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Evaluation of Antioxidant and Phytochemical Properties of *Pleurotus* sp. Mushroom Mycelium

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ABSTRACT

Pleurotus sp. mycelium is well-known for its therapeutic properties, since it contains a variety of bioactive compounds with antioxidant and phytochemical features. *Pleurotus* sp. mushroom research involves growing the mycelium in optimal conditions with sufficient nutrients to yield a large amount of biomass. The mushroom mycelium was grown using the submerged fermentation method, which provided enough nutrients to produce a large amount of mycelium. Hot water extraction yielded more extract and bioactive component than cold water extraction, with a dextrose equivalent of 0.913. To determine the effect of antioxidant properties on inhibiting the formation of free radicals and reactive oxygen species (ROS), phytochemical screening was used to identify bioactive components in mushroom extracts. Antioxidant effectiveness using the ABTS assay was critical when compared to ascorbic acid as the antioxidant standard. *Pleurotus* sp. mushroom mycelium inhibits free radicals and indicates the antioxidant and phytochemical activity.

1. Introduction

Mushrooms are non-green fungi with fruiting bodies that produce spores [1]. They grow from a network of hyphae, which collectively form the mycelium—a structure that can live for many years, sometimes up to a century [1]. The cell walls of mushrooms are made of chitin, a carbohydrate providing structural support similar to that in insect exoskeletons [2]. Mushrooms are classified based on their spore release: hypogeous fungi produce spores underground, while epigynous fungi release them above ground [3]. In ancient history, the Chinese believed that it is an "elixir of life" since it serves as functional food with high nutritional content and therapeutic use [4].

The *Pleurotus* species, commonly known as oyster mushrooms based on their distinctive appearance, has been recognized for its medicinal properties and nutritional value since around 1500 B.C. [5]. The *Pleurotus* genus is a rich source of dietary fiber, which contributes positively to health care and nutrition [5]. These mushrooms are capable of degrading various types of lignocellulosic substrates and can adapt to a wide range of climatic conditions [5]. They have a short life cycle,

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making cultivation relatively straightforward, often achieved through submerged fermentation with minimal resources [6].

Biologically active polysaccharides found in mushrooms contain valuable nutrients with a range of therapeutic activities, including anti-proliferative, anti-inflammatory, antioxidant, immune-stimulatory and anti-cancer effects [5]. Mushroom nutraceuticals, which encompass both nutritional and medicinal properties, offer antioxidant enzymes that can target human precancerous lesions, reduce oxidative damage and enhance resistance to chronic diseases [5].

The secondary metabolites and polysaccharides derived from mushroom mycelium vary based on their structure, molecular weight, solubility and monosaccharide composition, which influence the extraction methods used [7].These compounds have the potential to act as antioxidant agents by scavenging free radicals or reactive oxygen species (ROS), which can be beneficial in managing diseases. Additionally, they may enhance enzymatic activity to inhibit or break down unstable molecules or radicals, further supporting their therapeutic potential.

The *Pleurotus* sp. mushrooms offer significant benefits across various fields, including medicine, food, agriculture and biopharmaceuticals. However, there are challenges associated with extracting bioactive compounds, particularly polysaccharides, from mushroom mycelium [4]. Cultivating mycelium through submerged fermentation necessitates precise medium composition and controlled conditions [8]. Factors such as optimal temperature range, pH, humidity, and heat levels are crucial, as they can significantly influence the production of mycelium biomass and polysaccharides [4].

The purpose of this research is to cultivate and produce *Pleurotus* sp. mushroom mycelium using submerged liquid fermentation and extract the bioactive compounds from the dried mushroom mycelium of *Pleurotus* sp. The phytochemicals present in the extract will be assessed and their potential mechanisms of action underlying antioxidant will be detected through ABTS analysis.

Studying the carbohydrate content in mushrooms like *Pleurotus* sp. is essential for nutritional, industrial, and scientific advancements. Mushrooms are low-calorie, low-carb foods with polysaccharides such as β-glucans that offer health benefits like immune support and gut health. In the food industry, this research aids in developing low-carb products and enhancing their use as sustainable meat alternatives. Additionally, it supports eco-friendly practices by utilizing agricultural waste and boosts the economic value of mushrooms as functional foods. Scientific insights into mushroom carbohydrates also contribute to medical and biotechnological applications, including bioethanol production and biodegradable materials.

2. Methodology

2.1 Reagents

List of major reagents that are needed for the experiments include Phenol reagent, (System USA), concentrated sulfuric acid, (Fisher Scientific, Malaysia) ethanol, (HmbG Chemicals, UK), absolute alcohol, (HmbG Chemical, UK), distilled water, glucose, (Duchefa Biochemie, Netherland), yeast extract, (Duchefa Biochemie, Netherland), meat extract, (Sime Scientific, India), potassium dihydogen phosphate, (Duchefa Biochemie, Netherland), Dipotassium hydrogen phosphate, (Duchefa Biochemie, Netherland), ammonia chloride, (Duchefa Biochemie, Netherland), magnesium sulphate, (Duchefa Biochemie, Netherland).

