Asian Journal of Mycology 7(1): 47–54 (2024) ISSN 2651-1339
www.asianjournalofmycology.org

Article
Doi 10.5943/ajom/7/1/4

Screening for Bioactivities of Wild *Hymenopellis* spp., and *Volvariella pulla* in Thailand

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Niego AGT, Thongklang N, Charoensup R 2023 – Screening for bioactivities of wild *Hymenopellis* spp., and *Volvariella pulla* in Thailand. Asian Journal of Mycology 7(1), 47–54, Doi 10.5943/ajom/7/1/4

Abstract

This study investigates the potential medicinal properties of EtOAc and MeOH mycelial extracts obtained from *Hymenopellis* species and *Volvariella pulla*. The extracts were subjected to rigorous analysis to assess their inhibitory effects on bacterial growth, especially against opportunistic pathogens such as *M. luteus* and *C. albicans*. The study also unveils the antibacterial activity of the *Hymenopellis* taxa for the first time. Furthermore, the research reveals potent inhibitory activity against alpha-glucosidases exhibited by EtOAc extracts of *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0715, and *Volvariella pulla* T20-0714. This inhibition mechanism has implications for glucose absorption, vital for controlling blood glucose levels and managing diabetes. Notably, the EtOAc extracts displayed lower half-maximal inhibitory concentrations than standard acarbose, enhancing their potential therapeutic significance. Intriguingly, MeOH extracts from *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0715, and *Volvariella pulla* T20-0714 exhibited the capacity to promote glucose uptake in L6 cells, a crucial component of diabetes management. Considering the prevalence of diabetes mellitus and microbial infections, observed antidiabetic potential and antibacterial activity of these mushroom extracts hold promise for future therapeutic applications. The study not only contributes to understanding the medicinal properties of *Hymenopellis* and *Volvariella* but also underscores the significance of further research to explore their mechanisms and optimize their application in clinical contexts. Moreover, the absence of cytotoxicity in non-tumour cells may confirm the safety of *Hymenopellis* spp. and *Volvariella pulla* extracts for consumption.

Keywords – antimicrobial **–** antidiabetic **–** Basidiomycota **–** cytotoxicity **–** medicinal properties

Introduction

Hymenopellis is one of the most speciose genera of the oudemansielloid/xeruloid complex in the family Physalacriaceae (Niego et al. 2021b). Around 50 species have been documented for this genus (Petersen & Hughes 2010), with 51 epithets listed in the Species Fungorum (http://www.speciesfungorum.org). *Hymenopellis* is well known in eastern and northern America (Petersen & Hughes 2010, Woehrel & Light 2017), although only fifteen species are known from

Submitted 15 December 2023, Accepted 29 March 2024, Published 17 May 2024
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Asia. A few *Hymenopellis* species have been identified in Thailand, namely *H. raphanipes* and *H. radicata* recorded by Chandrasrikul et al. (2011), with the latter having no available sequences in the GenBank from Thailand. Niego et al. (2023b) also recorded *H. orientalis* and introduced two new species, *viz.*, *H. straminea* and *H. utriformis*, from Thailand.

Volvariella is another interesting genus placed within the family Pluteaceae (Justo et al. 2011); however, phylogenetically, it is closely related to *Camarophyllus* and *Cantharocybe*. Therefore, it has been suggested to be treated as *incertae sedis* until further studies are conducted to clarify its correct placement (Justo et al. 2011, Niego et al. 2021c). *Volvariella* comprises around 50 species (He et al. 2019), with 145 records in the Index Fungorum (http://www.indexfungorum.org). Eight species of *Volvariella* have been recorded in Thailand (Chandrasrikul et al. 2011, Niego et al. 2021c).

