fr. (Vargas-Isla et al. 2015).

In the Philippine context, studies on wild macrofungi have primarily focused on biodiversity documentation, ethnomycology, and preliminary bioactivity screening of selected taxa (De Leon et al. 2013, Dulay et al. 2016). For instance, the first successful domestication of *L. strigosus* was reported by Dulay et al. (2017), providing a foundation for subsequent investigations into its cultivation and bioactivity. Later studies showed that antioxidant and antimicrobial properties, as well as mycelial biomass production, are markedly shaped by the choice of culture system, whether solid-state or submerged (Dulay et al. 2020, 2021a). While solid-state fermentation remains an essential method in mushroom cultivation, particularly for fruiting body production, it often requires a lengthy incubation period that varies depending on the mushroom species and substrate used. In contrast, submerged fermentation offers a more efficient and scalable alternative, enabling faster production of mycelial biomass and bioactive metabolites. This method involves growing mushrooms in nutrient-enriched liquid media under controlled conditions, with continuous aeration through agitation (Bentil et al. 2018). Submerged fermentation is increasingly recognized for its ability to produce biological compounds rapidly and reproducibly, making it well-suited for meeting the growing demand for functional food ingredients (De Silva et al. 2013). Comparable findings have been described in other mushroom species, where culture conditions play a decisive role in metabolic output. Variables such as nutrient composition, pH, temperature, and aeration have consistently emerged as key determinants of both yield and metabolite activity (Marim et al. 2018, Zhang et al. 2019, Dulay et al. 2021b, Kalaw et al. 2021).

Despite these advances, the full spectrum of bioactive metabolites produced by *P. neostrigosus* under submerged fermentation remains unclear. To address this gap, the present study focused on optimizing submerged culture conditions, with particular attention to media type, pH, temperature, and agitation, for *P. neostrigosus* collected from the Philippines. Furthermore, it evaluates the bioactivities of their mycelial extracts through comprehensive enzyme-based screening for anti-inflammatory, anti-diabetic, and cholinesterase-inhibitory activities, as well as assays for embryotoxic, teratogenic, and cytotoxic properties. These findings are expected to enhance the understanding of *P. neostrigosus* as a viable candidate for future applications in the functional food, biotechnology, and pharmaceutical sectors, while contributing to the broader scientific literature on Philippine wild macrofungi.

Materials & Methods

Mushroom source and culture inoculant

Pure cultures of *P. neostrigosus* were sourced from the Culture Collections of the Center for Tropical Mushroom Research and Development at Central Luzon State University, located in the Science City of Muñoz, Nueva Ecija, Philippines. A block of agar from the pure culture of each mushroom was aseptically transferred onto sterilized potato dextrose agar (PDA) plates. The plates were incubated at 28 °C to promote mycelial growth. After a 7-day incubation period, mycelial discs were obtained using a flame-sterilized cork borer with a 10 mm diameter. These mycelial discs were used as inoculants to evaluate the optimum growth conditions.

Optimization studies for mycelial production

The effect of liquid culture media, namely, Indian mango (*Mangifera indica* L.) purée 25% (MP), mature coconut water (CW), and their combination, on the mycelial production of the mushrooms was assessed under submerged fermentation. The methods for evaluating culture media and pH conditions were adapted from Diaz et al. (2025). To determine the optimal pH, the pH of each medium was adjusted to 4, 5, 6, 7, 8, and 9 using 1.0 M sodium hydroxide (NaOH) or 1.0 M hydrochloric acid (HCl). A 100 mL volume of each medium was transferred into 250 mL Erlenmeyer flasks, sterilized at 121 °C and 15 psi for 30 minutes, and aseptically inoculated with mycelial discs. Cultures were incubated at 30 °C for 10 days in a shaking incubator (Model BJPX-2102C, Biobase, China) at 100 rpm. After incubation, mycelia were collected by filtration using Whatman® Grade 4 qualitative filter paper (pore size 20-25 µm, hydrophilic), rinsed with distilled water, and oven-dried (Oven Drying and Heating, Alpinum, Austria) and weighed accordingly. Biomass yield was expressed as milligrams dry weight per milliliter (mg DW/mL). To determine the optimal agitation condition, culture media adjusted to the previously identified optimal pH were inoculated with mycelial discs and incubated at 30 °C in a rotary shaker at agitation speeds of 100 and 150 rpm for 10 days. A separate static condition was also included. The effect of fermentation time on mycelial biomass was similarly evaluated. All experiments were conducted in triplicate. Data were analyzed using one-way analysis of variance (ANOVA), and treatment means were compared using Tukey's Honestly Significant Difference (HSD) test at a 5% level of significance.

Mass production of mycelia

Using the optimized culture conditions, mushroom mycelia were mass-produced for subsequent applications, including the extraction of bioactive compounds from the mycelia and culture (mycelia + spent culture liquid).

Ethanol extraction

The ethanol extraction procedure followed the protocols of Boukes et al. (2017) and Dulay et al. (2014), with minor modifications. Ten grams (10 g) of dried mushroom mycelia were subjected to extraction in 200 mL of 95% ethanol for 48 h at room temperature. After extraction, the mixture was filtered through Whatman® Grade 4 qualitative filter paper (pore size 20–25 µm, hydrophilic), and the resulting filtrates were oven dried. The dried extracts were stored at -8 °C until further use, and extract yields were recorded.

