



Antioxidant and Cytotoxic Activity of *Phellinus* Mushrooms from Northeast Thailand

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Abstract

Background and objectives: *Phellinus* belongs to the family of Hymenochaetaceae. In Traditional Chinese Medicine, it has been used as an ingredient for the treatment of different types of cancer, ischemia and skin diseases for thousands of years. The present study was aimed to evaluate and compare the mushroom constituents (total phenolic and flavonoid contents) and antioxidant and cytotoxic effect against cholangiocarcinoma cells. **Methods:** The samples of *Phellinus* mushrooms including *P. igniarius*, *P. linteus* and *P. nigricans* were prepared in two ways: macerated in 95% ethanol and decocted in distilled water. The antioxidant activity of the six extracts were evaluated using the DPPH, ABTS and FRAP assays. Total phenolics and flavonoids were determined using colorimetric tests. In addition, cytotoxic activities against cholangiocarcinoma cell lines (KKU-100 & KKU213A) were assessed by the SRB assay. **Results:** All ethanol extracts of samples showed significantly stronger antioxidant activity compared to aqueous extracts ($p < 0.05$), while the ethanol extracts contained higher total phenolic and flavonoid contents. *Phellinus linteus* showed the highest antioxidant activity and total phenolic content when compared to *P. igniarius* and *P. nigricans*. All samples showed high cytotoxicity against cholangiocarcinoma cell lines, particularly the ethanol extract of *P. linteus*. The cytotoxicity was correlated to the phytochemical contents and antioxidant activity of each *Phellinus* mushroom. **Conclusions:** The cytotoxicity and antioxidant activity are in proportion to the phenolic and flavonoid contents. Therefore, the antioxidant capacity of the mushroom extracts may advocate anti-cancer effects.

Keywords: antioxidant; cytotoxicity; *Phellinus* mushrooms

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Introduction

Phellinus is a group of medicinal mushroom belonging to the family Hymenochaetaceae [1]. The species decays heartwood, causes root cankers in live standing trees and destroy slash

and other woody residues [2]. The pilear surface is light to dark brown or black in color and the hymenial surface is poroid and, light to dark brown in color. Generative hyphae are subhyaline

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and pale yellow with, simple septate, thin to thick-walled, and branched. Basidiospores are subhyaline and golden yellow to golden brown and are thin-walled to thick-walled [3]. There are 479 species of *Phellinus* worldwide according to the Index Fungorum (2019) [4]. More than 31 species of *Phellinus* mushrooms exist in Thailand, and only two species (*Phellinus linteus* and *Phellinus igniarius*) have been reported for utilization as medication [5]. Traditional Chinese Medicine has used *Phellinus* as an ingredient for the treatment of cancers, herpes, earache, rash, ischemia and skin diseases for thousands of years in Asian countries [6].

Previous studies have shown that some species possess antioxidant [7,8], anti-inflammatory [9,10] and anticancer properties in the fruiting body of mushrooms belonging to the genus *Phellinus* [11,12]. The study of phytochemical constituents of *Phellinus* species have indicated that polysaccharides [4], triterpenoids [13], phenolics and flavonoids [6] were found. For example, scientific investigations demonstrated that hispolon isolated from *Phellinus igniarius* induced apoptosis of lung cancer by increasing apoptosis-related protein expressions, such as the cleavage form of caspase 3, caspase 8 and polymerase [14,15]. The polysaccharide isolated from *P. linteus* effectively inhibited proliferation and colony formation of hepatocellular carcinoma cells (HepG-2) via S-phase cell cycle arrest [16]. In addition, phenolic compounds and polysaccharides prevented cancer formation by their antioxidant effect [17] and immunomodulatory activity [18].

Antioxidants play an important role in neutralizing free radical reactions in the human body [19]. Free radicals have a potential oxidative stress to damage cells. The resultant cell damage contributes to human diseases such as cancer, diabetes mellitus and also inflammation [20]. Therefore, the discovery and research for potent bioactive substances with notable antioxidant activity and low cytotoxicity are important for the development of natural products. Phytochemicals that possess free radical scavenging activity can be used in the prevention and treatment of many inflammation involved diseases including cancer.

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States [21].

