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KEYWORDS: *Phellinus linteus*, *Phellinus igniarius*, natural mushroom, colon cancer, breast cancer

INTRODUCTION

Phellinus linteus (PL) and *Phellinus igniarius* (PI), both of which are natural mushrooms in the family Hymenochaetaceae of basidiomycetes, have been used as traditional medicine in Asian countries [1-2]. Many researches have shown the extract from these mushrooms to have anti-inflammatory, antioxidative and anti-tumor activities [2-3]. Especially, PL has also been demonstrated to be able to strongly suppress the growth of various tumors *in vitro* and *in vivo* [4-5].

The highest incidence rate of colon cancer for males was in Japan [6]. Breast cancer is the most common cancer in female worldwide, and is the second most common cancer in Thai women. The estimated incidence rate is 20.5 per 100,000 women [7]. Current knowledge about the anti-tumor activity of PL and PI on colon and breast cancer is still limited, especially their gene suppressive capability. In this study, human colon cancer cell lines (HCT-15) and human breast cancer cell lines (MCF-7) were treated by PL and PI extracts and detected by MTT assay, Western blot analysis and reverse transcription real-time PCR analysis to study anti-cancer effect of these extracts and their appropriate concentration that may be important to investigate the suppression of the apoptosis pathways.

MATERIALS AND METHODS

Sample preparation

Capsules and liquid extract from *P. linteus*, powdered polysaccharide extract from *P. linteus*, powdered triterpenoid extract from *P. igniarius* and powdered polysaccharide extract from *P. igniarius* were provided by Amazing Grace (Bangkok, Thailand). Crude polysaccharide extracts were prepared by hot water extraction and semi-purified by precipitation in 65% ethanol. The precipitate was dried at 42 °C, in vacuum and stored [8]. Crude triterpenoid extracts were prepared by 97% ethanol extraction at 40 °C for 200 hours. Hot water extraction from capsules, powdered polysaccharide extracts from *P. linteus* and *P. igniarius* were dissolved or suspended in distilled water at a concentration of 0.5 g/ml and diluted to 500, 250, 125, 62.5 and 31.25 µg/ml. Liquid extract was dissolved in distilled water at a concentration of 0.05 ml/ml and diluted to 1000, 500, 250, 125 and 62.5 µg/ml. Powdered triterpenoid extract from *P. igniarius* was dissolved in 97% ethanol at a concentration of 0.5 g/ml and diluted to 500, 250, 125, 62.5 and 31.25 µg/ml.

Cell cultures

Two human cancer cell lines, HCT-15 and MCF-7 (gifts from Dr Tomohiro Yano, Toyo University, Japan) were used. All cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 µg/ml of streptomycin (Gibco) and incubated at 37°C in 5% CO₂.

MTT assay

Cells were plated in 96-well microtiter plates and treated as described above. Thereafter, the medium was removed and 50 µl/ml of MTT solution in DMEM (Dulbecco's modified Eagle's medium); antibiotics (penicillin and streptomycin) were added. After 1-hr incubation, the MTT solution was replaced with 100 µl of DMSO to dissolve the tetrazolium crystals. Finally, the absorptions were read at a test wavelength of 540 nm and a reference wavelength of 650 nm with a Multiskan JX microplate reader (Thermo Labsystems, Cheshire, UK). Cell viability (%) was calculated as [optical density (OD) of the treated wells]/(OD of the control wells) x 100 [9].

Western blot analysis and reverse transcription real-time PCR analysis.

Cells were seeded in 6-well microtiter plates and treated as described above. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Tokyo, Japan). Protein extract (20 µg) was loaded onto a 7.5 or 12% SDS-polyacrylamide gel and separated by electrophoresis. The separated proteins were then transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). Immunoreactive bands were detected with an ECL detection reagent (Millipore, Billerica, MA, USA) [9]. PCR was performed with the PCR Thermal Cycler (Takara Bio Inc.) and Premix Ex Taq (Takara Bio Inc.). Primer sequences were IL-6 (180bp) and IL-8 (196bp) compared with the internal standard GAPDH (180bp). The PCR reaction was performed at 95°C for 5 min, followed by 35 cycles at 95°C for 45 sec, 59°C for 45 sec, 72°C for 60 sec, 72°C for 10 min and keep at 4°C. [9].