2.2 Equipment

List of major equipment's that are needed for the experiment include Blender, (Panasonic, Japan), Hotplate stirrer, (JLabTech), Whatman No.1 filter paper, (GE, Healthcare, UK), Incubator, pH meter, autoclave machine, Centrifuge, (Hettich, Switzerland), Micropipette, (Witeg digital, Germany), ELIZA Microplate reader, (Tecan, Switzerland), UV spectrophotometer (Cecil, Germany)

2.3 Microorganism

The *Pleurotus* sp. mushroom master plate obtained Manipal International University for this study.

2.4 Consumable Items

List of consumable items that are used for the experiment include Whatman No.1 filter paper, Falcon tube, distilled water, conical flasks, Petri plates, filter funnel, weighing boat, flacon tube racks, micropipette tips, cotton wool, aluminium foil, test tube and disposal pipette.

2.5 Subculture of Pleurotus sp. Mushroom Mycelium

The Potato Dextrose Agar (PDA) was prepared by dissolving 3 g of yeast extract and 39 g of PDA powder in 1 L of distilled water. The medium was sterilized at 121°C for 15 minutes and 100 kPa (15psi). Once autoclaved, it was poured into Petri plates to solidify. The mycelium was sub-cultured into 10 PDA with yeast extract after being roughly cut into a 1 cm square shape using a scalpel from the master plate. Following the completion of the subculture, each plate was parafilm-covered and allowed to grow for five to seven days in a dark environment [9].

2.6 Submerged Liquid Fermentation

Pleurotus sp. mycelium on PDA with yeast extract was kept at 4°C for prolonged used. Precise preparation was done for the basal medium, which included 30 g of glucose, 3 g of meat extract, 0.5 g of potassium dihydrogen phosphate (KH_2P0_4), 0.5 g of dipotassium hydrogen phosphate (K_2HPO_4), 4 g of ammonia chloride ($NHCl_4$), and 0.5 g of magnesium sulphate ($MgSO_4$) per liter. Following that, the medium's pH was raised to 5.6 and it was autoclaved for 20 minutes at 121°C and 100 kPa. Subsequently, 100 ml of autoclaved basal medium were placed into a flask, and 10 plugs of Pleurotus sp. mycelium were added. For the purpose of fostering mycelium growth, the culture flask was shaken for seven days at 28°C and 150 rpm. Following a week, the culture medium was centrifuged at 15000 x g (9000 rpm) at 4°C and then filtered in order to separate the pellet and supernatant. The pellet was dried in an oven at 60°C until a constant weight obtained.

2.7 Extraction Process

Hot water extraction was performed using 2 g of powdered mushroom mycelium mixed with 250 mL of distilled water. It was boiled for three hours at 100 °C in a double boiler water bath. After cooling to ambient temperature, the extract was filtered through Whatman No.1 filter paper to get the culture filtrate (extract). Carbohydrate was determined using the culture filtrate [10]. Cold-water extraction was performed using 2g of powdered mushroom mycelium mixed with 250 mL of distilled

water. The mixture was vigorously swirled for three hours. The extract was then filtered through Whatman No.1 filter paper to yield the culture filtrate (extract). The carbohydrate concentration was determined using the culture filtrate [11].

2.8 Phenol-Sulfuric Acid Assay

The phenol sulfuric acid assay was performed in flacon tubes using a glucose solution (1 g/10 ml) diluted to concentrations ranging from 1 to 5 mg/10 mL. The glucose stock is 100 mg/mL and is made by dissolving 1 g of glucose in 10 mL of distilled water, as stated in Table 1. Then, pipette 1 mL of sample solution into the test tube, followed by 5 mL of pure sulfuric acid. Following that, 1 mL of phenol reagent was pipetted into each mixture. It was set aside to cool to room temperature for 10 to 15 minutes before the absorbance at 492 nm was measured. The assay was carried out with a UV/Vis spectrophotometer by putting 1 mL of sample into a cuvette. A corresponding blank containing distilled water was tested in the same way as the samples. All samples were tested in triplicate (n=3) [12,13].

Table 1
Preparation of different glucose concentration for standard curve

Concentration (mg/mL)	Volume of stock added (mL)	Volume of distilled water added (mL)
1	0.1	9.9
2	0.2	9.8
3	0.3	9.7
4	0.4	9.6
5	0.5	9.5

2.9 Phytochemical Analysis

The extracted culture from hot and cold-water extractions was then tested for the presence of certain phytochemicals presents in the *Pleurotus* sp. mushroom mycelium.

2.9.1 Detection of saponins (Froth Test)

The test tube was filled with a single ml of mycelia extract and 2 mL of distilled water. The mixture was then gently shaken to mixed uniformly. The presence of foam indicates the presence of saponins was introduced by [14].

