



Research article

Investigating tiger milk mushroom (*Lignosus rhinocerus*) biochemical component yield and anticancer properties based on different casing materials

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Abstract

Importance of the work: The tiger milk mushroom (*Lignosus rhinocerus*) is rare and grows naturally in dense forests, severely limiting its availability as a health supplement. This scarcity has led to the development of casing-based tiger milk mushroom sclerotium culture, which mimics natural growing conditions to meet the growing demand.

Objectives: To investigate the effect of different casing materials on the growth characteristics and biological activities of tiger milk mushroom sclerotium.

Materials & Methods: The fully colonized tiger milk mushroom substrates were buried for 6 mth in 36 different casing materials consisting of coconut coir (CC), planting soil (PS), rice straw (RS), rubber wood sawdust (RWS), Hungary oyster mushroom spent substrate, Indian oyster spent mushroom substrate and various combinations of these materials. After harvesting, the sclerotium was sliced, dried and ground into a powder. The dried samples were extracted with boiling water and examined for biochemical components and biological activities.

Results: Sclerotium fresh weight was highest (96.53 ± 3.03 g) using the casing with CC. Casing with PS generated a high content of phenolic acids and flavonoids, as well as having high antioxidant and anti-lung cancer and anti-breast cancer activities. Casing with CC:RWS (1:1) provided a high content of β -glucan (81.50 ± 1.96 mg β -glucan/g extract).

Main finding: The growth characteristics and biological activities of tiger milk mushroom sclerotium varied depending on the casing material. The results of the study should assist in choosing effective casing materials capable of stimulating the desired bioactive components.

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Introduction

Nowadays, more people are aware of their health and are taking various supplements, as additional research supports the importance of supplements in boosting health and preventing disease (Hassen et al., 2022). Vitamin supplements derived from vegetables and fruits are classified as dietary supplements, with synthetic vitamins, medicinal plants and medicinal mushrooms considered as other popular natural dietary supplement options, since they are high in vitamins, minerals, polysaccharides and biologically active compounds with several health advantages (Dincheva et al., 2023; Łysakowska et al., 2023).

Tiger milk mushroom (*Lignosus rhinocerus*) is another kind of medical mushroom that is of research interest since important details on its medicinal properties are lacking. Tiger's milk mushrooms are usually found in dense evergreen forests in Thailand's south, along the Thai-Malaysian border, in Satun province and Betong district, Yala province (Nallathamby et al., 2018). According to legend, if some tiger's milk falls on the ground during cub feeding, the tiger milk mushroom blooms. Lau et al. (2015) described the various characteristics of tiger milk mushroom. It has a pileus similar to the reishi mushroom and an underground head, or rhizome (sclerotium), buried in the soil. The gathering of mushroom hyphae forms the sclerotium, and it has also been discovered that most of the essential compounds used to treat disease are present in this part. For example, polysaccharides (β -glucan) are a major component of the mushroom sclerotium, which also contains bioactive compounds such as phenolics, triterpenoids and sesquiterpenes (Lau et al., 2013; Wu et al., 2022).

The mushroom cultivation process must consider the quality of the mushroom mycelium and the conditions for mushroom development in addition to the nutrients supplied via the mushroom substrate. For example, increasing the layer of casing material required to attain high yields is required for some varieties of mushrooms—but not for all (Pardo-Giménez et al., 2020). The casing materials absorb water and enable gas exchange between the mushroom's surface and the surrounding environment (Pardo-Giménez et al., 2017). It aids in the prevention of mushroom drying and protects against insect and mushroom pests by encouraging the development of microorganisms that aid in mushroom blooming (McGee, 2018; Navarro et al., 2020). These factors enable mushrooms to transition from the vegetative (mycelial) phase to the reproductive (pileus) phase (Okhuoya and Okogbo, 1991).

Jamil et al. (2018) investigated growing tiger milk mushrooms in casing material using mushroom substrate containing 89% sawdust, 1% lime and 10% rice bran. The mycelium was fully colonized after adding tiger milk mushroom inoculum and incubating in a dark area at room temperature for 3–4 wk. Then, the mycelium was buried in soil containing 1% lime and 50–70% humidity for 3 mth. Each sclerotium was collected and extracted using boiling water. The amounts of nutrients and flavonoids in the casing-derived mushroom sclerotium was compared to those in the natural mushroom sclerotium. The results revealed that the mushroom sclerotium derived via casing contained higher contents of carbohydrate, protein, flavonoid and β -glucan than the natural mushroom sclerotium.

Tiger milk mushrooms are highly valued due to their numerous therapeutic characteristics and scarcity in the wild resulting in insufficient quantities to meet demand. Therefore, the current research aimed to develop casing cultivation methods for tiger milk mushroom cultivation to increase the yield and biological activity of the tiger milk mushroom sclerotium to help the development of its functional ingredients for dietary supplements and health-enhancing beverages to fulfill customer demand.

Materials and Methods

Source of mushroom sclerotium

The tiger milk mushroom was harvested from the Hala Bala Forest in Yala province and was subjected to strain isolation, with the subsequent strain identification performed using molecular identification based on sequencing the internal transcribed spacer region. Based on the results, the tiger milk mushroom was 92.95% identical to the strain *Lignosus rhinocerus* CH2.

Mushroom substrate preparation

The tiger milk mushroom substrate was prepared with ingredients of 100 kg rubber wood sawdust, 7 kg fine bran, 1 kg broken rice, 0.5 kg gypsum (calcium sulfate), 0.2 kg magnesium sulfate, 1 kg lime, 2–3 kg of phumite and 70–80 L of water for softening. Afterward, 900 g of the mixed materials were put in polypropylene bags, compressed tightly, placed in the neck of a bottle, fastened with a rubber band and closed with a cotton stopper. The substrate bags were sterilized

in an autoclave at 121°C for 15 min and then cooled to room temperature. The tiger milk mushroom inoculum in sorghum seeds was dropped into the sterilized bags of mushroom substrates. Then, the inoculated substrates were incubated on a farm at 25–30°C, allowing about 2 mth for the mycelia to reach maturity. After an entire spawn run, the substrates were further incubated for 2 mth to tighten the mycelium. The plastic bag was removed from the substrates and buried in various casing materials, where the casing is the layer added to the fully colonized substrate to retain moisture content.

Mushroom substrate casing

As shown in Table 1, the 36 different types of casing materials used in this experiment consisted of coconut coir (CC),

planting soil (PS), rice straw (RS), rubber wood sawdust (RWS), Hungary oyster mushroom spent substrate, Indian oyster spent mushroom substrate and various combinations of these materials (see Table 1). The fully colonized tiger milk mushroom substrates were buried separately for 6 mth in the various casing materials. After harvesting, each sclerotium sample was cleansed with tap water and dried at room temperature for 15 min on a cheesecloth.

Sclerotium preparation

The sclerotium was cut into 1.0 cm × 1.5 cm rectangles (0.2 cm thick) and dried at 70°C for 1 d in a hot-air oven until a constant weight was achieved. The dried samples were ground into a fine powder and kept in a dry, airtight space.

Table 1 List of 36 types of casing materials used to cover *L. rhinocerus* CH2 substrate