α-Glucosidase inhibition assay for anti-diabetic activity screening

A stock solution of 10,000 µg/mL and a 300 µg/mL working solution in dimethyl sulfoxide (DMSO) were prepared for each sample. The sample solutions were homogenized using a vortex mixer (Digisystem, Taiwan), sonicated at 40 kHz using a sonicator (Biobase, China), and centrifuged at 3,000 rpm (Biosan, Latvia). Each well of a 96-well quartz microplate consists of 190 µL of 50 mM sodium phosphate buffer solution (PBS) containing 100 mM sodium chloride (NaCl) at pH 6.8, 50 µL of 120 mU/mL enzyme solution, and 10 µL of working stock solution. Each working stock solution has two trials and two replicates ($n = 2$, $t = 2$). The 200 µL of acarbose (well concentration of 1000 µg/mL) was placed in the wells for the positive control and 200 µL of 5% DMSO in PBS for the negative control (solvent well concentration of 3.33%). After incubation for 10 min at 37 °C, 50 µL of 1.86 mM *p*-nitrophenyl- α -D-glucopyranoside was added to start the reaction. The absorbance of the liberated *p*-nitrophenol was measured at 405 nm every 30 seconds for 30 minutes using a Multiskan Go® UV/Vis Spectrophotometer (Thermo Fisher Scientific, Finland).

Cholinesterase inhibitory activity

For the acetylcholinesterase (AChE) inhibition assay, a sample stock solution of 10,000 µg/mL and a working solution of 2,000 µg/mL were prepared. Samples were homogenized using a vortex mixer, sonicated, and centrifuged. In a 96-well plate, the reaction mixture consists of 180 µL of 100 mM phosphate buffer at pH 8.0, 10 µL of 1.0 U/mL *Electrophorus electricus* acetylcholinesterase

(AChE) enzyme solution, 80 μ L of buffered Ellman's reagent (10.0 mM DTNB and 17.9 mM sodium bicarbonate (NaHCO_3) at pH 7.0), and 15 μ L of each working solution sample/100 μ g/mL positive control/negative control (methanol). Each test sample solution has two trials and two replicates ($n=2$, $t=2$). The sample, positive, and negative controls have a solvent well concentration of 5% methanol. After incubation for 15 minutes at 25 $^{\circ}\text{C}$, 15 μ L of 2.87 mM acetylthiocholine iodide (AChI) was added, and the absorbance was monitored at 420 nm using Multiskan (Thermo Scientific) for 31 readings with 25-s intervals.

COX-1 and COX-2 inhibition assays for anti-pain activity screening

A stock solution of 10,000 μ g/mL and a working solution of 200 μ g/mL were prepared using DMSO. Samples were homogenized using a vortex mixer, sonicator, and centrifuge. To a clean scintillation vial, 5184 μ L of 100 mM pH 8 Tris buffer was added. Then, 96 μ L of 250 U/mL COX enzyme (human recombinant enzyme for COX-2 and sheep enzyme for COX-1) and 480 μ L of 20 μ M Hematin were mixed and subsequently dispensed into the vial with the buffer. This makes the enzyme-cofactor solution. To each test well that had already been dispensed with 50 μ L of the same buffer, 120 μ L of the enzyme-cofactor mixture was added. Then, 10 μ L of sample/160 mM positive control (indomethacin)/negative control (DMSO) was added to the specified wells. Each working solution has two trials and two replicates ($n = 2$, $t = 2$). The sample, positive control, and negative control have a solvent well concentration of 5% DMSO. The mixture was incubated at 25 $^{\circ}\text{C}$ for 15 min. After incubation, 10 μ L of 200 μ M Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was added to each well, followed by 10 μ L of 2,000 μ M arachidonic acid. The reaction mixture was briefly mixed and purged with nitrogen (N_2). The reaction was monitored for 2 minutes using the CLARIOstar® plate reader (BMG Labtech, Germany) at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. Fluorescence intensity was measured at 12-s intervals.

Radical scavenging activity

The radical scavenging activity of the lipid extracts at 500 mg/L concentration was determined using the stable 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical following the standard method of Shimada et al. (1992) with modifications. A 100 μ L of test sample in ethanol was added to 5 μ L of DPPH solution (5 mg DPPH powder in 2 mL of ethanol) in 96-well microtiter plates. The mixture was shaken vigorously and left to stand in the dark for 30 minutes, after which the absorbance was measured at 517 nm. The inhibition of DPPH free radicals was calculated.

***Danio rerio* embryotoxicity and teratogenicity assay**

The embryotoxic and teratogenic potential of the extract was evaluated using zebrafish (*D. rerio*) embryos, following the method described by Dulay et al. (2012) with modifications. Test concentrations were prepared at 0.1, 1, 10, 100, 1,000, 10,000, and 100,000 μ g/mL using embryo water as the diluent. A negative control group containing only embryo water, and a positive control group treated with ethanol were included. For each treatment, 200 μ L of the prepared solution was dispensed into individual wells of a sterile, flat-bottom 96-well ELISA plate. Four embryos at the segmentation stage were transferred into each well. Hatchability and mortality rates were recorded using the resulting data, which were subjected to probit analysis to determine the median lethal concentration (LC_{50}).

Following the LC_{50} determination, a second experimental setup was conducted to assess sublethal and teratogenic effects using concentrations based on the LC_{50} value. Three working doses were prepared: a high dose (HD), a medium dose (MD), and a low dose (LD). In this setup, each treatment group, including the negative control, was tested in duplicate, with three embryos allocated per well. After incubation, embryos were examined under a compound microscope to assess morphological development. Embryos were classified based on the criteria of Nagel (2002) into three categories: lethal (coagulation, absent heartbeat), teratogenic (spinal curvature, pericardial edema, yolk deformities, head and tail malformations), and normal development (no abnormalities). In addition to morphological observations, heartbeat rate was measured by manually counting cardiac

contractions under the microscope for 10 seconds and multiplying by six to obtain beats per minute (bpm). The assay was deemed valid only if 100% of embryos in the negative control group developed normally without mortality or malformation.

