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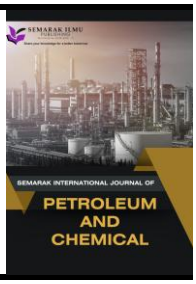

Study on Phytochemicals and Antioxidants of *Pleuratus* sp. Cultivated Through Submerged Fermentation

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ABSTRACT

This study investigates the bioactive compounds present in the genus *Pleurotus* sp. mushrooms, with a focus on their antioxidant properties. Hot water extraction of *Pleurotus* mycelium was performed at three temperatures: 30°C, 65°C, and 100°C. A total of 57.48 g of mycelium biomass was obtained through submerged fermentation in basal medium. The objective was to identify specific phytochemicals and evaluate the percentage of free radical inhibition. The mycelium was initially cultured on Potato Dextrose Agar plates and later subjected to fermentation. Six phytochemical tests were carried out to detect alkaloids, flavonoids, triterpenoids, saponins, phenolic compounds and anthraquinones. The analysis revealed the presence of triterpenoids, saponins, and phenolic compounds. Antioxidant activity was assessed using the DPPH and nitric oxide scavenging assays. The DPPH assay showed the 65°C extract had the highest inhibition of 97.92%, while the 100°C extract exhibited the highest inhibition at 82.67%, with the 65°C extract showing the lowest at 75.38%. The results suggest that extracts at 65°C demonstrated substantial antioxidant activity, comparable to synthetic antioxidants.

1. Introduction

Inside the developing world, particularly in Southern Asia and sub-Saharan Africa, malnutrition continues to be a major issue for the public health according to Schofield and Ashworth [1]. Diets in these regions often lack essential macronutrients like carbohydrates, proteins, and fats, as well as micronutrients such as minerals, vitamins, and electrolytes, or sometimes both. Malnutrition makes a person much more vulnerable to infections and contributes their severity, leading to higher illness and mortality rates as noted by Brabin and Coulter [2]. Furthermore, poverty the root cause of malnutrition, contributes to even more sickness and death.

To address this, researchers have been consistently exploring affordable, nutritious, and high-yield food sources. After years of research, mushrooms emerged as a viable alternative. Mushrooms

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can be consumed as functional foods that enhance health, and the β-glucan they contain can serve as a prebiotic in both human food and animal feed as highlighted by Jabir *et al.,* [3]. As they are cheap and easy to harvest, mushrooms are a very ideal food source that can replace others while also providing essential nutrients to the masses.

Arbaayah and Umi [4] mentions that various species of edible macro-fungi, such as the oyster mushroom (*Pleurotus* sp.), mound mushroom (*Termitomyces* sp.), paddy straw mushroom (*Volvariella volvacea*), shiitake (*Lentinula edodes*), split gill mushroom (*Schizophyllum commune*), bracket fungi (*Ganoderma* sp.), and monkey's ear or Jew's ear mushroom (*Auricularia* sp.), thrive in tropical regions characterized by warm and humid climates and that Malaysia is a prime example of such a region.

Malaysia has long been renowned for its abundant resources, such as energy and food sources. Today, as people begin to increasingly prioritize healthier lifestyles, healthy nutrient-rich foods like mushrooms have grown in popularity. The popularity of mushrooms can be heavily credited to their nutritional and medicinal properties, including proteins, carbohydrates, phytochemicals, antioxidants, anticancer, and more. Among cultivated macro-fungi, the button mushroom (Agaricus biporus) leads in global production volume, followed by the oyster mushroom (Pleurotus sp.), which accounts for 24.2% of world production according to findings by Aksu et al., [5].

The *Pleurotus* genus, commonly known as the white oyster mushroom, is a favourite among culinary enthusiasts. Its mild but chewy texture, delicate flavour, and unique aroma, the oyster mushroom is a highly sought after food. Additionally, its medicinal properties, such as antiviral, antidiabetic, antioxidant, antihypercholesterolic, anticancer, antitumor, and immunomodulatory effects, make it valuable to be used in pharmaceuticals as discussed by Deepalakshmi and Mirunalini [6]. Approximately 21,077 metric tons of mushrooms were imported for the local market in 2007 as noted by Ishak [7] based on data from the Department of Agriculture Malaysia. It is estimated that Malaysians consume about 324 grams of mushrooms per person per year, with this number expected to rise. The high consumer demand and the feasibility of large-scale cultivation using inexpensive methods such as growing mushrooms on sawdust, paddy straw, cotton, wheat straw, bamboo leaves, lawn grass as shown in the work of Kumari and Achal [8], yam peelings (*Dioscorea* sp.), cassava peelings (*Manihot* sp.), corn straw, oil palm fruit fibers, wild grass straw (*Pennisetum* sp.), banana pseudostem, and Bahia grass as proven by Martos *et al.,* [9] makemushroom cultivation a promising avenue.