Casing material	Casing material	Casing material
1. Coconut coir (CC)	2. Planting soil (PS)	3. Rice straw (RS)
4. Rubber wood sawdust (RWS)	5. Indian oyster spent mushroom substrate (IOSMS)	6. Hungarian oyster spent mushroom substrate (HOSMS)
7. Coconut coir:Planting soil (CC:PS; 1:1)	8. Coconut coir:Rice straw (CC:RS; 1:1)	9. Coconutcoir:Rubber wood sawdust (CC:RWS; 1:1)
10. Coconut coir:Indian oyster spent mushroom substrate (CC:IOSMS; 1:1)	11. Coconut coir:Hungary oyster spent mushroom substrate (CC:HOSMS; 1:1)	12. Planting soil:Rice straw (PS:RS; 1:1)
13. Planting soil:Rubber wood sawdust (PS:RWS; 1:1)	14. Planting soil:Indian oyster spent mushroom substrate (PS:IOSMS; 1:1)	15. Planting soil:Hungary oyster spent mushroom substrate (PS:HOSMS; 1:1)
16. Rice straw:Rubber wood sawdust (RS:RWS; 1:1)	17. Rice straw:Indian oyster spent mushroom substrate (RS:IOSMS; 1:1)	18. Rice straw:Hungary oyster spent mushroom substrate (RS:HOSMS; 1:1)
19. Rubber wood sawdust:Indian oyster spent mushroom substrate (RWS:IOSMS; 1:1)	20. Rubber wood sawdust:Hungary oyster spent mushroom substrate (RWS:HOSMS; 1:1)	21. Indian oyster spent mushroom substrate:Hungary oyster spent mushroom substrate (IOSMS:HOSMS; 1:1)
22. Coconut coir:Planting soil:Rice straw (CC:PS:RS; 1:1:1)	23. Coconut coir:Planting soil:Rubber wood sawdust (CC:PS:RWS; 1:1:1)	24. Coconut coir:Planting soil: Indian oyster spent mushroom substrate (CC:PS:IOSMS; 1:1:1)
25. Coconut coir:Planting soil: Hungary oyster spent mushroom substrate (CC:PS:HOSMS; 1:1:1)	26. Coconut coir:Rice straw:Rubber wood sawdust (CC:RS:RWS; 1:1:1)	27. Coconut coir:Rice straw:Indian oyster spent mushroom substrate (CC:RS:IOSMS; 1:1:1)
28. Coconut coir:Rice straw:Hungary oyster spent mushroom substrate (CC:RS:HOSMS; 1:1:1)	29. Coconut coir:Rubber wood sawdust: Indian oyster spent mushroom substrate (CC:RWS:IOSMS; 1:1:1)	30. Coconut coir:Rubber wood sawdust:Hungary oyster spent mushroom substrate (CC:RWS:HOSMS; 1:1:1)
31. Coconut coir:Indian oyster spent mushroom substrate:Hungarian oyster spent mushroom substrate (CC:IOSMS:HOSMS; 1:1:1)	32. Planting soil:Rice straw:Rubber wood sawdust (PS:RS:RWS; 1:1:1)	33. Planting soil:Rice straw:Indian oyster spent mushroom substrate (PS:RS:IOSMS; 1:1:1)
34. Planting soil:Rice straw: Hungary oyster spent mushroom substrate (PS:RS:HOSMS; 1:1:1)	35. Rice straw:Rubber wood sawdust: Indian oyster spent mushroom substrate (RS:RWS:IOSMS; 1:1:1)	36. Rice straw:Rubber wood sawdust: Hungary oyster spent mushroom substrate (RS:RWS:HOSMS; 1:1:1)

Color measurement

Based on the method of Shan et al. (2016), the color of sclerotium samples was determined by measuring the reflectance of a 100 g sample in terms of L^* (blackness/whiteness), a^* (greenness/redness) and b^* (blueness/yellowness) values of the CIELAB system using a ColorQuest XE colorimeter (Hunterlab; Reston, VA, USA). The apparatus was calibrated against black and white tiles before measuring the samples. Equations 1 and 2 were used to determine the total color difference (ΔE) between the dried sclerotium (L^* , a^* , b^*) and the fresh sclerotium (L^*_0 , a^*_0 , b^*_0) and the browning index (BI).

$$\Delta E = \sqrt{(L^*_0 - L^*)^2 + (a^*_0 - a^*)^2 + (b^*_0 - b^*)^2} \quad (1)$$

$$BI = \frac{[100(x-0.31)]}{0.172}, \text{ where } x = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)} \quad (2)$$

Extraction of sclerotium samples

After dissolving 0.5 g of the sclerotium powder in 20 mL of hot distilled water (100°C), the mixture was extracted using an orbital shaker running at 150 revolutions per minute for 15 min. The sediment was eliminated by filtering using the Whatman no. 1 paper. The prepared extracts were collected and kept at 4°C for further examination.

Determination of total phenolic content

The total phenol content (TPC) was determined according to Nora et al. (2016), with slight modification. First, 0.1 mL of the prepared extract was combined with 0.5 mL of Folin and Ciocalteu phenol reagent, then set aside for 5 min before adding 1 mL 7.5% (w/v) Na_2CO_3 solution to the reaction mixture before adjusting the volume to 10 mL with distilled water. The reaction mixture was kept in the dark for 90 min and subsequently, the absorbance at 765 nm was measured using an ultraviolet-visible spectrum (UV-Vis) spectrophotometer (GENESYS 10S UV-Vis; Thermo Fisher Scientific Inc.; Madison, WI, USA). The results were presented in milligrams of gallic acid equivalents (GAE) per gram of extract based on a calibration curve generated from a standard gallic acid solution (0–100 mg/L).

Determination of total flavonoid content

Roshanak et al. (2016) provided the method for quantifying the total flavonoid content (TFC) of the prepared extract. After adding 0.1 mL of the prepared extract to 2 mL of distilled water, 0.15 mL of 5% (weight per volume; w/v) NaNO_2 solution, 0.15 mL of 10% (w/v) AlCl_3 , 2 mL of 4% (w/v) NaOH were added and made up to 5 mL with distilled water. the absorbance of the reaction mixture was measured at 510 nm using the UV-Vis spectrophotometer. Various amounts of catechin (0–100 mg/L) were utilized as a standard. The results were presented in milligrams of catechin equivalents (CE) per gram of extract.

2,2-Diphenyl-1-picrylhydrazyl hydrate free radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging analysis was used to measure the free radical scavenging capacity of the prepared extract, according to Norra et al. (2016). A 0.1 mL sample of the prepared extract was mixed with 2.9 mL of 0.004% (w/v) DPPH in methanol. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer after the reaction mixture had been left at room temperature for 30 min. The percentage scavenging activity of DPPH radical was calculated using Equation 3:

$$\% \text{ DPPH inhibition} = \frac{(A_0 - A_s)}{A_0} \times 100 \quad (3)$$

where A_s is the absorbance of the sample solution and A_0 is the absorbance of the control. The concentration of the extract that scavenged 50% of DPPH was defined as the IC_{50} value.

Ferric reducing antioxidant power assay

The antioxidant potential of the prepared extract was determined using the ferric reducing antioxidant power (FRAP) assay, according to Çoklar and Akbulut (2017). A sample (300 mM) of acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) dissolved in 40 mM HCl , and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in a ratio of 10:1:1 to create fresh FRAP reagents. After that, the reaction was completed by mixing 1.5 mL of FRAP reagent with 0.1 mL of the prepared extract and incubating at 37°C for 4 min. The UV-Vis spectrophotometer was used to measure the absorbance at 593 nm. Calibration curves were established using ferrous sulfate (FeSO_4) at 0–1,000 $\mu\text{mol/L}$ values. For each gram of extract, FRAP values were expressed as micro moles of iron equivalent ($\mu\text{mol Fe}^{2+}$).

Determination of total triterpenoids

The total triterpenoid content was determined according to Gupta et al. (2018). In a boiling water bath set at 100°C, the prepared extract or the ursolic acid standard (0–100 mg/L) was evaporated until fully dry. After adding 0.3 mL 5% (w/v) vanillin/glacial acetic acid and 1 mL perchloric acid solution, the reaction was incubated in a water bath at 60°C for 15 min and then allowed to cool at room temperature. The absorbance at 548 nm was measured using the UV-Vis spectrophotometer after adding 5 mL of glacial acetic acid to the mixture. The triterpenoid content was expressed as milligrams of ursolic acid equivalent per gram of extract.

Determination of total protein content

The amount of total protein was measured according to Bradford (1976). A sample (1 mL) of Bradford's reagent and 0.1 mL of the prepared extract were mixed; then, the mixture was left at room temperature for 10 min. The protein content was calculated using the standard curve of bovine serum albumin (BSA; 0–1 mg/mL) after the absorbance of the sample had been measured at 595 nm using the UV-Vis spectrophotometer.

Determination of β -glucan content

A Megazyme Enzymatic Mushroom and Yeast β -Glucan Assay Kit (Megazyme International Ireland Ltd; Bray, Ireland) was used to determine the 1,3-1,6- β -d-glucan content in the prepared extract according to the manufacturer's instructions. The amount of β -glucan in each gram of extract was represented as milligrams of β -glucan equivalent.

