Macronutrient composition, antioxidant and antibacterial properties of *Pleurotus pulmonarius's* fruiting body vs. stem waste

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Abstract. The increasing cultivation of mushrooms has led to substantial waste production, ranging from 5-20% of total volume mushroom harvested. This study aimed to compare the macronutrient composition and bioactivities of oyster mushroom (*Pleurotus pulmonarius*), focusing on its stem waste versus fruiting bodies. Macronutrients analyzed include moisture, ash, fat, fiber, protein, and carbohydrates using proximate analysis. Antioxidant activity was assessed via DPPH and ABTS assays, while antimicrobial activity was evaluated using microdilution techniques. Results indicated that mushroom stem waste contained significantly higher levels of carbohydrates (46.2 ± 0.69%), ash (8.2 ± 0.04%), and fiber (15.4 ± 0.10%) compared to the fruiting bodies (carbohydrates: 38.1 ± 0.84%, ash: 6.7 ± 0.12%, fiber: 9.1 ± 0.02%). Conversely, the fruiting bodies exhibited superior antioxidant activity with IC50 values ranging from 166.1 to 503.5 µg/mL. Similar minimum inhibitory concentrations (MIC) were observed between both parts, except for E. coli, where the fruiting bodies showed an MIC of 2000 µg/mL compared to 1000 µg/mL for stem waste. In conclusion, this study highlights the bioactive potential of mushroom stem waste and further purification could enhance its suitability for diverse bioactive applications.

1 Introduction

Pleurotus pulmonarius or oyster mushroom is one of the 25 mushroom species that are extensively cultivated worldwide accounting 19% of world's output [1]. This species is cultivated indoors using an artificial log in which a plastic bag containing nutrient substrates which are made from lignocellulosic material, such as straws, cottonseed hulls, corn cobs, peanut shells, cotton from the textile industry, coffee pulp, paper, and leaves [2]. It is also

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well-known for its health-promoting properties, as it is high in protein, rich in essential amino acids and fibre, and low in fat, yet includes important fatty acids [3]. β-glucans, peptides, proteins, terpenes, fatty acid esters, and polyphenols have been shown to be the main group of compounds responsible for the antioxidant and antimicrobial properties of mushrooms [4]. Furthermore, [5] reported that oyster mushrooms are rich in vitamins and selenium, which are important natural antioxidants in the biological system.

Antioxidants are utilised by living cells to combat the toxicity of reactive oxygen species (ROS), reactive nitrogen species (RNA), and reactive sulphur species (RSS) caused by oxidant loading that lead to oxidative stress [6]. It is believed that oxidative stress is a crucial factor in the progression of numerous degenerative diseases, such as cancer and hepatotoxicity [7]. Some synthetic antioxidants used to prevent oxidative stress, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are known to have harmful health effects [8]. Thus, there is a need for natural antioxidants compounds, such as compounds in the *Pleurotus* extract that have been shown to improve catalase gene expression and reduced the frequency of free radical-induced protein oxidation in aged rats [9].

P. pulmonarius or grey oyster mushroom is widely cultivated in Malaysia with 98% (4,739.4 tans) of mushroom production in 2016 were supplied by 211 local agronomists [10]. The ease in the cultivation and the nutritional value of oyster mushroom have made this species popular and high in demand [11]. The rising number of mushroom productions has produced a large amount of stem waste, specifically the stem at the base of the fruiting body. [12] observed that after the cultivation process, the residual material amounted to 5 to 6 kg of by-product (eg. substrate and stem waste) per kilogram of mushrooms produced. Additionally, poor-quality mushrooms accounted for 5 to 20% of the total production volume, depending on the capacity of the mushroom farm. The increasing concern for environmental quality and the desire for a circular and stable economy has led to an interest in recycling mushroom waste. It has been pointed out that there is still lack of studies present on the waste from the mushroom farming industry, specifically the stem waste [12]. Therefore, this present study investigates the macronutrients composition and biological activities present in fruiting bodies and stem waste, which is important in discovering new active compounds for nutraceuticals, functional foods, and cosmetics.

2 Materials and methods

2.1 Sample preparation

Whole mushroom (*P. pulmonarius*) samples were collected from a mushroom farming house located in Universiti Malaysia Kelantan, Malaysia. The samples were cleaned and separated between its fruiting bodies and stem waste, accordingly (Fig. 1). Naturally, the stem waste was found under the substrate and harvested alongside the mushroom as it can interrupt the next cycle's growth if not removed. Samples were then dried in an oven (Jeio Tech) at 40 ºC for 48 h prior to grinding and sieving at 550 µm. Samples were kept in an airtight container until further analysis.