Pleurotus sp. mushroom has always been known as mushroom with huge number of bioactive compounds such as phytochemicals and polysaccharides, and it exhibits antibacterial, anticancer, antioxidant, antidiabetic, antiviral, antihypercholesterolic, antitumor and immunomodulatory properties which were discussed by Deepalakshmi and Mirunalini [6]. Hence, the purpose of this project is grown and produce mushroom mycelium through submerged liquid fermentation (SLF) and to investigate the phytochemicals from different extraction condition. Finally, antioxidant analysis will be done on the *Pleurotus* sp. mushroom extract to prove free radical scavenging activity by the mycelium extracts.

This study addresses a key research gap by examining specimens that were locally isolated from the area surrounding Universiti Sains Islam Malaysia in Nilai, Negeri Sembilan. This study also analyses the effects of different extraction temperatures of the sample. By focusing on these region-specific samples and unique extraction conditions, this research aims to provide a deeper understanding of the phytochemical composition and antioxidant properties of *Pleurotus* sp. within this unique local area along with the effects of differing extraction conditions.

2. Methodology

2.1 Overview of Research

The methodology employed in this study is shown in Figure 1. It involved several sequential steps to investigate the antioxidant properties of *Pleurotus* sp. mycelium. Initially, the mycelium was subcultured on Potato Dextrose Agar (PDA) supplemented with yeast extract to ensure optimal growth and maintain a pure culture. Subsequently, the actively growing mycelium was subjected to submerged fermentation in a basal liquid medium, facilitating large-scale biomass production and the synthesis of bioactive compounds. Following fermentation, the mycelium was harvested, and bioactive components were extracted using appropriate methods. The extracted compounds underwent phytochemical analysis to identify secondary metabolites, such as flavonoids and phenolic compounds. The antioxidant potential of the extracts was then evaluated through DPPH and nitric oxide radical scavenging assays to determine their free-radical scavenging activity. Finally, the experimental results were compiled, analyzed, and documented for further interpretation and reporting. This stepwise approach ensured a thorough and systematic evaluation of the antioxidant properties of *Pleurotus* sp. mycelium.

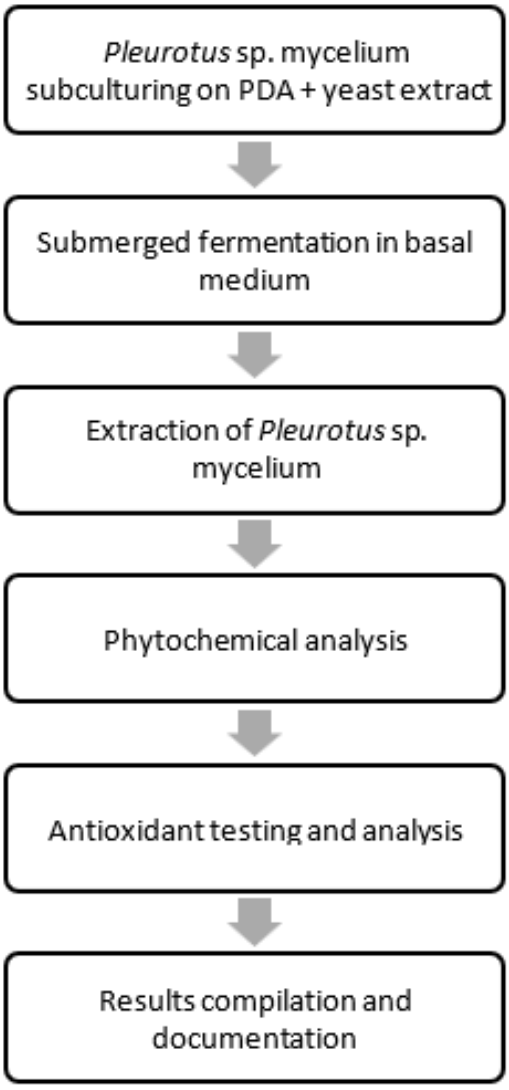

Fig. 1. Flowchart of the research processes and process

2.2 Maintenance of Mushroom Mycelium

In this study, locally isolated *Pleurotus* sp. mushroom mycelium was utilized. The mycelium was cultured on Potato Dextrose Agar (PDA) plates supplemented with 30 g/L of 2% yeast extract, which provided vitamins, minerals, and amino acids that were noted as essential by Fujita and Hashimoto [10]. The culture plates were stored at 4°C for long-term preservation. To obtain a pure culture, the *Pleurotus* mycelium was sub-cultured several times.