Analysis of in vitro anti-inflammatory activities inhibition of bovine serum albumin denaturation

BSA denaturation inhibition by the prepared extract was performed according to Williams et al. (2008), with minor modifications. Phosphate buffer pH 6.4 containing 1 mL of 1% (w/v) BSA and 0.1 mL of the prepared extract or the reference drug diclofenac sodium (0–1,000 mg/L) were combined. Then, reactions were incubated at 37°C for 20 min, followed by a second incubation at 70°C for 5 min. The absorbance of the sample was read at 660 nm using the UV-Vis spectrophotometer after the reaction mixture

had cooled to room temperature. The percentage inhibition of BSA denaturation was calculated using Equation 4:

$$\% \text{ Inhibition of BSA denaturation} = \frac{(A_0 - A_s)}{A_0} \times 100 \quad (4)$$

where A_s is the absorbance of the sample solution, and A_0 is the absorbance of the control. The concentration of the extract that inhibited 50% of BSA denaturation was defined as the IC_{50} value.

Proteinase inhibitory assay

The inhibition of proteinase inhibitory action was determined according to Gunathilake et al. (2018), with slight modification. An amount (0.06 mg) of trypsin, with 1 mL of 20 mM Tris HCl buffer (pH 7.4) and 0.5 mL of the prepared extract was added to the reaction mixture. Then, the mixture was incubated at 37°C for 5 min before adding 1 mL of 0.8% (w/v) bovine serum albumin. After incubating at 37°C for 20 min, the reaction was stopped by adding 2 mL 70% acetic acid. The supernatant was collected after centrifuging the reaction mixture at 10,000× g for 10 min. Next, the protein hydrolyzed in the supernatant was measured for absorbance at 210 nm. The percentage inhibition of proteinase activity was calculated using Equation 5:

$$\% \text{ Inhibition of proteinase activity} = \frac{(A_0 - A_s)}{A_0} \times 100 \quad (5)$$

where A_s is the absorbance of the sample solution and A_0 is the absorbance of the control. The concentration of the extract that inhibited 50% of proteinase activity was defined as the IC_{50} value.

3-(4,5-Dimethylthiazol-2-yl))-2,5 diphenyltetrazoliumbromide assay

The cytotoxic activity of the prepared extract on the A549 lung cancer and MCF-7 breast cancer cell lines was measured using an 3-(4,5-dimethylthiazol-2-yl))-2,5 diphenyltetrazoliumbromide (MTT) assay, according to Rossiana et al. (2018). Initially, the lung cancer cell line (A549) and breast cancer cell line (MCF-7) at 1×10^4 cells per well were cultivated in a 96-well culture plate and placed in a CO_2 incubator at 37°C with 5% carbon dioxide for 24 hr. After 24 hr of incubation, the old medium was removed from each well. Then, 100 μ L of the prepared extract at concentrations of 0, 20, 35, 70, 140, 300 and 500 μ g/mL

and the positive control or negative control were inserted into a 96-well culture plate, followed by an incubation period at 37°C under 5% CO₂ for 24 hr. The absorbance of the sample was read at 540 nm using a microplate reader, and the cell viability was calculated using Equation 6:

$$\% \text{ Cell viability} = \frac{A_{\text{treated}}}{A_{\text{untreated}}} \times 100 \quad (6)$$

The concentration of the extract that inhibited 50% of the growth of cancer cells was defined as the IC₅₀ value.

Synchrotron radiation-based Fourier transform infrared spectroscopy

Fine powder from each of the *L. rhinocerus* sclerotium samples was analyzed for prominent functional groups using synchrotron radiation-based Fourier transform infrared spectroscopy (SR-FTIR) at the Synchrotron Light Research Institute in Nakhon Ratchasima, Thailand, based on infrared spectroscopy and an Imaging BL4.1 end station. The process was carried out using a photon energy range of 0.01–0.5 eV with a 36X Schwarzschild Objective, a Bruker Vertex 70 spectrometer coupled with a Bruker Hyperion 2000 microscope and a 100 µm narrow-band, mercury-cadmium-telluride detector cooled using liquid nitrogen (Bruker Optics; Ettlingen, Germany). The FTIR spectral data collection was controlled using the OPUS software (version 7.8; Bruker Optics Ltd.; Ettlingen, Germany).

A small amount of fine dried sclerotium powder was carefully mounted onto a 0.8 mm diamond anvil cell lower window, then placed in the upper anvil cell window on top; the windows were sandwiched together with screws. The thin film sample sandwiched between the clear windows was observed under

a light stereomicroscope, based on triplicated samples for each group. In transmission mode, the samples were analyzed in the infrared spectral range of 4,000–900 cm⁻¹ with a 30× 30 µm square aperture. The spectrum of each selected sample area was recorded at 6 cm⁻¹ resolution and recorded as the average of 64 scans. Appropriate FTIR spectra with the polysaccharide intensity in the 0.2–1.2 abs range were extracted and selected from each measured sample image using the OPUS program. The Unscrambler X software (version 10.5; CAMO; Oslo, Norway) was used to carry out principal component analysis (PCA) to observe the glucan types in the sample groups. Data preprocessing was performed using the Savitzky-Golay transform and 17 smoothing points, with 3 polynomial orders; then, the baseline correction was offset and converted for the spectral region of 1,750–1,090 cm⁻¹ based on extended, multiplicative signal correction for further PCA analysis.

Statistical analysis

The results of three replicate experiments were expressed as mean ± SD values. The statistical analysis was performed using a one-factor analysis of variance. Significant differences were tested at *p* < 0.05. Pearson's correlation analysis (*r*) and multivariate PCA were performed using the Minitab statistical software (version 19; Minitab Inc.; State College, PA, USA).

Results

Yield of sclerotium

The effects of the different casing materials on the yield of tiger milk mushroom sclerotium are summarized in Table 2.

Table 2 Fresh weight, dry weight and browning index (BI) of sclerotium samples obtained from casing of mushroom substrate with various materials

Casing material	Fresh weight (g/900 g substrate)	Dry weight (g/900 g substrate)	BI
CC	96.53±3.03 ^a	57.24±2.51 ^a	26.37±0.49 ^e
PS	38.53±1.92 ^h	22.62±2.17 ^g	42.19±0.94 ^b
RS	34.42±1.14 ⁱ	20.78±1.39 ^h	23.78±0.16 ^f
CC:PS (1:1)	60.54±2.06 ^d	36.01±1.31 ^d	48.22±0.54 ^a
CC:RS (1:1)	58.59±2.93 ^e	31.37±1.52 ^e	32.48±0.43 ^c
CC:RWS (1:1)	68.22±2.62 ^c	38.51±2.98 ^c	26.32±0.52 ^e
CC:PS:RS (1:1:1)	47.31±3.12 ^f	25.79±1.61 ^f	26.08±0.61 ^e
CC:RS:RWS (1:1:1)	74.91±3.22 ^b	43.41±3.43 ^b	16.35±0.54 ^h
PS:RS:RWS (1:1:1)	41.47±2.47 ^g	20.34±1.89 ^h	28.38±0.14 ^d
Wild TM	30.25±1.36 ⁱ	19.12±1.05 ^h	19.18±0.63 ^g

CC = coconut coir; RWS = rubber wood sawdust; RS = rice straw; PS = planting soil; TM = tiger milk mushroom sclerotium.

Values (mean ± SD; *n* = 3) within rows superscripted by different lowercase letters are significantly (*p* < 0.05) different.

The highest fresh weight of sclerotium was produced using the CC casing (96.53 ± 3.03 g), followed by the mixtures CC:RS:RWS(1:1:1; 74.91 ± 3.22 g), CC:RWS(1:1; 68.22 ± 2.62 g), CC:PS (1:1; 60.54 ± 2.06 g), CC:RS (1:1; 58.59 ± 2.93 g) and PS (38.53 ± 1.92 g), respectively. Wild TM had the lowest weight of sclerotium (30.25 ± 1.26 g). Hungary oyster spent mushroom, Indian oyster spent mushroom and rubber wood sawdust, both individually and in combination with other casing materials, caused rot and decomposition of the tiger milk mushroom substrate. On the other hand, using CC:RWS (1:1) produced a good weight of sclerotium (68.22 ± 2.62 g). In addition, the weight of dried tiger milk mushroom sclerotium decreased by approximately 1.6–2.0 times its fresh weight after drying in a hot-air oven at 70°C for 12 hr.

