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Biological Activities of the Mycelial Crude and β-Glucan Extracts of *Auricularia cornea*

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Abstract : Selected wild jelly ear mushrooms were screened and identified by analyzing their mycelial crude and beta-glucan extracts. Fresh mushroom samples were collected from Dong Yai Forest, Amnat Charoen Province, Thailand. Auricularia specimens were isolated and identified as saprobic fungi for use in this study. The fungi were inoculated onto agar to permit fungal spore shooting, followed by cultivation in an enriched liquid medium to obtain fungal mycelia. The mycelia were then subjected to extraction and analyses of their contents and biological activities. All saprobic fungi grew well on both agar and liquid medium. They clearly contained different amounts of beta-glucans and phenolic compounds. Among the mushrooms, the mycelia of *Auricularia cornea* (RSPG00622, jelly ear fungus) displayed the highest beta-glucan content and strongest anti-oxidant activities. The extracted beta-glucans also exerted immunomodulating effects on THP-1monocytes exposed to *Escherichia coli* lipopolysaccharide.
Keywords : beta-glucans, fungal mycelium, immunomodulating agent, *Auricularia cornea.*

Introduction

At present, food provides humans with necessary nutrients and assists in maintaining optimal physical and psychological conditions. Food can also be used in disease prophylaxis. This idea has prompted the identification of functional foods that can influence human health. Functional foods include inter alia fungi, which represent an abounding source of many bioactive compounds such as β-glucans, polyphenols (including flavonoids), and ascorbic acid[1].

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β-Glucans are polysaccharides that feature D-glucoses connected by 1,3-β-glycosidic bonds. They are commonly found in the cell walls of yeasts, bacteria, fungi, algae, and grains. Various studies have revealed the beneficial effects of β-glucans in humans. The structures of β-glucans (including the tertiary structure), as well as their water solubility, molecular weight, and chain length, are varied; these differences influence their bioactivities. Owing to their benefits, the Food and Drug Administration has allowed their use in food products, but the agency requires labeling detailing their health benefits[2].β-Glucans have extensively been incorporated into human supplements to help reduce cholesterol and sugar levels in the blood[3]and improve skin health[4].Moreover, β-glucans have also been applied in animal feeds to stimulate growth and development[5], modulate the immune system[6], balance the gastrointestinal flora[7], and reduce odor and the release of ammonia from feces[8].

The named edible mushrooms, including *Schizophyllum*, *Lentinus*, and *Pleurotus* spp., have been widely described as potential β-glucan producers. Thus, the application of fungus-derived β-glucans in healthcare and cosmetics is of interest. The extraction of β-glucans requires special attention to obtain consistent raw material. The extraction of β-glucans from saprobic fungi can be performed using the fruiting body, mycelium, and sclerotium, which represent all stages of the macrofungus life cycle. However, the fungal mycelium is recommended as the source for β-glucan extraction in the event that a fungal strain of interest cannot be cultured from the fruiting body or if a shorter working period is required.

In addition to β-glucans, many mushroom crude extracts contain anti-aging anti-oxidants; possess anti-microbial, anti-cancer, and anti-inflammatory properties; promote immune system functioning[9]; and protect against coronary artery disease and diabetes mellitus[10].We thus studied the anti-oxidant and enzyme-inhibiting activities and determined the total phenolic content of mushrooms within an explored area. This information will be further used in the development of cosmetics and functional food supplements that will contribute to understanding the benefits of mushrooms.

Materials and Methods

Specimen and morphological observation

Jelly ear mushrooms were collected and isolated from Dong Yai Community Forest in Amnart Charoen Province, Thailand. *Auricularia cornea* (RSPG622) specimens were collected on May 30, 2017, whereas *Auricularia* specimens were collected on June 21, 2016. The expedition was led by the National Biotechnology for Development Agency or NSTDA along with the Biodiversity-Based Economy Development Office (Public Organization) and coordinated by the Plant Genetic Conservation Project Under The Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG Project). Mushrooms were photographed, and necessary information from the field was annotated. Specimens were dried at 45°C using a food dehydrator and preserved at BIOTEC Bangkok Herbarium (coded BBH42456)[11]. Macroscopic and microscopic features were observed, measured, and noted using dried specimens at the Mushroom Laboratory, National Biobank of Thailand[12, 13].

Molecular approaches

Genomic DNA was extracted from both dried specimens and culture plates to confirm the identity of mushrooms. The protocol and reagents obtained from the magLEAD Consumable kit were used for DNA extraction and purification[14]. The primer pair ITSF1 (CTTGGTCATTTAGAGGAAGTAA) and ITSF4 (TCCTCCGCTTATTGATATGC) was used to amplify the internal transcribed spacer (ITS) regions. Polymerase chain reaction (PCR) products were checked using 1% agarose gel electrophoresis. All successful PCR products were sequenced using an ABI 3500 Series Genetic Analyzer and Sanger sequencer (Thermo Fisher Scientific) at NBT.

Phylogenetic analyses

The quality of obtained sequence was first checked and manually edited using MEGA-X. The region of alignment region was generated and manually adjusted using MEGA-X[15] followed by alignment using the MUSCLE Multiple aliment program[16]. The distance matrices were clustered using the UPGMA strategy. The DNA sequences of 11 species of *Auricularia* retrieved from GenBank were included in this study. *Exidia recisa*

was used as the outgroup. For the phylogenetic algorithm, the maximum likelihood (ML) was used to infer the evolutionary relationships of all taxa. RAxML 8.2.10[17] was used to generate the best ML trees. All phylogenetic tools were used in the CIPRES Science Gateway[18].

Cultivation of fungal mycelia

All fresh mushroom samples of saprobic fungi collected from Dong Yai Forest, Amnat Charoen Province, Thailand were inoculated on potato dextrose agar (PDA; 200 g of potato, 20 g of glucose, 20 g of agar, and 1000 ml of water) to permit fungal spore shooting. The isolated fungi were re-streaked on PDA and incubated at room temperature (28 ± 2°C) for 5–8 days or until the fungal mycelia covered the entire surface of the agar. A cork borer was used to cut the growing edge of the mycelia for further cultivation in potato dextrose broth (PDB; 200 g of potato, 20 g of glucose, and 1000 ml of water) to obtain fungal cells, which then were subjected to further study.