2.3 Chemicals and Reagents

Distilled water, absolute ethanol, methanol, Potato Dextrose Agar, yeast extract, meat extract, potassium dihydrogen phosphate, magnesium sulphate, Dragendorff's reagent, sodium hydroxide solution, acetic anhydride solution, neutral 5% ferric chloride solution, benzene, 10% ammonium hydroxide solution, 0.1 mM 1,1-diphenyl-2- picrylhydrazyl solution, sodium nitroprusside solution, ascorbic acid, Griess reagent, butylated hydroxytoluene.

2.4 mycelium Biomas Cultivation

The locally isolated *Pleurotus* sp. mushroom mycelium was maintained on Potato Dextrose Agar (PDA) plates with the addition of 30 g/L of 2% yeast extract as a stock culture. The mycelium stock was then stored at 4°C for long-term use. Subsequently, submerged liquid fermentation was performed to produce mycelium biomass using the culture broth. The optimized medium conditions as shown in Table 1 were applied in this study. From the stock culture, 10 plugs of *Pleurotus* sp. mushroom mycelium were inoculated into each flask containing 100 mL of basal medium, and fermentation was conducted at 28°C with a rotation speed of 150 rpm for 7 days. After fermentation, the samples were filtered to separate the mycelium biomass from the culture broth. To measure the dry weight of the mycelium, the pellet was washed multiple times with distilled water and dried in an oven at 50°C until a constant weight was reached. The dried mycelium was then ground into powder and stored for future use, following a procedure detailed by Ahmad *et al.,* [11].

Table 1
Compositions of optimized medium for submerged liquid fermentation of Pleurotus sp. mushroom mycelium

Materials	Concentration (g/L)
Glucose	40.0
Meat extract	4.0
Magnesium sulphate (MgSO4)	0.005
Potassium dihydrogen phosphate (KH2PO4)	1.0

2.5 Hot Water Extraction Process

3.3 grams of mycelium powder were mixed with 50 mL of distilled water. The mixture was heated in a water bath at 30°C, 65°C, and 100°C. The resulting extracts were filtered through Whatman #1 filter paper following a procedure established by Zhang *et al.,* [12]. The filtrates were then used for subsequent analysis of phytochemicals and antioxidant activities.

2.6 Phytochemical Compound Analysis

The extracts obtained through various hot water extraction processes were analyzed for specific phytochemicals produced in the *Pleurotus* sp. mushroom. The tests conducted were based on tests previously utilized by Adebayo & Ishola [13], highlighted in a guide by Harborne [14] and are as follows:

2.6.1 Test for Alkaloids:Dragendorff's test

1 to 2 mL of Dragendorff's reagent was added to 5mL of the extract. Prominent yellow or orange precipitation confirms the test as positive.

2.6.2 Test for Flavonoids:Alkaline Reagent test

Few drops of sodium hydroxide were added into the test solution. In order to further prove the positivity of the result, few drops of dilute acid were added into the test solution. Formation of intense yellow colour will occur, and if it turns colourless upon addition of a few drops of dilute acid, the test is positive.

2.6.3 Test for Triterpenoids: Libermann-Burchar's test

5 mL of extract was mixed with a few drops of acetic anhydride, boiled, and allowed to cool. Sulfuric acid was then introduced to the side of the test tube. The appearance of a deep red color suggests the presence of terpenes.

2.6.4 Test for Phenols:Ferric Chloride test

To 5 ml of extract, few drops of neutral 5% ferric chloride solution were added. Dark green colour indicates the presence of phenols.

2.6.5 Test for Saponins: Froth test

5 mL of mushroom extract was vigorously shaken with 8 mL of distilled water in a test tube for 30 seconds, then left undisturbed for 20 minutes. Persistent froth indicates the presence of saponins.

2.6.6 Test for Anthraquinones: Ammonium hydroxide test

2 mL of the extract was shaken with 10 mL of benzene, after which the mixture was filtered. Then, 5 mL of 10% ammonium hydroxide was added to the filtrate. A pink, red, or violet color in the ammoniacal phase indicates a positive test.

2.7 Antioxidant Assay
2.7.1 DPPH free radical scavenging assay

A spectrophotometric assay was performed using 1,1- diphenyl-2-picrylhydrazyl (DPPH), a stable radical, as the reagent. A 0.1 mM DPPH solution in methanol was prepared, and 1 mL of this solution was mixed with 3 mL of extracts from *Pleurotus* sp. mushroom mycelium (1 g/L). The mixture was shaken vigorously and allowed to stand for 30 minutes at room temperature. Absorbance was

measured using ELIZA at 517 nm. The proficiency in scavenging the DPPH radical was calculated using Eq. (1).

$$DPPH\ Scavenging\ Activity\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where
A_0= Absorbance of control
A_1 = Absorbance of the presence of the mushroom extract

2.7.2 Nitric oxide radical scavenging assay

To determine the nitric oxide radical scavenging activity of *Pleurotus* sp., 2 mL of 10 mM sodium nitroprusside prepared in phosphate buffer saline at pH 7.4 was combined with 0.5 mL of *Pleurotus* sp. extract (1 g/L) and ascorbic acid. The mixture was then combined with 0.5 mL of Griess reagent and incubated for 30 minutes. Finally, the absorbance was measured at 540 nm using ELIZA in accordance with the work of Garrat [15].