Macrofungi have been explored for years, not only for their nutritional value but also for their medicinal properties (Zhang et al. 2016). They have been recognized in recent years for their various bioactivities (Sandargo et al. 2019), such as anticancer, antidiabetic, antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and immunomodulatory properties (Niego et al. 2021a). Most macrofungi have not been fully explored due to the slow growth of mycelia and various nutritional preferences and are often neglected as a source of important bioactive compounds (Karwehl & Stadler 2016, Lu et al. 2020). Although *Hymenopellis* is quite common, there are limited studies in terms of its bioactivities. For instance, only *Hymenopellis radicata* has been documented to produce some important bioactive compounds such as oudenone, lectin, mucidin, and polysaccharides with antifungal, antioxidant, anti-inflammatory, hemagglutinating activity, and lung protective effects (Anke et al. 1990, Liu et al. 2013, 2021, Gao et al. 2017). *Volvariella* species, especially *Volvariella volvacea,* are one of the most cultivated mushroom species in Southeast Asia as a high-quality food source (Bao & Wang 2015). *Volvariella volvacea* is also well documented for its bioactivities, with beta-glucan as the major polysaccharide (Kishida et al. 1989, Sangthong et al. 2022). Other compounds from this species with bioactivities, such as proteins and lectins, were also documented (Yao et al. 1998, Liu et al. 2011). *Volvariella volvacea* is found to have bioactivities such as antioxidant, antitumor, immunosuppressive, and immunoregulatory activities (Kishida et al. 1989, Liu et al. 2011, Ruksiriwanich et al. 2014).

Both *Hymenopellis* and *Volvariella* are speciose genera; however, only *H. radicata* and *V. volvaceae* were explored for their bioactivities. In this study, *Hymenopellis* sp. and *Volvariella pulla*, previously identified in taxonomic and phylogenetic studies (Niego et al. 2021c, 2023a, 2023b), were used to check for their bioactivities. We explore the possibility that other species from these genera also exhibit promising bioactivities for future medicinal, nutraceutical, and pharmaceutical applications.

Materials & Methods

Crude Extraction of Samples

The mycelia of the mushroom samples were subcultured on malt extract agar (MEA) using agar plugs. Each strain was grown on 70 plates and incubated at room temperature for 14 days or until the mycelium covered more than half the area of the plate. Agar plates with fungal cultures were macerated with 30 mL of HPLC-grade ethyl acetate or methanol (EtOAc or MeOH) using a homogenizer (Ultra-turraxP®). This process was carried out three times as follows: The agar slurry (mushroom mycelia) was left to soak overnight for the first extraction. It was then separated into a liquid layer and a solid layer. To the solid layer, 30 mL of HPLC-grade ethyl acetate was added again and soaked for eight hours each time for the second and third extractions. The solid layer was discarded. The liquid layers (crude extracts) were pulled together into the pre-weighed vial. It was then air-dried to allow the liquid component to evaporate. The remaining solid component (powder) was the crude extract. HPLC-grade methanol was then added at a concentration of 1 mg/mL. These were used for different bioactivity assays. Sample concentrations of 100 µm/ml were used in all bioactivity screenings.

Antimicrobial Assay

Pure cultures of gram-positive bacteria (*Streptococcus pyogenes* ATCC19615, *Bacillus cereus* ATCC11778, *Listeria monocytogenes* F2365, *Micrococcus luteus* DMST15503, *Enterococcus faecalis* ATCC29212, and *Staphylococcus aureus* ATCC25923 and MRSA NPRC 001R) and gram-negative bacteria (*Acinetobacter baumanii* ATCC19606, *Escherichia coli* TISTR780, *Klebsiella pneumoniae* ATCC700603, *Proteus mirabilis* DMST8216, *Pseudomonas aeruginosa* ATCC10145, *Shigella flexneri* DMST4423, *Salmonella typhimurium* DMST562, and *Salmonella typhi* DMST22842) and yeast (*Candida albicans*), which were used as test organisms in the study, were obtained from a stock culture collection of Mae Fah Luang University, Chiang Rai, Thailand. Standard protocols and procedures were followed in handling these microorganisms. An antimicrobial test was performed following the Kirby-Bauer Disc Diffusion Method (Clear Zone).