Mycelial crude extraction

The fungal mycelia harvested from the PDB cultures were washed with distilled water and oven-dried at 45°C for 16 h. Extraction was then performed as described by Wu and Hansen[19]. Specifically, extraction was performed using two types of solvents, namely 80% methanol and deionized water at a solid:liquid ratio of 0.02:1, at 80°C for 30 min. Subsequently, the mixture was centrifuged at 6000 rpm for 15 min, and the obtained supernatant and pellets were separated and stored at 4°C for further analysis.

Mycelial β-glucan extraction (adapted from Borchani et al.[20])

The fungal cells from the aforementioned broth cultures were collected via centrifugation. Water was added to obtain a cells-to-water ratio of 1:3, and the mixture was incubated at −20°C for 72 h. The cells were then separated by centrifugation. Water was added to the cells (cells:water = 1:1), and the cells were boiled at 100°C for 10 min and centrifuged. The cells were re-suspended in water (cells:water = 1:1). Protease at a concentration of 0.3% was added, and the mixture was incubated at 55°C for 5 h. The precipitate was separated by centrifugation. Ethanol was added at a ratio of 1:2 (precipitate:ethanol) and left for 24 h. The resulting precipitate (β-glucan extract) was collected by centrifugation and oven-dried at 70°C.

Total phenolic content analysis

The analysis was performed by modifying the Folin–Ciocalteu colorimetric method as described by Wolfe et al[21]. Each crude extract of the 10 mushroom species that contained the highest β-glucan contents in the present study was added at a volume of 125 µl to a test tube containing 500 µl of distilled water. Folin–Ciocalteu reagent was added at a volume of 125 µl and left for 6 min. Then, 1250 µl of 7% sodium carbonate and 1000 µl of distilled water were added. After 90 min at room temperature, the absorbance at 760 nm was measured, compared with the standard curve of gallic acid, and calculated as mg gallic acid equivalents (GAE)/g sample.

β-Glucan extract characterization

A quantitative analysis of β-glucan extracts was performed using a Megazyme Enzymatic Mushroom and Yeast Beta-Glucan Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland). To analyze the total glucan level, approximately 90 mg of mushroom mycelia were placed into a culture tube, and approximately 2 ml of 12 M sulfuric acid were then added. The tube cap was replaced, and the tube contents were mixed using a vortex mixer. The tube was then immersed in an ice-water bath for approximately 2 h. The contents were mixed using a vortex mixer, 4 ml of distilled water were then added, and the contents were mixed again for approximately 10 s. After that, an additional 6 ml of distilled water were added and mixed well using a vortex mixer. The cap was then loosened, and the tube was placed in a water bath at approximately 100°C for 5 min. The cap was tightened, and the tube was incubated for 2 h. It was then removed from the water bath and left to cool to room temperature. The entire suspension was transferred to a 100-ml volumetric flask and rinsed with 200 mM sodium acetate buffer (pH 5.0). Six milliliters of 10 M KOH were added, and the total volume adjusted to 100 ml using sodium acetate buffer and mixed using a vortex mixer. Following centrifugation, the supernatant was collected to determine the level of D-glucose. Specifically, 0.1 ml of supernatant were added to a culture tube, and 0.1 ml of exo-1,3-β-glucanase + β-glucosidase were added. The mixture was mixed well

using a vortex mixer. The tube was incubated in a water bath at 40°C for 60 min. Three milliliters of glucose oxidase/peroxidase were added, and the tube was further incubated for 20 min. The absorbance at 510 nm was determined. Regarding the determination of α-glucans, approximately 100 mg of mushroom mycelia were placed in a culture tube, and 2 ml of 2 M KOH were added. The mixture was mixed by shaking and placed in an ice-water bath for approximately 20 min. Eight milliliters of 1.2 M sodium acetate buffer (pH 3.8) were subsequently added to the tube, which was mixed via shaking. Amyloglucosidase (1630 U/ml) + invertase (500 U/ml) in a volume of 0.2 ml were added and mixed well. The tube was incubated in a water bath at 40°C for 30 min with periodic shaking. Following centrifugation, 0.1 ml of supernatant were transferred to another culture tube. GOPOD (0.1 ml) was added, and the tube was incubated in a water bath for 20 min. The absorbance of the tube contents at 510 nm was determined. β-Glucan contents were calculated using the following equation:

β-Glucans (% w/w) = total glucans (% w/w) − α-glucans.

The contents of moisture, protein, lipid, ash, fiber, and carbohydrates were analyzed according to the Association of Official Agricultural Chemists (AOAC, 2000). The anti-oxidant activities of β-glucan extract were assayed using the 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay[22]and ferric reducing anti-oxidant power (FRAP) assay[23]. The toxicity and anti-inflammatory properties of the extract were tested as described by Chanput et al[24].

Analysis of anti-oxidant properties

The mycelial crude and β-glucan extracts were tested for their anti-oxidant effects using various methods with the anti-oxidants ascorbic acid (vitamin C), α-tocopherol (vitamin E), and butylated hydroxytoluene as control references. The crude extract was analyzed using DPPH, FRAP, and nitric oxide methods. Concerning the β-glucan extract, 10 ml of distilled water were added to 0.1 g of the extract. The mixture was autoclaved at 121°C for 15 min and then centrifuged at 6000 rpm for 10 min. The obtained supernatant was tested for anti-oxidant properties using DPPH and FRAP methods.

DPPH radical scavenging assay

One milliliter of 0.1 mM DPPH in ethanol was mixed with 1 ml of 10 mg/ml crude or β-glucan extract. The mixture was left in the dark for 30 min, and then its absorbance at 517 nm was measured using a UV-visible spectrophotometer (Shimadzu UV-160A, Japan). The test was performed in triplicate. The measured absorbance was calculated to yield the percent radical scavenging activity as follows:

% DPPH radical scavenging activity = [(A0 − A1)/A0] × 100,

where A0 is the absorbance of the control sample and A1 is the absorbance of the test sample.

