

Protein-Bound Polysaccharide K (PSK)-Rich Extract of *Lentinus Polychrous* Exhibits Anticancer Activity by Apoptosis Induction and Cell Cycle Arrest in Cholangiocarcinoma Cells

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Abstract

Cholangiocarcinoma (CCA) is an uncommon adenocarcinoma of the bile duct epithelial. There is no standard regimen yet for patients with adequate performance status. Therefore, effective agents are necessary. Recently, protein-bound polysaccharide K (PSK) has shown a great anticancer potential. *L. polychrous* is a Thai local edible mushroom that traditionally used as a folk medicine for treatments of fever and inflammation. This study aimed to identify the PSK from hot water extract of *L. polychrous* and examine the anticancer activity against a CCA cell line KKU-213A. The PSK were quantified by ELISA and characterized using SDS-PAGE, glycoprotein staining kit, and western blot analysis. The antioxidant activity was assessed by DPPH assay. Cytotoxic effect against KKU-213A was assessed by MTT assay. Apoptosis and cell cycle were analyzed by flow cytometry. The highest PSK levels were found in *L. polychrous* extract compared with the other 14 mushroom species which purified PSK is approximately 70 kDa of glycoprotein. The hot water extract of *L. polychrous* showed strong antioxidant activity. The cell viability of KKU-213A cells was significantly inhibited with *L. polychrous* extract at an IC₅₀ value of 3.31 ± 0.09 mg/mL. In addition, the induction of cell cycle arrest at S and G2/M phases was observed together with induction of apoptosis, and mitochondrial membrane dysfunction in concentration-dependent manner. Hence the *L. polychrous* extract is the great source of PSK with strong antioxidant activity and exhibits anti-CCA effects through cell cycle arrest and apoptotic induction.

Keywords: Cholangiocarcinoma, *Lentinus polychrous*, Anticancer, Apoptosis, Cell cycle, Protein-bound polysaccharide K (PSK), Antioxidant

Introduction

CCA is a fatal malignancy originated from the biliary epithelial cells and is a major public health problem in many Asian countries [1]. The highest incidence rate of CCA in the world is observed in the northeast region of Thailand, where the highest prevalence of *Opisthorchis viverrini* (OV) infection is reported [2]. The serious clinical challenge of CCA is due to a high rate of recurrence and metastasis. Patients with unresectable and/or metastasis are treated with palliative care, radiotherapy, chemotherapy, immunotherapy, and/or targeted therapy. The combination treatment of chemotherapy and radiotherapy had rather severe adverse effects [3]. CCA is usually insensitive to chemotherapy by 5-fluorouracil (5-FU) and gemcitabine where the combination of gemcitabine and cisplatin are currently the standard treatment for 1st-line chemotherapy in advanced cases [4,5]. Thus, the alternative treatments of CCA with improving efficacy are critical and urgent.

Plant-derived compounds have emerged as a promising approach in cancer therapy. Mushrooms have been recognized for food and medical properties for thousands of years. There are various bioactive compounds including polysaccharide, protein, and some small molecules which have become popular sources of antitumor, antiviral, antimicrobial, antioxidative, and immunoenhancing agents. Immunological-based pharmaceutical agents which are isolated from more than 30 mushroom species have shown anticancer effects in animal model studies. All are chemically *b-D*-glucan in nature (i.e., linear polymers of *D*-glucose with other monosaccharides). *b-D*-glucans linked to proteins (so-called polysaccharide-peptides, more formally termed "proteoglycans" [6].

PSK, also known as polysaccharide K or Krestin, is proteoglycans or protein-bound polysaccharide which is isolated from the mushroom named *Coriolus versicolor* (CM101) by hot water extraction [7]. The mushroom extracted with hot water has been used in

traditional Chinese medicine for decades. In Japan, PSK has been used for more than 30 years as adjuvant therapy together with standard surgery, chemotherapy, and radiation therapy [8]. Apart from the immunomodulatory effects, PSK have exerted the anticancer activity by induction of apoptosis and inhibition of metastasis [9-12]. Several clinical trials demonstrated that PSK significantly extended survival at 5 years or beyond in stomach, colon-rectal, esophageal, nasopharynx, non-small cell lung, and breast cancers [8,13,14]. Moreover, PSK has reported the antitumor effects against MethA fibrosarcomas implanted in immunodeficient NOD/SCID mice [15]. These findings suggest the potential roles in anticancer activity of PSK in several cancers, but its effect against CCA has never been elucidated.

L. polychrous is a Thai local edible mushroom that traditionally used as a folk medicine for treatments of fever and inflammation that caused from snake or scorpion envenomation [16]. The extract of *L. polychrous* mycelia showed the anti-inflammatory activity both *in vivo* and *in vitro* [17] and immunomodulatory effects in cell lines [18]. The methanolic extract and crude polysaccharides from fruiting bodies and mycelia of *L. polychrous* were reported the antioxidative activity [19] and the anti-proliferative effect on MCF-7 breast cancer cell lines [20,21]. The bioactive substances of *L. polychrous* consist of several phenolic compounds (e.g. antioxidant-catechin), ergosterols, polysaccharides and proteins (e.g. anticancer protein-polysaccharide peptide) [22,23]. A number of antitumor substances have been identified in various edible mushroom species. Accumulating findings indicate the antitumor properties of PSK and its synergistic effect in combined therapies [10,14,24,25]. However, the bioactive substances including PSK, and the anti-CCA activity of *L. polychrous* extract have not been clearly elucidated yet.