Color changes

The results from investigating the effects of the casing materials on the color of the sclerotium showed that the different casing materials resulted in varying browning index (BI) values (Table 2). Casing the mushroom substrate with CC:PS (1:1) provided the highest BI value (48.22 ± 0.54), followed by casing with PS (42.19 ± 0.94), while casing with CC:RS:RWS (1:1:1) produced the lowest BI values (16.35 ± 0.54).

Total phenolic and flavonoid contents of sclerotium

Analysis of the TPC and TFC in the tiger milk mushroom sclerotium resulting from casing the mushroom substrate with various materials showed that CC yielded the lowest values for the TPC (12.74 ± 0.19 mg GAE/g extract) and TFC (3.74 ± 0.27 mg CE/g extract), as shown in Table 3. The highest TPC value was in the sclerotium with PS casing (26.63 ± 0.16 mg GAE/g extract), followed by CC:RWS (1:1; 24.53 ± 0.36 mg GAE/g extract), CC:PS (1:1; 21.48 ± 0.63 mg GAE/g extract) and CC:RS:RWS (1:1:1; 20.64 ± 0.34 mg GAE/g extract), respectively. In comparison, casing with CC:RS (1:1) provided the highest TFC value (9.32 ± 0.41 mg CE/g extract), followed by PS (9.12 ± 0.19 mg CE/g extract), PS:RS:RWS (1:1:1; 7.85 ± 0.16 mg CE/g extract) and CC:PS:RS (1:1:1; 7.74 ± 0.12 mg CE/g extract), respectively.

Antioxidant activity of sclerotium

The IC_{50} of DPPH radical scavenging activity was the lowest for casing the mushroom substrate with PS:RS:RWS (1:1:1; 19.46 ± 0.09 mg/mL), as shown in Table 3. The use of PS as a casing material produced the highest FRAP value (23.75 ± 0.51 μM Fe(II)/g extract), followed by CC:RS (1:1; 20.65 ± 0.63 μM Fe(II)/g extract), PS:RS:RWS (1:1:1; 19.34 ± 0.78 μM Fe(II)/g extract) and CC:PS (1:1; 18.43 ± 0.72 μM Fe(II)/g extract), respectively, as shown in Fig. 4B. The lowest FRAP value was produced with wild TM (11.36 ± 0.83 μM Fe(II)/g extract).

Table 3 Total phenolic and total flavonoid contents, DPPH activity and FRAP value of sclerotium extract obtained from the casing of mushroom substrate with various materials

Casing material	Total phenolics (mg GAE/g extract)	Total flavonoids (mg CE/g extract)	DPPH activity (IC_{50} ; mg/mL)	FRAP value (μM Fe(II)/g extract)
CC	12.74 ± 0.19^g	3.74 ± 0.27^{fg}	28.48 ± 0.35^b	14.76 ± 0.59^d
PS	26.63 ± 0.16^a	9.12 ± 0.19^{ab}	20.59 ± 0.34^f	23.75 ± 0.51^a
RS	15.84 ± 0.26^e	4.73 ± 0.17^{ef}	26.74 ± 0.76^c	15.79 ± 0.76^d
CC:PS (1:1)	21.48 ± 0.63^c	6.36 ± 0.30^{cd}	24.47 ± 0.14^d	18.43 ± 0.72^c
CC:RS (1:1)	16.57 ± 0.69^e	9.32 ± 0.41^a	22.48 ± 0.35^e	20.65 ± 0.63^b
CC:RWS (1:1)	24.53 ± 0.36^b	4.46 ± 0.21^f	28.57 ± 0.71^b	14.35 ± 0.43^d
CC:PS:RS (1:1:1)	17.92 ± 0.39^d	7.74 ± 0.12^{bc}	23.73 ± 0.68^{de}	17.85 ± 0.11^c
CC:RS:RWS (1:1:1)	20.64 ± 0.34^c	5.89 ± 0.21^{de}	26.37 ± 0.36^c	15.88 ± 0.37^d
PS:RS:RWS (1:1:1)	18.63 ± 0.58^d	7.85 ± 0.16^b	19.46 ± 0.09^f	19.34 ± 0.78^{bc}
Wild TM	14.34 ± 0.42^f	2.66 ± 0.25^g	30.75 ± 0.23^a	11.36 ± 0.83^e

CC = coconut coir; RWS = rubber wood sawdust; RS = rice straw; PS = planting soil; TM = tiger milk mushroom sclerotium; DPPH = 2,2'-diphenyl-1-picrylhydrazyl radical scavenging activity; FRAP = Ferric ion reducing antioxidant power; GAE = gallic acid equivalents; CE = catechin equivalents; IC_{50} = concentration of extract scavenging 50% of 2,2'-diphenyl-1-picrylhydrazyl activity.

Values (mean $\pm$ SD; $n = 3$) within rows superscripted by different lowercase letters are significantly ($p < 0.05$) different.

Total protein, β -glucan and triterpenoid contents of sclerotium

The protein contents in sclerotium obtained from casing substrate with CC:PS:RS (1:1:1), CC:RS (1:1) and PS were 2.87 ± 0.08 mg BSA/g extract, 2.57 ± 0.03 mg BSA/g extract and 2.37 ± 0.07 mg BSA/g extract, respectively (Table 4). Casing with RS and CC:RWS (1:1) resulted in the protein content being as low as 1.42 ± 0.04 mg BSA/g extract and 1.23 ± 0.06 mg BSA/g extract, respectively.

The β -glucan contents were in the range 38.40 ± 1.50 – 81.50 ± 1.96 mg β -glucan/g extract, as shown in Table 4. Sclerotium derived from the CC:RWS (1:1) casing had the highest β -glucan content (81.50 ± 1.96 mg β -glucan/g extract), followed by CC:RS:RWS (1:1:1; 59.90 ± 1.68 mg β -glucan/g extract), wild TM (55.40 ± 1.81 mg β -glucan/g extract) and RS (56.10 ± 1.63 mg β -glucan/g extract), respectively. The PS casing contained the lowest β -glucan content (38.40 ± 1.50 mg β -glucan/g extract).

The casing with CC:RS (1:1) stimulated an increase in total triterpenoid content to a maximum value of 9.74 ± 0.12 mg ursolic acid/g extract, followed by CC:PS (1:1; 8.28 ± 0.24 mg ursolic acid/g extract) and PS (6.46 ± 0.43 mg ursolic acid/g extract), as shown in Table 4. The lowest total triterpenoid content was found when the substrate was cased with CC:PS:RS (1:1:1) (3.60 ± 0.25 mg ursolic acid/g extract).

In vitro anti-inflammatory activity of sclerotium

The IC_{50} values for the inhibition of BSA denaturation and proteinase activity were in the ranges 84.62 ± 1.44 – 289.537 ± 2.75 and 138.46 ± 6.76 – 321.75 ± 6.87 μ g/mL, respectively (Table 5). Casing mushroom substrate with PS produced the highest IC_{50} values for both the inhibition of BSA denaturation (289.537 ± 2.75 μ g/mL) and proteinase activity (321.75 ± 6.87 μ g/mL). In contrast, wild TM had the lowest IC_{50} values for both the inhibition of BSA denaturation (84.62 ± 1.44 μ g/mL) and proteinase activity (138.46 ± 6.76 μ g/mL), indicating its high anti-inflammatory activity.

In vitro anticancer activity of sclerotium

The MTT assay against the A549 lung cancer cell line revealed that sclerotium produced by casing with CC:RS:RWS (1:1:1), CC and RS had high IC_{50} values for anti-lung cancer activity (598.37 ± 12.25 – 679.35 ± 10.25 μ g/mL), indicating low anti-lung cancer activity (Table 5). In contrast, casing with PS, PS:RS:RWS (1:1:1) and CC:PS (1:1) resulted in the sclerotium extract having high cytotoxic activity against the A549 lung cancer cell line (IC_{50} values 127.49 ± 10.30 – 174.75 ± 8.99 μ g/mL). The cytotoxicity analysis of the MCF-7 breast cancer cell line revealed that sclerotia obtained by casing with PS had the highest cytotoxic activity against breast cancer cells (IC_{50} value 367.54 ± 9.69 μ g/mL), as shown in Table 5. On the other hand, casing with CC:RS:RWS (1:1:1), CC and RS resulted in low cytotoxic activity against the breast cancer cell line (IC_{50} values 694.72 ± 12.77 – 896.32 ± 9.98 μ g/mL).