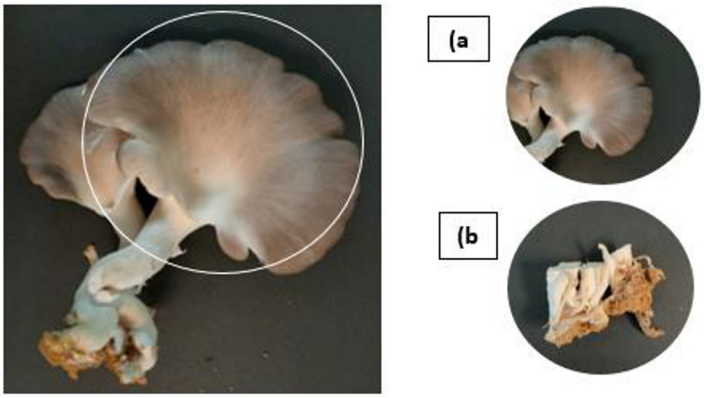

Fig. 1. Classification of mushroom parts: a) fruiting bodies, and b) stem waste.

2.2 Preparation of the extracts

Ground fruiting bodies (FB) and stem waste (SW) were extracted using 80 % (v/v) ethanol, with a ratio of 1:10 using the Soxhlet apparatus (Favorit®) for 6 hours at 78 °C. The extracts were collected (liquid form) in the round bottom flask was then cooled down to room temperature. Later, the extract was filtered with a Whatman No. 1 filter paper, evaporated with a rotary evaporator at 50 °C, freeze-dried, and stored at -80 °C until further analysis.

2.3 Proximate Analysis

The fruiting bodies and stem waste were dried at 40 °C and the samples were analyzed for macronutrient composition according to the Association of Official Analytical Chemists (AOAC) 1999, 1990, 1984, and Pearson 1981. These include the determination of carbohydrate, fat, protein, crude fiber, ash, and moisture content. The results were expressed in percent dry weight (mean ±SD).

2.4 Antioxidant analysis

2.4.1 Total Phenolic Content (TPC)

The total phenolic content was determined using the Folin-Ciocalteu method, as described in [13]. A 1 ml sample was mixed with 1 ml of Folin-Ciocalteu reagent, followed by 1 ml of 20% sodium carbonate. The mixture was diluted to 10 ml with deionized water and stored in the dark for 90 minutes. Absorbance was measured at 725 nm using a UV-1900 Shimadzu spectrophotometer. Results were expressed as mg gallic acid equivalents (GAE) per 100 g of extract, and gallic acid as a reference.

2.4.2 Total Flavonoid Content (TFC)

The flavonoid content was determined using a previously described method [14]. A 0.25 ml sample was mixed with 1.25 ml deionized water and 75 µl of 5% sodium nitrite. After 6 minutes, 150 µl of 10% aluminum chloride hexahydrate, 0.5 ml of 1 M sodium hydroxide, and 2.5 ml of deionized water were added. The mixture was incubated for 5 minutes, and absorbance was measured at 510 nm using a UV-1900 Shimadzu spectrophotometer. Results were expressed as mg quercetin equivalents (QE) per 100 g of extract with quercetin as the reference

2.4.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The antioxidant activity of the samples was measured using the DPPH assay, as modified from [8]. A 0.1 mM DPPH solution in ethanol was mixed with 1 ml of the sample, shaken, and left at room temperature for 30 minutes. Absorbance was recorded at 517 nm using a UV-1900 Shimadzu spectrophotometer. The assay was conducted in triplicate, with ascorbic acid as the reference compound, and the DPPH scavenging capacity was calculated using a formula 1:

$$Percentage\ of\ inhibition\ (\%) = [(A0-A1)/A0]\ x100, \quad (1)$$

where A0 was the absorbance of the control reaction and A1 was the absorbance of the sample. The results were expressed as IC50 value (µg/mL), concentration where percentage inhibition was at 50%.

2.4.4 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Assay

The antioxidant potential was assessed using the ABTS assay, modified from [8]. ABTS and potassium persulfate solutions were prepared, incubated, and diluted to an absorbance of 0.700 ± 0.002 at 734 nm. For the assay, 1 ml of the sample was mixed with 2 ml of the ABTS solution, incubated for 20 minutes, and absorbance was measured at 734 nm. The experiment was conducted in triplicate, with ascorbic acid as a reference, and percentage inhibition of antioxidant activity was calculated using formula 1.