RESULTS AND DISCUSSION

The *in vitro* effect of hot water extract from *P. linteus* on the growth of two different RCC cancer cell lines, MCF-7 and HCT-15, was evaluated. In both cell lines, cell growth was inhibited in a dose-dependent manner by all treatments. Figures 1 and 2 showed the results of PI-triterpenoids and PL-polysaccharide treatments on MCF-7 breast cancer cell lines (a) and HCT-15 colon cancer cell lines (b). Cells were treated with 5 concentrations of samples and control. Cell viability was determined by MTT assay after 24, 48 and 72 hrs.

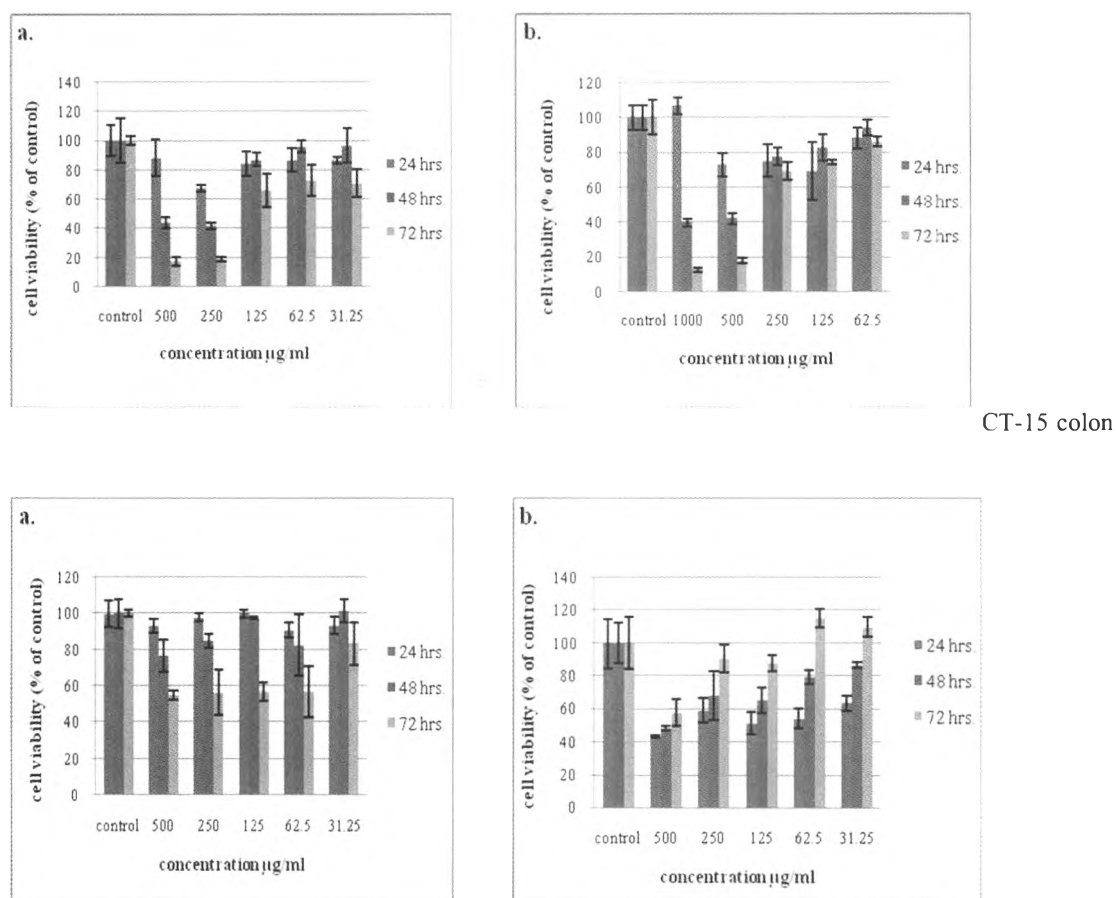


Figure 2 The results of PL-polysaccharide treatment on MCF-7 breast cancer cell (a) and HCT-15 colon cancer cell line (b) (n=3).