Cholangiocarcinoma, a cancer of bile duct, is a significant public health burden in the world, especially in developing countries such as Thailand. The lack of early diagnosis, resistance to chemo and radiotherapies are some of the difficulties and challenges encountered during the treatment [22]. At present, anti-cancer drugs have displayed several side-effects and complications when compared to natural anticancer materials. The natural products are effective and less-toxic agents which are keys in the development of new drugs for the treatment of cancers [23]. Moreover, phytochemicals are gaining popularity in order to be used as prevention and treatment of cancers [24]. A number of bioactive substances show potential to prevent cancer by molecular mechanisms and prove effect against various stages in the neoplastic process [25]. The study of bioactive compounds and development of new drugs for the treatment of cholangiocarcinoma is prioritized; therefore, the objectives of the present study were to evaluate total phenolic and flavonoid contents, antioxidant and cytotoxic activities of *Phellinus* mushroom extracts collected from northeast, Thailand against cholangiocarcinoma.

Materials and Methods

Ethical considerations

The research proposal was approved by the Institutional Biosafety Committee of Mahasarakham University, Mahasarakham District, Thailand (Code; IBC12-13/2563, Date approved; September 17, 2020).

Chemicals and reagents

Cell culture medium including Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic, trypsin, phosphate buffer saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco (USA); dimethyl sulfoxide (DMSO), 2,4,6-tri-(2-pyridyl)-s-triazine (A0382300), Folin-Ciocalteu reagent (LM0821611), methanol (HPLC grade), Rutin (A0257221), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, BCBV9734), DPPH (1.1-diphenyl-2-picrylhydrazyl), STBB0828V) were purchased from Sigma Chemicals (Germany).

Mushroom samples and cell lines

The samples including *P. igniarius* (MSUT2931),

P. linteus (MSUT2712), and *P. nigricans* (MSUT2707), were obtained from the Natural Medicinal Mushroom Museum, Faculty of Science, Maharakham University, Thailand (MSUT) in June 2019. Cell lines (KKU-100 & KKU-213A) were obtained from Cholangiocarcinoma Research Institute, KhonKaen University, KhonKaen Maharakham District, Thailand.

Extraction

Aqueous extracts

The powder of dried fruit bodies of each sample (77 g) was boiled in distilled water (310 mL) for 4h. After filtration, the water extracts were dried using a freeze dryer at -98 °C for 26 h. The dried extracts were then kept in a refrigerator at 4 °C for further studies.

Ethanol extract

The powder of each sample (150 g) was extracted sequentially by maceration at room temperature with 95% ethanol (600 mL) in a big glass flask for 3 days (150 × 600 mL, 3 days each). The solution was filtered using gauze and Whatman No. 01 filter paper. The solvents used in the extraction were evaporated by a rotary evaporator and further concentrated by heating at 60 °C in a water bath, then kept in a refrigerator at 4 °C [26].

Bioassays

Antioxidant activity

Antioxidant activity of samples were determined using the DPPH, ABTS, and FRAP assays.

DPPH free radical scavenging activity

DPPH assay was used to assign free radical scavenging activity, which was described previously by Amid et al. [27]. The samples were prepared at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL, respectively. Twenty µL of samples and 180 µL of DPPH (300 µM) were added to a 96-well plate, incubated at 37 °C for 30 min in a dark place. The absorbance was further measured at 517 nm. The negative control was 95% ethanol and ascorbic acid was used as the positive control. Ethanol was replaced instead of the DPPH solution as a blank. The radical scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = [100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}]$$

Where A_{blank} = absorbance of blank, A_{sample} = absorbance of the sample

ABTS radical scavenging activity

ABTS was produced through the chemical oxidation reaction with potassium persulfate as mentioned by Payet et al. [28]. The blue-green ABTS solution was adjusted with water to obtain an absorbance of 0.7 ± 0.02 at 734 nm. The samples were prepared by mixing 20 µL of the samples (250 µg/mL) with 280 µL ABTS dissolved in water and incubated for 5 min at room temperature. The absorbance was measured at 734 nm using a microplate reader. Ascorbic acid was dissolved in distilled water and used as the positive control. The inhibition percentage of the radical scavenging activity was calculated by using the following equation:

$$\% \text{ inhibition} = [100 \times (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}]$$

Where A_{blank} = absorbance of blank, A_{sample} = absorbance of the sample

FRAP determination

The FRAP assay (ferric reducing antioxidant power) was used to measure the antioxidant power of mushroom extracts. The reduction of ferric tripyridyltriazine [Fe(III)-TPTZ] complex to ferrous tripyridyltriazine [Fe(II)-TPTZ] at low pH is indicated by blue color. The absorbance of the Fe (II)-TPTZ complex was recorded at 593 nm and ferrous sulfate was used as the reference standard [29].