The Kirby-Bauer method was used to determine the antimicrobial activity of crude mushroom extracts. The crude extracts were prepared at a concentration of 20 mg/mL in HPLC-grade methanol. A 6-mm sterilized filter paper disk was impregnated with 20 µL of each crude extract prior to bioassays. Petri dishes of MH agar (NA) were inoculated with 1000 µL of bacterial or fungal suspensions (density of 5 x 10^5 cfu/mL) using the spread plate method. The prepared extract discs were placed in MH agar Petri dishes. Bacteria or fungi mixed with methanol served as a negative control, and amoxicillin was used as a positive control for bacteria and ketoconazole for fungi. The inoculated Petri dishes were incubated at 37 ˚C for 24 hours in three replicates. The zone of inhibition represents the lethality of a bacterial or fungal test organism.

Alpha-glucosidase Inhibitory Assay

The alpha-glucosidase inhibitory test by Phukhatmuen et al. (2020) was modified and used. One hundred (100 mg/ml) sample solutions were diluted in phosphate buffer (pH 6.8) with 100% dimethyl sulfoxide (DMSO), and 50 ml of each sample was pipetted and mixed in vitro with 100 µL of alpha-glucosidase enzyme (0.35 unit/ml). Following a 10-minute pre-incubation at 37 °C, 100 µL of 1.5 mM p-NPG was added, and the sample was incubated for another 20 minutes at 37 °C. The reaction was then stopped by adding 1000 µL of Na_2CO_3 (1M). As a positive control, acarbose was employed. A microplate reader detected the absorbance at 405 nm (PerkinElmer, lnc., USA).

Glucose Uptake Activity Assay

The L6 cells were cultivated in 12-well plates, washed twice with serum-free MEM, and incubated for two hours at 37 °C, with 0.5 ml of the same medium. The cells were rinsed three times with KRP buffer before being treated for 30 minutes with 0.9 ml of KRP buffer at 37 °C. Subsequently, sample concentrations of 100 m/ml were added to the wells and incubated at 37 °C for 20 minutes. To begin glucose absorption, 0.1 ml of KRP buffer containing 0.037 mM/l 2-deoxy-D-[3H] glucose and 0.001 mmol/l glucose was added. The test ended 15 minutes after washing the cells three times with cold PBS. At 37 °C for 20 minutes, the cells were lysed with 0.7 ml of 1% Triton X-l00, and the OD value at 417 nm was measured. Glucose uptake increase rate (%) = (As/A0) ×100.

The same applies to the glucose uptake of the samples at 100 µm/ml concentrations, and A0 is the glucose uptake of the control.

Cytotoxicity Test

The 3T3-L1 preadipocytes were grown in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum. The cells were incubated in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C.

The evaluation of mushroom cytotoxicity in 3T3-L1 cells was carried out by reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The cells were seeded in a 96-well plate (1x 10 cells/ well) and left to attach to the medium for 48–72 hours before being exposed to mushroom extract. A microscopic examination was carried out, and observations were

made every 24 hours intervals. After 72 hours, various concentrations of the samples in 0.1% DMSO were added and incubated for 24 hours in a 5% CO_2 incubator, and the images were viewed. Cells incubated with MTT (20 µl, 5 mg/mL) in phosphate-buffered saline solution were added. After four hours of incubation, 1 ml of DMSO was added. Viable cells were determined by absorbance at 570 nm. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC50) was graphically determined. The effect of the samples on the proliferation of 3T3-L1 cells was expressed as a percentage of viable cells using the following formula: % cell viability = (A570 treated cells / A570 control cells) × 100%.

Results

Species Confirmation

Other taxonomic and cultivation studies confirmed the identity of *Hymenopellis* sp. T20-0713 and *H. utriformis* T20-0715 (Niego et al. 2023a, b). The blastn results of ITS and nrLSU of *Volvariella pulla* T20-0714 were 100% similar to the previously recorded *Volvariella pulla* in Thailand (Niego et al. 2021c).