2.9.2 Detection of polyphenols (Ferric Chloride Test)

The test tube was filled with one mL of mycelia extract and 2 mL of distilled water. Three drops of 10 % aqueous ferric (III) chloride ($FeCl_3$) were added afterwards, followed by three drops of freshly produced potassium ferrocyanide ($K_4[Fe(CN)_6]$). Green or black precipitation indicates the presence of polyphenols in mycelium extractions [15].

2.9.3 Detection of reducing sugars (Benedict's Test)

Two mL of mycelium extract was added to the test tube, followed by 0.5 mL of Benedict's reagents [16]. The combinations were then cooked for around 10 to 15 minutes, until the blue or purple color turned orange or yellow. Colour variations were determined by the concentration of reducing sugars.

2.9.4 Detection of lipids (Sudan Test)

Two ml of mycelium extract were put to a test tube, followed by five drops of Biuret reagent. It was gently whirled until the mixture was evenly combined [17]. The blue color of the solutions remains unaltered, demonstrating the absence of proteins in the sample. On the other hand, the solutions changed the color to purple or pink, indicating the presence of protein in the sample. Due to the quantity of lipids in the extract, color changes may be too faint to detect.

2.9.5 Detection of flavonoids (Alkaline reagents)

One mL of mycelium extract was added to the test tube, followed by the alkaline reagent [18]. The mixture was then swirled thoroughly to combine. Yellow or orange precipitate indicates the presence of flavonoids, implying positive results [16].

2.9.6 Detection of protein and amino acids (Ninhydrin Test)

Two drops of 10 mg Ninhydrin solution in 200 mL of acetone were mixed with 2 mL of mycelium extract culture filtrate [17]. Purple colour indicates the presence of proteins and free amino acids in the sample [17,19].

2.10 Antioxidant Analysis

2.10.1 ABTS preparation

ABTS stock solution (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) was made by adding 9.5 mL of ABTS solution (7 mM) to a clean falcon tube. The ABTS buffer was then made by combining 245 uL potassium persulfate (100 mM) with 10 ml of distilled water to form a standard molarity of 2.45 mM. Both solutions were mixed in tubes covered with aluminum foil in a 1:1 ratio to generate the ABTS stock solution, which was then incubated in a dark environment for 18 hours. Working solution of ABTS was generated from the stock by diluting with distilled water for the UV spectrophotometer absorbance at 734 nm, with a reading in the range of 0.07 [20].

2.10.2 ABTS assay

The working concentration of the samples was prepared in test tubes by mixing 1 mL of ABTS working solution in the dark with the samples as shown in Table 2. The absorbance was measured at 734 nm [20].

Table 2
Preparation of samples in ABTS assay

Tube	Samples (mL)	Distilled water (mL)
A	0.1	0.9
B	0.2	0.8
C	0.3	0.7
D	0.4	0.6
E	0.5	0.5
F	0.6	0.4
G	0.7	0.3
H	0.8	0.2
I	0.9	0.1
J	1.0	0.0

The radical scavenging capacity were calculated using the Eq. (1) below:

$$Inhibition\ (\%) = \frac{(\text{Absorbance control} - \text{Absorbance sample})}{\text{Absorbance control}} x\ 100 \quad (1)$$

3. Results

3.1 Cultivation of Pleurotus sp. Mycelium

Pleurotus sp. mushrooms belong to the fungi kingdom because they have unique traits that set them apart from others. The oyster mushroom is also known as basidiocarp due to its phylum Basidiomycota. In the family of Pleurotaceae, species of genus *Pleurotus* is well-known for their profuse new mycochemicals, which have been cultivated in Germany.

It may grow saprophytically at temperatures ranging from 1 to 32°C in the tropical region climat e [5]. *Pleurotus* is characterized by sideways stem growth in relation to the cap and appears in a variety of colors with a lateral stripe [6]. As a result, the "oyster" is represented as a cap-shaped mushroom. It is primarily produced in tropical regions with a large temperature range, such as Eastern Asia, China, South Korea, Japan and Indonesia, as well as North America and Europe. *Pleurotus ostereatus*, *Pleurotus florida*, *Pleurotus eryngii* and other exotic oyster mushrooms are a few examples. These have unique traits and attributes, including colors, stalked or sessile growth, epiphytic or parasitic behaviour, subterranean or above growth, and other characteristics [5]. In this study we have successfully cultivated the newly isolated *Pleurotus* sp. mushroom mycelium onto PDA plates as shown in Figure 1.