3. Result and Discussion

3.1 Submerged Fermentation of Pleurotus sp. Mycelium

In each flask, roughly 10 plugs of *Pleurotus* sp. mushroom mycelium were collected from the culture plates and inoculated into the basal medium. The flasks were then incubated in a shaker at 28°C and 150 rpm, for about a week for the first six batches of submerged fermentation, and four to five days for the final two batches after adding more glucose and meat extract. The mycelium biomass was then separated from the supernatant by filtration, dried, weighed, and ground into powder.

Table 2 showcases the amount of *Pleurotus* sp. mycelium biomass obtained from submerged fermentation in basal medium containing meat extract, the amount gradually increased batch by batch from 3.22 g in the first batch (Batch 1) to 15.88 g in the last batch (Batch 8). At the end of the entire submerged fermentation process, a total of 57.48 g of *Pleurotus* sp. mycelium biomass was obtained.

For the metabolism of the *Pleurotus* mycelium, the vital components in submerged fermentation are glucose, meat extract, potassium dihydrogen phosphate (K2PO4), and magnesium sulphate (MgSO4), which are key constituents of the basal medium. Specifically, 40 g/L of glucose, 5 g/L of meat extract, 1 g/L of potassium dihydrogen phosphate, and 0.5 mg/L of magnesium sulphate were diluted and mixed with 1 L of distilled water to produce the basal medium.

Fermentation is generally classified into solid and liquid types. Liquid fermentation, or submerged fermentation, utilizes liquid substrates such as broth and molasses. Throughout the fermentation process, bioactive compounds secreted by bacteria or fungi are found in the fermentation broth, providing a nutritious environment. During metabolism, microorganisms quickly utilize substrates, necessitating a continuous replenishment of nutrients. This method is especially suitable for moisture-loving microorganisms like bacteria. Subramaniyam and Vimala [16] mentions that one key advantage of liquid fermentation is the simplified purification process. Furthermore, this technique is primarily employed for extracting secondary metabolites intended for liquid form.

Table 2
Total amount of Pleurotus sp. mycelium biomass obtained through submerged fermentation

Batch Number	Mycelium Biomass (g)
1	3.22
2	4.47
3	5.61
4	6.36
5	5.58
6	6.36
7	10.00
8	15.88
Total	57.48

Submerged liquid fermentation is a method that converts complex biological substances into simpler compounds through various microorganisms, including fungi and bacteria. This process enables metabolic breakdown, resulting in the release of additional by-products beyond the usual fermentation products. These by-products are known as secondary metabolites, which can encompass antibiotics, enzymes, growth factors, and more according to Balakrishnan and Pandey [17], Machado *et al.,* [18] and Robinson *et al.,* [19]. Often referred to as bioactive compounds, these secondary metabolites have significant biological activity. Recent studies have indicated their economic and industrial relevance, leading to their use in pharmaceutical and food research, particularly in prebiotics and probiotics.

3.2 Phytochemical Analysis of Pleurotus Mycelium Extract

As observed in Table 3, which shows the various tests conducted to detect the presence of different phytochemicals in the extract of *Pleurotus* sp. mushroom, the results from the three replicates were consistent for each test. A total of six phytochemical tests were carried out, namely Dragendorff's test, alkaline reagent test, Libermann-Burchard's test, ferric chloride test, froth test, and anthraquinones test. These tests identify alkaloids, flavonoids, triterpenoids and steroids, phenolic compounds and tannins, saponins, and anthraquinones, respectively. The *Pleurotus* mycelium biomass extract, prepared at 65°C for 4 hours, was used as the standard for all tests. Depending on the test, the sample solution exhibited various reactions, including colour changes, froth formation, or ring formation. Table 3 exhibited the phytochemicals present in *Pleurotus* sp. mycelium extracts at 65°C extraction condition. To achieve optimal outcomes and prevent false positives that might compromise the research data, three replicates were performed for each test.