FRAP assay

FRAP reagent was prepared by mixing 10 µl of 300 mM acetate buffer with 1.0 ml of 20 µM $FeCl_3 \cdot 6H_2O$, 1.0 ml of 40 µM 2,4,6-tris(2-pyridyl)-s-triazine in HCl, and 1.2 ml of water. Sixty microliters of 10 mg/ml β-glucan extract or 1 mg/ml crude extract in 95% ethanol were added to a test tube, followed by 180 µl of water and 1.8 ml of FRAP reagent. The mixture was left at 37°C for 4 min, and the absorbance at 595 nm was then measured using a UV-visible spectrophotometer. The anti-oxidant effects were determined by the ability of the extract to provide free electrons (as a reducing agent). The presence of this property was demonstrated by its absorbance in comparison with that of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) as the standard in the range of 0.10–1.00 mmol/L and reported as µmol of Fe(II)/g test sample. The test was performed in triplicate.

Nitric oxide scavenging assay

This assay was performed as described by Sumanont et al[25]. Specifically, 100 µl of mycelial crude extract at various concentrations were added to 500 ml of 100 mM sodium nitroprusside, which is a nitric oxide generator. The mixture was left at 25°C for 180 min. Five hundred microliters of 1% sulfanilamide solution were then added, and the mixture was left in the dark for 10 min. Five hundred microliters of 0.1% naphthylethylenediamine dihydrochloride solution were then added, and the mixture was left in the dark again for 10 min. Any tubes containing large amounts of anti-oxidants would reduce the nitric oxide in the tubes,

resulting in fading of the color. The color was measured using a spectrophotometer at 540 nm. The results are reported as percent inhibition using the following equation:

Anti-nitric oxide oxidant (%) = [(A0 −A1)/A0] × 100,

where A0 is the absorbance of the control sample and A1 is the absorbance of the test sample.

Cytotoxicity and immunostimulation tests

Lipopolysaccharide (LPS) contamination detection

The β-glucan extract was checked for LPS contamination using a GenScript test kit (USA).

THP-1 cell culture

THP-1 monocytes (American Type Culture Collection, USA) were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin in a CO_2 incubator under 5% CO_2 at 37°C.

MTT assay of monocyte proliferation

THP-1 monocytes were incubated with the β-glucan extract at concentrations of 0, 10, 25, 50, 100, 250, 500, and 1000 μg/ml in 96-well plates for 24 h. The medium was removed, and following the addition of MTT, the plate was incubated for 2 h. Dimethyl sulfoxide:ethanol (1:1) was then added, and the plates gently shaken horizontally for 5 min before the measurement of absorbance at 570 nm. The value obtained from the cells without the test substance was used as a control, and the values after incubation with different concentrations of the test substance were compared with the control value.

Anti-inflammatory properties of the -glucan extract in THP-1 monocytes

- Resting state

THP-1 monocytes in exponential growth not exceeding the 25th passage were incubated with the β-glucan extract at concentrations of 0, 2, 10, 25, and 50 μg/ml for 3 h. The expression of genes related to inflammation, namely the pro-inflammatory genes interleukin (IL)-8, IL-1β, and tumor necrosis factor (TNF-α) and the anti-inflammatory gene IL-10, was determined using real-time PCR with glyceraldehyde 3-phosphate dehydrogenase as a reference gene.

- Inflamed state

THP-1 monocytes in exponential growth not exceeding the 25th passage were stimulated with 100 ng/ml Escherichia coli endotoxin (LPS) for 3 h before incubation and the determination of gene expression as described previously.

Analysis of tyrosinase inhibition

The analysis of the tyrosinase-inhibiting activity of the extract was performed by modifying the methods of Kubo et al[26] and Saewan et al[27]. The crude extract was prepared at various concentrations. One milliliter of 2.5 mM L-dopa was mixed with 1.8 ml of 0.1 M sodium phosphate buffer (pH 6.8), and the mixture was incubated for 10 min. Next, 0.1 ml of the test substance and 0.1 ml of tyrosinase (138 U) were added, and the mixture was incubated again for 10 min. The reaction was detected by measuring dopachrome via its absorbance at 475 nm using a spectrophotometer. The measured values were used in determining the percent tyrosinase inhibition using the following equation:

% tyrosinase inhibition = [A − (B − C)]/A × 100

where A is the absorbance of the control sample (no test substance), B is the absorbance of the test sample with tyrosinase, and C is the absorbance of the test sample without tyrosinase.

Results

Taxonomy and phylogeny

From 23 jelly ear mushroom samples, 12 were identified at the species level, and all isolates of saprobic fungi (classified according to their role in habitats) were cultured in the laboratory. Sample RSPG336 was 30 mm in diameter, and it exhibited a round to fan shape, a smooth surface, and chocolate brown-colored fruiting bodies. RSPG662 was smaller than RSPG336, (7 mm in diameter), and it displayed an unsymmetrical shape, a smooth surface with a wrinkled lower part, margins lacking hairs, a thick texture, and a light brown color. Specimens were attached to the decaying wood in the dry deciduous of the community forest. These two species were distinguished according to the length and shape of the hair surface. RSPG662 had a short hair length (100–150 µm) and a club shape with a 1–2-µm-thick cell wall, whereas RSPG336 had a longer hair length (200–300 µm), a club shape, and a thick, almost solid cell wall (3–5 µm).

RSPG622 *Auricularia cornea* (upper left & right)
RSPG336 *Auricularia* sp. (lower left & right)

The phylogenetic analysis of the ITS dataset comprised 61sequences of *Auricularia* species from around the world. Strong bootstrap support was observed for 19species in the tree. This tree revealed the sequences of RSPG622, which was identified as A. cornea with high bootstrap supported among three other sequences from Thailand and Australia (Figure1).