2.5 Antibacterial Assay

The antibacterial assay was measured using microdilution technique according to [15] with slight modification. All the samples were prepared at 8000 mg/mL concentration in Phosphate-buffered saline (PBS): Dimethyl sulfoxide (DMSO) with the ratio 1:1. Then, 100 µL of Muller Hinton Broth (MHB) was dispensed into each well of 96-well microtiter plate. To prepare serial dilutions, 100 µL of the sample was separately dispensed into MHB-containing microplate wells and mixed well. Next, 100 µL of this mixture was pipetted from the first well into a second well and repeated to generate a dilution series (8000 to 125 µg/mL). Finally, 100 µL of each bacterial suspension was separately added to each well containing the MHB and the sample mixture. The absorbance of was measured using microplate reader (iMark) at 595±5 nm. After the first reading was finished, all the plates were covered and incubated at 37 °C for 24 h afterward, and the absorbance was measured again. The inhibition percentage was calculated using the equation below:

$$Percentage\ of\ inhibition\ (\%) = [(Ac - At)/ Ac]\ x\ 100, \quad (2)$$

where Ac was the absorbance of the negative control and At was the differential absorbance of the first and second samples. The minimum percentage inhibition is indicated as MIC value in µg/mL. The experiment was performed in triplicates and Ampicillin (100 µg/mL) was used as a standard.

2.5 Statistical Analysis

Data were analyzed using GraphPad Prism software version 9.3.1. Mean differences for the tested parameters were assessed with a two-tailed t-test, where a p-value of ≤0.05 indicated statistical significance.

3 Result and Discussion

3.1 Macronutrient composition

The composition of fiber, fat, protein, ash and carbohydrate showed significant differences ($p \leq 0.05$) between FB vs. SW, except for moisture content (Table 1). The results showed that SW had significantly higher carbohydrate, ash, and fiber content compared to FB. Meanwhile, the composition of protein and fat in FB was significantly higher compared with SW. However, there is no significant difference ($p \geq 0.05$) in moisture content composition with 12.57±0.02 % for FB and 12.61±0.02 % for SW. This is because the analysis was performed using dried samples where the fresh samples were oven-dried before analysis. The composition of fat in FB were higher than in SW with 2.03±0.12 % vs. 1.19±0.02 %, and the protein content was 31.57±0.95 % (FB) and 16.38±0.70 % (SW). However, the carbohydrate, ash, and fiber contents were higher in FB (46.20±0.69%, 8.18±0.04%, 15.44±0.10%, respectively) compared to SW (38.10±0.83%, 6.67±0.12%, 9.06±0.02%, respectively).

Table 1: Nutrient composition between fruiting body (FB) and stem waste (SW). * Indicates a significant difference between FB vs. SW with $p < 0.05$ considered as significant.

Nutrient Composition	Mean ± SD (%)		*p*-value
	Fruiting Body (FB)	Stem Waste (SW)	
Crude Fiber	9.1 ± 0.02	15.4 ± 0.10	*0.0001
Fat	2.0 ± 0.12	1.2 ± 0.02	*0.0070
Protein	31.6 ± 0.95	16.4 ± 0.70	*0.0030
Ash	6.7 ± 0.12	8.2 ± 0.04	*0.0010
Moisture content	12.6 ± 0.02	12.6 ± 0.02	0.0600
Carbohydrate	38.1 ± 0.84	46.2 ± 0.69	*0.0100

Note: p-value less than 0.05 considered as a significant difference

The results were in agreement with the study performed by [16], where *Pleurotus spp*. contained 36 % to 60 % carbohydrate, 11 % to 42 % of protein, and 0.2 % to 8 % fat Meanwhile, the fiber content was similar to [17] with 4 % to 30 % soluble and insoluble

fibers in the *Pleutorus spp*. The ash and moisture content data were supported by the results found by [18] with 10 % and 8 % ash and moisture respectively.

The high amount of fiber in SW probably due to component of the mushroom's cell wall especially the non-starch polysaccharides that vary in the different developmental stages of the mushroom life cycle [19]. As mushrooms develop, their fiber content increases due to the accumulation of complex polysaccharides, such as beta-glucans and chitin, in their cell walls, which strengthens and supports the structure of the mushroom. Our data suggest SW, which mostly consists of mushroom stalks, contains a higher percentage of carbohydrate content due to the bulk of sugar compounds such as pentoses, hexoses, and polymeric carbohydrates including glycogen that act as an energy reserve compound. Polysaccharides like cellulose, glucose, and starch is also very important during the growth and development of the mushroom as nutrients and contribute to the structure development. This correlates with the finding from [20], who found the percentage of the carbohydrate was higher in stalk (61.80±0.12) compared to cap and stalk (51.9±0.25) because the glucans act as a reserve and usable polysaccharides were concentrated mostly in the stalks. All of these macronutrient compositions are depending on the type of mushroom and other parameters including harvest, growth, and storage conditions [21].