The term "phyto-" in phytochemicals comes from the Greek word phyton, meaning plant. Essentially, all chemicals found in plants are considered phytochemicals, including polysaccharides, phytosterols, phenols, alkaloids, flavonoids, organosulfurs, and carotenoids, each with its own specific functions. Typically, the term phytochemical refers to the wide variety of biologically active compounds found in plants. Additionally, phytochemicals contribute to the color, flavor, and natural defensive measures of plants against harmful pests and insects. These compounds also have certain

disease-preventive properties as reported by Chede [20]. For example, phytochemicals from medicinal plants can help maintain balance in the brain by affecting the neurotransmitter receptors that are involved in major inhibitory functions as observed by Kumar and Khanum [21]. Moreover, phytochemicals have the potential to lower the risk of cancer and cardiovascular diseases. As a result, foods containing phytochemicals are highly beneficial for consumers due to their remarkable medicinal and disease-preventive qualities.

Table 3
Detection of phytochemicals in Pleurotus sp. mycelium extracts

Phytochemical Tests	Replicate 1	Replicate 2	Replicate 3
Tests for alkaloids (Dragendorff's Test)	-	-	-
Test for flavonoids (Alkaline Reagent Test)	-	-	-
Test for triterpenoids and steroids (Libermann-Burhcar's Test)	+	+	+
Test for phenolic compounds and tannins (Ferric Chloride Test)	+	+	+
Test for saponins (Froth Test)	+	+	+
Test for anthraquinones	-	-	-

Note: (+) indicates positive result/detected and (-) indicates negative result/not detected

In most vegetation, including plants and fungi, phytochemicals play significant roles by providing color, flavor, and protection. Phytochemicals refer to any chemical substances found in plants. In this study, *Pleurotus* mycelium extract was utilized to test for the presence of various phytochemicals. The tests conducted were Dragendorff's test, alkaline reagent test, Libermann- Burchard's test, ferric chloride test, froth test, and anthraquinone test. The aim of these tests was to detect alkaloids, flavonoids, triterpenoids and steroids, phenolic compounds and tannins, saponins, and anthraquinones, respectively.

As shown in Figure 2(a), which illustrates the Dragendorff's test, no reaction was observed in the extract. Typically, if the test is positive, a noticeable yellow or orange precipitate would form, but no such reaction was seen. According to the research by Parihar *et al.,* [22], Dragendorff's test was positive in aqueous solution but not in methanol. Since water was the main solvent used for extraction, a positive reaction was expected. The lack of reaction could be attributed to the low alkaloid content in the extract. In Figure 2(b), the alkaline reagent test for flavonoids was conducted, but the result was negative. The extract should have turned an intense yellow upon the addition of a basic solution and then become colorless with the addition of dilute acid, but no color change occurred. Although this test has been proven positive in other studies, the data here showed no result, likely due to an insufficient number of flavonoids in the extract, similar to the Dragendorff's test.

For the Libermann-Burchard's test, where triterpenoids and steroids are tested, the formation of a brown ring at the junction of the two layers and the top layer turning green indicates the presence of steroids. On the other hand, the extract will turn deep red if triterpenoids are present. Since the extract turned slightly reddish, as shown in Figure 2(c), this suggests that triterpenoids are present in *Pleurotus*, though in a small amount, while the test for steroids was negative. In the ferric chloride test, which detects phenolic compounds and tannins, the extract will become dark green if phenolic compounds are present. As seen in Figure 2(d), the extract turned blackish-green, confirming the presence of phenolic compounds in *Pleurotus* mushroom. For the froth test, which tests for saponins, a froth will form and persist for some time. Figure 2(e) clearly shows a froth layer on top of the extract, which persisted for over 20 minutes. Lastly, as observed in Figure 2(f), the test for anthraquinones was performed to detect their presence. Typically, a pink, violet, or red color in the

ammoniacal phase confirms anthraquinones, but no such color was observed. Even in the research conducted by Parihar *et al.,* [22], this test produced negative results.