In this study, we developed indirect ELISA tool using polyclonal antibody to determine the PSK levels from the hot water extract of 15 Thai edible mushrooms including *L. polychrous*. The PSK was purified and characterized then compared to the commercial PSK. The *L. polychrous* extract was assessed the antioxidant activity. Furthermore, the extract was used to examine the antitumor cytotoxic activity in KKU-213A CCA cell line. Finally, the mechanism of cell death in CCA cells was investigated.

Materials and methods

Cell line and cell culture

The human CCA cell line, KKU-213A (well-differentiated adenocarcinoma) was established from Thai CCA patients and obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Gibco, NY) supplemented with 10 % fetal bovine serum and 1 % antibiotic-antimycotic (Gibco). Cells were maintained at 37 °C in a 5 % CO₂ atmosphere with 95 % humidity.

Preparation of mushroom extracts

Fruiting bodies of 15 mushrooms were collected from Phitsanulok (location: X684 + C6C, Tha Chang, Phrom Phiram District), Nakorn Ratchaseema (location: W2F9 + 558 Suranari, Mueang Nakhon Ratchasima District), and Trang Provinces (location: PMXW + F26, Pak Chaem, Huai Yot District) of Thailand. The identification of mushrooms was kindly supported by Associate Prof. Sureelak Rodthong from Suranaree University of Technology, Thailand. Those fresh fruiting bodies were prepared by homogenizing in hot water at 80 °C. The hot water extracts were precipitated with 90 % ethanol/ammonium sulfate in order to harvest extracts and measure the immunoreactive PSK level using ELISA assay.

Development of indirect ELISA to quantify immunoreactive PSK of mushrooms

To quantify immunoreactive PSK amounts in fruiting body extracts of mushrooms, indirect ELISA was performed following the procedure described previously in quantitation of Vt and Vg in ovary and hemolymph of prawns [23]. Briefly, the commercial PSK and hot water extracts from fruiting body were coated on 96 well-plates at room temperature. Anti-PSK antibody specific to commercial PSK was used as a primary antibody at a dilution of 1:500. The enzyme reaction was carried out by o-phenylenediamine after the addition of anti-rabbit IgG-HRP (dilution 1:5,000). PSK concentrations in the samples were calculated on the basis of PSK concentrations according to a standard curve. The sensitivity of the assay for immunoreactive PSK was from 0.1 - 100 ng per assay.

Preparation and characterization of immunoreactive PSK

The hot water extracts of fruiting bodies from mushrooms were dialyzed against PBS, pH 7.5 containing 1 mM protease inhibitor cocktail D. The dialyzed extract was first precipitated and concentrated by viva spin column. Then, the concentrated solution was purified by affinity chromatography on CNBr-activated sepharose 4B column which conjugated with anti-PSK antibody as ligand. The absorbed PSK fraction in column were eluted with ammonium acetate pH 5. Protein concentration was determined by Bradford assay. Purity of PSK was confirmed by SDS-PAGE, stained with silver and glycoprotein staining kit.

Western blot analysis

Western blotting was carried out basically as previously reported [23]. Briefly, after reducing SDS-PAGE and transferring to a PVDF membrane, PSK was bound to anti-PSK and PSK antibody (dilution 1:1,000) followed by a secondary horseradish peroxidase (HRP) conjugated-goat anti-rabbit IgG (dilution 1:10,000).

Immunoreactivity was demonstrated by enhanced chemiluminescence (ECL) and developed protein bands by film.

DPPH assay

The DPPH (2,2-diphenylpicrylhydrazyl) assay was used to determine the free radical scavenging activity of mushroom extracts. BHA and the mushroom extract were mixed with 1 mL of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in dark condition, and the absorbance was then measured at 512 nm.

$$\% \text{DPPH Scavenging assay (Inhibition)} = \left[\frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})}{\text{OD}_{\text{control}}} \right] \times 10 \quad (1)$$

Cell viability assay

KKU-213A cells (5×10^3 cells/well) were seeded in 96-well plate and culture overnight. The cells were treated with various concentrations of extract (1, 2, 3, and 4 mg/mL) or sterile DW (control group). After 48 h, cell viability was determined by MTT assay. Briefly, the cells were incubated with 0.5 mg/mL of MTT for 4 h at 37 °C. The medium was aspirated and dissolved the pellet with 100 % dimethyl sulfoxide (DMSO), and the plate was read at a wavelength of 540 nm using microplate reader to determine the cell viability.

Detection of apoptosis by Annexin V/7-AAD staining

KKU-213A cells (2×10^5 cells/well) were seeded in 6-well plate and culture overnight. Cells were treated with 0, 1, and 2 mg/mL extracts for 48 h. Cells were washed twice and analyzed by Muse™ Annexin V & Dead Cell Kit (Merck Millipore, Germany). Briefly, cells were resuspended in Muse™ Annexin V & Dead Cell reagent for 20 min at room temperature. Cells were then analyzed using Muse Cell Analyzer flow cytometry

(Merck Millipore). The early apoptotic (Annexin V^{High}/7-AAD^{Low}) and late apoptotic (Annexin V^{High}/7-AAD^{High}) cells were counted as the total apoptosis.