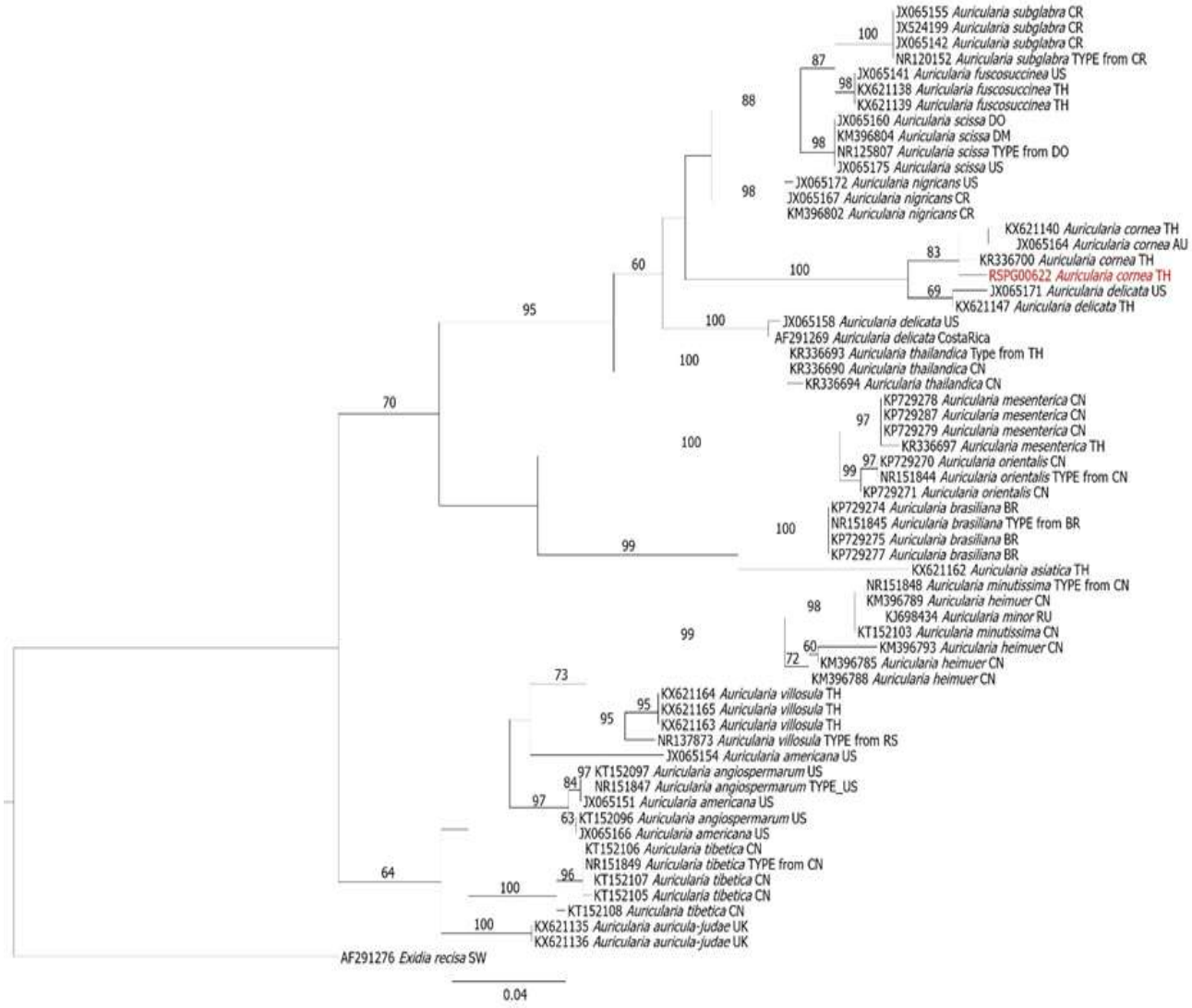


Figure1.Phylograms inferred from maximum likelihood analyses of the internal transcribed spacer sequences of *Auricularia*. Species names are followed by the GenBank accession numbers. The target sequence is presented in red. Bootstrap support values of the maximum likelihood analysis exceeding 60% are presented above each node. The tree was rooted with *Exidia recisa* (AF291276), or it was an outgroup

From the PDB cultures over a 21-day period, different types of mushrooms yielded different quantities of mycelia, including *Auricularia* sp. (RSPG336) and *A. cornea* (RSPG622) (Table 1).

Table 1: Mycelial outputs (mean ± SD) from broth cultures

Sample	Output (g dry weight)
Auricularia sp. RSPG336	0.30 ± 0.12^{c}
Auricularia cornea RSPG622	0.55 ± 0.05^{b}

*The superscripts denote the results of statistical analysis using the t-test, in which the same and different characters denote insignificant and significant ($p < 0.05$) differences, respectively.

The mycelial crude extracts were analyzed for their total phenolic contents. The methanol extracts contained higher total phenolic content than the aqueous extracts, whereas the *Perenniporia subadusta* (RSPG139) methanol extract contained the maximal amount of phenolic compounds at 6.55 mg GAE/g sample (Table 2). From the analysis of the anti-oxidant properties of crude extracts using the DPPH and FRAP assays, the mushroom species and solvents used in the extraction significantly affected the ability of the extracts to

scavenge DPPH free radicals and their electron donor activity (FRAP assay). The methanol extracts possessed higher anti-oxidant capabilities than the aqueous extracts, and the *P. subadusta* (RSPG139) methanol extract had the best ability to scavenge DPPH, followed by the *Auricularia* sp. (RSPG336) methanol extract. The *Lentinus* sp. (RSPG278) methanol extract was the most capable electron donor, followed by the *P. subadusta* (RSPG139) methanol extract (Table 2).