Table 4 Total protein, β -glucan and total triterpenoid contents of sclerotium extract obtained from casing of mushroom substrate with various materials

Casing material	Total proteins (mg BSA/g extract)	β -glucan (mg β -glucan /g extract)	Total triterpenoids (mg ursolic acid/g extract)
CC	1.86 ± 0.06^{ab}	49.00 ± 2.38^f	4.25 ± 0.20^e
PS	2.37 ± 0.07^{ab}	38.40 ± 1.50^j	6.46 ± 0.43^c
RS	1.42 ± 0.04^{ab}	56.10 ± 1.63^c	5.36 ± 0.23^d
CC:PS (1:1)	1.87 ± 0.05^{ab}	41.90 ± 0.91^i	8.28 ± 0.24^b
CC:RS (1:1)	2.57 ± 0.03^{ab}	50.00 ± 2.06^e	9.74 ± 0.12^a
CC:RWS (1:1)	1.23 ± 0.06^b	81.50 ± 1.96^a	4.27 ± 0.28^e
CC:PS:RS (1:1:1)	2.87 ± 0.08^a	45.40 ± 0.83^h	3.60 ± 0.25^e
CC:RS:RWS (1:1:1)	2.15 ± 0.07^{ab}	59.90 ± 1.68^b	4.24 ± 0.13^e
PS:RS:RWS (1:1:1)	2.25 ± 0.02^{ab}	46.20 ± 1.59^g	6.12 ± 0.25^{cd}
Wild TM	2.15 ± 0.04^{ab}	55.40 ± 1.81^d	5.37 ± 0.17^d

CC = coconut coir; RWS = rubber wood sawdust; RS = rice straw; PS = planting soil; TM = tiger milk mushroom sclerotium.

Values (mean $\pm$ SD; $n = 3$) within rows superscripted by different lowercase letters are significantly ($p < 0.05$) different.

Table 5 Concentration of extract scavenging 50% (IC₅₀) values of anti-inflammatory activity (BSA denaturation inhibition and proteinase inhibition) and anticancer activity (A549 anti-lung cancer and MCF-7 anti-breast cancer) of sclerotium extract obtained from casing of mushroom substrate with various materials

Casing material	BSA denaturation inhibition (IC ₅₀ ; µg/mL)	Proteinase inhibition (IC ₅₀ ; µg/mL)	A549 anticancer (IC ₅₀ ; µg/mL)	MCF-7 anticancer (IC ₅₀ ; µg/mL)
CC	187.47±3.12 ^f	169.65±3.30 ^f	662.51±14.64 ^b	749.70±14.26 ^b
PS	289.57±2.75 ^a	321.75±6.87 ^a	127.49±10.30 ⁱ	367.54±9.69 ⁱ
RS	249.58±8.04 ^c	276.56±7.00 ^b	679.35±10.25 ^a	896.32±9.98 ^a
CC:PS (1:1)	264.28±4.92 ^b	243.59±4.93 ^c	174.75±8.99 ^b	467.53±13.56 ^g
CC:RS (1:1)	276.39±2.08 ^b	288.61±7.10 ^b	438.58±12.17 ^f	669.86±11.04 ^d
CC:RWS (1:1)	269.53±4.40 ^b	274.86±7.50 ^b	461.73±11.46 ^c	625.10±7.95 ^{ef}
CC:PS:RS (1:1:1)	184.23±2.99 ^f	176.54±4.22 ^{ef}	521.16±16.01 ^d	617.84±14.30 ^f
CC:RS:RWS (1:1:1)	231.39±4.64 ^d	220.68±5.75 ^d	598.37±12.25 ^c	694.72±12.77 ^c
PS:RS:RWS (1:1:1)	205.48±2.93 ^c	188.96±6.07 ^c	158.37±14.91 ^h	395.38±14.67 ^h
Wild TM	84.62±1.44 ^g	138.46±6.76 ^e	693.58±18.46 ^a	884.74±13.72 ^a

CC = coconut coir; RWS = rubber wood sawdust; RS = rice straw; PS = planting soil; TM = tiger milk mushroom sclerotium.

Values (mean ± SD; *n* = 3) within rows superscripted by different lowercase letters are significantly (*p* < 0.05) different.

Pearson's correlation analysis

The statistical analysis in Table 6 indicated moderately positive correlations between the yield and IC₅₀ of DPPH (*r* = 0.695, *p* < 0.05). Low and moderate negative correlations were evident between the yield and TPC (*r* = -0.281), the yield and FRAP activity (*r* = -0.573) and the yield and TFC (*r* = -0.603). These results suggested that a high sclerotium yield resulted in less antioxidant activity. The BI had a positive correlation with TPC (*r* = 0.402), TFC (*r* = 0.416), total triterpenoid content (*r* = 0.666, *p* < 0.05), and FRAP activity (*r* = 0.633). The BI had a negative correlation with

the IC₅₀ of DPPH (*r* = -0.434), the IC₅₀ of A549 anti-lung cancer activity (*r* = -0.775, *p* < 0.05) and the IC₅₀ of MCF-7 anti-breast cancer activity (*r* = -0.640). The BI of sclerotium was related to the total phenolic, total flavonoid and total triterpenoid contents, as well as to the antioxidant and anticancer properties. The TPC and triterpenoid content had positive correlations with the IC₅₀ of BSA denaturation inhibition (*r* = 0.633 and *r* = 0.600, respectively) and the IC₅₀ of proteinase inhibition activity (*r* = 0.574 and *r* = 0.494), indicating the anti-inflammatory effects of the phenolic and triterpenoid compounds. In contrast, the TPC, TFC, total triterpenoid and β-glucan contents had negative correlations with

Table 6 Correlation between phenolic contents, antioxidant activity, anti-inflammatory activity, and anticancer activity of sclerotium obtained from casing of mushroom substrate with various materials

	Yield	BI	TPC	TFC	DPPH	FRAP	Protein	BGC	TP	BSA	Proteinase	MCF-7	A549
BI	-0.211												
TPC	-0.281	0.402											
TFC	-0.603	0.416	0.297										
DPPH	0.695***	-0.434	-0.252	-0.896*									
FRAP	-0.573	0.633	0.396	0.914*	-0.883**								
Protein	-0.261	0.113	-0.080	0.794***	-0.667***	0.625							
BGC	0.282	-0.550	0.149	-0.569	0.662***	-0.692**	-0.654						
TP	-0.246	0.666***	0.056	0.565	-0.489	0.606	0.195	-0.394					
BSA	-0.252	0.505	0.633	0.289	-0.130	0.427	-0.260	0.142	0.600				
Proteinase	-0.370	0.397	0.574	0.308	-0.143	0.453	-0.225	0.126	0.494*	0.960*			
MCF-7	0.325	-0.640	-0.637	-0.603***	0.734	-0.688**	-0.368	0.432	-0.304	-0.206	-0.099		
A549	0.412	-0.775***	-0.597	-0.605***	0.755**	-0.736**	-0.252	0.470	-0.511	-0.368	-0.255	0.956*	

BI = browning index; TPC = total phenolic content; TFC = total flavonoid content; DPPH = IC₅₀ of 2,2'-diphenyl-1-picrylhydrazyl activity; FRAP = Ferric ion reducing antioxidant power; BGC = β-glucan content; TP = total triterpenoid content; BSA = IC₅₀ of BSA denaturation inhibition; Proteinase = IC₅₀ of proteinase inhibition activity; A549 = IC₅₀ of A549 anti-lung cancer activity; MCF-7 = IC₅₀ of MCF-7 anti-breast cancer activity.

* = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001.

the IC₅₀ of A549 anti-lung cancer activity ($r = 0.597$, $r = 0.605$, $r = 0.511$ and $r = 0.470$, respectively) and the IC₅₀ of MCF-7 anti-breast cancer activity ($r = 0.637$, $r = 0.603$, $r = 0.304$ and $r = 0.432$, respectively). The anti-lung cancer and breast cancer activity of sclerotium is thought to be due to its TPC, TFC, total triterpenoid and β -glucan contents.