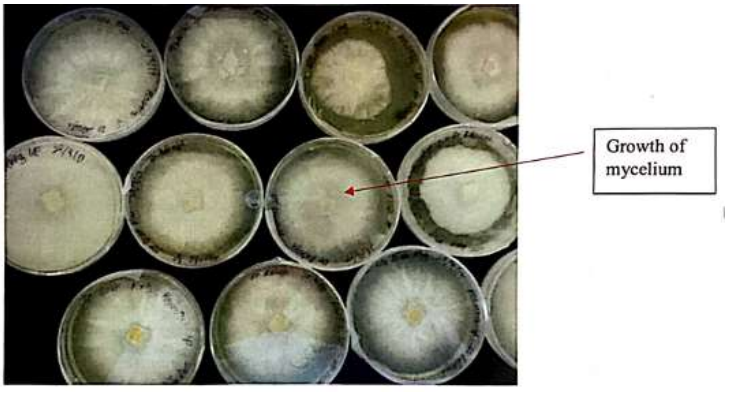

Fig. 1. Pleurotus sp. mycelium growth onto PDA plates

3.2 Submerged Liquid Fermentation

Table 3 shows the amount of mushroom mycelium biomass produced by *Pleurotus* sp. during submerged fermentation. The experiment consists of four batches. The first batch of submerged fermentation contains 3.08 g of mushroom mycelium biomass, the least quantity of biomass among the other batches. The final or fourth batch of submerged fermentation for *Pleurotus* sp. mushroom mycelium yielded the largest mushroom mycelium biomass (6.62 g). The total biomass of *Pleurotus* sp. mycelium obtained after one week of fermentation was 18.96 g.

Table 3
Total amount of mushroom mycelium biomass of Pleurotus sp. obtained through submerged liquid fermentation

Submerged Liquid Fermentation Batch	Mushroom mycelium biomass (g)
1	3.08
2	4.01
3	5.25
4	6.62
Total	18.96

The benefit of submerged fermentation is that it is primarily used to manage the extraction of secondary metabolites in liquid form and makes product purification easier. Additionally, it aids in scaling up processes and adjusting various process parameters for concentration, pH and other factors [21]. In submerged liquid fermentation, the bioactive compounds are secreted into the fermentation broth. Once the nutrients in a substrate are depleted, it needs to be replenished based on nutrient profiling. This fermentation technique is particularly suited for microorganisms and bacteria that require a high moisture content [21].

In addition, it is more affordable, practical and widely accessible than alternative techniques for producing mushroom mycelium in large quantities. Used widely in the commercial manufacture of antibiotics, amino acids, ethanol, organic acid and other substances, it is mostly favoured for large-scale fermentation. Additionally, it provides better mycelium production in a constrained area within a brief period of time with minimal contamination risks. As a result, there is an adequate quantity of polysaccharide and mycelium biomass in just a short amount of time [21].

Submerged fermentation is the most efficient approach to generate vast amounts of mushroom mycelium. It is because the basal medium has the necessary components and sources for mushroom mycelium growth. The amount of yield of the mushroom mycelium gradually increases from batch to batch as the components of (4 g/L) ammonia chloride ($NHCl_4$) and (0.5 g/L) dipotassium hydrogen phosphate (K_2HPO_4) were added into the basal medium, as shown in Table 4. The comparison was made from batch to batch, and the inclusion of $NHC1_4$ and $KHPO_4$ resulted in a much higher yield of mycelium cultivation. As a result, the final batch of submerged fermentation obviously generated the maximum production of mycelium biomass [11]. With the optimum conditions of 5.6 pH and 28°C at 150 RPM, as well as the addition of the components, the third batch began to provide outstanding yield as the mycelium biomass gradually increased. The production of *Pleurotus* sp. mushroom mycelium was at its peak, with a substantial output that could be harvested in less than one week. This is due to an alteration in the amount of basal medium. The amount of meat extract was reduced from 3 g/L to 1 g/L, and components of $NHCl_4$ and $KHPO_4$ were added to promote mushroom mycelium growth. These sources promote fungal growth and provide more biomass for mushroom mycelium. The meat extract is required in the precise amount of 1 g/L due to its high nitrogen content

in comparison to yeast extract, which directly leads to increased biomass synthesis; nevertheless, the amount of meat extract must not exceed 3 g/L. It is because it may increase the rate of contamination and create unwanted growth of another organism.

According to Table 3, the amount of *Pleurotus* sp. mycelium biomass obtained from submerged fermentation in basal medium increased with the addition of $NHCl_4$ and $KHPO_4$ from batch to batch. The initial batch (Batch 1) had 3.08 g of mushroom mycelium biomass, which subsequently rose to 6.62 g by the last batch. *Pleurotus* sp. mushroom mycelium biomass of 18.96 g was collected at the end of the entire submerged fermentation procedure.

Furthermore, if there is more nitrogen source than carbon source in the basal medium, the mycelium growth is reduced [22]. A large amount of beef extract can be hazardous to the mushroom mycelium. The yield of mushroom mycelium biomass will be impacted. This is indirectly related to pH, which must be optimal for mushroom mycelium growth. Lower pH of the basal media causes mushroom mycelium to not grow, indicating that it is unsuitable for cultivation [23].