Table 2: Total phenolic compounds and anti-oxidant properties of mycelial crude extracts

Sample	Solvent	Total phenolic compounds (mgGAE/g sample)	% scavenging based on the DPPH assay	FRAP value (μmol Fe (II)/g sample)
Auricularia sp. RSPG336	Methanol	5.20 ± 0.06^{a}	57.47 ± 0.86^{a}	16.27 ± 0.78^{a}
Auricularia sp. RSPG336	Water	1.56 ± 0.01^{b}	17.66 ± 0.30^{b}	5.89 ± 0.18^{c}
A. cornea RSPG622	Methanol	4.27 ± 0.02^{ab}	34.72 ± 0.15^{ab}	10.72 ± 0.10^{b}
A. cornea RSPG622	Water	1.22 ± 0.05^{b}	13.54 ± 0.45^{b}	4.08 ± 0.20^{c}

*All values are presented as the mean ± SD. The superscripts denote the results of statistical analysis within each column per the t-test, in which the same and different characters indicate insignificant and significant ($p < 0.05$) differences, respectively. GAE, gallic acid equivalents; DPPH, 1,1-diphenyl-2-picrylhydrazine; FRAP, ferric reducing anti-oxidant power.

The results for tyrosinase inhibition by the crude extracts were consistent with those of the anti-oxidant assays. The methanol extracts inhibited this enzyme more effectively than the aqueous extracts, as presented in Table 3. The *Auricularia* sp. (RSPG00336) and *Coriolopsis byrsina* (RSPG00722) methanol extracts had the strongest inhibitory effects on tyrosinase activity, followed by the *P. subadusta* (RSPG00139) methanol extract.

Table 3: The tyrosinase-inhibiting properties of mycelial crude extracts

Sample	**Solvent**	**Tyrosinase-inhibiting activity (% inhibition)**
*Coriolopsis byrsina*RSPG722	Methanol	13.44 ± 1.08^{a}
Auricularia sp. RSPG336	Methanol	13.29 ± 0.70^{a}
A. cornea RSPG622	Methanol	13.18 ± 0.66^{a}
*Perenniporia subadusta*RSPG139	Methanol	7.57 ± 0.52^{b}
*C. byrsina*RSPG722	Water	6.78 ± 0.36^{b}
A. cornea RSPG622	Water	5.01 ± 0.28^{c}
Auricularia sp. RSPG336	Water	4.11 ± 0.26^{c}
Lentinus sp. RSPG278	Water	3.97 ± 0.46^{c}
Lentinus sp. RSPG278	Methanol	3.63 ± 0.82^{c}
Phellinus sp. RSPG48	Water	3.24 ± 0.62^{c}
L. sajor-caju RSPG224	Water	1.41 ± 0.23^{d}
*Perenniporia subadusta*RSPG139	Water	0.97 ± 0.32^{d}
L. sajor-caju RSPG224	Methanol	0.70 ± 0.10^{de}
Phellinus sp. RSPG48	Methanol	NA

*The superscripts denote the results of statistical analysis within each column per the t-test, in which the same and different characters indicate insignificant and significant ($p < 0.05$) differences, respectively

The 93 isolates of saprobic fungi cultured in the laboratory were also screened for their ability to produce β-glucans. The 10 isolates with the highest β-glucan contents were as follows: *A. cornea* (RSPG622, jelly ear fungus), 42.27%; *Resupinatus* sp. (RSPG00581), 40.28%; *Hohenbuehelia* sp. (RSPG00580), 39.16%;

Phellinus sp. (RSPG00048), 38.86%; *Auricularia* sp. (RSPG00336), 38.21%; *C. byrsina* (RSPG00722), 38.10%; *Lentinus* sp. (RSPG00278), 36.71%; *Perenniporia* sp., 35.78%; *L. sajor-caju* (RSPG00224), 35.77%; and *L. fasciatus* (RSPG00590), 35.41% (Figure 2).

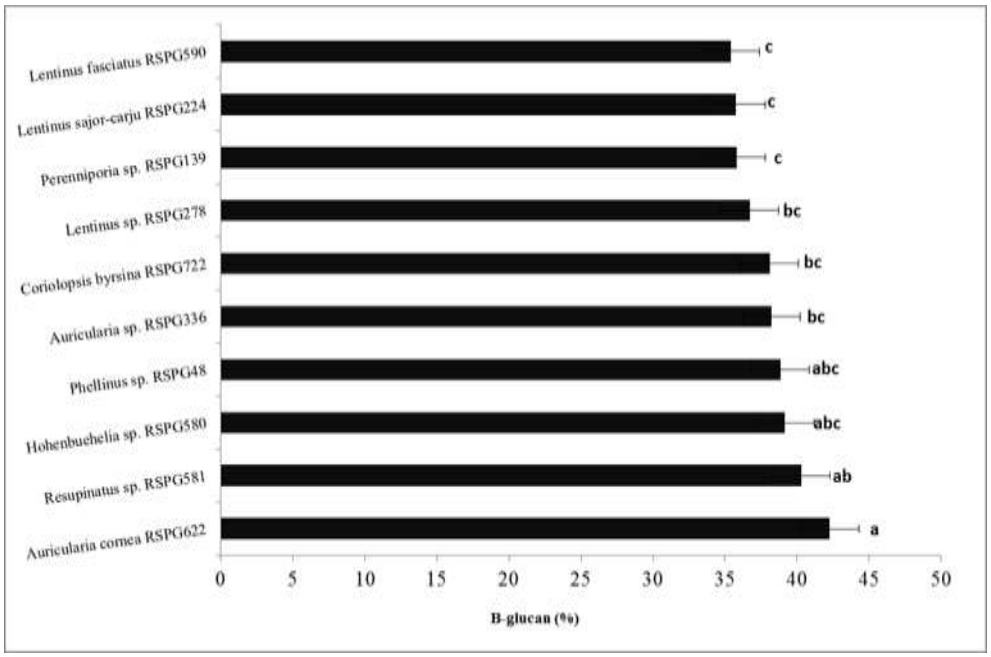


Figure2.β-Glucan contents in the selected fungal mycelia

β-Glucan extracts were analyzed for their anti-oxidant activities. The 10 β-glucan extracts possessing the strongest anti-oxidant activities are presented in Table 4.