***Artemia salina* lethality assay**

The cytotoxic potential of the mushroom extract was evaluated using the brine shrimp (*A. salina*) lethality assay. Brine shrimp larvae (nauplii) were obtained by hatching eggs in artificial seawater, prepared by dissolving 25 g of salt in 1 L of distilled water. Test solutions were prepared at concentrations of 1, 10, 100, 1,000, 10,000, and 100,000 µg/mL. Ten nauplii were introduced into each well of the ELISA plate containing the respective treatment solutions, while artificial seawater was used as the control. After 12 hours of exposure, the number of dead nauplii was recorded, and the percentage mortality was calculated. The median lethal concentration (LC₅₀) was determined using Probit analysis. Toxicity levels were interpreted based on the criteria described by Bastos et al. (2009): LC₅₀ > 1000 µg/mL was considered non-toxic, LC₅₀ between 500 and 1000 µg/mL indicated weak toxicity, and LC₅₀ < 500 µg/mL was classified as toxic.

Results

Optimal submerged culture condition for mycelial biomass production

The influence of culture media, pH, temperature, and the effect of agitation on the mycelial biomass yield of *P. neostrigosus* in submerged fermentation was evaluated. The mycelial biomass yields of *P. neostrigosus*, as affected by different factors, are shown in Table 1.

Table 1 Mycelial biomass yield of *P. neostrigosus* as affected by liquid culture media, pH, temperature, agitation, and incubation time.

Factors	Treatments	Biomass yield (mg d.w /100 mL)
Culture media	Indian Mango (M)	196.67 ± 11.55 ^b
	Coconut water (CW)	386.67 ± 15.29 ^a
	Combination (M + CW)	170.00 ± 17.32 ^b
pH	pH 4	54.00 ± 11.14 ^c
	pH 5	95.33 ± 95.33 ^d
	pH 6	385.67 ± 5.13 ^a
	pH 7	399.00 ± 4.58 ^a
	pH 8	312.33 ± 30.92 ^b
	pH 9	165.33 ± 8.10 ^c
Temperature	10°C	0.00 ± 0.00 ^c
	20°C	203.00 ± 17.06 ^b
	30°C	400.67 ± 11.59 ^a
Agitation	0 rpm (static)	414.67 ± 12.65 ^b
	100 rpm	517.67 ± 32.05 ^b
	150 rpm	849.67 ± 61.45 ^a

Means with the same letters of superscript in each factor are not significantly different at 5% level of significance. Values in boldface indicate the highest biomass yield within each factor.

Among liquid culture media, coconut water (CW) produced the significantly highest biomass yield of 386.67 mg DW / 100 mL after 10 days of incubation, followed by 25% Indian mango puree (M) and their combination. In terms of pH, CW with pH 7 registered the highest biomass yield (399.00 mg DW / 100 mL) of *P. neostrigosus* but did not significantly differ from those grown in CW with pH 6. In contrast, the significantly lowest biomass yield was recorded at pH 4, followed by pH 5 and pH 9. The effect of temperature was evaluated using CW with pH 7, and the mycelial biomass yields are also presented in Table 1. Noticeably, *P. neostrigosus* produced the significantly

highest biomass yield when incubated at 30 °C, followed by those incubated at 20 °C. However, no mycelial growth was observed in those incubated at 10 °C. Incubation of the culture at 150 rpm agitation significantly doubled the mycelial biomass yield of *P. neostrigosus* to 849.67 mg DW/100 mL.

Enzyme-based assays

Enzyme-based assays were conducted to assess the inhibitory activity of *P. neostrigosus* extract against metabolic and inflammatory enzymes (Table 2). At 10 µg/mL, the *P. neostrigosus* extract exhibited low inhibitory activity against α-glucosidase and acetylcholinesterase (AChE), showing no significant difference compared to their respective negative controls. In contrast, the extract showed selective cyclooxygenase (COX) inhibition. While no inhibition of COX-2 was observed, a moderate inhibition of COX-1 was detected, which was significantly different from the negative control ($p < 0.05$). Overall, these findings suggest that *P. neostrigosus* has potential as an α-glucosidase or AChE inhibitor and may exert anti-inflammatory effects through partial COX-1 inhibition. In addition to enzyme inhibition, antioxidant potential was assessed using the DPPH radical scavenging assay. At 1000 ppm, *P. neostrigosus* extract exhibited 29.77% scavenging activity, indicating a modest but measurable antioxidant capacity.

Table 2 Inhibitory activities of *P. neostrigosus* mycelial ethanolic extract against α-glucosidase, acetylcholinesterase, cyclooxygenase, and DPPH radical scavenging assay.

Assay	Extract	% Inhibition	
α-glucosidase	<i>P. neostrigosus</i> (10 µg/mL ⁻¹)	5.84 ± 5.16 ^{*a}	
	Acarbose (10 µg/mL ⁻¹)	88.76 ± 0.68	
	DMSO (33000 µg/mL ⁻¹)	0.00 ± 0.00	
Acetylcholinesterase	<i>P. neostrigosus</i> (10 µg/mL ⁻¹)	2.45 ± 6.00 ^{*a}	
	Galantamine (10 µg/mL ⁻¹)	98.03 ± 0.04	
	Methanol (10 µg/mL ⁻¹)	0.00 ± 0.00	
		COX-1	COX-2
Cyclooxygenase	<i>P. neostrigosus</i> (10 µg/mL ⁻¹)	20.11 ± 12.78 ^{*a}	0.00 ± 0.00
	Indomethacin (2862 µg/mL ⁻¹)	93.98 ± 1.85	91.86 ± 4.40
	DMSO (10 µg/mL ⁻¹)	0.00 ± 0.00	0.00 ± 0.00
DPPH	<i>P. neostrigosus</i> (1000 µg/mL ⁻¹)	29.77 ± 2.91 ^{*a}	
	Ascorbic acid	86.88 ± 4.09	
	DPPH + Ethanol	0.00 ± 0.00	

Values are expressed as mean ± SD. Asterisk (*) denotes significant difference from ($p < 0.05$) of mushroom sample compared with negative control. Superscript (a) denotes significant difference ($p < 0.05$) of mushroom sample compared with positive control.