The submerged fermentation process was successful once all the crucial components was measured accurately and appropriately to sustain the growth of the *Pleurotus* sp. mushroom mycelium. The key to the excellent growth of mushroom mycelium in the specific time interval with the optimization factor of the temperature 28°C with 5.6 pH and 150 rpm. The factor of temperature and pH able to affect the growth of mushroom mycelium whereas the constant orbital shaking keeps the basal medium with the plugs mushroom mycelium better growth as the nutrient is evenly distributed using shaker.

3.3 Total Carbohydrate Content

The phenol sulfuric acid assay was performed on mushroom mycelium extracts, and a shift in color to brownish orange was noted after the addition of concentrated sulfuric acid and phenol reagent. The reaction is very exothermic due to the heat released by the chemical reaction between concentrated sulfuric acid and phenol. The brownish orange color intensity was determined for absorbance readings at 492 mm using a UV spectrophotometer. The darker color of the brownish orange is diluted 10-fold, depending on the intensity of the color. However, the solution becomes very dark black, indicating that the component, polysaccharide, has been burned. The phenol sulfuric acid assay response can only last three hours, beyond which it varies and is inaccurate. The sample's absorbance was measured in triplicate and recorded.

The total carbohydrate content was evaluated using phenol sulfuric acid assay at 492 nm using D-glucose as a standard. The range of D-Glucose concentration for standard curve listed as 1, 2, 3, 4 and 5 mg/10 mL, respectively (Table 4). The standard curve was used to calculate the carbohydrate content of mushroom extract. It also compares the obtained carbohydrate content to the glucose standard curve. The greatest absorbance reading in a UV spectrophotometer for glucose is 21.67 nm at a concentration of 5 mg/10 ml. While the concentration of glucose at 1 mg/10 ml had the lowest absorbance measurement in a UV spectrophotometer of 6.13 nm. The linear regression equation of the glucose solution's standard curve was utilized to generate the hot and cold water extract samples. The R^2 value is 0.9922. The standard curve of D-glucose in phenol sulfuric acid assay is shown in Figure 2. The phenol sulfuric acid assay used the average value of the extracts' absorbance readings.

Table 4
The absorbance of different concentration of D-glucose concentration at 492 nm in phenol sulfuric acid assay

Concentration (mg/mL)	Absorbance (nm)
Blank	0.00
1	6.13
2	8.51
3	13.03
4	17.57
5	21.67

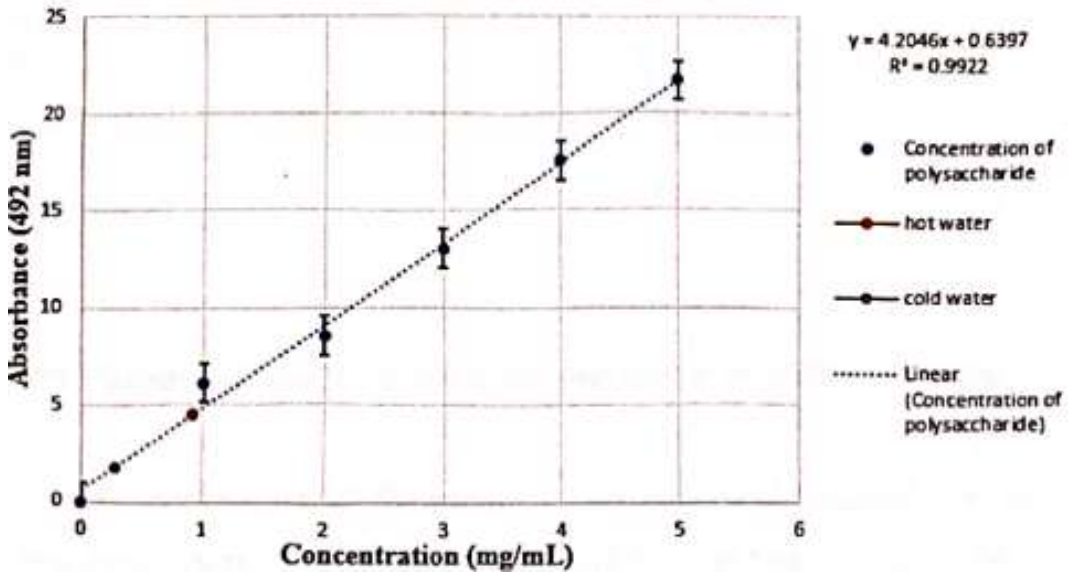

Fig. 2. Standard curve of D-glucose in phenol sulfuric acid assay

The crude extracts of the hot and cold water samples were dried using a rotary evaporator. Both extract yields are diluted to a concentration of 2 g/mL. The hot water extracts showed highest concentration of carbohydrate with 4.48 mg/mL and cold water extract exhibited with 1.82 mg/mL of carbohydrate content as shown in Table 5.