Table 4:Anti-oxidant properties of β-glucan extracts as tested using the DPPH and FRAP assays

β-glucan extract	**DPPH assay (IC_{50}, mg/ml)**	**FRAP assay (μmol Fe (II)/g sample)**
*Auricularia cornea*RSPG622	5.79 ± 0.29^{a}	3.53 ± 0.07^{a}
Hohenbuehelia sp. RSPG580	7.32 ± 0.20^{ab}	2.81 ± 0.17^{b}
Resupinatus sp. RSPG581	8.57 ± 0.40^{b}	3.53 ± 0.29^{a}
Phellinus sp. RSPG48	11.71 ± 0.22^{c}	1.26 ± 0.07^{c}
Lentinus sp. RSPG278	15.69 ± 1.59^{d}	1.38 ± 0.02^{c}
L. fasciatus RSPG590	16.19 ± 0.73^{d}	0.74 ± 0.02^{e}
*L. sajor-caju*RSPG224	16.24 ± 1.07^{d}	1.20 ± 0.01^{cd}
*Perenniporia subadusta*RSPG139	17.07 ± 0.45^{d}	1.03 ± 0.07^{d}
Auricularia sp. RSPG336	20.16 ± 1.78^{e}	0.68 ± 0.02^{e}
*Coriolopsis byrsina*RSPG722	20.38 ± 2.73^{e}	0.46 ± 0.02^{f}
BHT	0.23 ± 0.00	2620.01 ± 73.82

*All values are presented as the mean ± SD. The superscripts denote the results of statistical analysis within each column per the t-test, in which the same and different characters indicate insignificant and significant ($p < 0.05$) differences, respectively. DPPH, 1,1-diphenyl-2-picrylhydrazine; FRAP, ferric reducing antioxidant power; IC50, half maximal inhibitory concentration; BHT, butylated hydroxytoluene.

In this research, the β-glucan extract from *A. cornea* RSPG00622 cells was selected for further analysis. Its approximate composition is presented in Table 5. In total, carbohydrates comprised 59.54% of the extract content (of which 47.25% were β-glucans), whereas 32.83% of the biomass was proteins.

Table 5:Approximate composition of the β-glucan extract from *Auricularia cornea* RSPG00622

Composition	% based on dry weight (mean ± SD)
Moisture	1.29 ± 0.07
Lipid	3.70 ± 0.10
Protein	32.83 ± 0.27
Carbohydrate	59.54 ± 0.59
Ash	2.12 ± 0.04
Fiber	0.52 ± 0.04

The cytotoxicity and inflammation-related activities of the β-glucan extract from *A. cornea* RSPG00622 were examined. Figure3 presents the percent viability of THP-1 monocytes following incubation with the β-glucan extract for 3 h. More than 90% of the cells survived at every tested concentration of β-glucans, suggesting that these polysaccharides had no cytotoxicity in these cells. The effects of β-glucans on inflammation-related gene expression were then studied in a resting state and in the presence of LPS-induced inflammation, as presented in Figures4(A) and 4(B), respectively. The results illustrated that in the resting state, β-glucans at concentrations of ≥10 μg/ml significantly increased the expression of IL-8 and IL-10 but not IL-1β or TNF-α. In the inflamed state, β-glucans at concentrations of 2–25 and 50 μg/ml significantly upregulated IL-1β and TNF-α gene expression, respectively.

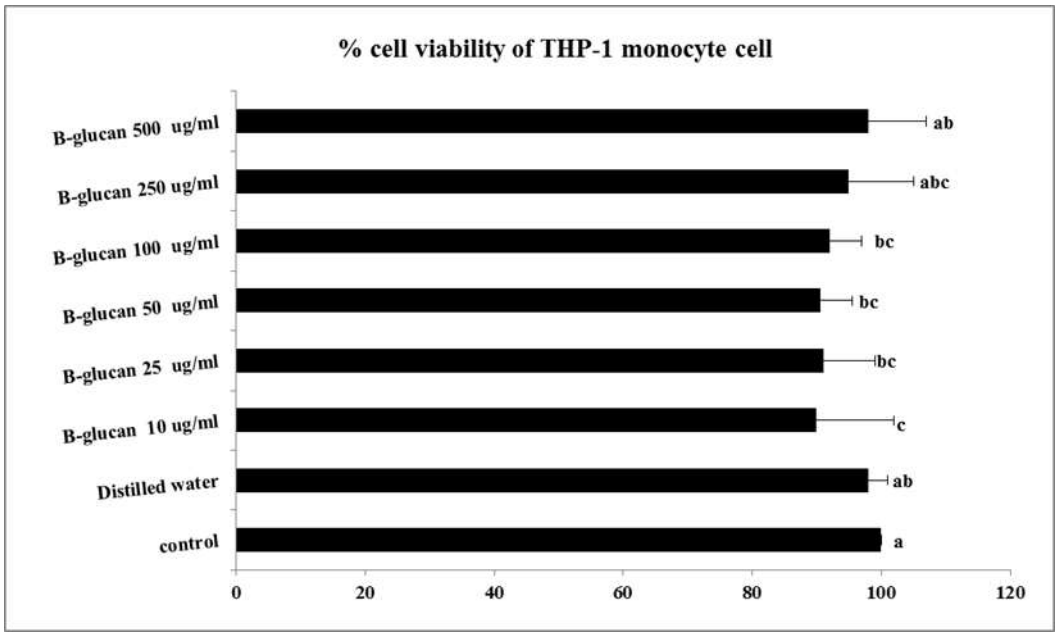
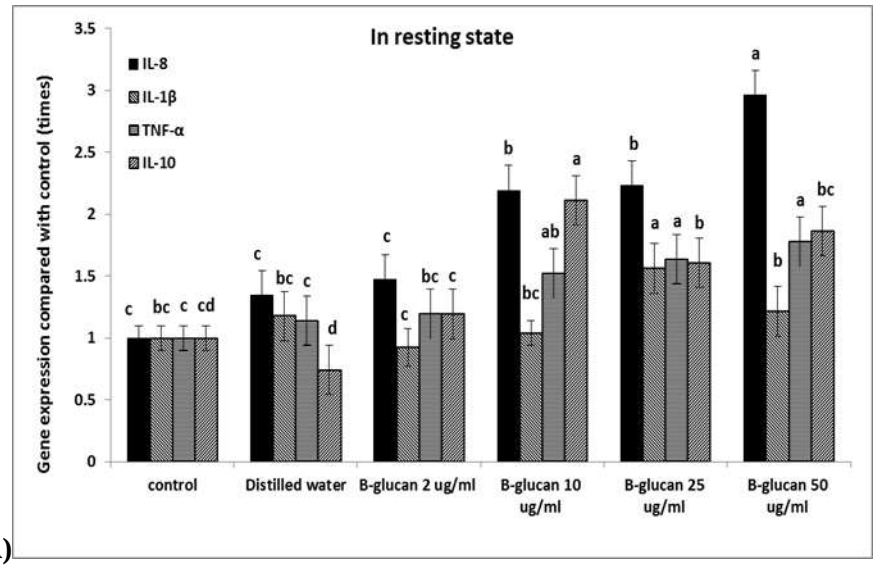
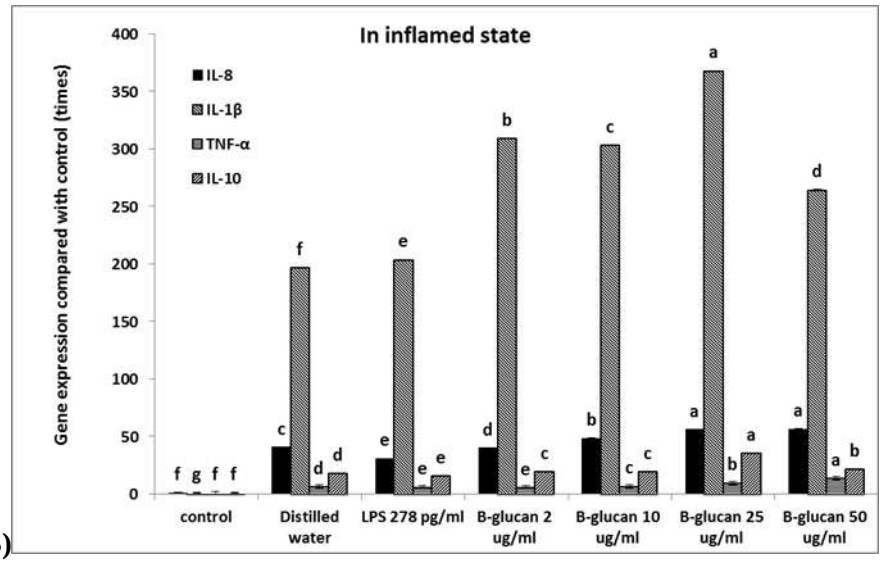