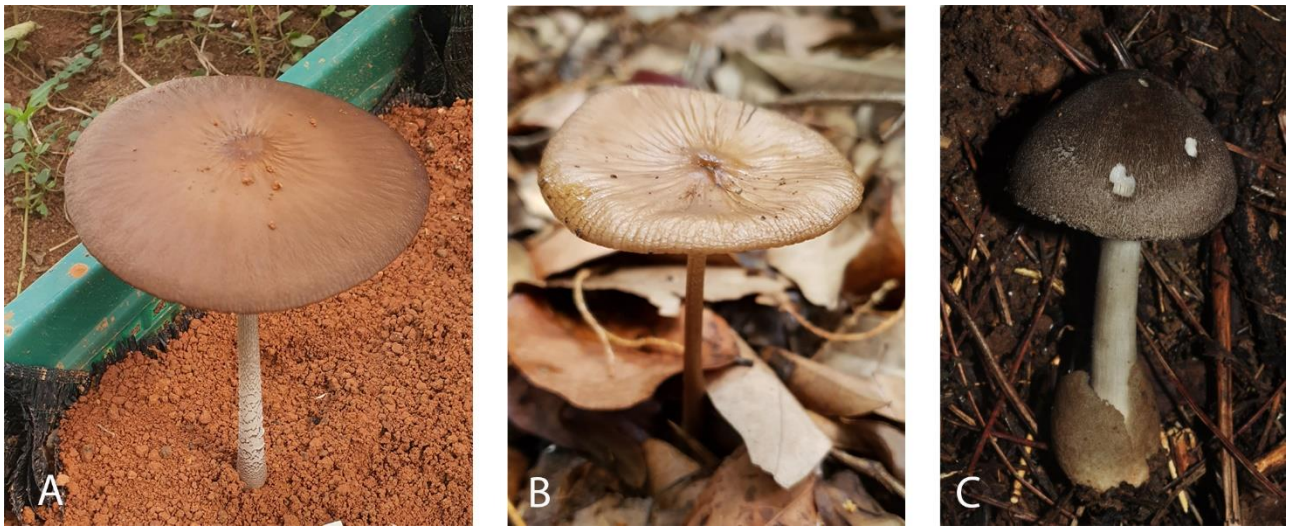

Fig. 1 – Mushroom samples. A. *Hymenopellis* sp. T20-0713. B. *Hymenopellis utriformis* T20-0715. C. *Volvariella pulla* T20-0714.

Antimicrobial Capacity of Extracts

The antimicrobial activities of two *Hymenopellis* species and *Volvariella pulla* extracts against seven gram-positive and eight gram-negative bacteria and yeast, *C. albicans*, are shown in Table 1. The 16 test organisms were used in the Kirby Bauer assay against the ethyl acetate and methanolic extracts of *Hymenopellis* and *Volvariella pulla* strains. *Volvariella pulla* T20-0714 did not exhibit inhibition against any test organism.

Most of the extracts did not have antibacterial or antifungal activities, except for the EtOH extracts of *Hymenopellis* sp. T20-0713 and *Hymenopellis utriformis* T20-0715, which showed weak activity against *M. luteus* with 1.3 cm and 1.2 cm clearance, respectively. The EtOH extract of *Hymenopellis* sp. T20-0713 was the only extract with antifungal activity against *C. albicans*, with an average inhibition zone of 3.0 cm diameter.

Determination of Alpha-glucosidase Inhibition

The EtOAc and MeOH extracts of *Hymenopellis* spp. and *Volvariella pulla* strains were determined to have alpha-glucosidase inhibitory activity. The EtOAc extracts of *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0714, and *Volvariella pulla* T20-0714 exhibited the inhibition of alpha-glucosidase at 79.30±0.97%, 92.55±3.82%, and 82.64±9.37%, respectively. The IC_{50} of

Hymenopellis sp. T20-0713 (621 µg/mL), *H. utriformis* T20-0715 (95 µg/mL), and *Volvariella pulla* T20-0714 (288 µg/mL) were lower compared to acarbose (660 µg/mL). However, MeOH extracts from mushroom samples exhibit a lower inhibition of alpha-glucosidase less than 50%.