The effect of hot water extract from *P. linteus* on the growth of cancer cell lines *in vitro* was assayed with two different cancer cell lines: MCF-7 and HCT-15. In both cell lines, cell growth was inhibited in a dose-dependent manner by all treatments. Moreover, both cancer cell lines were very strongly inhibited by PI-polysaccharide (Fig. 3).

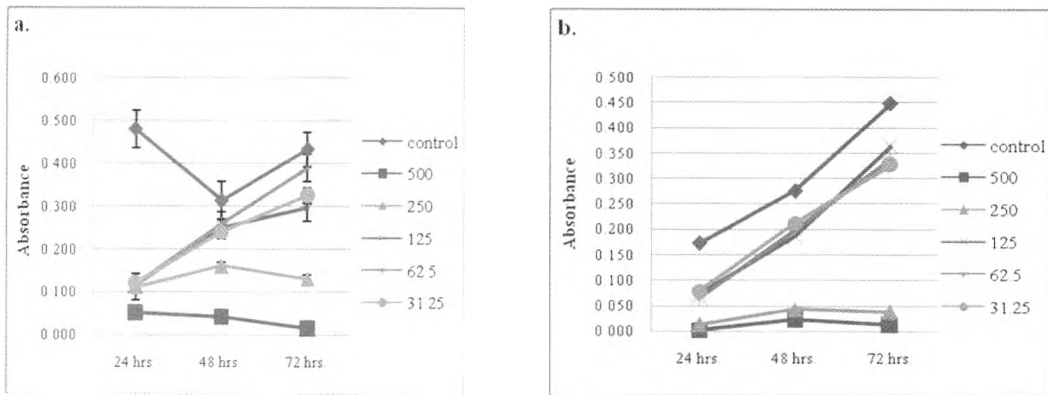


Figure 3 The results of PI-polysaccharide treatment on MCF-7 breast cancer cell line (a) and HCT-15 colon cancer cell line (b) (n=3).

Moreover, we determined the protein expression levels of several additional factors involved in the cell cycle and apoptosis as well as the factors. However the expression of Bax was increased by PI-125 in MCF-7 and slightly increased by PI-62.5 in HCT-15. The expression of the pro-apoptotic Bax was suppressed but that of anti-apoptotic Bcl-2 remained unchanged (Fig. 4). We can therefore speculate that apoptosis could be potentiated by PI-125 in MCF-7. All PCR analysis indicated the DNA expression of IL-6 and IL-8 to be unaltered. PI-polysaccharide at 125 $\mu\text{g/ml}$ and 62.5 $\mu\text{g/ml}$ did not affect DNA expression of IL-6 and IL-8 (Fig. 5). Analysis by HPLC showed crude extracts from *P. linteus* and *P. igniarius* as consisting of monosaccharides such as rhamnose, mannose, arabinose, galactose, xylose, glucose and triterpenoids.