Principal component analysis

Fig. 1 shows the outcome of PCA, where the main principal components (PC1 and PC2) accounted for 92.55% of the overall variance in the data set. In this context, BSA denaturation inhibition, proteinase inhibition activity, A549 anti-lung cancer activity, MCF-7 anti-breast cancer activity and DPPH activity were observed in the negative region of both PC1 and PC2, according to the PCA biplot of the IC₅₀ value. The PCA biplot results showed that casing tiger milk mushroom substrate with PS, CC:PS (1:1), CC:RS (1:1) or PS:RS:RWS (1:1:1) increased the sclerotium BI values, TPC, TFC, total triterpenoid content, total protein content, antioxidant activity (DPPH and FRAP) and anticancer activity (A549 and MCF-7 cell lines). The sclerotium yield, β -glucan content and anti-inflammatory activity (BSA denaturation inhibition and proteinase inhibition activity) increased with casing using CC, RS, CC:RWS (1:1), CC:RS:RWS (1:1:1) and wild TM.

Semi-quantitative β -glucan analysis by a combination of synchrotron radiation-based Fourier transform infrared spectroscopy and principal component analysis

Fungal fruiting bodies and mycelia contain two forms of glucans: α -D-glucans and β -D-glucans. A chemical molecule's vibrational spectrum is one of its unique characteristics. SR- FTIR microspectroscopy is an effective and sensitive technique for determining glycosidic linkage positions and the anomeric configuration in glucans. Polysaccharide FTIR spectral regions can be characterized as: 3,000–2,500 cm⁻¹ is assigned to symmetrical and asymmetrical stretching vibrations of backbone CH and CH₂, 1,800–1,500 cm⁻¹ is double bond stretching of COO⁻, 1,500–1,200 cm⁻¹ is considered to represent CH₂ and the numerous C-OH deformations and 1,200–800 cm⁻¹ is related to glycosidic linkage formation in polysaccharides (Hong et al., 2021). Two types of glucans can be distinguished based on absorption fingerprint bands at 1,155 and 1,023 cm⁻¹, which are responsible for vibration of C-O-C and C-OH found in α -D-glucans (Choong et al., 2014), whereas peaks at 1,160, 1,078 (C-O stretching vibration in a pyranose ring C-O-C) and 1,044 cm⁻¹ peaks are represent β -D-glucans (Synytsya and Novak, 2014; Usuldin et al., 2020).

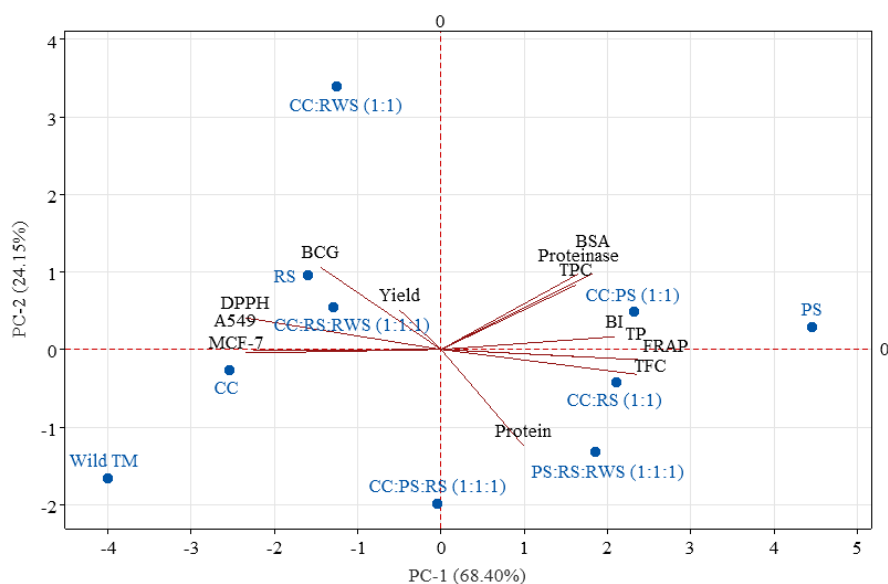


Fig. 1 Principal component (PC) analysis biplot of physio-biochemical properties of sclerotium obtained from the casing of mushroom substrate with various materials, where CC = coconut coir; RWS = rubber wood sawdust; RS = rice straw; PS = planting soil; TM = tiger milk mushroom sclerotium; BI = browning index; TPC = total phenolic content; TFC = total flavonoid content; DPPH = concentration of extract scavenging 50% (IC₅₀) of 2,2'-diphenyl-1-picrylhydrazyl activity; FRAP = Ferric ion reducing antioxidant power; BGC = β -glucan content; TP = total triterpenoid content; BSA = IC₅₀ of BSA denaturation inhibition; Proteinase = IC₅₀ of proteinase inhibition activity; A549 = IC₅₀ of A549 anti-lung cancer activity; MCF-7 = IC₅₀ of MCF-7 anti-breast cancer activity.

SR-FTIR microspectroscopy and PCA were applied to observe glucans produced in *L. rhinocerus* using spectral regions between 3,000–2,800 cm^{-1} and 1,750–950 cm^{-1} , respectively, as X-variables (Figs. 2A and 2B). Although all samples had slightly different lipid and amide I absorption regions, the polysaccharide region was notable. PC-1 separated analytical samples into two groups with a high score level of 72% by loadings at 1,157 and 1,080 cm^{-1} . This causes samples to be grouped in (-)PC-1, such as RS, CC:RS:RWS (1:1:1), CC:PS:RS (1:1:1), and PS:RS:RWS (1:1:1), while the remainder were in (+)PC-1 and the loadings of PC-2 indicated all samples were different from each other at absorption peaks 1,162 cm^{-1} , 1,072 cm^{-1} and 1,037 cm^{-1} . Spectral averaging with subsequent secondary derivatizing of all sample groups was performed for further comparison, as shown in Fig. 2C. Notably, there were infrared absorption fingerprints of both α - and β -glucans at around 1,160 and 1,040 cm^{-1} , in which those belonging to β -glucans were slightly shifted to higher vibrational wavenumbers. Thus, these two peaks could be combinations of both α - and β -glucans produced by *L. rhinocerus*. Therefore, the absorption

peak at 1,079 cm^{-1} was the main characteristic of β -glucans (Patel et al., 2020) in these samples that was used for relatively close comparison (Fig. 2D). It was clear that all the samples contained β -glucans. The first four samples with the highest β -glucan contents were CC:PS:RS (1:1:1), CC:RS:RWS (1:1:1), PS:RS:RWS (1:1:1) and CC:RWS (1:1).

Discussion

The influence of the casing material on the yield and biological activity of tiger milk mushroom sclerotium was studied, and it was discovered that casing mushroom substrates with different casing materials resulted in different yields and levels of biological activity. The difference in the casing material may have been dependent on water absorption, air ventilation and chemical composition, such as the amounts of cellulose, hemicellulose and lignin. The sclerotium weight was the highest when the casing substrate contained coconut coir (CC) (96.53 ± 3.03 g) and was the lowest with wild TM (30.25 ± 1.26 g).