Danio rerio embryotoxicity and teratogenicity

Exposure of zebrafish embryos to *P. neostrigosus* mycelial extract revealed concentration and time-dependent increases in mortality rates (Table 3). Notably, ethanol (positive control) consistently caused 100% mortality across all time points, while the negative control (embryo water) showed no mortality, confirming the assay's validity.

At both 24- and 48-hours post-treatment exposure (hpte), 100% mortality was observed at the highest concentrations tested (100,000, 10,000, and 1,000 µg/mL), indicating strong toxicity at these levels. At intermediate concentrations (100 and 10 µg/mL), mortality was lower, but still notable by 48 hpte, particularly at 100 µg/mL, where mortality reached $83.33 \pm 14.43\%$. In contrast, the lowest concentrations (1 and 0.1 µg/mL) showed minimal or no mortality, closely resembling the negative control. The LC₅₀ value calculated at 42.49 µg/mL further supports the moderate toxicity profile of the extract. Hatchability was also strongly affected in a dose-dependent manner. No hatching occurred at concentrations of 100 µg/mL and above, which coincided with complete mortality.

Embryos exposed to lower concentrations (<100 µg/mL) exhibited normal to slightly delayed hatching. Conversely, partial hatchability was observed at 10 and 1 µg/mL, while the highest hatchability rate occurred at the lowest concentration (0.1 µg/mL) and in the negative control group.

Table 3 Mortality and hatchability rates of zebrafish embryos after 24 and 48h of exposure to the different concentrations of mycelial extract of *P. neostrigosus*.

Extract (µg/mL)	Mortality (%)		LC ₅₀ value**	Hatchability (%)
	24 hpte*	48 hpte		
100,000	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a	42.49 µg/mL	0.00 ± 0.00 ^a
10,000	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a		0.00 ± 0.00 ^a
1,000	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a		0.00 ± 0.00 ^a
100	0.00 ± 0.00 ^b	83.33 ± 14.43 ^a		0.00 ± 0.00 ^a
10	0.00 ± 0.00 ^b	75.00 ± 0.00 ^a		25.00 ± 0.00 ^{ab}
1	0.00 ± 0.00 ^b	25.00 ± 25.00 ^b		41.67 ± 38.19 ^{ab}
0.1	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b		91.67 ± 14.43 ^b
Embryo water	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b		100.00 ± 0.00 ^b
Ethanol	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a		0.00 ± 0.00 ^a

Each value represents the mean ± SD of triplicate tests (n=3). In each column, means with the same letter of superscript are not significantly different from each other at 5% level of significance using Tukey's HSD.

*hpte = hours post-treatment exposure

**LC₅₀ value was computed based on the mortality at 48 hpte.

The heartbeat rate and observable teratogenic effects of zebrafish embryos at the pharyngula stage were assessed following exposure to different concentrations of mycelial extracts of *P. neostrigosus*, based on the computed LC₅₀ values (Table 4). Embryos exposed to the control treatment (embryo water) exhibited the highest mean heartbeat rate (123.00 ± 59.23 bpm), which was not significantly different ($p > 0.05$) from those exposed to low doses (LD) of the mycelial extract (117.00 ± 56.35 bpm). In contrast, embryos exposed to medium (MD) and high doses (HD) of the extract showed significantly lower heartbeat rates of 78.00 ± 37.53 bpm and 84.00 ± 40.41 bpm, respectively.

Table 4 Teratogenic effects and heartbeat rate at pharyngula phase zebrafish embryos exposed to different concentrations of mycelial extract of *P. neostrigosus* at 72h of exposure.

Treatments	Heartbeat rate (min ⁻¹)	NH	TM	HM	PE	YD	DG
HD (4.25 µg/mL)	78.00 ± 37.53 ^b	-	-	-	+	+	+
MD (2.12 µg/mL)	84.00 ± 40.41 ^b	-	-	-	+	+	+
LD (1.06 µg/mL)	117.00 ± 56.35 ^a	-	-	-	-	+	-
Embryo water	123.00 ± 59.23 ^a	-	-	-	-	-	-

*HD= High dose, MD= Medium dose, LD= Low dose (based on LC₅₀ value).

*Each value of the heartbeat rate represents the mean ± SD of duplicate tests (n=2).

In each column, means with the same letter of superscript are not significantly different from each other at 5% level of significance using Tukey's HSD.

*(+) and (-) indicate the presence and absence of teratogenic effects of the different treatments, respectively. NH, No heartbeat; TM, Tail malformation; HM, Head malformation; PE, Pericardial edema; YD, Yolk deformity; DG, Delayed growth.

In terms of teratogenic endpoints (Fig. 1), pericardial edema, yolk deformities, and delayed growth were consistently observed at HD and MD levels of the extract, with fewer abnormalities

seen in the LD groups. The embryos exposed to ethanol showed a complete absence of heartbeat without morphological abnormalities due to embryo coagulation.