Figure 3. Percent viability of THP-1 monocytes following incubation with β-glucan extract from *A. cornea* RSPG00622. The control was unstimulated cells



(A)



(B)

Figure 4. Effects of β-glucan extract from *A. cornea* RSPG00622 on inflammatory gene expression in (A) the resting state and (B) in the presence of lipopolysaccharide-induced inflammation. The control was unstimulated cells

Discussion

The present study focused on mushrooms from the Dong Yai Forest because the forest is a pilot area in the plant genetic conservation program initiated by Her Royal Highness Princess Sirindhorn, which has the objectives of compiling a body of knowledge and resources and utilizing such resources. For this study, saprobic fungi were chosen because they can easily be cultured as mycelia in the laboratory, and previous studies revealed their high β-glucan contents and ability to produce anti-oxidant phytochemicals[28, 29].

The 80% methanol extracts of mycelia contained various amounts of phenolic compounds. The methanol extract of P. *subadusta* RSPG00139 contained the highest phenolic levels among all analyzed mushroom types. Methanol extracts also possessed biological activities, namely anti-oxidation and tyrosinase inhibition. These findings are in accordance with the results reported by Punitha and Rajasekaran[1], who found that the methanol extract of *Volvariella volvacea* (Bull. ex Fr.) (straw mushroom) yielded high amounts of active substances, namely phenols (including flavonoids) and ascorbic acid, and the extract was a good anti-oxidant as tested using DPPH, 2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), superoxide, and nitric oxide assays. A study of the phenolic compounds of *Ganoderma lucidum* (Lingzhi mushroom) and *Trametes hirsuta* (hairy bracket) extracted using water found that their total phenolic contents were 30.916 ±