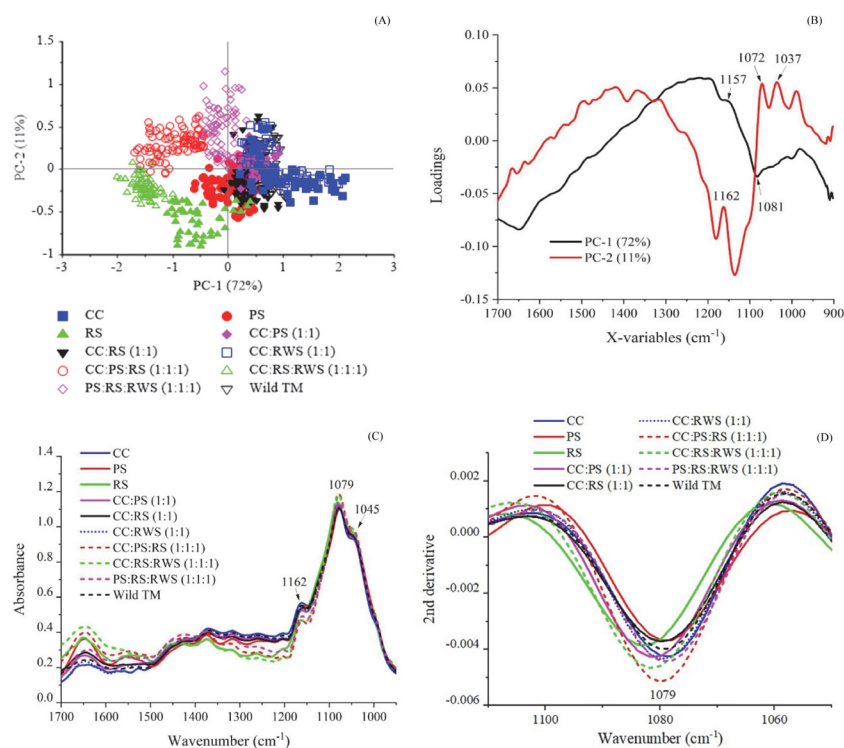


Fig. 2 (A) Principal component (PC) analysis score plot of 1700–900 cm^{-1} synchrotron radiation-based Fourier transform infrared spectroscopy (SR-FTIR) spectral range measured from *L. rhinocerus* sclerotium grown in different casings correlated to loadings plot; (B) major X-variable differences at 1,157 and 1,080 cm^{-1} in PC-1 and at 1,162, 1,072 and 1,037 cm^{-1} in PC-2; (C) three main glucan peaks at 1,162, 1,079, and 1,045 cm^{-1} located in polysaccharide region based on SR-FTIR spectra; (D) Relative quantitative analysis based on overlaying 2nd derivative spectra of all samples in range 1,200–1,050 cm^{-1} , where CC = coconut coir; RWS = rubber wood sawdust; RS = rice straw; PS = planting soil; TM = tiger milk mushroom sclerotium

The highest sclerotium weight with the coconut coir was probably due to its ability to hold water (78.77%) and its porosity (89.70%) which allowed for good ventilation. In contrast, planting soil had lower values of 43.37% for water holding capacity and 59.84% for porosity (Rangel et al., 2006). Furthermore, the coconut fiber was composed mainly of cellulose, hemicellulose and lignin that provided essential carbon (lignocellulosic substrate). Rice straw is also a major carbon source (lignocellulosic substrate), similar to coconut coir; however, it only holds 20% of the water (Rashad and Hussien, 2013). As a result, the casing material had less moisture, reducing the sclerotium weight. This was consistent with Dong et al. (2022), who reported that *Sclerotium rolfsii* sclerotium did not grow in an environment with a low moisture content (such as 0.20%) rather requiring a level in the range 40–100%. The porosity and water-holding capacity of soil were both moderate. Soil contains minerals, organic substances, microbes, air, water and other components; consequently, the amount of carbon is not as great, though the soil can provide moisture and transfers minerals from the soil to the sclerotium. Ramakrishna and Ravishankar (2011) reported that soil played an important role in the production of primary and secondary metabolites by plants. Muscolo et al. (2019) noted that the soil chemistry and biochemical properties influenced antioxidant performance, as well as carotenoid (carotenoids) and glucosinolates production in *Brassica rupestris* Raf, a vegetable in the kale and cabbage family. Consequently, the soil casing resulted in a low sclerotium weight (38.53 ± 1.92 g) due to its lack of a major carbon source, although there were abundant levels of phenolic acids and flavonoids. Furthermore, activation of the soil chemistry and biochemical properties resulted in high antioxidant activity and anti-lung cancer and breast-cancer activities. Cao et al. (2021) studied the sclerotium formation of fuling mushrooms (*Wolfiporia cocos*) at the gene level. They found that sclerotium synthesis involved many genes, such as genes for polysaccharide synthesis (α -1,3-glucan synthase and β -1,3-glucan synthase), triterpenoid synthesis (cytochrome P450 group) and also for polysaccharide degradation (CAZymes group: carbohydrate-active enzymes). Kumar and Gopal (2015) reported that microorganisms were essential in agricultural residue decomposition, producing high-value metabolites and nutrient flow that were beneficial to crops. Jarial et al. (2005) found that mushrooms would not fruit if buried in sterile casing material. Cho et al. (2008) found high levels of *Flavobacterium* sp. in sterile casing material, while *Pseudomonas* was abundant in non-sterile casing material. They also discovered that *Pseudomonas* sp.

stimulated the fruiting of the Hungarian oyster mushroom (*Pleurotus ostreatus*) in soil cultivation and increased mushroom yield. As a result, microorganisms in the casing material may influence the sclerotium growth of tiger milk mushrooms. Infrared (IR) is one of the most widely used methods to identify chemical compounds and to elucidate their chemical structure based on the fingerprinting features of their IR profile (Zhang et al., 2023) and was applied in the current study to identify β -glucan in the *L. rhinocerus* sclerotium using the SR-FTIR technique, with these results and those from the spectrophotometric techniques showing similar trends. The casing mushroom substrate with CC:RWS (1:1) had the highest amount of β -glucan, while the casing mushroom substrate with PS had the least amount of β -glucan (Fig. 2 and Table 4). However, using the SR-FTIR technique in this experiment provided only a preliminary analysis because it is a fast and non-destructive method (Wang et al., 2015). Confirmation analysis is required using more complex techniques such as nuclear magnetic resonance spectroscopy. In addition, more research is needed to examine the enzyme activity that occurs during the casing of mushroom substrates involving various materials to determine which enzymes are involved and to verify the potential of the microorganisms in these casing materials to produce tiger milk mushroom sclerotium.