Cell cycle analysis

To detect the proportion of cells in different phases of the cell cycle, the DNA content was detected by Muse™ Cell cycle kit (Merck Millipore). KKU-213A cells were seeded in 6-well plates overnight and incubated with 0, 1, and 2 mg/mL extracts for 48 h. Then, cells were collected, washed with PBS, and fixed with 70 % ethanol at least 3 h at -20 °C. Cells were centrifuged and washed once with PBS. Cells were added by Muse™ Cell cycle reagent for 30 min at room temperature and analyzed using Muse™ Cell Analyzer flow cytometry.

Mitochondrial membrane potential ($\Delta\Psi_m$) assay

To elucidate the role of mitochondria played in KKU-213A cell death following treatment with extracts, changes of the mitochondrial membrane potential or depolarization were detected by flow cytometry using the Muse™ mitoPotential kit (Merck Millipore). Briefly, cells were treated with extracts for 48 h then collected and washed twice with PBS. Cells were then incubated with Muse™ MitoPotential working solution and Muse™ MitoPotential 7-AAD for 5 min at room temperature. Then cells were analyzed on a Muse™ Cell Analyzer flow cytometry.

Statistical analysis

Mean values were from 3 independent experiments. Statistical analysis was performed on GraphPad Software (San Diego, CA, USA) using 1-way ANOVA followed by Dunnett's multiple comparisons test. A *p*-value of less than 0.05 was considered statistically significant.

Results and discussion

Identification of immunoreactive PSK in mushroom extracts

The PSK levels of 15 mushroom extracts were determined by calculating from PSK standard curve using ELISA method. The high levels of immunoreactive PSK were found in hot water extraction of fruiting body from 7 mushroom extracts which are *Termitomyces fuliginosus* (7), *Russula foetens* (8), *Canharellus cibarius* (9), *Lactarius flavidulus* (10), *Russula emetia* (11), *Russula alboareolata* (12), and *L. polychrous* (15). Interestingly, *L. polychrous* showed the highest level of immunoreactive PSK as shown in **Figure 1**. Table data as supplementary material shown in supplement data.

The extract of *L. polychrous* was selected for purification by affinity chromatography on CNBr-

activated sepharose 4B column which conjugated with anti-PSK antibody as ligand. The molecular weight of protein from *L. polychrous* extract was approximately 70 kDa which its molecular mass was similar to the commercial PSK using SDS-PAGE and western blotting as shown in **Figures 2(A) - 2(B)**. The protein band displayed glycoprotein content by glycoprotein staining kit as shown in **Figure 2(C)**. The *L. polychrous* extract also inhibited DPPH free radicals at 60 % while BHA, an antioxidant compound, showed 76 % as shown in **Figure 2(D)**. Table data as supplementary material shown in supplement data. These results indicate that *L. polychrous* is the great source of PSK and may exert a direct anticancer activity. Therefore, the anticancer effects of *L. polychrous* were further investigated against KKV-M213A cell line including its related mechanisms.

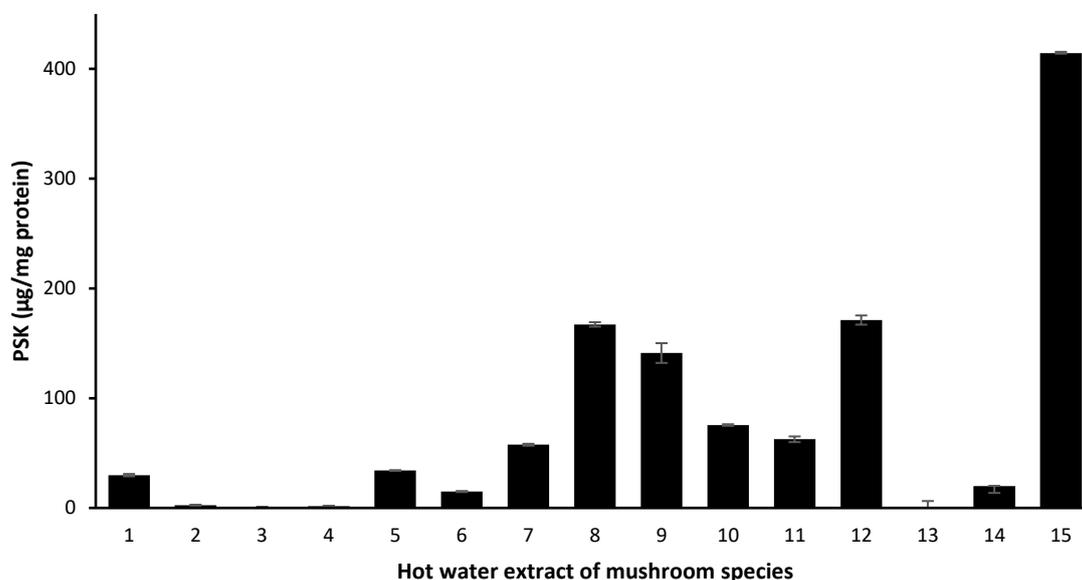


Figure 1 PSK levels of mushroom extracts using indirect ELISA. Hot water extract from 15 mushroom species; (1) *Lentinus edodes* (2) *Pleurotus flabellatus* (3) *Pleurotus citrinopileatus* (4) *Pleurotus cystidiosus* (5) *Bloletus griseipurpureus* (6) *Fistulina hepatica* (7) *Termitomyces fuliginosus* (8) *Russula foetens* (9) *Canharellus cibarius* (10) *Lactarius flavidulus* (11) *Russula emetia* (12) *Russula alboareolata* (13) *Russula virescens* (14) *Russula delica* (15) *L. polychrous*. Values are mean \pm SD of independent triplicate experiments.