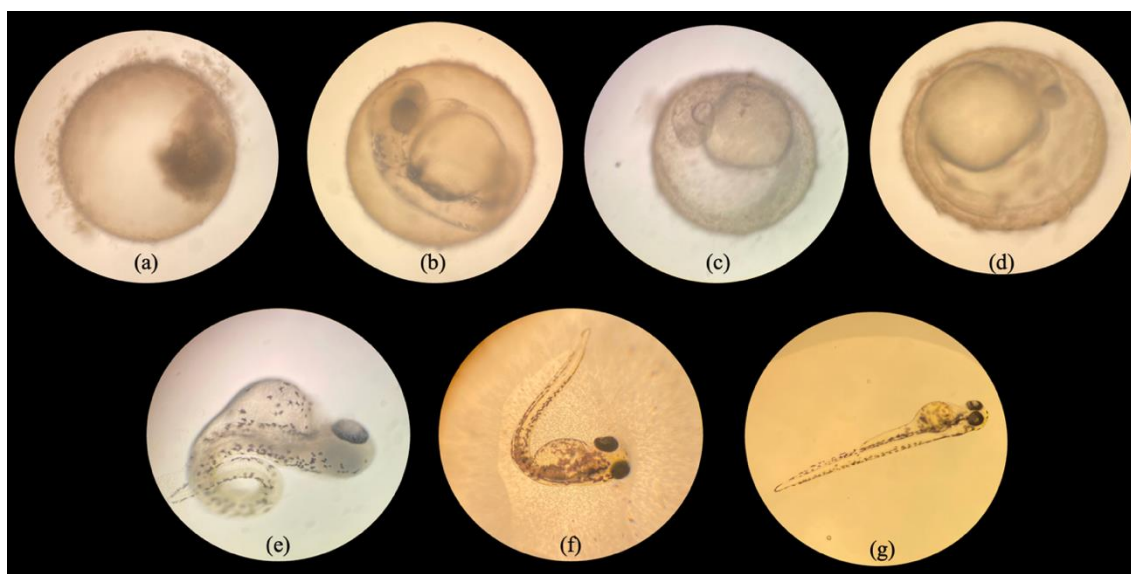


Fig. 1 – Morphological endpoints of zebrafish embryos exposed to the different concentrations of mycelial extracts of *P. neostrigosus*. Coagulated embryos exposed to ethanol (a) delayed growth embryo observed at HD of mycelial extract (b-c) yolk deformity at HD (d) pericardial edema HD, and MD (e-f), and a normal embryo in embryo water (g).

Artemia salina cytotoxicity

The brine shrimp (*A. salina*) lethality assay similarly revealed a dose-dependent increase in mortality for the mycelial extract of *P. neostrigosus* after 12 hours of exposure (Table 5).

Table 5 Mortality of *A. salina* nauplii after 12h of exposure to the different concentrations of mycelial extract of *P. neostrigosus*.

Extract (µg/mL)	Mortality (%)	LC ₅₀ value
100,000	100.00 ± 0.00 ^a	16.83 µg/mL
10,000	100.00 ± 0.00 ^a	
1,000	93.33 ± 5.77 ^a	
100	40.00 ± 10.00 ^b	
10	33.33 ± 5.77 ^b	
1	26.67 ± 5.77 ^b	
0.1	15.67 ± 12.50 ^c	
Embryo water	0.00 ± 0.00 ^c	
Ethanol	100.00 ± 0.00 ^a	

Each value represents the mean ± SD of triplicate tests (n=3). In each column, means with the same letter of superscript are not significantly different from each other at 5% level of significance using Tukey's HSD. *hpte = hours post-treatment exposure.

The mycelial extract exhibited significant lethality at higher concentrations, with mortality decreasing progressively at lower doses. Mortality was absolute (100%) at the highest concentrations of 100,000 µg/mL and 10,000 µg/mL, and remained notably high (93.33 ± 5.77%) at 1,000 µg/mL. At moderate to low concentrations (100, 10, and 1 µg/mL), mortality rates decreased to 40.00 ± 10.00%, 33.33 ± 5.77%, and 26.67 ± 5.77%, respectively. At the lowest tested concentration

(0.1 µg/mL), mortality further declined to $15.67 \pm 12.50\%$. The calculated LC₅₀ value of 16.83 µg/mL underscores the extract's toxicity at relatively low concentrations.

Discussion

The genus *Panus* Fr. comprises wood-rotting basidiomycetes recognized for their ecological role in lignocellulosic decomposition and their potential economic importance. Its taxonomic history has been complex and extensively revised over time. According to current taxonomic databases, approximately 113 taxa have been assigned to the genus, including species, varieties, and morphotypes (Index Fungorum, 2024). However, most records originate from Europe and North America, whereas relatively fewer species have been formally documented from tropical Asian regions. The circumscription of *Panus* and its allied genera has long been controversial, resulting in taxonomic instability and frequent reclassification. Historically, the genus has been assigned to multiple families, including Agaricaceae, Polyporaceae, Tricholomataceae, and Meruliaceae, reflecting the challenges associated with morphological classification (Imai 1938, Singer 1962, Yue et al. 2024). More recent phylogenetic studies have proposed the recognition of Panaceae as a distinct family to accommodate *Panus* and closely related taxa (Justo et al. 2017), further illustrating the dynamic nature of fungal systematics in the genomic era. Nevertheless, comprehensive investigations into the biological activities of *P. neostrigosus* remain scarce.

In the present study, key parameters influencing the submerged cultivation of *P. neostrigosus* were investigated, with particular emphasis on optimizing biomass production under varying nutritional and physicochemical conditions. Among the three indigenous liquid media evaluated, coconut water proved to be the most effective in supporting mycelial biomass production. Coconut water contains both organic and inorganic compounds, encompassing nearly all minerals commonly found in food. Coconut water is composed of about 95.5% water, with the remaining fraction consisting of carbohydrates, proteins, fats, amino acids, vitamins, and mineral salts (Lamdande et al. 2020). Moreover, it is also a natural source of sugars such as glucose, fructose, and sucrose (Ogundiya 1991, Divya et al. 2022). It also provides several amino acids, including alanine, arginine, cysteine, and serine, at concentrations reported to be even higher than those in cow's milk (Prades et al. 2012). Yannam et al. (2020) further demonstrated that coconut water contains nine essential amino acids, with tryptophan being the most abundant, while glutamine, alanine, and tyrosine were identified as primary constituents. Coconut water is a rich source of vitamin C along with several water-soluble B vitamins, including thiamine (B1), riboflavin (B2), pantothenic acid (B5), pyridoxine (B6), biotin (B7), and folate (B9) (Thampan & Rethinam 2004, Yong et al. 2009). It also provides a broad mineral profile, with potassium as the dominant element, complemented by sodium, calcium, magnesium, zinc, iron, and phosphorus (Richter et al. 2005, Uphade et al. 2008, Kumar et al. 2021). This distinctive mix of sugars, amino acids, vitamins, and minerals likely accounts for the enhanced mycelial biomass production of *P. neostrigosus* when grown in coconut water.