Conclusion

Different casing materials resulted in different growth characteristics and biological activities of tiger milk mushroom sclerotium. Casing mushroom substrate with CC produced the highest sclerotium weight while casing with CC:RWS (1:1) produced the highest β -glucan content. Casing with CC:RS (1:1) contained the most triterpenoids. Casing with PS provided high levels of phenolic, flavonoid and antioxidant activity, as well as anti-lung cancer and breast cancer properties, whereas the wild TM had a high level of anti-inflammatory activity. As a result, casing materials should be chosen based on the type of bioactive component or biological activity desired.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254. doi.org/10.1016/0003-2697(76)90527-3
- Cao, S., Yang, Y., Bi, G., et al. 2021. Genomic and transcriptomic insight of giant sclerotium formation of wood-decay fungi. *Front Microbiol.* 12: 746121. doi.org/10.3389/fmicb.2021.746121
- Cho, Y.S., Weon, H.Y., Joh, J.H., Lim, J.H., Kim, K.Y., Son, E.S., Lee, C.S., Cho, B.G. 2008. Effect of casing layer on growth promotion of the edible mushroom *Pleurotus ostreatus*. *Mycobiology* 36: 40–44.
- Choong, Y.-K., Xu, C.-H., Lan, J., Chen, X.-D., Jamal, J.A. 2014. Identification of geographical origin of *Lignosus* samples using Fourier transform infrared and two-dimensional infrared correlation spectroscopy. *J. Mol. Struct.* 1069: 188–195. doi.org/10.1016/j.molstruc.2014.04.001
- Çoklar, H., Akbulut, M. 2017. Effect of sun, oven and freeze-drying on anthocyanins, phenolic compounds and antioxidant activity of black grape (Eksikara) (*Vitis vinifera* L.). *S. Afr. J. Enol. Vitic.* 38: 264–272. doi.org/10.21548/38-2-2127
- Dincheva, I., Badjakov, I., Galunska, B. 2023. New insights into the research of bioactive compounds from plant origins with nutraceutical and pharmaceutical potential. *Plants* 12: 258. doi.org/10.3390/plants12020258
- Dong, X.L., Gao, C.Y., Li, P.L., Lian, S., Zhou, S.Y., Li, B.H. 2022. Effects of temperature, moisture, substrates and soil coverage on sclerotium germination and hyphal growth of Southern blight of apple in China. *Eur. J. Plant Pathol.* 162: 477–487. doi.org/10.1007/s10658-021-02418-1
- Gunathilake, K.D.P.P., Ranaweera, K.K.D.S., Rupasinghe, H.P.V. 2018. Influence of boiling, steaming and frying of selected leafy vegetables on the in vitro anti-inflammation associated biological activities. *Plants (Basel)*. 7: 22. doi.org/10.3390/plants7010022
- Gupta, A., Maheta, P., Chauhan, R., Pandey, S., Yadav, J.S., Shah, S. 2018. Simultaneous quantification of bioactive triterpene acids (ursolic acid and oleanolic acid) in different extracts of *Eucalyptus globulus* (L.) by HPTLC method. *Phcog J.* 10: 179–185. doi.org/10.5530/pj.2018.1.30
- Hassen, G., Belete, G., Carrera, K.G., et al. 2022. Clinical implications of herbal supplements in conventional medical practice: A US perspective. *Cureus* 14: e26893. doi.org/10.7759/cureus.26893
- Hong, T., Yin, J.Y., Nie, S.P., Xie, M.Y. 2021. Applications of infrared spectroscopy in polysaccharide structural analysis: Progress, challenge and perspective. *Food Chem. X.* 12: 100168. doi.org/10.1016/j.fochx.2021.100168
- Jamil, N.A.M., Rashid, N.M.N., Hamid, M.H.A., Rahmad, N., Al-Obaidi, J.R. 2018. Comparative nutritional and mycochemical contents, biological activities and LC/MS screening of tuber from new recipe cultivation technique with wild type tuber of tiger's milk mushroom of species *Lignosus rhinoceros*. *World J. Microbiol. Biotechnol.* 34: 1. doi.org/10.1007/s11274-017-2385-4
- Jarial, R.S., Shandilya, T.R., Jarial, K. 2005. Casing in mushroom beds—A review. *Agric. Rev.* 26: 261–271.
- Kumar, B.L., Gopal, D.V.R.S. 2015. Effective role of indigenous microorganisms for sustainable environment. *3Biotech* 5: 867–876. doi.org/10.1007/s13205-015-0293-6
- Lau, B.F., Abdullah, N., Aminudin, N. 2013. Chemical composition of the tiger's milk mushroom, *Lignosus rhinocerotis* (Cooke) Ryvarden, from different developmental stages. *J. Agric. Food. Chem.* 61: 4890–4897. doi: 10.1021/jf4002507
- Lau, B.F., Abdullah, N., Aminudin, N., Lee, H.B., Tan, P.J. 2015. Ethnomedicinal uses, pharmacological activities, and cultivation of *Lignosus* spp. (tigers milk mushrooms) in Malaysia—A review. *J. Ethnopharmacol.* 169: 441–458. doi.org/10.1016/j.jep.2015.04.042
- Lysakowska, P., Sobota, A., Wirkijowska, A. 2023. Medicinal mushrooms: Their bioactive components, nutritional value and application in functional food production—A review. *Mol.* 28: 5393. doi.org/10.3390/molecules28145393
- McGee, C.F. 2018. Microbial ecology of the *Agaricus bisporus* mushroom cropping process. *Appl. Microbiol. Biotechnol.* 102: 1075–1083. doi.org/10.1007/s00253-017-8683-9
- Muscolo, A., Sidari, M., Settineri, G., Papalia, T., Mallamaci, C., Attinà, E. 2019. Influence of soil properties on bioactive compounds and antioxidant capacity of *Brassica rupestris* Raf. *J. Soil Sci. Plant Nutr.* 19: 808–815. doi.org/10.1007/s42729-019-00080-5
- Nallathamby, N., Phan, C.W., Seow, S.L., Baskaran, A., Lakshmanan, H., Abd Malek, S.N., Sabaratnam, V. 2018. A status review of the bioactive activities of tiger milk mushroom *Lignosus rhinocerotis* (Cooke) Ryvarden. *Front. Pharmacol.* 8: 998. doi.org/10.3389/fphar.2017.00998.
- Navarro, M.J., Gea, F.J., Pardo-Giménez, A., Martínez, A., Raz, D., Levanon, D., Danay, O. 2020. Agronomical valuation of a drip irrigation system in a commercial mushroom farm. *Sci. Hortic.* 265: 109234. doi.org/10.1016/j.scienta.2020.109234
- Norra, I., Aminah, A., Suri, R. 2016. Effects of drying methods, solvent extraction and particle size of Malaysian brown seaweed, *Sargassum* sp. on the total phenolic and free radical scavenging activity. *Int. Food Res. J.* 23: 1558–1563.
- Okhuoya, J.A., Okogbo, F.O. 1991. Cultivation of *Pleurotus tuber-regium* (Fr) Sing on various farm wastes. *Proc. Okla. Acad. Sci.* 71: 1–3.
- Pardo-Giménez, A., Pardo, J.E., Zied, D.C. 2017. Casing materials and techniques in *Agaricus bisporus* cultivation. In: Diego, C.Z. Pardo-Giménez, A. (Eds.). *Edible and medicinal mushrooms: Technology and applications*. John Wiley & Sons Inc. Chichester, UK, pp. 149–174. doi.org/10.1002/9781119149446.ch7

- Pardo-Giménez, A., Jaime, C., Pardo, J.E., Alvarez-Orti, M., Zied, D.C. 2020. Influence of substrate density and cropping conditions on the cultivation of sun mushroom. *Span. J. Agric. Res.* 18: e0902. doi.org/10.5424/sjar/2020182-16037
- Ramakrishna, A., Ravishankar, G.A. 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav.* 6: 1720–1731.
- Rangel, J.I., Leal, H., Palacios-Mayorga, S., Sánchez, S., Ramirez, R., Méndez-García, T. 2006. Coconut fiber as casing material for mushroom production. *Terra Latinoam.* 24: 207–213.
- Rashad, R.T., Hussien, R.A. 2013. Studying the use of cellulose, silica and lignin extracted from rice straw as sandy soil conditioners. *Int. J. Agron. Agri. Res.* 3: 21–35.
- Roshanak, S., Rahimmalek, M., Goli, S.A.H. 2016. Evaluation of seven different drying treatments in respect to total flavonoid, phenolic, vitamin C content, chlorophyll, antioxidant activity and color of green tea (*Camellia sinensis* or *C. assamica*) leaves. *J. Food Sci. Technol.* 53: 721–729. doi.org/10.1007/s13197-015-2030-x
- Rossiana, N., Madihah, Nur, A.A., Mayawatie, B., Andayaningsih, P. 2018. Cytotoxicity assay of ethyl acetate extract shimeji (*Lyophyllum shimeji* (Kawam.) Hongo) and white oyster mushroom (*Pleurotus ostreatus* Jacq.) against HCT-116 cell line. *IOP Conf. Ser. Earth Environ. Sci.* 197: 012007. doi.org/10.1088/1755-1315/197/1/012007
- Shan, O.E., Zzaman, W., Yang, T.A. 2016. Physical changes of robusta coffee bean during superheated steam roasting. *Pertanika J. Trop. Agric. Sci.* 39: 311–320.
- Synytsya, A., Novak, M. 2014. Structural analysis of glucans. *Ann. Transl. Med.* 2: 17. doi:10.3978/j.issn.2305-5839.2014.02.07
- Usulidin, S.R.A., Mahmud, N., Ilham, Z., Khairul Ikram, N.K., Ahmad, R., Wan-Mohtar, W.A.A.Q.I. 2020. In-depth spectral characterization of antioxidative (1,3)- β -D-glucan from the mycelium of an identified tiger milk mushroom *Lignosus rhinocerus* strain ABI in a stirred-tank bioreactor. *Biocatal. Agric. Biotechnol.* 23: 101455. doi.org/10.1016/j.bcab.2019.101455
- Wang, M., Lu, X., Yin, X., et al. 2015. Synchrotron radiation-based Fourier-transform infrared spectromicroscopy for characterization of the protein/peptide distribution in single microspheres. *Acta Pharm. Sin. B.* 5: 270–276. doi.org/10.1016/j.apsb.2015.03.008
- Williams, L.A.D., O'Connar, A., Latore, L., Dennis, O., Ringer, S., Whittaker, J.A., Conrad, J., Vogler, B., Rosner, H., Kraus, W. 2008. The *in vitro* anti-denaturation effects induced by natural products and non-steroidal compounds in heat treated (immunogenic) bovine serum albumin is proposed as a screening assay for the detection of anti-inflammatory compounds, without the use of animals, in the early stages of the drug discovery process. *West Indian Med. J.* 57: 327–331.
- Wu, B., Wang, Y., Zeng, L., et al. 2022. Comparative analysis of the metabolites and biological activity of cultivated and wild *Lignosus rhinocerotis*. *Biomed Res. Int.* 2022: 5752575. doi.org/10.1155/2022/5752575
- Zhang, S., Qi, Y., Tan, S.P.H., Bi, R., Olivo, M. 2023. Molecular fingerprint detection using raman and infrared spectroscopy technologies for cancer detection: A progress review. *Biosens.* 13: 557. doi.org/10.3390/bios13050557