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Non-starch polysaccharides from different developmental stages of *Pleurotus tuber-regium* inhibited the growth of human acute promyelocytic leukemia HL-60 cells by cell-cycle arrest and/or apoptotic induction

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Abstract

The chemical compositions and *in vitro* anti-proliferative activities of the water-soluble non-starch polysaccharides (NSPs) extracted from the fruiting body, mycelium and culture medium (coded as HWE, EDP and CEP, respectively) of a novel edible mushroom *Pleurotus tuber-regium* (PTR) were compared. HWE is likely to be a heteropolysaccharide–protein complex with a high molecular weight of 186×10^4. Glucose-rich EDP had a moderate MW of 50.9×10^4 while mannose-rich CEP had a low MW of 4.4×10^4. Among all PTR NSPs, HWE had the strongest cytotoxicity (approximate IC_{50}: 25 μg/ml) and exerted effective anti-proliferative activity at 200 μg/ml against human acute promyelocytic leukemia cells (HL-60). All PTR NSPs induced apoptosis in HL-60 cells with an increase in the ratio of Bax/Bcl-2. Analysis from flow cytometry and Western blot demonstrated that EDP caused G_2/M arrest in HL-60 cells by lowering the Cdk1 expression while HWE caused S arrest in the HL-60 cells by a depletion of Cdk2 and an increase in cyclin E expression.

Keywords: Non-starch polysaccharides; *Pleurotus tuber-regium*; Cell-cycle arrest; Apoptosis; Mushroom sclerotia; HL-60 cells

1. Introduction

Nowadays, development of non-invasive treatments of cancer is required as the traditional cancer treatments are often toxic to normal cells and can cause serious side effects (Wasser, 2002). Mushroom non-starch polysaccharides (NSPs) have been considered as natural anti-tumor agent having tumor-specific and immunomodulatory effects (Wasser, 2002). The NSPs derived from different developmental stages of mushroom have been used as nutraceuticals and alternative medicine in Japan, China, Russia, US and Canada (Mizuno, 1999; Wasser & Weis, 1999). The most common ones are Lentinan extracted from the fruiting body of *Lentinus edodes*, Schizophyllan isolated from the culture medium product of *Schizophyllum commune*, and PSK obtained from the mycelium of *Coriolus versicolor* (Miles & Chang, 1997; Mizuno, 1996; Wasser & Weis, 1999). It is generally suggested that mushroom NSPs having anti-tumor activity are homoglycans with a triple-helical $(1 \rightarrow 6)$ branched $(1 \rightarrow 3)$-β-D-glucan structure of high molecular mass (Wasser, 2002). Nevertheless, anti-tumor mushroom NSPs consisting of hetero-polysaccharides and polysaccharide–protein complexes have also been reported (Ooi & Liu, 2000; Wang, Liu, Ng, Ooi, & Chang, 1995). The potency of anti-tumor mushroom polysaccharides could be affected by their structural characteristics, including glycosidic linkages, conformation and molecular mass (Bohn & BeMiller, 1995; Mischnick, 1995). However, the detailed relationships between the structure of mushroom NSPs and their anti-tumor activities are still unclear. Recently, many mushroom NSPs have been shown to exert a direct cytotoxic effect on cancer cells

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in vitro. For instance, polysaccharide–peptide (PSP) isolated from the fruiting body of *Coriolus versicolor* (Lin et al., 2004), polysaccharides obtained from the fruiting body of *Ganoderma lucidum* (Jiang, Slivova, Valachovicova, Harvey, & Sliva, 2004), and glucans extracted from the sclerotium of *Poria cocos* (Zhang, Chiu, Cheung, & Ooi, 2006), have all been shown to have growth inhibitory effects on cancer cells mediated by cell cycle arrest and/or induction of apoptosis.