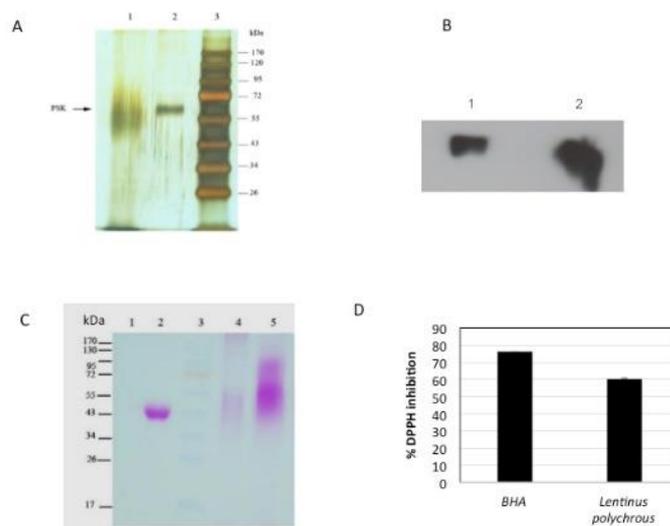


Figure 2 Immunoreactive PSK in *L. polychrous*. (A) SDS-PAGE 15 % with silver staining. Lane 1; commercial PSK. Lane 2; Purified immunoreactive PSK. Lane 3; Marker protein. (B) SDS-PAGE 15 % and western blot analysis. Lane 1; Purified protein from hot fruiting body extract of *L. polychrous*. Lane 2; Commercial PSK. (C) SDS-PAGE 15 % and Glycoprotein staining. Lane 1; Negative control (soybean trypsin inhibitor). Lane 2; Positive control (Horseradish peroxidase). Lane 3; Marker. Lane 4; Commercial PSK. Lane 5; Immunoreactive PSK of *L. polychrous* hot water extract. (D) *L. polychrous* extract shows DPPH inhibition compare with positive control, BHA. Values are mean \pm SD of independent triplicate experiments.

***L. polychrous* extract suppresses cell viability of KKKU-213A CCA cell line**

To determine the effect of *L. polychrous* extract on cell viability of KKKU-213A cells, the experiment was performed using MTT assay. As shown in **Figure 3**, treatment with *L. polychrous* extracts at 0, 1, 2, 3, and 4 mg/mL for 48 h significantly inhibited the cell viability of KKKU-213A cells in a dose-dependent manner compared to the untreated or control cells. The IC_{50} value of *L. polychrous* extract for KKKU-213A cells was 3.31 ± 0.09 mg/mL. These data suggested that *L. polychrous* extract suppressed cell viability of KKKU-213A cells. Table data as supplementary material shown in supplement data. The results are consistent with previous reports which demonstrated that the methanolic extract of *L. polychrous* mycelia showed cytotoxic effect in MCF-7 breast cancer cells with IC_{50} value greater than 500 μ g/mL with antioxidant activity [20]. The crude polysaccharides from fresh and dried fruit bodies of *L. polychrous* at concentration of 1

mg/mL showed cytotoxic effects at 38 and 45 %, respectively against MCF-7 [19]. Moreover, PSK was found the cytotoxic activity in leukemias, melanomas, fibrosarcoma and cervical, lung, pancreas, and gastric cancers with a wide range from 22 to 84 %. These data suggested that *L. polychrous* may exert inhibitory activity in a cell type-specific manner. However, the bioactive constituents in the crude extract could be varied by the extraction method due to the solubility with the solvent. In addition to PSK, the hot water *L. polychrous* extracts may contain other bioactive compounds that may show synergistic or antagonistic effects. For example, an anticancer protein polysaccharide peptide (PSP) was found in the extract of *L. polychrous* [23]. In this study, we did not examine the cytotoxic effect in the normal cell line. Previous report demonstrated that the high dose (2.5 g/kg BW) of the crude polysaccharide extract of *L. polychrous* fruiting bodies were not toxic in the rat model [26] indicating the safety of this edible mushroom.