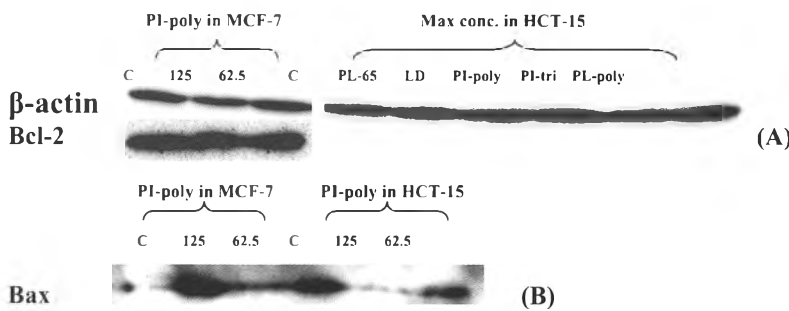
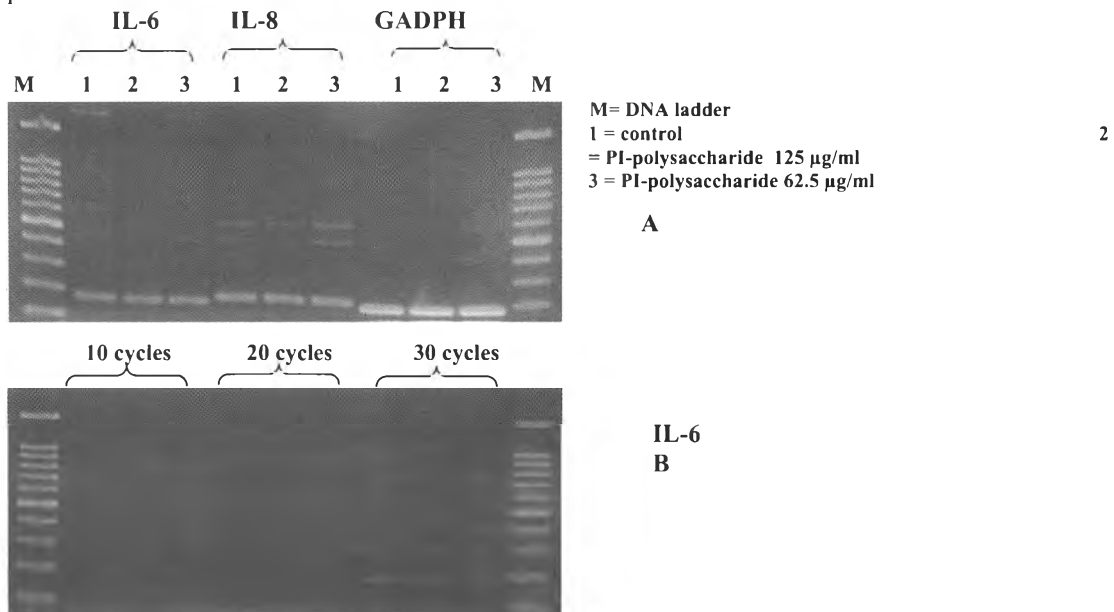


Figure 4 Effect of the treatment on the protein expression in MCF-7 and HCT-15 cells. Whole cell lysates were analyzed by Western blotting using the indicated antibodies. (A) Effect of PI-polysaccharide on the expression of cell cycle-associated proteins in MCF-7 cells. C, control; 125, PI-poly conc. 125 $\mu\text{g/ml}$; 62.5, PI-poly conc. 62.5 $\mu\text{g/ml}$ and effect of all samples on the expression of cell cycle-associated proteins in HCT-15 cells at maximum conc. C, control; PL-65; LD; PI-poly, PI-polysaccharide; PI-tri, PI-triterpenoids; PL-poly, PL-polysaccharide. (B) Effect of PI-polysaccharide on the expression of cell cycle-associated proteins in MCF-7 and HCT-15 cell lines.



M= DNA ladder
 1 = control
 2 = PI-polysaccharide 125 $\mu\text{g/ml}$
 3 = PI-polysaccharide 62.5 $\mu\text{g/ml}$

A

IL-6
 B

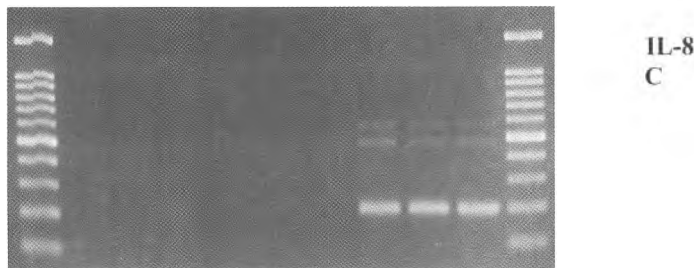


Figure 5 The results of PCR analysis on the DNA expression of IL-6, IL-8 and GADPH in MCF-7 cells. (A). Cells were treated with 0, 125 and 62.5 µg/ml PI-polysaccharide for 24 h. (B) The results of PCR analysis on the DNA expression of IL-6 in MCF-7 cells with 0, 125 and 62.5 µg/ml PI-polysaccharide for 24 h. was performed with the PCR Thermal Cycler 10, 20 and 30 cycles. (C) The results of PCR analysis on the DNA expression of IL-8 in MCF-7 cells with 0, 125 and 62.5 µg/ml PI-polysaccharide for 24 h. was performed with the PCR Thermal Cycler 10, 20 and 30 cycles.

CONCLUSION

The results demonstrated that crude extracts of *Phellinus linteus* (PL) and *Phellinus igniarius* (PI) mushrooms have the cytotoxic activities on colon and breast cancer cell lines.

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