Table 5
The concentration of carbohydrate of hot and cold water extraction

Extracts	Concentration of carbohydrate (mg/mL)
Hot water	4.48 ± 0.05
Cold water	1.82 ± 0.07

It is clearly shown that the hot water extraction process exhibited high production of carbohydrate content. The extraction is carried out with the purpose of breaking the cell wall of the outer membrane of the mushroom mycelium. As a result, using an adequate extraction procedure produces more polysaccharide. Hot water extraction is easier to accomplish than other extraction techniques, and the extraction results are stronger and more efficient. Polysaccharides have strong antiproliferative properties because the chemical structure differs between homopolymers and heteropolymers. Polysaccharides are mostly fungal cell wall structural materials derived from two types of polysaccharides: Cellulose and matrix-like B-glucan, a-glucan and glycoproteins. The extraction procedure is based on the cell wall structure for efficient extraction [21]. The extraction methods are determined by the structure and solubility of the water. In addition, low molecular

compounds will be eliminated from the mushroom mycelium during extraction by using either 100 % ethanol or 95 % ethanol, hot water or cold water [11].

3.4 Phytochemical Analysis

The antioxidant qualities of the crude extract are a result of its phytochemical composition, with the majority of phytochemicals playing important roles in these characteristics. The test results demonstrate a variety of reactions and outcomes in the sample extract solution, ranging from colour changes to foam production and they corroborate the favourable findings from the other researcher's investigation. The phytochemical test shows that the culture filtrate extract contains bioactive substances such as carbohydrates, alkaloids, proteins and amino acids. The hot water extraction may contain different phytochemical substances than the cold water extract; thus, several phytochemical tests were performed to acquire results based on the test reactions.

The word phytochemical comes from the Greek word phyton, which means plant. From the results in Table 6 hot water extracts from *Pleurotus* sp. mushroom mycelium exhibited positive results for all the tested phytochemicals test. It was reported that *Pleurotus* sp. mushroom are rich sources of bioactive compounds such as polysaccharides, anthroquinones, steroids, tannins, phenols, alkaloids, flavonoids and others [24]. Each phytochemical serves a purpose in relation to color, flavor, signaling molecules in plants, and inherent defense against insects and pests [24]. It also contains medicinal compounds used to treat cancer and cardiovascular ailments. Examples of therapeutic plants with phytochemicals that may be used to treat a range of illnesses include neurotransmitter inhibition, which lowers the likelihood of developing chronic illness. Thus, any plant or food containing phytochemicals may have significant therapeutic, pharmacological or biological effects on humans [25].

Table 6
Phytochemicals presents in hot and cold water extracts of Pleurotus sp. mushroom mycelium

Phytochemical test	Positive result appearance	Hot water extract	Cold water extract
Test for protein & amino acid (Ninhydrin Test)	Purple colour (Senthamarai *et al.,* 2012)	+	+
Test for saponins (Froth Test)	Layer of foam (Senthamarai *et al.,* 2012)	+	+
Test for polyphenols (Feeric chloride Test)	Dark Green Colour (Banu and Catherine 2015)	+	+
Test for reducing sugar (Benedict's Test)	Red colour precipitate (Parihar *et al.,* 2015)	+	+
Test for lipids (Sudan Test)	Layer of fats (Kokate *et al.,* 1971)	-	-
Test for protein (Biuret's Test)	Pinkish or purple violet (Sharma *et al.,* 2017)	+	+
Test for flavonoids	Yellow to orange colour (Kumar *et al.,* 2012)	+	+

Table 6 displays the results of the phytochemical test that was conducted using *Pleurotus* sp. extract culture filtrate in both hot and cold water. Alkaloids, phenolics, flavonoids, amino acids, protein, and carbohydrates may be present in *Pleurotus* sp. culture filtrate. The results of the phytochemical screening show the presence of many alkaloids, carbohydrates, saponins, polyphenols and other substances. After the tests' reaction, the results of the phytochemical tests were noted and examined. The saponin test for froth was conducted, and the results showed that the extract for

cold water did not create froth as expected within a few minutes. A coating of froth that has been present on the extract's surface for more than 15 minutes is clearly visible in hot water extracts. The extract coloured dark green when phenolic components were present in the ferric chloride test, which measures polyphenol, phenolic and tannin compounds. For the polyphenol test, the extract changed colour from dark green in the cold water extract to light green in the hot water extract, suggesting the presence of phenolic component. The kind of phenolic structure determines the colour of the phenolic chemical. The cold water extracts shows dark green colour due to the cold water extraction methods that does not destroy the phenolics compounds. However, because the heat energy denatures the chemicals, hot water extracts have a lighter green colour. As a result, its colour is marginally paler than that of cold water extracts.