Determination of total phenolics content

The Folin-Ciocalteu colorimetric method was used to investigate the total phenolic content in mushroom extracts [30]. Two hundred µL of Folin-Ciocalteu reagent mixture (1:10 diluted with distilled water) was mixed with 20 µL of a sample (1000 µg/mL) and incubated for 5 min at room temperature; 160 µL of sodium bicarbonate (Na_2CO_3) solution (75g/L) was added to the mixture and incubated at 25 °C for 30 min. Finally, the absorbance of the solutions was recorded at 630 nm. Gallic acid solution was used to obtain the standard calibration curve (10-125 µg/mL) and total phenolic results were presented as milligrams of gallic acid equivalents (GAE) per gram of dried extract.

Determination of total flavonoids content

Total flavonoids content in the mushroom extracts were determined using the aluminum chloride colorimetric assay [31]. Extracts (100 μ L) or standard solution of rutin (10, 20, 40, 60, 80 mg/mL) were added to 30 μ L of 5% NaNO₂ and 30 μ L of 10 % AlCl₃. In the 6th min, the total volume was made up to 1 mL by 200 μ L of 1M NaOH and distilled water. The absorbance was measured against the prepared blank reagent at 415 nm. The total flavonoids content of the sample was expressed as milligrams of rutin equivalents (RE) per gram of dried extract.

Cell culture

Cholangiocarcinoma cell lines, KKU-100 and KKU-213A [32,33], were cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic (100 IU/mL of penicillin-streptomycin solution) in a humidified atmosphere of 5% CO₂ at 37 °C for 72 h before experiments.

Determination of cytotoxicity against cholangiocarcinoma cells

Cholangiocarcinoma cells were plated to 96-well plates (2×10^3 cells/well) and incubated. After 24 h, the cells were treated with samples (100 μ L, 500 μ g/mL) and incubated at periods of 24, 48, 72 h. The concentration of DMSO in a vehicle control was 0.5% in DMEM. SRB assay was used to determine the cancer cell viability after treatment of mushroom extracts.

Ten percent of trichloroacetic acid was used to fix the cells in the refrigerator at 4 °C for 30 min. The plate was then washed with distilled water, dried and stained with SRB solution for 30 min and further washed with 1% acetic acid to remove the unbound dye. The bound protein stain in the plate was solubilized using the Tris base. The absorbance was measured at 540 nm [34,35]. The percentage of inhibition was measured as $[1 - (\text{optical density of test} / \text{optical density of vehicle control})] \times 100$

Statistical analysis

The experiments of the study were performed in triplicates. The results have been shown as mean \pm SD. The differences between the groups were considered significant when $p < 0.05$. The Student's t-test was used to define statistical significance among two groups and one-way analysis of variance (ANOVA) followed by

Duncan post hoc test was compared for multiple groups.

Results and Discussion

The antioxidant activity, total phenolic and total flavonoid contents of the *Phellinus* mushroom extracts are shown in Table 1. In this study, the ethanol extracts demonstrated significantly higher activities than the water extracts ($p < 0.05$) in all assays. In the DPPH assay, the ethanol extract of *P. linteus* showed the highest antioxidant activity compared to other extracts ($p < 0.05$, IC₅₀ value 28.85 \pm 0.56 μ g/mL). The ethanol extracts from *P. linteus* and *P. nigricans* exhibited the highest antioxidant activity in the ABTS assay radical, with IC₅₀ values of 14.06 \pm 0.08 μ g/mL and 15.41 \pm 0.40 μ g/mL, respectively; whereas the ethanol extracts from *P. igniarius* displayed moderate antioxidant activity in response to ABTS radicals with IC₅₀ values of 18.44 \pm 0.48 μ g/mL. The total flavonoids content was found to be higher in *P. igniarius* whereas it was less than other extracts (353.30 \pm 1.87 μ g/mL; 145.53 \pm 2.10 μ g/mL). In the FRAP assay, the ethanol extract from *P. linteus* and *P. igniarius* exhibited the highest antioxidant activity ($p < 0.05$) while *Phellinus* mushroom extracts from both mushrooms demonstrated the highest total phenolics and flavanoidd contents, respectively. The greatest total phenolics content was observed in ethanol extract of *P. linteus* (184.80 \pm 5.54 mg GAE/g) while the ethanol extract from *P. igniarius* showed the highest amount of total flavonoid content (353.30 \pm 1.87 mg RE/g) ($p < 0.05$).

The present study suggested that the ethanol extract of *P. linteus* demonstrated the highest capacity of antioxidant activity in all assays. The reports by Samchai et al. [36] which revealed that the ethanol extract of *P. linteus* showed high free radical scavenging capacity with IC₅₀ value of 29.18 \pm 0.20 μ g/mL, while Seephonkai et al. [37] reported lower free radical scavenging capacity (IC₅₀ = 59.24 \pm 0.31 μ g/mL). The results of the present study also suggested that there was a direct correlation between total phenolics content and antioxidant activity which was observed in the ethanol extract of *P. linteus*. The previous study of Laovachirasuwan et al. [38] suggested that the ethanol extracts of *Phellinus* mushroom possessed higher antioxidant activity than the water extracts which was similar to those observed in the present study.