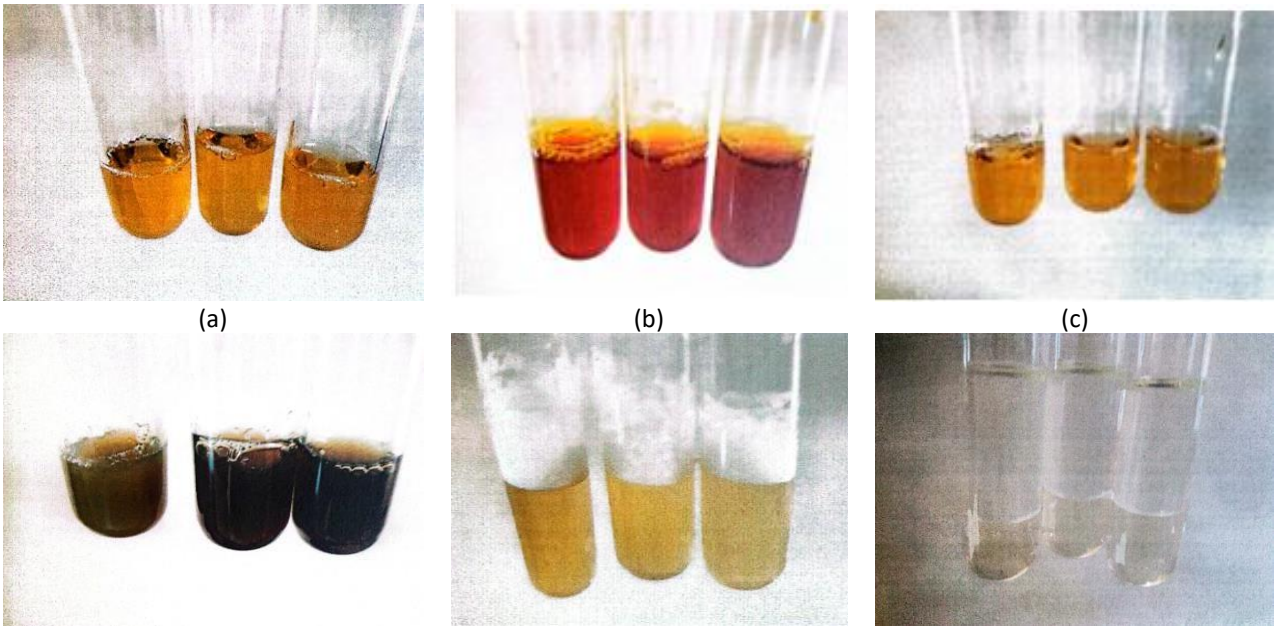

Fig. 2. Test results. (a) Dragendorff's test for alkaloids (b) Alkaline reagent test for flavonoids (c) Libermann-Burchar's test for triterpenoids and steroids (d) Ferric chloride test for phenolic compounds (e) Ferric chloride test for phenolic compounds (f) Froth test for saponins and test for anthraquinones

3.3 Antioxidant Analysis of Pleurotus Mycelium Extract at Three Different Extraction Temperatures Using ELISA (Enzyme-Linked Immunosorbent Assay)

3.3.1 Pleurotus mycelium biomass extraction at different temperatures for 4 hours

According to Table 4, hot water extraction was conducted using 3.3 g/50 mL of dried *Pleurotus* sp. mycelium biomass powder at temperatures of 30°C, 65°C, and 100°C for 4 hours. The colour of the extract darkened as the temperature increased, indicating that more phytochemicals from the *Pleurotus* sp. mushroom were being extracted. Subsequently, the extract was utilized for various antioxidant analyses.

Table 4
Different extraction condition of *Pleurotus* sp. mycelium

Sample	Temperature (°C)
1	30
2	65
3	100

3.3.2 Determination of DPPH Free Radical Scavenging Activity

A DPPH assay was performed to evaluate the ability of *Pleurotus* to inhibit free radicals, with results compared to a standard curve that was created based on the values in Table 5. The assay utilized extracts from *Pleurotus* at three different extraction temperatures: 30°C, 65°C, and 100°C. As indicated in Table 4, Sample 2, representing the *Pleurotus* extract at 65°C, demonstrated the highest

average inhibition percentage of 97.92%. Following this, Sample 1 (30°C extraction temperature) had an average inhibition of 94.21%, while Sample 3 (100°C extraction temperature) showed an average inhibition percentage of 61.91%. The values for the percentage of inhibition of the DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical of the ascorbic acid were the average of the three replicates using Eq. (1).

Free radicals are continuously produced by cells as part of their normal functioning. However, when produced in excess, these free radicals can lead to various complications, damaging essential components like DNA, protein, and cell membrane through oxidation, a process where electrons are removed by the free radicals. Free radicals originating from both exogenous and endogenous sources can lead to numerous diseases. This is where antioxidants are crucial. As mentioned, free radicals inflict damage through oxidation, while antioxidants counteract this process. Moreover, antioxidants can prevent the formation of radicals, scavenge them, and facilitate their decomposition. Thus, the damage to tissue induced by these free radicals can be prevented as acknowledged by Young and Woodside [23]. In essence, an antioxidant can be defined as any substance that can delay or inhibit the oxidation of a substance when present in low concentration compared to the oxidizable substrate as outlined by Halliwell and Gutteridge [24].