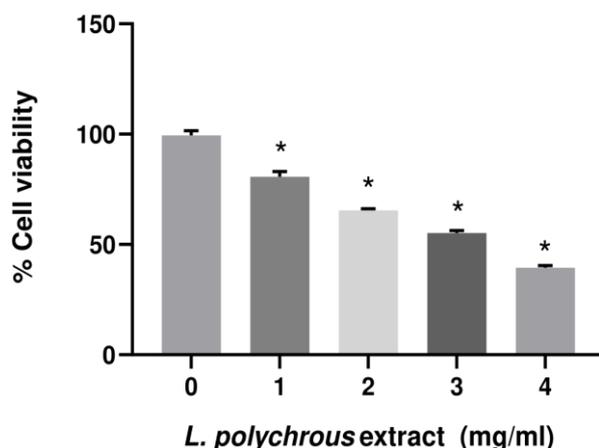


Figure 3 The effect of *L. polychrous* extract on cell viability of KKU-213A cells. The cell viability of KKU-213A treated with *L. polychrous* extract at various concentrations for 48 h was determined by MTT assay. Values are mean \pm SD of independent triplicate experiments. * $p \leq 0.05$ versus non-treated cell.

***L. polychrous* extract induced apoptosis in KKU-213A**

Apoptosis is viewed as a mechanism through which a variety of naturally active compound inhibit tumor growth. To elucidate whether the cytotoxicity of *L. polychrous* extract in KKU-213A cells is due to apoptosis induction, the number of apoptotic cells was assessed by AnnexinV and 7-AAD double staining by flow cytometry. KKU-213A cells were treated with *L. polychrous* extract at 0, 1, and 2 mg/mL for 48 h. After treatment, the number of an early apoptosis was increase at the dose of 1 mg/mL and the number of late apoptosis

was also increase at higher dose of 2 mg/mL as shown in **Figure 4**. Table data as supplementary material shown in supplement data. The percentage of apoptotic cells were significantly elevated in a dose-dependent manner. Recent studies have shown that PSK exhibited the anti-proliferative actions associated with upregulated pro-apoptotic protein Bax levels, resulting in induction of apoptosis in pancreatic cancer cells [27]. Our results indicate that the inhibitory effects of *L. polychrous* extract on KKU-213A cells may be associated with induction of apoptosis.

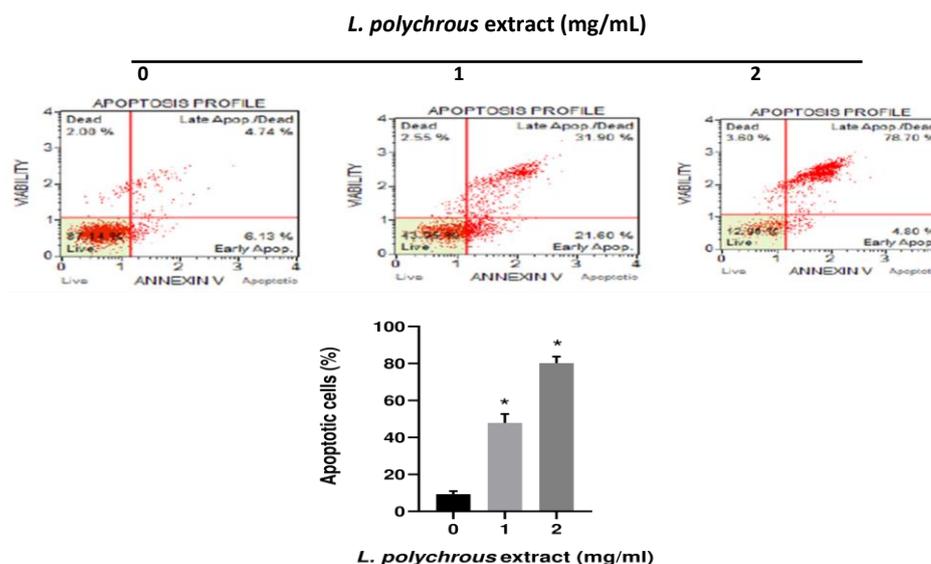


Figure 4 The effect of *L. polychrous* extract on cell apoptosis of KKU-213A cells. KKU-213A cells were treated with *L. polychrous* extract at 0, 1, and 2 mg/mL for 48 h, double stained with Annexin V and 7-AAD and analyzed by flow cytometry. The total number of early and late apoptotic/dead cells was shown as the percentage of apoptotic cells. Values are mean \pm SD of independent triplicate experiments. * $p \leq 0.05$ versus non-treated cell.

***L. polychrous* extract induced cell cycle arrest of KKU-213A cells**

Several naturally occurring compounds have been reported to suppress the growth of cancer cells through disruption of cell cycle progression [28,29]. Evading cell cycle arrest is the most frequently observed phenomenon in tumor development. The cell cycle includes a number of checkpoints that act as surveillance mechanisms. Upon cellular stress or DNA damage, these mechanisms induce cells to undergo either cell cycle arrest, activation of repair systems, or apoptotic induction. To examine the effect of *L. polychrous* extract on cell cycle progression, KKU-213A cells were incubated for 24 h in the absence or presence of 1 and 2 mg/mL of *L. polychrous* extract. Cells were stained with PI and the percentage of cells in each phase of the cell cycle were determined by flow cytometry. As shown in **Figure 5**, there was an alteration of cell numbers in different cell cycle phases compared with the control. In fact, treatment with *L. polychrous* extract induced an elevation of the number of cells in the S and G2/M phase of the cell cycle, while the number of cells in the G1