Depending on the environmental and culturing conditions, an edible mushroom *Pleurotus tuber-regium* (PTR), which belongs to the class *Basidiomycetes*, can exist as mycelium, fruiting body and sclerotium which is a solid mass of hyphae in its life cycle (Oso, 1997; Ude, Ezenwugo, & Agu, 2003; Zoberi, 1973). The fruiting body of PTR is high in protein (Kadiri & Fasidi, 1990) whereas its sclerotium has over 80% of NSPs (Cheung & Lee, 1998). Glucose is the major sugar in both the mycelial and sclerotial PTR NSPs. A hot alkali-soluble and hot water-soluble PTR sclerotial NSPs have been shown to have a main chain of (1 → 3)-β-D-glucan with branching occurred at every third glucose having a (1 → 6)-β-D-glucopyranosyl unit (Deng et al., 2000; Zhang et al., 2001). Previous findings have demonstrated that hot alkali-soluble and hot water-soluble sclerotial NSPs, as well as hot water-soluble PTR mycelial NSPs had potent *in vitro* anti-proliferative activities (Zhang, Cheung, Zhang, Chiu, & Ooi, 2004a; Zhang, Zhang, Cheung, & Ooi, 2004b). However, its mechanism is still poorly understood. Moreover, antitumor activity of PTR NSPs extracted from its fruiting body and culture medium (extra-cellular polysaccharides) has not yet been reported. Therefore, PTR NSPs isolated from different developmental stages of the mushroom were evaluated for their *in vitro* anti-proliferative activities and the mechanisms focusing on cell cycle regulation and apoptotic pathway with an emphasis on the structure–activity relationship were determined.

2. Materials and methods

2.1. Mushroom and cell lines

Fruiting bodies of *Pleurotus tuber-regium* (PTR) were cultivated by the Sanming Mycological Institute in Fujan of China and the mycelia were produced from submerged fermentation using a basal medium that contained 4 g/L yeast extract, 1 g/L KH_2PO_4, 0.6 g/L $MgSO_4 \cdot 7H_2O$ and 30 g/L glucose, according to our previous studies (Wu, Cheung, Wong, & Huang, 2003, 2004).

Human acute promyelocytic leukemia HL-60, human foreskin Hs68 and monkey normal kidney Vero cell lines were purchased from the American Type Culture Collection (ATCC) Manassas, VA. The HL-60 and Vero cells were grown in RPMI 1640 medium and Hs68 cells were grown in DMEM. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, incubating under an atmosphere of 5% carbon dioxide at 37 °C.

2.2. Extraction of mushroom non-starch polysaccharides (NSPs)

The powders of PTR fruiting bodies were boiled in distilled water (1:25 w/v) at 95 °C for 2 h. The extraction mixture was centrifuged at 14000 rpm for 40 min and the supernatant was dialyzed against ultra-pure water until the total dissolved solids (TDS) in the dialysate was below 10 μg/ml. The retentate in the dialysis was lyophilized to give the hot water-soluble PTR fruiting body NSPs designated as HWE.

Extraction of PTR mycelial NSPs was similar to that of the PTR fruiting bodies. The PTR mycelial NSPs dissolved in the hot water extract were precipitated by adding 4 volumes of 95% ethanol and the mixture was left overnight. The precipitates were collected by centrifugation at 14000 rpm for 40 min and re-dissolved in water before the solution was then dialyzed against ultra-pure water until the TDS in the dialysate was below 10 μg/ml. The retentate was lyophilized to give the hot water-soluble PTR mycelial NSPs designated as EDP.

Water-soluble extra-cellular polysaccharides were isolated from culture medium of PTR mycelia by centrifugation using a Pall Gelman Maximate System with a cassette of MW10,000 cut off. One liter of the water-soluble extra-cellular polysaccharide extract was concentrated by ultra-filtration and washed with repeated addition of distilled water until the TDS in the filtrate was below 5 μg/ml. The final concentrate was lyophilized to give the PTR culture medium water-soluble extra-cellular polysaccharides coded as CEP.

2.3. Chemical composition and molecular weight of PTR NSPs

Monosaccharide composition of the PTR NSPs was determined by the alditol acetates of the sugar derivatives using gas chromatography (GC) as previously described (Theander, Aman, Westerlund, Andersson, & Petersson, 1995). In brief, the NSPs were hydrolyzed by sulfuric acid and reduced by sodium borohydride and acetylated by acetic anhydride. The alditol acetate derivatives of the samples were analyzed by a GC chromatograph (Hewlett-Packard 6890, CA) with an DB-225 column (15 m × 0.25 mm i.d., 0.25 μm film, Alltech, IL). The initial temperature of the oven was at 170 °C, followed by a temperature rise of 2 °C/min to 220 °C with a final hold of 10 min. The temperature of the injector and detector were at 270 °C. Helium was used as the carrier gas and the sugar derivatives were detected by flame ionization (Zhang, Zhang, Cheung, & Ooi, 2004b). Allose was used as the internal standard to correct losses in derivatization and monosaccharide standards were used to correct for detector responses.