Table 5
Percentage of inhibition of ascorbic acid at different concentrations for DPPH assay

Concentration (g/L)	Percentage of inhibition (%)
Blank	0
0.1	20.90
0.2	30.68
0.3	40.04
0.4	50.16
0.5	62.12
0.6	73.75
0.7	83.79
0.8	89.61
0.9	99.61
1.0	106.42

Note: The values of percentage inhibition (%) are the average of three replicates

In this research project, a total of two antioxidant assays were conducted: the DPPH assay and the nitric oxide free radical scavenging assay. For both tests, a standard curve or calibration curve was established based on the inhibition percentage values of ascorbic acid (for the DPPH free radical scavenging assay) and butylated hydroxytoluene (for the nitric oxide free radical scavenging assay) at various concentrations ranging from 0.1 to 1.0 g/L. According to Table 5, the highest percentage of inhibition for ascorbic acid occurs at a concentration of 1.0 g/L, yielding a value of 106.42% for the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. Using these values, the standard curve was plotted, as illustrated in Figure 3. In Table 6, the average percentage of inhibition of *Pleurotus* sp. mycelium at three different extraction temperatures (30°C, 65°C, and 100°C) was calculated, with Sample 2 at 65°C demonstrating the highest average inhibition percentage of 97.92% from three replicates. Sample 1, extracted at 30°C, followed closely with an average of 94.21%. Sample 3, extracted at 100°C, exhibited the lowest average percentage of inhibition at 61.91%.

Typically, an increase in temperature correlates with enhanced antioxidant activity in conjunction with concentration; however, this is not universally applicable.

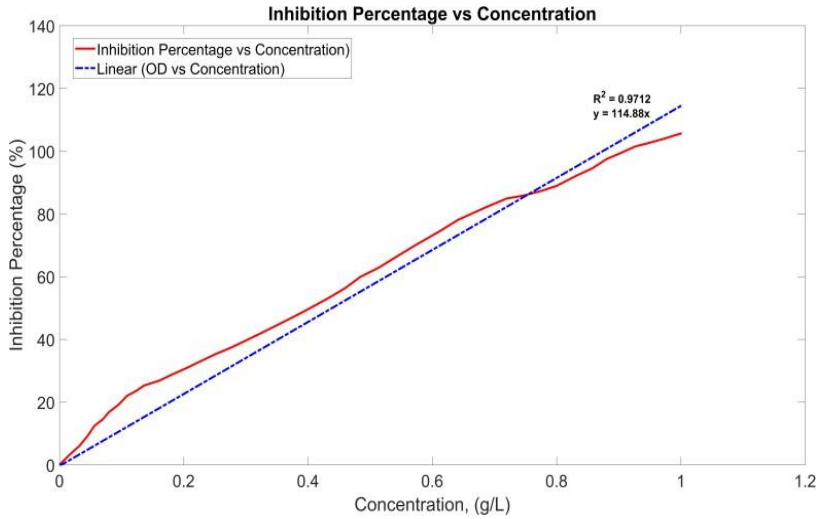

Fig. 3. Standard curve of ascorbic acid for DPPH free radical scavenging assay

Table 6
DPPH free radical scavenging activity of *Pleurotus* sp. mycelium extract at different extraction temperatures

Trial	Percentage of inhibition (%)		
	Sample 1 (30°C)	Sample 2 (65°C)	Sample 3 (100°C)
1	85.69	86.26	52.48
2	94.88	110.18	69.43
3	102.05	97.33	63.81
Average	94.21	97.92	61.91

According to Réblová [25], extremely high temperatures can diminish the antioxidant activity of the sample. Thus, Sample 2 (extraction temperature 65°C) demonstrates the greatest ability to scavenge and detect free radicals. As the scavenging activity of *Pleurotus* increases, the absorbance reading or optical density (OD) decreases. This reduction is due to the diminishing quantity of DPPH radicals as the antioxidants in the *Pleurotus* extract lose hydrogen, leading to a change in structural conformation as shown by Shimada *et al.,* [26] and Fukumoto and Mazza [27]. The free hydrogen subsequently binds to the DPPH radical, resulting in the formation of diphenylhydrazine (DPPH-H) molecules. As the DPPH radicals are reduced, discoloration occurs as reported by Molyneux [28]. When comparing the free radical inhibition percentages of *Pleurotus* with the synthetic antioxidant ascorbic acid, the latter demonstrates a higher inhibition percentage. This indicates that Sample 2 possesses superior free radical scavenging ability amongst the three tested extracts.

3.3.3 Determination of Nitric Oxide Free Radical Scavenging Activity

A nitric oxide free radical scavenging assay was performed to determine the scavenging ability of *Pleurotus*, with three replicates per sample, and the results were compared to a standard curve shown in Figure 4 that was created based on the values in Table 7. The samples, each a *Pleurotus* mycelium extract, were extracted at different temperatures: Sample 1 at 30°C, Sample 2 at 65°C, and Sample 3

at 100°C. As indicated by the data in Table 8, Sample 3 (100°C extraction) had the highest scavenging ability with an average of 82.67%, while Sample 2 (65°C extraction) displayed the lowest scavenging ability at 75.38%. Sample 1 (30°C extraction) ranked second with an average of 79.53%.