3.2 Antioxidant Analysis

3.2.1 Total phenolic and flavonoids content

According to [22], the antioxidant activity of the mushroom is strongly affected by the total phenolic and flavonoid content. The phenolic content of the samples was determined based on the gallic acid standard curve and expressed as gallic acid equivalents (GAE) per gram of dry weight. Meanwhile, the total flavonoid was presented as mg quercetin equivalent (QE) per gram of dry weight. Based on Table 2, it can be observed that the total phenolics of the FB (4.31±0.68 mg GAE/g) is higher than SW (2.12±0.22 mg GAE/g). This data recommends that the phenolic content of the FB is higher than SW. The amount obtained for this test were in ranged compared to the previous studies which are 6.27 mg GAE /g [23], 4.47 ± 0.22 mg GAE /g [21] and 0.015 - 0.075 mg GAE/g [24].

Table 2: Total phenolic and total flavonoid of the fruiting body (FB) and stem waste (SW).

Sample	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)
Fruiting Body (FB)	4.31±0.68	7.79±0.95
Stem Waste (SW)	2.12±0.22	4.75±0.73

*Data obtained in triplicate and expressed as mean ± SD.

The total flavonoid content of the FB (7.79±0.95 mg QE/g) is higher compared to SW (4.75±0.73 mg QE/g). Other researchers have reported that the flavonoid content ranged from 0.025 - 0.131 mg QE/g [24], 1.88 mg CAE/g [25] and 6.38±0.07- 7.79±0.04 mg CAE/g [26]. Thus, the amount of flavonoid obtained in this study is higher compare to other report. The difference results from the earlier study could be explained by the drying method used, different extraction process and solvent used during extraction. The drying method and differences in the extraction process and solvents can affect the preservation of active compounds, as certain methods and solvents are more effective at extracting specific compounds.

3.2.1 DPPH+ and ABTS+ assays

The antioxidant potential was measured using colorimetric assays that resulted in color changes after the reaction took place and the absorbance readings were taken at specific wavelengths. The linear regressions and the IC50 of the samples and standards were shown in Table 3. The antioxidant activity of the samples has been expressed as IC50 value (µg/mL). This value represents the concentration of the sample needed to scavenge 50% of free radical compounds. The IC50 value is inversely proportional to the antioxidant property, which means the lower the IC50 value the higher the antioxidant activity. It can be observed that the IC50 value of the FB (274.8±7.37 µg/mL) is lower than SW (503.5±4.60 µg/mL) in the DPPH assay. In addition, the IC50 values of FB (166.1±1.13 µg/mL) in the ABTS assay is also lower compared to SW (279.5±1.32 µg/mL). This suggests that FB contains higher antioxidant activity than SW. However, compared to ascorbic acid, those mushroom extracts exhibit lower radical scavenging activity.

Table 3: Linear regression and IC50 values of fruiting body (FB), stem waste (SW), and ascorbic acid standard for DPPH and ABTS assays.

Samples	**Linear regression for DPPH (R^2)**	**DPPH (IC_{50} µg/ml)**	**Linear regression for ABTS (R^2)**	**ABTS (IC_{50} µg/ml)**
Fruiting body (FB)	Y= 0.18 x- 0.92 (0.95)	274.8±7.37	Y=0.28x+ 3.05 (0.88)	166.1±1.13
Stem Waste (SW)	Y= 0.10x-2.14 (0.97)	503.5±4.60	Y= 0.16x+4.30 (0.95)	279.5±1.32
Ascorbic acid (AA)	Y= 2.51x + 8.62 (0.90)	16.4±0.25	Y=1.91x + 23.5 (0.99)	13.9±0.06

In the present study, the IC50 values showed that the samples had higher antioxidant activities compared to the previous study by [27], who found the IC50 values of 2.75 - 12 mg/ml for most of the tested *Pleurotus spp.*This may be due to the glucan and phenolic content that naturally acts as a mushroom defense system, such as contributing to the development of macrophage function and can protect the host from any harmful microorganism [28]. In addition, [5] reported that the high content of vitamins and selenium also contribute to the excellent antioxidant activity of the mushroom.