Table 1. Antioxidant activity, total phenolics and total flavonoids contents of *Phellinus* mushroom extracts

Samples	DPPH	ABTS	FRAP	Total phenolics content	Total flavonoids content
	IC ₅₀ (µg/mL)	IC ₅₀ (µg/mL)	(mM Fe ²⁺ / 100 mg)	(mg GAE/g of sample)	(mg RE/g of sample)
Ethanol extracts					
<i>P. nigricans</i>	32.46 ± 0.30 ^{B*}	15.41 ± 0.40 ^{A*}	22.70 ± 5.04 ^{ab}	148.86 ± 3.50 ^b	183.55 ± 5.93 ^c
<i>P. linteus</i>	28.85 ± 0.56 ^{A*}	14.06 ± 0.08 ^{A*}	36.90 ± 0.97 ^a	184.86 ± 5.54 ^a	223.67 ± 6.91 ^b
<i>P. igniarius</i>	32.33 ± 0.54 ^{B*}	18.44 ± 0.48 ^{B*}	37.57 ± 6.21 ^a	145.53 ± 2.10 ^b	353.30 ± 1.87 ^a
Aqueous extracts					
<i>P. nigricans</i>	81.90 ± 0.25 ^{C*}	24.59 ± 1.03 ^{C*}	11.19 ± 0.79 ^{bc}	58.70 ± 2.76 ^c	75.46 ± 1.15 ^e
<i>P. linteus</i>	176.68 ± 0.77 ^{E*}	29.99 ± 1.62 ^{D*}	12.39 ± 0.61 ^{bc}	50.43 ± 1.65 ^c	74.81 ± 3.52 ^e
<i>P. igniarius</i>	84.62 ± 1.13 ^{D*}	25.21 ± 1.13 ^{C*}	12.23 ± 1.08 ^{bc}	54.48 ± 2.97 ^c	96.76 ± 4.29 ^d
Ascorbic acid	4.53 ± 0.33	5.34 ± 0.37	38.37 ± 1.55	-	-

*Statistically significant difference when compared to ascorbic acid (within column), $p < 0.05$; different letters indicate statistically significant difference within columns (uppercase letters, $p < 0.05$; lowercase letters, $p < 0.001$); mM: millimole equivalent; GAE: gallic acid equivalent; RE: rutin equivalent.

Maingam et al. [39] reported antioxidant activity of crude hot boiling-water extract from cultured mycelia of *P. linteus*, which inhibited DPPH radical scavenging with the IC₅₀ value of 243.25±30.82 µg/mL. The growth of cholangiocarcinoma cell lines (KKU-100 & KKU-213A) was evaluated using the sulforhodamine B (SRB) assay modified from the US National Cancer Institute (NCI) [32,35]. In the current study, three ethanol extracts of *Phellinus* mushrooms which presented as the highest antioxidant activity, total phenolics content and total flavonoids content were selected for cytotoxicity against. Percent cell inhibition of each extract is presented in Table 2. As presented in Table 2, the ethanol extract of *P. linteus* had the greatest cytotoxicity against in both KKU-100 and KKU-213A cells when compared to other extracts at all incubation periods (24, 48, and 72 h) ($p < 0.05$). After treating with *P. linteus*, percent cell inhibition of KKU-100 ranged from 74.22 ± 2.08 to 95.52 ± 0.14 and 91.42 ± 1.22 to 98.92 ± 0.22 for KKU-213A cells. This is the first report of *Phellinus* mushroom extracts on cytotoxicity against cholangiocarcinoma cell lines. The study of Park et al. [40] revealed that the combination of the ethanol extract of *P. linteus* and a monoclonal antibody, cetuximab, increased the sensitivity of KRAS mutated colon cancer cells to cetuximab which indicated the potential of *Phellinus* mushroom extracts as a medical supplement against colon cancer.

The ethanol extract of *P. igniarius* showed the second highest cytotoxicity in both cells and in all incubation periods with percent cell inhibition ranging from 46.61 ± 2.16 to 83.46 ± 0.5 for KKU-100 and 75.53 ± 3.08 to 91.83 ± 0.79 for KKU-213A cells. Song et al. [41] reported that

the ethanol extract of *P. igniarius* displayed antiproliferative and antimetastatic effects in human hepatocellular carcinoma (SK-Hep-1) and rat heart vascular endothelial (RHE) cells. This exhibited the potential of *P. igniarius* extract as an adjuvant for cancer chemotherapy.