Total carbohydrate content of the PTR NSPs was quantified by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebes, & Smith, 1956). In brief, 0.5 ml of aqueous sample solution was vortex-mixed with 0.5 ml of 5%

phenol in water. Concentrated sulphuric acid (18 M, 2.5 ml) was added rapidly and the mixture was allowed to stand for 30 min at room temperature. The absorbance of the reaction mixture was measured by a spectrophotometer (Genesys G5, Spectronic, Leeds, UK) at 490 nm and the total carbohydrate content was quantified in reference to a calibration curve of glucose standards.

Protein content of the PTR NSPs was quantified by the Lowry method (Lowry, Rosebrough, Lewis, & Randall, 1951) using a protein assay kit (Sigma, St Louis, MO). Briefly, 1 ml of Lowry reagent solution was mixed with 1 ml of aqueous sample solution. The mixture was stood at room temperature for 20 min before 0.5 ml of Folin and Ciocalteu's phenol reagent was added immediately. After standing for 30 min at room temperature, the absorbance of the purple color solution was measured at 750 nm. The protein content of the samples was calculated from a standard curve of bovine serum albumin.

The molecular weight and the homogeneity of the PTR NSPs were estimated by Size-Exclusion Chromatography (SEC) using a high pressure liquid chromatography (HPLC) system (Waters 600E, Waters, Dublin, CA) with a TSK gel G5000 PW column (30 cm × 7.5 mm i.d., Supelco, Bellefonte, PA) with PWH Guard column (7.5 cm × 7.5 mm i.d., Supelco, Bellefonte, PA). The samples dissolved in 0.2 M sodium chloride were injected into the column with 0.2 M sodium chloride solution as solvent at a flow rate of 3.0 ml/min at room temperature. Molecular weight of the PTR NSPs was calculated from a calibration curve obtained from pullulan standards (Shodex Standard P-82, Showa Denko, Japan) using the Empower software (Waters). Detection of the peaks was done by refractive index and ultraviolet absorbance at 280 nm.

2.4. Cell viability assay

Cell viability of treated suspended HL-60 cells was measured by trypan blue dye exclusion method based on the ability of viable cells to exclude the dye. An amount of 2.5×10^3 cells was seeded in each well of a 96-well microplate. The PTR NSPs at final concentrations of 12.5, 25, 50, 100, 200 and 400 μg/ml were incubated with the cells for 72 h at 37 °C. The number of viable cells that excluded the trypan blue dye was counted using a hemacytometer. The samples were compared with control without any NSPs added. All tested samples were carried out in five replicates. The result was expressed as % growth inhibition calculated as follows:

$$[(\text{Number of control cells} - \text{number of treatment cells})/\text{number of control cells}] \times 100\%$$

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method was applied for adherent Hs68 and Vero cells. An amount of 2.5×10^3 cells was seeded in each well of a 96-well microplate. After 24 h of acclimization, the PTR NSPs at final concentrations of 12.5, 25, 50, 100, 200 and 400 μg/ml were incubated with the cells for 72 h. MTT (20 μl/well) was added to the wells and incubated for 5 h at 37 °C. The blue formazan product was leaked out after mixing with 150 μl DMSO. The cell viability was reflected by the amount of the formazan detected by a microplate reader (SpectraMAX 250, Molecular Devices, Sunnyvale, CA) as described by Mosmann (1983). The samples were compared with controls without any PTR NSPs. All tested samples were carried out in five replicates. The result was expressed as % growth inhibition calculated as:

$$[(\text{Absorbance of control cells} - \text{absorbance of treatment cells})/\text{absorbance of control cells}] \times 100\%$$

2.5. Cell proliferation assay

Cell proliferation ELISA BrdU incorporation assay (chemiluminescent) is a method based on the incorporation of pyrimidine analogue, 5-bromo-2-deoxyuridine, (BrdU) into the DNA of proliferating cells during S phase. The procedures used were those described in the commercial kit (Roche Applied Science, East Sussex, UK) (Du, Lin, Xu, & Wang, 2006). In brief, 1×10^4 cells/ml were seeded in each well of a 96-well microplate. The NSP samples at concentrations of 12.5, 25, 50, 100, 200 and 400 μg/ml were incubated with the cells for 72 h. BrdU-labeling solution (10 μl/well) was then added and the plate was further incubated for 2 h to allow incorporation of BrdU into the DNA-synthesizing cells. The cells were fixed with the FixDenat solution provided and were then incubated with the anti-BrdU-POD working solution (100 μl/well). The cells were washed and then incubated with the substrate component solution. The chemiluminescent light emitted from the reaction mixture was measured by a luminometer (ML3000 microtiter® plate luminometer, Dynatech Laboratories, Chantilly, VA). The samples were compared with control without any NSPs added. All tested samples were carried out in five replicates. The results were expressed as % proliferating cells calculated as:

$$[\text{Relative light unit/second (rlu/s) of treated cells/rlu/s of control cells}] \times 100\%$$