phase decreased compared with the control. These results indicated that *L. polychrous* extract treatment caused cell cycle arrest at S and G2/M phases of KKU-M213A cells. Checkpoint at G1/S and G2/M transitions are essential regulatory gates during cell cycle progression [30]. Several studies have shown a common feature in which exposure to PSK induces cell cycle arrest at G1 phase in various cancers derived from leukemias, melanomas, fibrosarcoma and cervical, lung, pancreatic, and gastric cancers [10]. Interestingly, we found that S phase and G2/M phase arrest of KKU-M213A cells was observed after treatment with *L. polychrous* extract for 24 h. This finding suggests that the mode of with *L. polychrous* extract depends on the cell type. Presently, the molecular mechanisms of *L. polychrous* extract-induced cell cycle arrest in KKU-M213A cells are unknown and require further investigation. In addition, the prior results of apoptotic induction, the suppression of KKU-M213A cell growth by *L. polychrous* extract may be caused by cell cycle arrest leading to apoptotic cell death.

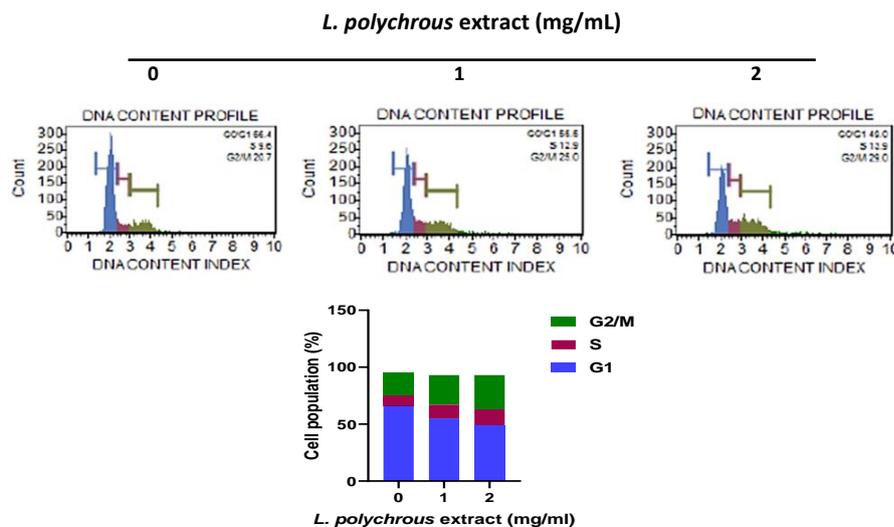


Figure 5 The effect of *L. polychrous* extract on cell cycle of K KU-213A cells. K KU-213A cells were treated with *L. polychrous* extract at 0, 1, and 2 mg/mL for 48 h, stained with PI and analyzed by flow cytometry. The percentage of cells in different cycle phases was shown. Values are means of independent triplicate experiments.

L. polychrous extract induced depletion of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial-initiated responses are involved in regulation of the intrinsic apoptotic pathway [31]. The loss of mitochondrial membrane potential ($\Delta\Psi_m$) is a key event to eliminate cancer cells [32]. To determine whether a mitochondrial response was involved in the *L. polychrous* extract-induced apoptotic pathway of K KU-M213A cells, cells were treated with *L. polychrous* extract (0, 1, and 2 mg/mL) for 24 h, labeled with Muse™ MitoPotential reagent and examined the changes of mitochondrial membrane potential ($\Delta\Psi_m$) or depolarization by flow cytometry. As shown in **Figure 6**, the number of cells with depolarization was increased remarkably in a dose-dependent manner in *L. polychrous* extract-treated cells compared with the control. The percentage of cells with depolarization increased from 35 to 66 % when *L. polychrous* extract increased from 1 to 2 mg/mL. These results indicate that the dysfunction of the mitochondrial membrane in K KU-M213A cells existed after treatment with *L. polychrous* extract.

The metabolic activities of mitochondria in cancer cells are distinct from those in normal cells and are

considered to be a biologically significant source of apoptotic failure that is closely associated with chemo- and radio-resistances in cancer therapy. Previously, PSK induced apoptosis via the mitochondrial or intrinsic pathway in HL-60 promyelomonocytic leukemia cells [11]. These studies are consistent with our observation in which K KU-M213A cells treated with *L. polychrous* extract underwent loss of the mitochondrial membrane potential by increase the depolarization. Once the mitochondrial outer membrane is permeable, proapoptotic proteins such as cytochrome C are released into the cytosol from the intermembrane space, resulting in activation of caspase-9 and -3. The other route of apoptosis, the extrinsic pathway, is initiated by the binding of ligands to death receptors. The transmembrane death signal induces formation of the DISC through adaptor proteins and subsequently leads to activation of caspase 8. Our data did not show the caspase activation, so this part should be further explored. However, our data clearly reveals that mitochondria is feasible target of *L. polychrous* extract for apoptosis of human CCA cells.