Dulay et al. (2017) first reported the successful isolation and culture of a native *P. neostrigosus* strain, noting that both coconut water agar and Sabouraud dextrose agar supported rapid mycelial growth. In a later study, Dulay et al. (2021a) found that several *Lentinus* isolates achieved optimal yields in submerged cultures prepared with coconut water-based media, an outcome attributed to the medium's naturally high sugar and mineral content. Similar results have been observed in other species. For example, Chutimanukul et al. (2023) showed that coconut water enhanced enzymatic degradation of complex substrates in *Hericium erinaceus* (Bull.) Pers., which improved nutrient assimilation and sustained vigorous mycelial growth. Collectively, these findings suggest that coconut water serves not only as a nutrient source but also as a stimulant of enzymatic activity and substrate utilization in mushrooms.

As pH plays a crucial role in fungal development, it can affect nutrient solubility, membrane transport, and the stability of key enzymes. Wood-rotting fungi, including *Panus* spp., secrete ligninolytic enzymes whose catalytic activity is generally higher under acidic conditions (Pointing 2001). The present finding that *P. neostrigosus* grows well at pH 6.0–7.0, therefore, highlights its ecological versatility and potential for cultivation on locally available agro-industrial residues, which

often buffer toward neutrality. This observation is consistent with reports on related *Lentinus* species cultivated in the Philippines. Kalaw et al. (2021) reported that *Lentinus* isolates attained their highest mycelial density in media adjusted near neutral pH, with growth inhibition observed under strongly acidic or alkaline conditions. This suggests that *P. neostrigosus*, is adapted to moderately acidic environments but maintains tolerance up to neutrality, likely reflecting the chemical properties of decaying wood substrates in its natural habitat. Interestingly, Philippine isolates of *P. neostrigosus* characterized by Dulay et al. (2022) showed genetic clustering distinct from other *Lentinus* spp., which may partly explain their relatively broader pH tolerance. This variation may reflect adaptive evolution of Philippine strains to the more fluctuating conditions typical of tropical environments. Similar results have been reported for *Pleurotus ostreatus* (Jacq.) P. Kumm., which also tolerates near-neutral pH, although its radial growth declines once pH exceeds 7.0 (Chang & Miles 2004). Likewise, Shim et al. (2005) identified pH 7 as the optimal condition for *Macrolepiota procera* (Scop.) Singer, reinforcing the view that many basidiomycetes favor neutrality for maximum mycelial development.

Among environmental factors, temperature is particularly critical in mushroom cultivation, as it regulates mycelial growth, metabolic activity, and sporulation. For basidiomycetes such as *Panus*, maintaining culture within the optimal thermal range supports rapid substrate colonization and efficient energy utilization. Moderate increases in temperature often stimulate enzymatic activity, but excessive heat can denature enzymes and inhibit growth (Chang & Miles 2004). In the present study, *P. neostrigosus* produced the highest mycelial biomass at 30 °C, indicating that this temperature provides favorable growth conditions. This finding is consistent with earlier reports on fourteen *Lentinus* isolates from Luzon, Philippines (Dulay et al. 2021b). A similar pattern has been described in related genera: *Pleurotus* and *Lentinus* species generally grow best at around 25 °C, though some can withstand temperatures as high as 45 °C (Gbolagade et al. 2006, Vargas-Isla & Ishikawa 2008). Such findings point to the adaptation of *P. neostrigosus* to moderately warm environments, consistent with the tropical conditions where it naturally occurs. Temperature not only shapes growth but also influences the activity of extracellular enzymes such as laccases and peroxidases, which drive lignocellulose breakdown in white-rot fungi. Elisashvili et al. (2008) noted that production of these ligninolytic enzymes peaks between 28 °C and 30 °C, promoting faster substrate colonization. Once temperatures drift outside this range, enzyme activity declines, offering a likely explanation for the reduced biomass yield of *P. neostrigosus* under non-optimal conditions. Parallel results have been documented in other mushrooms: *P. ostreatus* grows best at 28–30 °C, with reduced biomass below 25 °C and inhibition above 35 °C (Bellettini et al. 2019), while *Ganoderma lucidum* (Curtis) P. Karst. shows maximum mycelial density at 30°C, though fruiting requires slightly lower temperatures (Baby et al. 2015). The tropical straw mushroom *Volvariella volvacea* (Bull.) Singer thrives even higher, at 30–32 °C, reflecting its adaptation to hot climates (Chang & Miles 2004). In contrast, temperate mushrooms such as *Lentinula edodes* (Berk.) Singer and *Agaricus bisporus* (J.E Lange) Imbach prefer cooler conditions of 20–25 °C (Bellettini et al. 2019). Collectively, these differences underscore the species-specific adaptations of mushrooms and confirm the suitability of *P. neostrigosus* for cultivation in tropical regions.