Table 1. Antimicrobial activities of *Hymenopellis* strains collected from Chiang Rai, Thailand

No.	Test organisms	***Hymenopellis* sp. T20-0713**		***H. utriformis* T20-0715**		**Control**	
		EtOAc	**MeOH**	**EtOAc**	**MeOH**	**(+)**	**(-)**
	Gram positive (+) bacteria						
1	*S. pyogenes* ATCC19615	0	0	0	0	5.4	0
2	*B. cerues* ATCC11778	0	0	0	0	5.8	0
3	*L. monocytogenes* F2365	0	0	0	0	5.5	0
4	*M. luteus* DMST15503	**1.3**	0	**1.2**	0	6.0	0
5	*E. faccalis* ATCC29212	0	0	0	0	5.5	0
6	*S. aureus* ATCC25923	0	0	0	0	6.0	0
7	MRSA NPRC 001R	0	0	0	0	6.0	0
	Gram negative (-) bacteria						
8	*K.pneumoniae* ATCC700603	0	0	0	0	5.5	0
9	*P. mirabilis* DMST8216	0	0	0	0	6.0	0
10	*A. baumanii* ATCC19606	0	0	0	0	5.8	0
11	*S. flexneri* DMST4423	0	0	0	0	5.8	0
12	*S. typhimurium* DMST562	0	0	0	0	5.8	0
13	*S. typhi* DMST22842	0	0	0	0	5.4	0
14	*P. aeruginosa* ATCC10145	0	0	0	0	6.0	0
15	*E. coli* TISTR780	0	0	0	0	6.0	0
	Yeast						
16	*C. albicans*	**3.0**	0	0	0	4.0	0

Determination of Glucose Uptake

The ability of MeOH extracts of *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0715, and *Volvariella pulla* T20-0714 to take in glucose was higher than that of the control by 38.7±17.7%, 12.6±2.0%, and 14.7±5.6%, respectively. In contrast, the EtOAc extracts displayed no glucose uptake.

Cytotoxicity on 3T3-L1

The cytotoxic activity of EtOAc and MeOH extracts was tested on 3T3-L1 cell lines at 100 µg/ml concentration. Evaluation of the cytotoxicity of the extracts was performed by reducing 3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Viable cells were determined by absorbance at 540 nm. The results showed that the EtoAC and MeOH extracts have 100% cell viability and, therefore, no cytotoxic effects on 3T3-L1 cell proliferation.

Discussion

Most EtOAc and MeOH extracts from mushroom samples did not inhibit the growth of most bacterial strains. Only EtOAc extracts of *Hymenopellis* sp. T20-0713 and *H. utriformis* T20-0715 exhibited inhibition of *M. luteus* growth. *Micrococcus luteus* is a gram-positive bacterium and an opportunistic pathogen in immunocompromised people or those with indwelling catheters (Zhu et al. 2021). The EtOAc extract of *Hymenopellis* sp. T20-0713 also prevented the growth of *C. albicans*. *Candida albicans* is an opportunistically pathogenic yeast that can cause candidiasis. Both *M. luteus* and *C. albicans* are normal microbiota in the body; however, within immunocompromised individuals, they can turn into pathogens, resulting in diseases (Talapko et al. 2021).

Hymenopellis species, especially *Hymenopellis radicata,* were previously documented to have antifungal activity (Anke et al. 1990, Rosa et al. 2003). This species can produce Oudemansin,

an antifungal E-β-methoxyacrylate (Anke et al. 1990). However, *Hymenopellis* species have never been documented for their antibacterial activity; therefore, this study is the first documentation of the antibacterial activity of this genus.