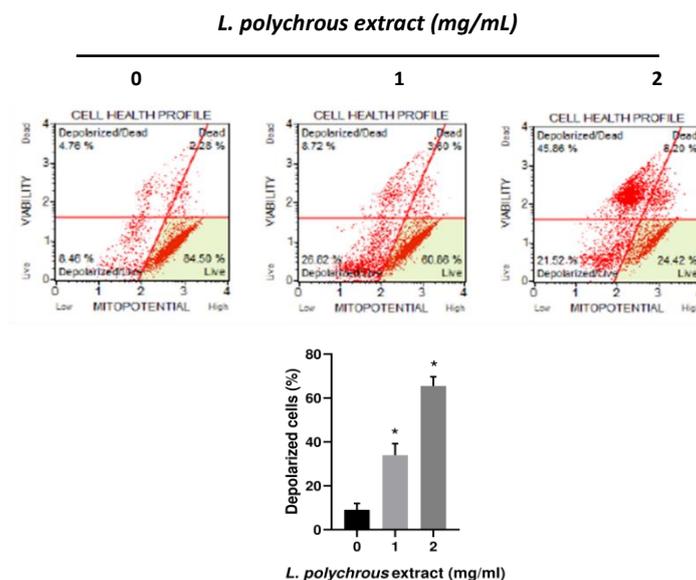


Figure 6 The effect of *L. polychrous* extract on mitochondrial membrane potential ($\Delta\Psi_m$) of K KU-213A cells. K KU-213A cells were treated with *L. polychrous* extract at 0, 1, and 2 mg/mL for 48 h, stained using the Muse™ mitoPotential kit and analyzed by flow cytometry. The percentage of depolarized cells was shown. Values are mean \pm SD of independent triplicate experiments. * $p \leq 0.05$ versus non-treated cell.

Conclusions

The findings in this study highlight the medicinal value of edible mushroom *L. polychrous* with high content of PSK and strong antioxidant activity. Interestingly, the hot water crude extract of *L. polychrous* exerts anti-proliferative and pro-apoptotic effects in K KU-213A CCA cell line primarily mediated cell cycle arrest at S and G2/M phase and mitochondrial dysfunction. Thus, *L. polychrous* extract is a potential agent for further study in development of CCA preventive and therapeutic agent. The effect of *L. polychrous* extract in other CCA cell lines or *in vivo* study warrants further research. Progress in metagenomics, proteomics, heterologous gene expressions and nanotechnology, combined with the use of *L. polychrous* extract may lead to the discovery and design of novel anticancer molecules.

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References

- [1] B Sripa, JM Bethony, P Sithithaworn, S Kaewkes, E Mairiang, A Loukas, J Mulvenna, T Laha, PJ Hotez and PJ Brindley. Opisthoriasis and *Opisthorchis*-associated cholangiocarcinoma in Thailand and Laos. *Acta Trop.* 2011; **120**, S158-S168.
- [2] S Sriamporn, P Pisani, V Pipitgool, K Suwanrungruang, S Kamsa-Ard and DM Parkin. Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. *Trop. Med. Int. Health* 2004; **9**, 588-94.

- [3] AE Sirica. Cholangiocarcinoma: Molecular targeting strategies for chemoprevention and therapy. *Hepatology* 2005; **41**, 5-15.
- [4] M Hejna, M Pruckmayer and M Raderer. The role of chemotherapy and radiation in the management of biliary cancer: A review of the literature. *Eur. J. Cancer* 1998; **34**, 977-86.
- [5] T Sasaki, T Takeda, T Okamoto, M Ozaka and N Sasahira. Chemotherapy for biliary tract cancer in 2021. *J. Clin. Med.* 2021; **10**, 3108.
- [6] R Zhou, ZK Liu, YN Zhang, JH Wong, TB Ng and F Liu. Research progress of bioactive proteins from the edible and medicinal mushrooms. *Curr. Protein Pept. Sci.* 2019; **20**, 196-219.
- [7] S Tsukagoshi, Y Hashimoto, G Fujii, H Kobayashi, K Nomoto and K Orita. Krestin (PSK). *Cancer Treat. Rev.* 1984; **11**, 131-55.
- [8] H Nakazato, A Koike, S Saji, N Ogawa and J Sakamoto. Efficacy of immunochemotherapy as adjuvant treatment after curative resection of gastric cancer. *Lancet* 1994; **343**, 1122-6.
- [9] TS Hattori, N Komatsu, S Shichijo and K Toh. Protein-bound polysaccharide K induced apoptosis of the human Burkitt lymphoma cell line, Namalwa. *Biomed. Pharmacother.* 2004; **58**, 226-30.
- [10] E Jiménez-Medina, E Berruguilla, I Romero, I Algarra, A Collado, F Garrido and A Garcia-Loracorresponding. The immunomodulator PSK induces *in vitro* cytotoxic activity in tumor cell lines via arrest of cell cycle and induction of apoptosis. *BMC Cancer* 2008; **8**, 78.
- [11] N Hirahara, M Fujioka, T Edamatsu, A Fujieda, F Sekine, T Wada and T Tanaka. Protein-bound polysaccharide-K (PSK) induces apoptosis and inhibits proliferation of promyelocytic leukemia HL-60 cells. *Anticancer Res.* 2011; **31**, 2733-8.
- [12] Y Maehara, S Tsujitani, H Saeki, E Oki, K Yoshinaga, Y Emi, M Morita, S Kohnoe, Y Kakeji, T Yano and H Baba. Biological mechanism and clinical effect of protein-bound polysaccharide K (KRESTIN®): Review of development and future perspectives. *Surg. Today* 2012; **42**, 8-28.
- [13] J Sakamoto, S Morita, K Oba, T Matsui, M Kobayashi, H Nakazato and Y Ohashi. Efficacy of adjuvant immunochemotherapy with polysaccharide K for patients with curatively resected colorectal cancer: A meta-analysis of centrally randomized controlled clinical trials. *Cancer Immunol. Immunother.* 2006; **55**, 404-11.
- [14] K Oba, S Teramukai, M Kobayashi, T Matsui, Y Kodaera and J Sakamoto. Efficacy of adjuvant immunochemotherapy with polysaccharide K for patients with curative resections of gastric cancer. *Cancer Immunol. Immunother.* 2007; **56**, 905-11.
- [15] H Hoshi, H Saito, H Iijima, M Uchida, T Wada, G Ito, H Tanaka, T Sawada and K Hirakawa. Anti-protein-bound polysaccharide-K monoclonal antibody binds the active structure and neutralizes direct antitumor action of the compound. *Oncol. Rep.* 2011; **25**, 905-13.
- [16] U Klinhom and W Klinhom. *Traditional mushrooms*. Thai Health Foundation, Bangkok, Thailand, 2006.
- [17] N Fangkrathok, J Junlatat and B Sripanidkulchai. *In vivo* and *in vitro* anti-inflammatory activity of *Lentinus polychrous* extract. *J. Ethnopharmacol.* 2013; **147**, 631-7.
- [18] N Fangkrathok, J Junlatat, K Umehara, H Noguchi and B Sripanidkulchai. Cytotoxic and immunomodulatory effects of polyhydroxyoctane isolated from *Lentinus polychrous* mycelia. *J. Nat. Med.* 2014; **68**, 302-9.
- [19] C Thetsrimuang, S Khammuang and R Sarnthima. Antioxidant activity of crude polysaccharides from edible fresh and dry mushroom fruiting bodies of *Lentinus sp.* Strain RJ-2. *Int. J. Pharmacol.* 2011; **7**, 58-65.