Additionally, in the Ninhydrin test, where positive results seem to indicate the presence of amino acids and display a purple or blue colour. Protein and amino acids are absent since the results of the hot and cold water extracts did not match the positive outcome. The *Pleurotus* sp. mushroom mycelium is devoid of protein and amino acid-containing substances. The reason behind this could be the inefficiency of the extraction process or the low levels of protein and amino acids in the *Pleurotus* sp. mushroom mycelium. When protein and amino acids are present, the extract in the Biuret test turns purple or pink and the extracts of hot and cold water both become slightly purple, suggesting the presence of modest amounts of protein and amino acid compounds in the extract. Benedict's test for the presence of sugar and carbohydrates, where the amount of reducing sugar determines whether the sample turns red or takes on a particular colour. In this test the extract of hot water gets yellow and the extract of cold water turns orange. Both extracts have reducing sugar, but the type of reducing sugar differs, hence the colour changes because the non-reducing sugar results stay blue. The extract turned into a noticeable orange or yellow precipitate, indicating a positive flavonoid test. Hot water extract turns dark orange, while cold water extract turns lighter orange where both extracts have flavonoids, although their colours vary according to the type of flavonoid present [19].

The Sudan test finds lipids, and positive results indicate that lipids are forming in both extracts. In this test neither extract produced a fat layer, the result negative. It results from inadequate extract samples or extraction techniques. The methods of extraction rely on water. As a result, it is hydrophobic in relation to lipids. The hot water extracts are generally more visible when comparing the outcomes of the culture filtrates of cold and hot water. This is due to the presence of flavonoids, protein, amino acids, saponins, and reducing sugar, but not lipids or significantly less polyphenol. The antioxidant properties for hot water extract exhibit higher scavenging percentage which makes it better than cold water extract.

Since phytochemicals demonstrate how biological qualities for antioxidants are carried out by the substances, they are closely correlated with antioxidant properties. Water-based extraction is a superior extraction method since it is non-toxic when compared to solvent-based extraction.

3.5 Antioxidant Analysis: ABTS Assay

The ABTS assay was used to determine the antioxidant properties. The powerful oxidizing agent of potassium persulfate within the ABTS is reacted with varying concentrations of hot and cold water in the ABTS assay mechanism. Therefore, the blue green color solution will become decolorized as a result. The hydrogen is the cause of the bluish green solution's decolorization. The antioxidant activity was measured by providing antioxidant that stayed steady for 30 minutes in order to assess the absorbance reading. The decrease in antioxidant responses with ABTS is the reason of the absorbance reading loss.

Antioxidant properties quantitatively through ABTS assay. The absorbance measurements were taken in triplicate for each concentration, with each trial containing one set of triplicate extracts at varying concentrations. Based on Figure 3, the R^2 value is 0.9877 where the R^2 value is near to one. As a result, the absorbance readings for ascorbic acids are accurate and trustworthy. The equation of the linear graph was utilized to establish the scavenging activity that is equivalent to the ascorbic acid standard curve.

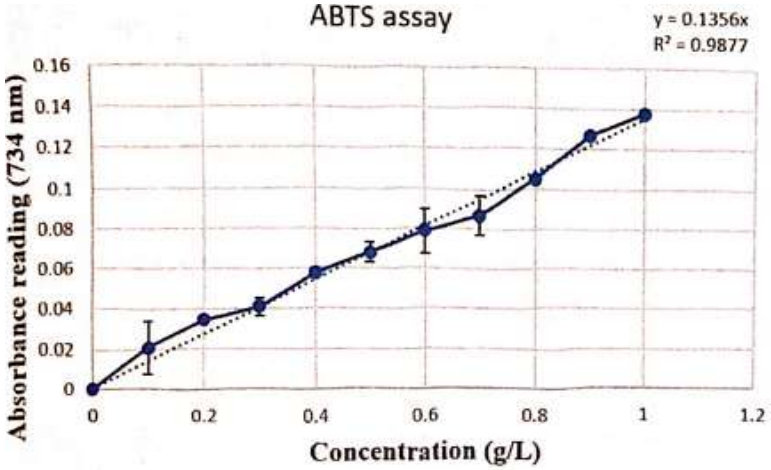

Fig. 3. Standard curve of ABTS assay using ascorbic acid at absorbance of 734 nm

Figure 4 depicts the standard curve of the ABTS assay of hot water and cold extracts of *Pleurotus* sp. mushroom mycelium. The percentage of scavenging activity for each set of trials was estimated using the calculation in the technique. The scavenging activity for both hot and cold water extract gradually increased as the concentration increased. It was clearly shown in the graph that the scavenging activity of hot water extract is higher as compared to cold water extract. Ascorbic acid was used as a standard for comparison with the various extracts of hot and cold water. The IC_{50} was determined and shows a direct relationship between concentration and percentage of scavenging activity. The hot water extract's IC_{50} is 0.5 g/L at 50 % scavenging activity, whereas the cold water extract's IC_{50} is 0.6 g/L.