The EtOAc extracts of *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0715, and *Volvariella pulla* T20-0715 also exhibited strong inhibitory activity against alpha-glucosidase. Mushroom samples inhibit alpha-glucosidase by reducing glucose absorption as a result of decreased carbohydrate digestion. Inhibition of the α-glucosidase enzyme can help delay the digestion of carbohydrates, thus reducing glucose levels in the blood (Kashtoh & Baek 2022). The EtOAc extracts also have a half-maximal inhibitory concentration lower than that of standard acarbose. *Hymenopellis utriformis* T20-0715 has the lowest concentration of 95 μg/mL, followed by *Volvariella pulla* T20-0714 (288 μg/mL) and *Hymenopellis* sp. T20-0713 (621 μg/mL).

The MeOH extracts of *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0715, and *Volvariella pulla* T20-0714 promoted glucose uptake of L6 cells, a rat skeletal muscle cell line. *Hymenopellis* sp. T20-0713 has the highest glucose uptake percentage of 38.7±17.7%. It is well known that antidiabetic activity is linked to a greater capacity of adipocytes and skeletal muscle cells to absorb glucose (Kumar et al. 2018). Skeletal muscles carry out about 75% of our body's insulin-stimulated glucose absorption (Honka et al. 2018, Merz & Thurmond 2020). A high level of blood sugar is the result of impaired glucose absorption in skeletal muscle tissue (Kumar et al. 2018).

Diabetes mellitus is a serious condition in which the blood glucose level is too high, which has deadly consequences (Chowdhury & Paul 2020). Both EtOAc and MeOH extracts of *Hymenopellis* and *Volvariella* have antidiabetic activity and can be promising in the treatment of diabetes mellitus. There are no existing previous studies on the antidiabetic activity of *Hymenopellis* species; however, some articles have documented the antidiabetic activity of *Volvariella*, especially *V. volvacea* (Singh et al. 2017, Pushpa et al. 2021).

The cytotoxicity of the EtOAc and MeOH extracts of *Hymenopellis* sp. T20-0713, *H. utriformis* T20-0715, and *Volvariella pulla* T20-0714 were also tested against 3T3-L1 cell lines. The results should show that all extracts did not exhibit toxicity against non-tumor cells with a cell viability of 100%. Since species from these genera are consumed in other Asian countries, this result may support the edibility of *Hymenopellis* and *Volvariella* by confirming their non-toxicity.

Conclusion

In conclusion, the evaluation of EtOAc and MeOH extracts from various mushroom samples revealed intriguing findings regarding their potential medicinal properties. Although most of the extracts did not show inhibitory effects against bacterial growth, the EtOAc extracts of *Hymenopellis* sp. T20-0713 and *H. utriformis* T20-0715 exhibited promising inhibition of *M. luteus*, a gram-positive bacterium recognized as an opportunistic pathogen. Additionally, the same extract demonstrated inhibitory activity against *C. albicans*, a pathogenic yeast responsible for candidiasis. The genus *Hymenopellis*, particularly *H. radicata*, had previously been noted for antifungal activity, and this study establishes its antibacterial properties for the first time.

Given the increasing prevalence of diabetes mellitus and its associated complications, the antidiabetic potential of these mushroom extracts holds promise for future therapeutic applications. The absence of cytotoxicity against non-tumor cells suggests the safety of *Hymenopellis* spp. and *Volvariella pulla* as edible mushrooms. Altogether, this study sheds light on the diverse medicinal properties of these mushrooms and underscores their potential significance in treating various health conditions, particularly diabetes and microbial infections. Further research is warranted to elucidate the underlying mechanisms and optimize their application in clinical settings.

Acknowledgement

This article is supported by the Thailand Science Research and Innovation grant “Macrofungi diversity research from the Lancang-Mekong Watershed and surrounding areas (Grant No. DBG6280009) and the Thailand Research Fund “Study of saprobic Agaricales in Thailand to find new industrial mushroom products” (Grant No. DBG6180015). We thank the people who helped

and assisted in the lab work at M3 for the bioactivity analyses: Dr. Wuttichai Jaidee, Ms. Narawadee Rujanapun, Ms. Kulawadee Kumpeera, Ms. Enjoy, Ms. Chalinee Janta, Mr. Supphanat Khumthian, and Mr. Pornprom Amantrakul from the Medicinal Plants Innovation Center of Mae Fah Luang University.

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