In the present study, *P. nigricans* was reported to have the least cytotoxic activity in this study when compared to other *Phellinus* mushroom extracts, the percent cell inhibition of *P. nigricans* ranged from 49.22 ± 1.98 to 51.13 ± 1.06 for KKU-100 and 52.20 ± 0.86 to 79.98 ± 0.5 for KKU-213A cells. Li et al. [42] reported that proteoglycans isolated from the mycelium of *P. nigricans* displayed antitumor, and immunomodulating activities. The cytotoxic activity of *Phellinus* mushroom extracts depends on various factors e.g. bioactive compounds in the extracts, incubation times and type of cell lines. A previous study reported that KKU-213A cell line was more sensitive to cancer chemotherapeutic agents than KKU-100, [43] similar result was observed in the present study. For example *P. igniarius* and *P. nigricans* showed weak cytotoxic activity at 24 and 48h when compared with *P. linteus*. The difference of bioactive compounds of *Phellinus* mushroom is an important factor involved in cytotoxicity. In a previous study, hispolon isolated from fruiting bodies of *P. igniarius* exhibited cytotoxicity on lung cancer cells (A549 & H661) and decreased cell viability in a concentration- and time-dependent manner [15].

Sarfraz et al. reported that hispolon from *P. linteus* induced apoptosis and sensitized human cancer cells to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through upregulation of death receptors [44].

Table 2. Effects of the *Phellinus* mushroom extracts on growth of cholangiocarcinoma cell lines at different times

Samples/ time point	CCA cell line (% inhibition at 500 µg/mL)					
	KKU-100			KKU-213A		
Time (h)	24	48	72	24	48	72
<i>P. nigricans</i>	49.22 ± 1.98 ^B	47.46 ± 3.94 ^C	51.13 ± 1.06 ^{a*}	52.20 ± 0.86 ^{a*}	74.04 ± 0.91 ^a	79.98 ± 0.50 ^{a*}
<i>P. linteus</i>	74.22 ± 2.08 [*]	96.68 ± 0.35 [*]	95.52 ± 0.14 ^{b*}	91.42 ± 1.22 ^{b*}	96.74 ± 0.46 [*]	98.92 ± 0.22 [*]
<i>P. igniarius</i>	49.72 ± 2.12 ^A	46.61 ± 2.16 ^D	83.46 ± 0.50 ^{c*}	75.53 ± 3.08 [*]	78.23 ± 3.22 [*]	91.83 ± 0.79 ^{c*}

Star (*) indicate statistically significant difference among mushrooms, $p < 0.05$; different lowercase letters indicate statistically significant difference among time points, $p < 0.001$; Different uppercase letters (A,B,C) indicate statistically not significant difference among mushrooms and time points, $p < 0.05$

Phellinus mushroom extracts is rich in biologically active compounds with therapeutic potential. Polysaccharide, especially β -glucan, steroid, flavonoid, and phenolic are believed to be responsible for the biological activities as observed in the *Phellinus* mushroom [4]. The results on antioxidant and growth inhibition effect of ethanol extracts from *P. linteus*, *P. igniarius*, *P. nigricans* showed their potentials for further bioactivity investigation and may be candidates for development of natural commercial products in the further.

Conclusion

The present study demonstrated that all samples showed antioxidant activity and cytotoxicity against cholangiocarcinoma cells. Both biological activities have relation to phytochemical of *Phellinus* mushroom extracts such as total phenolics content and total flavonoids content. Therefore, the mechanisms of antioxidant capacities may advocate anti-cancer effects; *Phellinus* mushroom may be candidates for discovery of new drugs in the future.

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Author contributions

Mushroom sample preparation, extraction, biological tests, chemical reaction testing and drafting of the manuscript were carried out by Sonesay Thammavong. Methin Phadungkit and Pornpun Laovachirasuwan were responsible for

plan setting and recommendation of biological tests, they were responsible for manuscript modification and editing. Khwanyuruan Naksuwankul was responsible for designing the study, providing mushroom samples, identifying samples and manuscript editing. Waraporn Saentaweek assisted with some biological tests. Atit Silsirivanit and Sopit Wongkham advised and guided some experiments.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

PE: *Phellinus* mushroom extract; CCA: cholangiocarcinoma; HepG-2: hepatocellular carcinoma cells; DMEM: Dulbecco's modified eagle medium; PBS: phosphate

buffer solution; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; ABTS: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); DPPH: 1.1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing antioxidant power; TPC: total phenolic content; TFC: total flavonoid content, RE: rutin equivalents.