The percentage of inhibition of ascorbic acid at various concentration (0.1-1.0 g/L) were the average of three replicates, and its values were used to create the standard curve. The values of the inhibition percentage were calculated based on Eq. (2).

$$Nitric\ Oxide\ Scavenging\ Activity\ \ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (2)$$

Table 7
Scavenging activity of BHT at different concentrations for nitric oxide assay

Concentration (g/L)	Percentage of inhibition (%)
Blank	0
0.1	6.38
0.2	19.19
0.3	28.46
0.4	40.51
0.5	52.67
0.6	60.29
0.7	69.72
0.8	81.13
0.9	87.34
1.0	99.72

Note: The values of percentage of inhibition are the average of three replicates

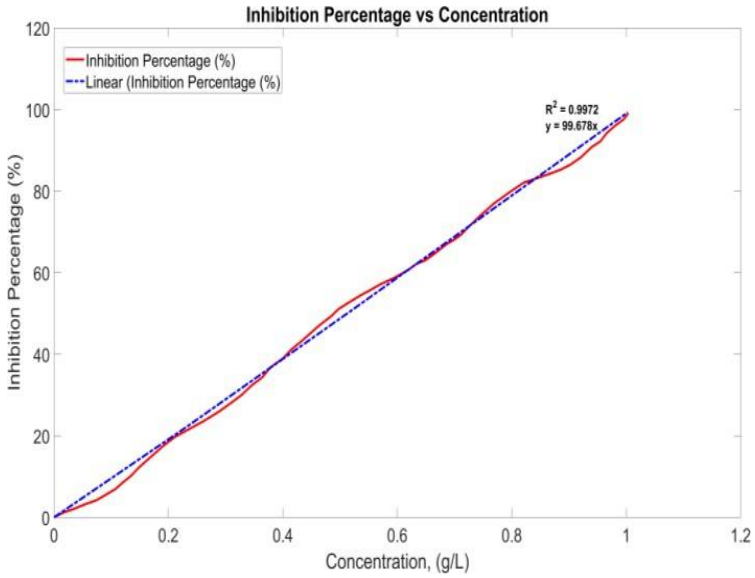

Fig. 4. Standard curve of butylated hydroxytoluene (BHT) for nitric oxide radical scavenging assay

For the nitric oxide radical scavenging assay, butylated hydroxytoluene (BHT) was used. At a concentration of 1.0 g/L for the BHT, an inhibition percentage of 99.72% was observed. This indicates that at this concentration, BHT effectively detects free radicals. Subsequently, a standard curve was plotted based on these values, as shown in Figure 4. In Table 8, Sample 3, extracted at 100°C,

demonstrates the highest inhibition percentage at 82.67%, while Sample 2, extracted at 65°C, shows the lowest inhibition percentage at 75.38%. This assay illustrates the extract's capacity, at different extraction temperatures, to scavenge free radicals such as nitric oxide (NO) and reactive nitrogen species (RNS) derived from the interaction with reactive oxygen species (ROS) which was also explored in the study by Tsai *et al.*, [29]. As the antioxidants in *Pleurotus* donate protons to the nitrite free radicals, the absorbance value decreases. This decrease in absorbance can be used to measure the extent of the nitric oxide radical scavenging activity of *Pleurotus* sp. mushroom as demonstrated by Turkoglu *et al.*, [30].

Table 8
Nitric oxide radical scavenging activity of *Pleurotus* sp. mycelium extract at different extraction time

Trial	Percentage of inhibition (%)		
	Sample 1 (30°C)	Sampl 2 (65°C)	Sample 3 (100°C)
1	79.19	75.64	83.33
2	79.55	73.95	72.51
3	79.85	76.54	92.18
Average	79.53	75.38	82.67

4. Conclusion

Submerged fermentation was conducted using basal medium as a platform for the growth of *Pleurotus* sp. mycelium, enabling the production of numerous economically beneficial products. The various phytochemical tests indicated that *Pleurotus* sp. mushrooms contain a range of beneficial bioactive metabolites that may help prevent various diseases. Specifically, out of the six tests performed, three yielded positive results: triterpenoids, phenolic compounds, and saponins. Furthermore, the antioxidant properties of the *Pleurotus* mushroom were assessed by determining the percentage of inhibition of free radicals through DPPH scavenging assay and nitric oxide free radical scavenging assay using the ELIZA method. In the DPPH assay, the *Pleurotus* mycelium extract at an extraction temperature of 65°C exhibited the highest percentage of inhibition at 97.92%. Conversely, in the nitric oxide assay, the extract at an extraction temperature of 100°C demonstrated the highest inhibition percentage of 82.67%.

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