The presence of polyphenolic compounds such as flavonoids and the aromatic ring structure of phenolics might contribute to the mushroom scavenging ability against free radicals [29]. The existence of these compounds in the oyster mushroom may lead the way in the cosmetics industry where the study has been performed by [9] found that *Pleurotus* spp. the extract improved catalase gene expression and reduced the frequency of free radical-induced protein oxidation in aged rats. This protection could prevent the cells from aging caused by the oxidative damage of free radicals and boost skin nutrition.

3.4 Antibacterial Activity

The antibacterial potentials of the mushroom extracts were estimated from their ability to inhibit different pathogenic bacteria. By using the microdilution method, the optical

density (OD) of the extract against the pathogenic bacteria after 24 h of incubation was read at 595±5nm. The SW extract inhibits more pathogenic bacteria compared to FB in terms of the percentage of inhibition as shown in Table 4. However, there is no significant difference in MIC values between the FB and SW except for *E. coli*. The antibacterial activity of the samples has been expressed as MIC value (µg/mL). This value represents the lowest concentration of the antibacterial agent that can prevent the visible growth of pathogenic bacteria. It can be observed that the lowest MIC value are both extracts from FB and SW (125 µg/mL) against the *B. subtilis* followed by *P. aeruginosa* (250 µg/mL). Next, the MIC value for SW extract (1000 µg/mL) was lower compared to FB extract (2000 µg/mL) against the *E. coli*, while the highest MIC value is 4000 µg/mL for both FB and SW against *S. aureus* bacteria. Nevertheless, [30] have found that the lowest inhibition zone diameter is *P. aeruginosa* (5.6±0.4mm), which may be due to the variances in bioactive compositions or concentrations, type of solvent and extraction used also the mechanism of action of active ingredients in the extracts.

Table 4: Minimum inhibition concentration (MIC) value of the fruiting body (FB) and stem waste (SW) against the tested bacteria. Ampicillin was used as control at 100 µg/ml.

Bacteria	MIC (µg/ml)	
	FB	SW
P. aeruginosa	250	250
E. coli	2000	1000*
S. aureus	4000	4000
B. subtilis	125	125

*Shows the significant difference of MIC value between FB and SW ($p \leq 0.05$).

In addition, the MIC values of FB and SW (250 µg/ml) against gram-negative bacteria, *P. aeruginosa* were lower compared to *E. coli* for both FB (2000 µg/ml) and SW (1000 µg/ml). Furthermore, the percentage of the inhibition for these bacteria at 2000 µg/ml showed a lower inhibition in *E. coli* for FB (10.49±0.25%) and SW (6.22±0.76%) compared to FB (20.72±0.17%) and SW (13.15±0.61%) for *P. aeruginosa*. This data was interrelated to the previous study by [31], who found the inhibition zone of the *E. coli* (3.00 ±1.00) is lower than *P. aeruginosa* (10.00 ±1.00). This finding suggests that *E. coli* is highly resistant to these extracts due to the outer membrane of the bacteria that may prevent the entrance of many antimicrobial agents including the mushroom extracts [32].

The detected antimicrobial activity from this experiment could be from the alkaloids and flavonoids content in these mushroom extracts which have been shown to possess antimicrobial properties [33]. Additionally, the polysaccharide content mainly composed of β-glucans and α-glucans in the cell wall of mushrooms contributed to the antibacterial potential. According to [5], the β-D Glucan (Pleuran) isolated from the fruiting bodies of Pleurotus spp. also helped the survival of mice against bacterial infections. The presence of these secondary metabolites may produce antibacterial activity with the mechanism of action by disruption of the cell membrane, prevention of protein synthesis, enzymes, and microbial adhesion [34].

4 Conclusion

As a conclusion, the stem waste (SW) has potential nutrients in terms of carbohydrate, fiber, and ash that contain inorganic salts and non-volatile organic compounds which may benefit in several applications such as in the food and supplement industry. In addition, it may contribute to the pharmaceutical and cosmetics industry by improving human health as it shows the capability of the antioxidant defense system in fighting against the production of free radicals. Moreover, it also has shown its potential in antimicrobial properties by inhibiting some pathogens including gram-positive and gram-negative bacteria. Although in recent years the number of studies on the health-promoting effects of the mushrooms has increased, there have been relatively few studies involving the SW.

Funding/financial disclosures

This research was funded by the Ministry of Higher Education (MOHE), Malaysia (FRGS/1/2021/STG03/UMK/02/3) in collaboration with internal project funded by Universiti Malaysia Kelantan (R/FUND/A0700/00830A/002/2022/01006).

Conflict of interest

None.

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