During submerged fermentation, biomass yield was maximized at an agitation speed of 150 rpm. Agitation is an important parameter in liquid fermentation, as it regulates oxygen transfer, nutrient distribution, and fungal morphology. Insufficient agitation can lead to poor aeration, nutrient gradients, and compact pellet formation, while excessive agitation may cause shear stress, hyphal fragmentation, and reduced yields (Felse & Panda 2000). In submerged cultures, proper agitation ensures uniform delivery of both nutrients and oxygen (Cho et al. 2006). It has been shown to stimulate biomass accumulation as well as exopolysaccharide production (Xu et al. 2006). In this study, 150 rpm provided an effective balance, promoting vigorous mycelial growth without evidence of stress or inhibition. Comparable findings have been reported in other fungi. For instance, *G. lucidum* (Curtis) P. Karst reached its highest biomass and polysaccharide yields at around 150 rpm, though productivity declined at higher speeds (Park et al. 2002). Similarly, Tang and Zhong (2003) observed that while increasing agitation improves aeration, it also alters fungal morphology,

often leading to dense pellets that hinder mass transfer. Papagianni (2004) further emphasized that agitation intensity influences the balance between pelletized and dispersed growth, with moderate levels favoring intermediate pellet sizes that optimize oxygen and nutrient uptake.

Beyond growth, agitation has also been linked to secondary metabolism. Fang and Zhong (2002) demonstrated that *G. lucidum* grown under controlled agitation produced substantially higher levels of intracellular polysaccharides and ganoderic acids compared with static cultures. These findings suggest that agitation not only promotes biomass formation but may also stimulate bioactive metabolite production, likely through improved mass transfer and shear-induced activation of metabolic pathways.

To explore potential antidiabetic effects, the mycelial extract was tested using the α -glucosidase inhibitory assay. In this study, *P. neostrigosus* extract displayed only weak activity, with 5.84% inhibition observed at 10 μ g/mL. In contrast, Kim et al. (2017) reported markedly stronger effects from water extracts of *Cordyceps militaris* (L.) Fr., which achieved 45.77% inhibition at 0.1 mg/mL, 54.76% at 0.25 mg/mL, 58.74% at 0.5 mg/mL, and 60.5% at 1.0 mg/mL. Similarly, Fatmawati et al. (2013) reported that *G. lucidum* extracts exhibited strong α -glucosidase inhibition, largely attributed to triterpenoids. In contrast, *P. ostreatus* showed only moderate activity, which was associated with its phenolic and polysaccharide fractions (Jayakumar et al. 2009). Markedly stronger α -glucosidase inhibition has been observed in *Inonotus obliquus* (Fr.) Pilát, with Ma et al. (2013) attributing this activity to its high phenolic and flavonoid content. More recently, Shi et al. (2024) showed that acid hydrolysates of *Auricularia auricula-judae* (Bull.) Quél polysaccharides improved glucose metabolism in mice by reducing oxidative stress, thereby alleviating diabetic symptoms. The evidence shows that α -glucosidase inhibition differs widely among mushroom species. This variation depends mostly on metabolite composition, especially triterpenoids, flavonoids, and phenolic acids. In *P. neostrigosus*, the weak inhibition seen in this study may reflect the species' unique metabolite profile. It could also be due to limits in the extraction method or to active compounds being present only at low levels.

Acetylcholinesterase (AChE) is an enzyme that breaks down acetylcholine and is closely linked to the development of neurodegenerative disorders. To explore the possible neuroprotective role of *P. neostrigosus*, its mycelial extract was tested for AChE inhibition. At the examined concentration, the extract produced only 2.45% inhibition. This weak effect suggests limited ability to slow acetylcholine breakdown, a mechanism often targeted in Alzheimer's therapy. In comparison, Tel et al. (2015) reported much stronger inhibition in wild mushrooms such as *Lactarius deliciosus* (L. Gray), *Boletus edulis* Bull., and *Cantharellus cibarius* Fr., with values above 30%. These effects were linked mainly to phenolic and flavonoid compounds. Similar relationships between enzyme inhibition and phenolics or terpenoids were also noted by Ferreira et al. (2006) and Barros et al. (2010). *Hericium erinaceus* known for its neurotrophic and cognition-enhancing effects, has shown mild cholinesterase inhibition as well. However, its benefits are thought to involve broader neuroactive pathways rather than direct AChE suppression (Szućko-Kociuba et al. 2023). Although *P. neostrigosus* showed minimal inhibition in this study, stronger effects may emerge at higher concentrations or with improved extraction and fractionation. It is also worth noting that mushroom-derived neuroprotection often relies not only on cholinesterase inhibition but also on other mechanisms, especially antioxidant and anti-inflammatory actions.

Moreover, cyclooxygenase (COX) enzymes are crucial in the production of prostaglandins. They serve as signaling molecules that play a major role in controlling inflammation and related physiological processes. COX-1 is constitutively expressed and plays physiological roles in gastric protection and platelet aggregation, while COX-2 is inducible and primarily associated with inflammation and pain. Natural COX inhibitors are of pharmacological interest because of their potential as anti-inflammatory and analgesic agents. *Panus neostrigosus* mycelial extract inhibited COX-1 activity by 20.11% at the tested concentration, whereas no detectable inhibition of COX-2 was observed. These findings suggest a selective inhibitory effect toward COX-1, though at a relatively modest level compared with other mushroom species reported in the literature. Several edible and medicinal mushrooms are known to modulate COX activity, often through phenolic

compounds, terpenoids, or polysaccharides. Wasser (2014) summarized that *G. lucidum* contains triterpenes that strongly inhibit both COX-1 and COX-2, contributing to its traditional use as an anti-inflammatory remedy. *Inonotus obliquus* extracts were found to strongly suppress COX-2 activity in macrophages stimulated with LPS, which was consistent with lower levels of nitric oxide and prostaglandins (Ma et al. 2018). In comparison, *P. ostreatus* extracts showed a moderate ability to inhibit COX, an effect linked to their phenolic antioxidants and compounds with lovastatin-like properties (Jayakumar et al. 2010). The observation that *P. neostrigosus* selectively inhibited COX-1 indicates that its metabolites may interact more strongly with constitutive isoforms than with inducible ones. Since COX-1 inhibition is commonly associated with antiplatelet activity and gastric side effects, this raises different therapeutic implications. By contrast, most drug development has targeted COX-2, as its inhibition controls inflammation with a lower risk of gastrointestinal complications. The absence of detectable COX-2 inhibition in *P. neostrigosus* therefore suggests a more limited anti-inflammatory potential compared with mushrooms such as *Ganoderma* and *Inonotus*, which are better known for their stronger and more COX-2-specific actions. The COX-1 selectivity observed here is consistent with earlier reports showing that certain fungal metabolites, such as terpenoids and polysaccharides, can modulate inflammation through cyclooxygenase pathways (Su et al. 2015). Although direct evidence of COX-1 inhibition in *P. neostrigosus* is still lacking, insights from related species provide useful context.