- [20] N Armassa, O Pongchompu, S Rayan, S Leethong, N Weerapreeyakul and S Machana. Antioxidant activity and cytotoxicity in breast cancer cells line of mushrooms extracts; *Lentinus polychrous* Lev. compared to *Ganoderma lucidum* (Fr.) Karst. *Isan J. Pharmaceut. Sci.* 2009; **5**, 243-50.
- [21] C Thetsrimuang, S Khammuang, K Chiablaem, C Srisomsap and R Sarnthima. Antioxidant properties and cytotoxicity of crude polysaccharides from *Lentinus polychrous* Lév. *Food Chem.* 2011; **128**, 634-9.
- [22] N Fangkrathok. Chemical constituents and pharmacological activities of *Lentinus polychrous* Lév. *Isan J. Pharmaceut. Sci.* 2018; **15**, 49-57.
- [23] J Attarat and T Phermthai. Bioactive compounds in three edible *Lentinus* mushrooms. *Walailak J. Sci. Tech.* 2015; **12**, 491-504.
- [24] JH Choi, YB Kim, HY Lim, JS Park, HC Kim, YK Cho, SW Han, MW Kim and HJ Joo. 5-fluorouracil, mitomycin-C, and polysaccharide-K adjuvant chemioimmunotherapy for locally advanced gastric cancer: The prognostic significance of frequent perineural invasion. *Hepato Gastroenterology* 2007; **54**, 290-7.
- [25] R Katoh and M Ooshiro. Enhancement of antitumor effect of tegafur/uracil (UFT) plus leucovorin by combined treatment with protein-bound polysaccharide, PSK, in mouse models. *Cell. Mol. Immunol.* 2007; **4**, 295-9.
- [26] C Mekjaruskul, N Fangkrathok and B Sripanidkulchai. Acute toxicity investigation of polysaccharide extracts of *Lentinus polychrous* in rats. *Songklanakarin J. Sci. Tech.* 2015; **34**, 433-9.
- [27] AH Rosendahl, C Sun, D Wu and R Andersson. Polysaccharide-K (PSK) increases p21(WAF/Cip1) and promotes apoptosis in pancreatic cancer cells. *Pancreatology* 2012; **12**, 467-74.
- [28] C Chen and ANT Kong. Dietary cancer-chemopreventive compounds: From signaling and gene expression to pharmacological effects. *Trends Pharmacol. Sci.* 2005; **26**, 318-26.
- [29] BB Aggarwal, Y Takada and OV Oommen. From chemoprevention to chemotherapy: Common targets and common goals. *Expert Opin. Invest. Drugs* 2004; **13**, 1327-38.
- [30] HK Matthews, C Bertoli and RAMD Bruin. Cell cycle control in cancer. *Nat. Rev. Mol. Cell Biol.* 2022; **23**, 74-88.
- [31] MO Hengartner. The biochemistry of apoptosis. *Nature* 2000; **407**, 770-6.
- [32] V Gogvadze, S Orrenius and B Zhivotovsky. Mitochondria in cancer cells: What is so special about them? *Trends Cell. Biol.* 2008; **18**, 165-73.