0.057 and 38.25 ± 0.135 mg GAE/g extract, respectively[30]. The abundant active ingredients included ascorbic acid, chlorogenic acid, cinnamic acid, and vanillin, which possess substantial anti-oxidant effects. Not surprisingly, both types of mushroom extracts were active anti-oxidants against DPPH, superoxide, and nitric oxide. Likewise, a study by Rajasekaran and Kalaimagal[31]found that *G. lucidum* was a mushroom possessing medicinal properties. Its ethanol extract contained phenolic compounds in the amount of 42.41 ± 2.21 mg GAE/g extract, and it dramatically inhibited many types of free radicals, namely hydroxyl, superoxide, and nitric oxide. This mushroom has high potential for use as a raw material in medicine and dietary supplement production. Unfortunately, the present study did not determine the total phenolic content in this mushroom or compare the level with those of other mushroom types from the Dong Yai Forest. A study on the wild mushroom *Volvopluteus gloiocephalus* (big sheath mushroom) found that biologically active compounds detected in high amounts were also phenolic compounds. The total phenolic acid content in this mushroom's extract was 404.37 μg/100 g dry weight. The most common phenolic acids in the extract were p-coumaric acid, p-hydroxybenzoic acid, gallic acid, and cinnamic acid. Taveepanich and Toomkam [32] studied the anti-oxidant effects of 20 wild edible mushroom extracts from Ubon Ratchathani province using three solvents, namely hexane, ethyl acetate, and ethanol, and three methods consisting of DPPH radical scavenging activity, ABTS radical cation scavenging activity, and FRAP. Taveepanich and Toomkam[32] also determined the total phenolic content using the Folin–Ciocalteu test. The DPPH, ABTS, and FRAP methods revealed that the wild mushroom extracts with the strongest anti-oxidant effects were the crude ethanol extract of *Astraeus hygrometricus* (barometer earthstar) at 50.27 ± 0.88%, the crude ethanol extract of *Russula mairei* Sing. at 57.61 ± 0.71%, and the crude ethyl acetate extract of *Alpova tappei* Fogel at 1558.20 mg. The highest total phenolic level was found in the crude ethanol extract of *A. hygrometricus* at 10.53 mg tannic acid equivalents/g extract, and the relationship between the phenolic content and anti-oxidant activity was demonstrated using the DPPH method. Baowthongkum and Promboon[33] studied the total phenolic contents and anti-oxidant properties of five species of wild mushrooms in Phetchabun province, namely *Thaeogyroporus porentosus*, *Amanita princes*, *R. virescens* (greencracked brittlegill), *Rhizopus nigricans* (blackening brittlegill), and *R. emetica* (sickener). The mushrooms were extracted using three solvents: ethyl acetate, methanol, and water. The A. princes ethyl acetate extract displayed the strongest anti-oxidant effect at a concentration of 0.01 mg/ml. This property was in line with its high total phenolic content of 67.91 mg GAE/g extract. Ayyasamy et al. [34] studied the anti-oxidant properties of the methanol extract of *P. florida* at a concentration of 100 μg/ml using DPPH, superoxide, and nitric oxide radical scavenging assays and assessed its metal-chelating ability and reducing power. The extract inhibited DPPH free radical production by 78%, and its IC50 was 50 μg/ml. The mushroom extract at a concentration of 500 μg/ml exhibited stronger reducing potency than ascorbic acid. At a concentration of 1000 μg/ml, the extract inhibited nitric oxide accumulation by 81.8%, superoxide free radical accumulation by 65%, ferrous ion accumulation by 64%, and hydroxyl free radical accumulation by 59%. The present study did not include these aforementioned mushrooms, but many other mushroom types were covered. The fact that large amounts of phenols were detected in mushroom extracts with ethanol or methanol is not surprising, considering the polarity of phenolic compounds.

Tyrosinase is an important enzyme in the production of melanin. Inhibition of this enzyme as observed in the present study suggests that a test substance can inhibit the new production of melanin. Tyrosinase inhibition may be mediated by phenolic compounds in mycelial extracts. Phenols contain hydroxyl groups in their structure, which is associated with L-dopa, the substrate of this enzyme, and thus, they may inhibit the enzyme by acting as a competitive inhibitor[35]. Further studies are warranted to elaborate the implications of this effect in skin pigmentation in healthy people and in those with melanoma.

Wahab et al.[36]studied the hypoglycemic effect of grey oyster mushrooms (*P. pulmonarius*) via the inhibition of the two enzymes: α-glucosidase and α-amylase. They found that the mushroom protein obtained via sedimentation with ammonium sulfate could inhibit α-glucosidase activity by 24.18% and α-amylase activity by 41.80%. The experiment suggests that certain mushrooms may be used in the treatment of type 2 diabetes. Further study is warranted concerning the mushroom species from Dong Yai Forest, as mentioned in the present study.

In summary, regarding mycelial extracts, they exhibit high potential for use as natural anti-oxidants and tyrosinase inhibitors, and they could be applied in the development of products because the cultivation of mycelia is easy and large quantities can be yielded. In particular, the mycelia of *P. subadusta* RSPG00139

extracted with methanol contain large amounts of phenolic compounds and good anti-oxidant and anti-tyrosinase effects. Therefore, they may be good options for further development into skincare products.

In terms of β-glucans, *A cornea* RSPG00622 mycelia can be extracted to yield extremely high β-glucan contents. Based on the results of anti-oxidant and inflammation-related gene expression analyses, β-glucans appear to possess moderately potent anti-oxidant and immunomodulating effects. The latter is implied by the fact that they upregulate the gene expression of IL-10, which is an anti-inflammatory cytokine, in monocytes in the resting state, whereas they upregulate the gene expression of IL-1β and TNF-α in response to E. coli LPS. Both latter cytokines are key mediators of inflammatory responses and are crucial for host defense responses to infection and injury. This mushroom species is popular in China, Japan, and Korea. The peculiar-looking mushrooms with a soft, jelly-like texture and ear-shaped fruiting bodies commonly grow on the wood of deciduous trees and shrubs[37]. TKihey have primarily been recognized as functional dietary supplements, especially in Asian countries, because of their wide spectrum of health properties, including anti-inflammatory, anti-oxidant, and anti-microbial activities. Hot water extraction is a popular approach for obtaining water-soluble polysaccharides[38]. Qin et al.[39]similarly reported that mushroom-derived glucans could act as immunomodulators by activating host immune cells (such as cytotoxic macrophages) or chemical messengers (cytokines such as ILs). In 2013, Zhou et al.[40]demonstrated the anti-mutagenic activity of β-glucans against the in vivo DNA-damaging effect of the alkylating agent cyclophosphamide. It was assumed that β-glucans probably modulated the response of the immune system. To date, many attempts have been made to extract β-glucans from this fungal species in the form of its fruiting body grown on a solid culture, but to date, only the present study has reported its extraction from its mycelia. The extraction method breaks the cell wall and removes impurities under mild-to-strong conditions. Based on this research, β-glucans can be extracted from *A. cornea* RSPG00622 mycelial cells through several steps, and the extracts can be further developed into supplements in certain dosage forms such as capsules.

Conclusion

The present research screened and identified saprobic fungi found at a forest in Thailand. *P. subadusta* RSPG00139 contains a high amount of total phenolic compounds in the form of *A. cornea* RSPG00622 β-glucans. The easy, convenient techniques for cultivating and extracting fungal mycelia are promising for the use of crude and β-glucan extracts as the primary ingredients in healthcare products. β-glucan extracts appear to contain anti-oxidant and immunomodulating activities. Thus, they may be developed for various applications in nutraceutical or cosmetic industries. The effects of the extraction process on the rheology, viscosity, gel formation, and molecular weight profile of β-glucans require further determination.

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