Basidiomycete mushrooms are also well known for their strong antioxidant capacity. This stems from the wide range of bioactive compounds they produce. Among these, phenolic compounds are often cited as the main contributors, acting as hydrogen donors and efficient radical scavengers. Flavonoids complement this activity by stabilizing oxidative reactions and shielding cells from oxidative damage (Ferreira et al. 2009). Polysaccharides, particularly β -glucans, add another protective mechanism by enhancing antioxidant enzyme activity while also directly neutralizing reactive oxygen species (Heleno et al. 2015). Mushrooms also provide a mix of fat-soluble antioxidants, such as tocopherols and carotenoids, and water-soluble ones, including vitamin C and glutathione. Together, these metabolites play an important role in reducing oxidative stress, which explains much of the therapeutic interest in mushrooms (Valentão et al. 2005). Because of this biochemical diversity, mushrooms are valued not only as nutrient-dense foods but also as natural candidates for managing oxidative stress-related conditions, such as cardiovascular disease, neurodegenerative disorders, and some cancers. Notable examples include *G. lucidum*, *P. ostreatus*, and *L. edodes*, all of which consistently show strong antioxidant activity. The antioxidant activity of *P. neostrigosus* was assessed through the DPPH radical scavenging assay. At 1000 ppm, the extract showed 29.77% activity, reflecting a detectable ability to quench free radicals. Comparable assays in other basidiomycetes have yielded higher values, with *A. bisporus* and *L. edodes* often exceeding 60% (Barros et al. 2007). Stronger activity has also been reported in several wild edible mushrooms, largely attributed to their phenolic and flavonoid content (Elmastaş et al. 2007). Likewise, *G. lucidum* is frequently highlighted for its pronounced antioxidant potential, which has been linked to triterpenes, polysaccharides, and phenolic compounds (Heleno et al. 2010).

Exposure of zebrafish embryos to the mycelial extract of *P. neostrigosus* produced clear dose- and time-dependent survival trends, suggesting toxicity at higher concentrations and with longer exposure. At low doses, survival rates were similar to the negative control, but mortality rose steadily as both concentration and incubation time increased. By 48 hours, complete mortality occurred at concentrations of 1,000 $\mu\text{g/mL}$ and above, while lower concentrations caused little to no effect. These results highlight the sensitivity of zebrafish embryos as a model for evaluating the toxicity of natural products (Kimmel et al. 1995). Comparable patterns have been noted in other mushrooms, such as aqueous extracts of *P. neostrigosus*, which showed dose- and time-dependent embryotoxicity (Dulay et al. 2018), and extracts of *Termitomyces clypeatus* R. Heim produced lethality and developmental defects at elevated concentrations (De Castro et al. 2016). Effects were also evident in hatching success. Embryos exposed to lower concentrations hatched normally within 48 hours, whereas higher concentrations markedly reduced hatchability. Such inhibition may reflect delayed embryonic development, a teratogenic effect reported in other mushrooms such as *V. volvacea*, where slowed

embryogenesis was linked to disrupted metabolism or interference with critical developmental enzymes (Asasira et al. 2025).

Additionally, the brine shrimp assay gave further evidence that the *P. neostrigosus* extract carries cytotoxic effects. Mortality was revealed to be directly proportional to the extract dosage. This pattern mirrors what has been seen in other mushrooms. Younis et al. (2014) showed that *L. edodes* extracts suppressed the growth of HepG2 liver carcinoma cells in a dose-dependent way, linking the effect to antitumor metabolites. While our work relied on *Artemia salina* instead of mammalian systems, the rising lethality with concentration reflects the same underlying idea that the extract contains constituents capable of disrupting cell viability. De Leon et al. (2020) reached a similar conclusion with *Fomitopsis feei* (Fr.), another Philippine macrofungus whose ethanolic extracts proved lethal to brine shrimp. Taken together, these findings suggest that *P. neostrigosus* may also produce metabolites with therapeutic potential, though deeper work on purified fractions and mammalian models will be needed to know for certain.

Conclusions

Extracts from the culture showed the ability to inhibit α -glucosidase and acetylcholinesterase, and displayed moderate COX-1 inhibition, pointing to possible antidiabetic and anti-inflammatory potential. Just as notable, however, the mycelial extract produced clear, dose-dependent toxic effects in both zebrafish embryos and brine shrimp, including embryotoxicity, teratogenicity, and cytotoxicity. These results highlight *P. neostrigosus* as a promising source of bioactive compounds, particularly those with cytotoxic potential. Further studies focusing on compound isolation, mechanism of action, and potential therapeutic applications are recommended to fully explore its value in pharmaceutical and biomedical research.

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Ethical Statement

Ethical considerations were observed throughout the conduct of the study involving zebrafish embryos. Experimental procedures followed internationally accepted guidelines for the care and use of laboratory animals. The use of early-stage zebrafish embryos was selected to minimize ethical concerns associated with vertebrate testing. All handling procedures were performed carefully to reduce stress and ensure humane experimental conditions. After experimentation, embryos were disposed of following standard laboratory biosafety and institutional protocols.

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