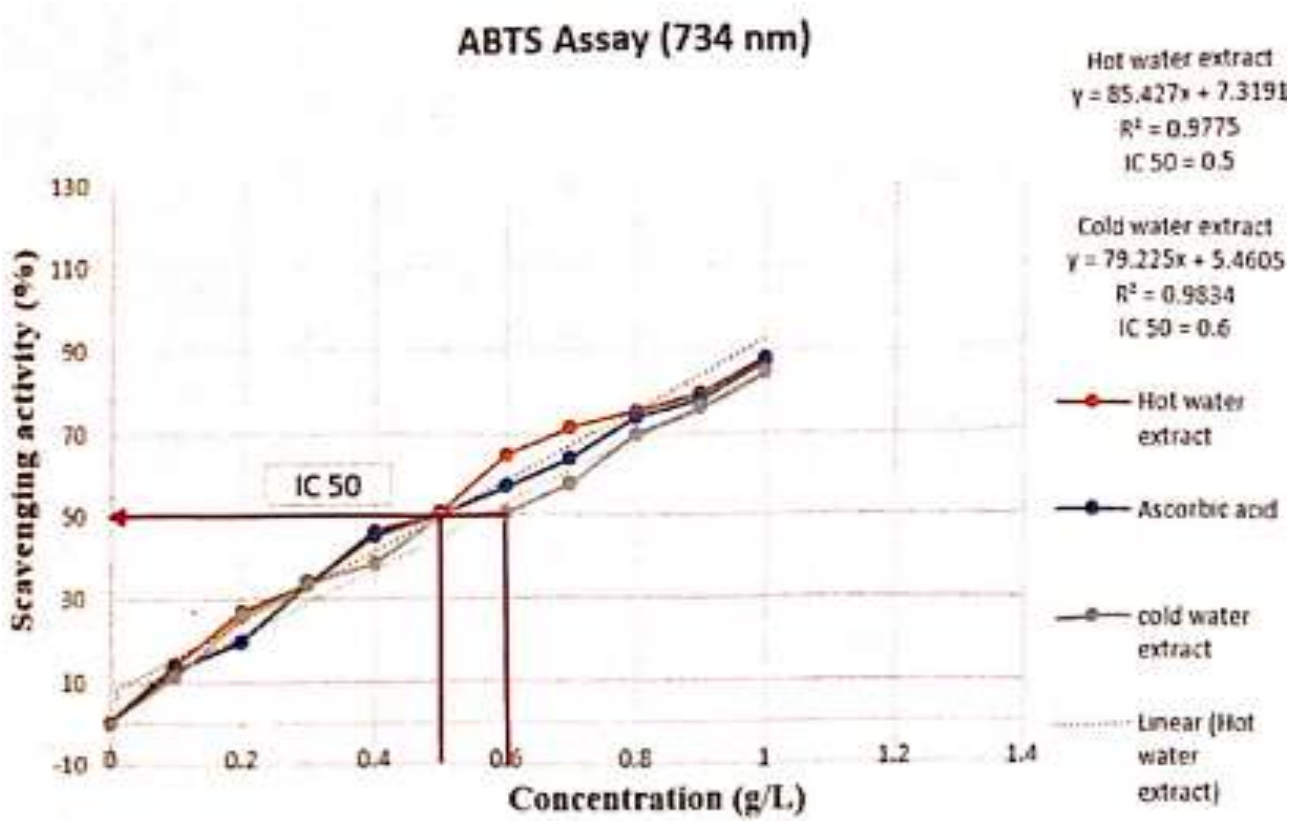

Fig. 4. Scavenging activity of hot and cold water extracts from Pleurotus sp. mushroom mycelium in ABTS assay

Because the bioactive component was extracted using heat energy, it was shown to have stronger antioxidant activities in the extract. Heat may have an impact on antioxidant qualities since it can degrade the bioactive component in the culture filtrate extract. However, compared to cold water extract, heat extraction can provide a larger yield of extract component and a higher amount of phytochemical compound that results in significant antioxidant activity. The reason for this is that effective extraction techniques can yield a substantial amount of extracts, as heat energy extraction produced extracts that were more concentrated than extracts made with cold water. By scavenging more free radicals, this indirectly contributes to a larger sample size of extracts for phytochemical substances. Based on the higher concentration of phytochemicals and bioactive compounds extracted, it can be concluded that the hot water extract has higher antioxidant properties and can with stand heat [26]. This results implies that hot water extract consist of antioxidant compound which potentially becomes sources for antioxidant products.

4. Conclusions

The mycelium of *Plerotus* sp. mushroom mycelium was successfully cultivated onto PDA agar plates. Submerged liquid fermentation of *Plerotus* sp. mushroom mycelium product high concentration of mycelium biomass. Phytochemical test exhibited that hot and cold water extracts exhibited positive results for the presence of amino acids, saponins, polyphenols, reducing sugars, proteins and flavonoids. Hot water extracts showed high efficiency of scavenging capacity in ABTS assay as compared to cold water extract. Hot water extracts of *Plerotus* sp. mushroom mycelium is potentially becomes the antioxidant sources for pharmaceutical and nutraceutical products.

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