2.6. Flow cytometric analysis of cell cycle

Effect of the PTR NSPs on the cell cycle was assessed by flow cytometry. The cells (2.5×10^4 cells/ml) were incubated with PTR NSPs (CEP at 300 μg/ml, EDP at 400 μg/ml and HWE at 200 μg/ml) for 24, 48 and 72 h, respectively. After the incubation, the cells were washed with phosphate buffer saline (PBS) twice and fixed in 70% cold ethanol and stored at −20 °C overnight. The fixed cells were washed with PBS twice and re-suspended in 1 ml of propidium iodide (PI) solution containing RNAase at a concentration of 1 mg/100 ml and kept at 4 °C overnight. The stained

cells were analyzed by a EPICS-XL flow cytometer (Beckman Coulter, Miami, FL). Cell-cycle phase distribution was analyzed using the Multi-Cycle software (Phoenix Flow Systems, San Diego, CA). All tested samples were carried out in triplicates.

2.7. Protein expressions by Western blot

The cells were incubated with PTR NSPs (CEP at 300 μg/ml, EDP at 400 μg/ml and HWE at 200 μg/ml) for 24, 48 and 72 h, respectively. The cell lysate was prepared by mixing with 0.1 ml of ice-cold lysis buffer containing 2 μl of protease inhibitor cocktail (BD Biosciences Pharmingen, San Diego, CA) for 1 h. The protein content in the cell lysate was quantified by the BCA protein assay using BSA protein standard through the measurement of absorbance at 562 nm by a microplate reader. Protein in the lysate was resolved by 12% SDS-polyacrylamide gel. The resolved proteins were transferred to a PVDF membrane (Bio-rad, Hercules, CA) and then the membrane was blocked with 0.5% Aurora blocking reagent (ICN Biomedicals, OH) for 1 h. The membrane was then probed with 3 ml of the corresponding primary antibody overnight. After that, the membrane was probed with 3 ml of the AP-conjugated goat anti-mouse secondary antibody for 1 h. The protein of interest was visualized by the chemiluminent protein detection system (Bio-rad, Hercules, CA). β-actin was used as the loading control. A densitometer was used to measure the relative intensity of the protein bands.

2.8. Statistics

Data expressed as mean values were statistically evaluated by the Student's *t*-test and differences between means with a *p*-value of less than 0.05 were considered significant.

3. Results

3.1. Chemical composition and molecular weight of PTR NSPs

The carbohydrate content of CEP and EDP (83.4% and 87.3%, respectively) was almost double of that of HWE (45.3%) (Table 1). Consistently, HWE had much higher protein content (30.4%) than that of CEP and EDP (15.9% and 3.24%, respectively). Three kinds of monosaccharides including glucose, mannose, galactose were detected in all PTR NSPs but in different proportions. Glucose was dominant in HWE and EDP while mannose was the major sugar in CEP. *N*-acetylglucosamine derived from chitin, a structural cell wall polysaccharide in fungi was only found in CEP and EDP (Table 1).

The results of SEC showed a large difference in the molecular weight (MW) between the PTR NSPs (Table 1). CEP had the smallest MW of 4.40×10^4 dalton while HWE had the largest MW of 186×10^4 dalton. Strong UV absorbance was observed in the SEC chromatogram of HWE (data not shown) which was consistent with the high protein content found in the chemical analysis of HWE (Table 1). Therefore, PTR NSPs isolated from different developmental stages varied considerably both in terms of chemical composition and molecular weight.

3.2. The effect of PTR NSPs on HL-60 cell viability

All PTR NSPs, particularly HWE, could inhibit the growth of HL-60 cells significantly ($p < 0.05$) in a dose dependent manner (Fig. 1). The approximate IC_{50} value for HWE, CEP and EDP against HL-60 cells was about 25, 300 and 300 μg/ml, respectively. For EDP, there was less than 50% growth inhibition at all concentrations except 400 μg/ml (Fig. 1). However, no cytotoxic effect of all PTR NSPs at concentration up to 400 μg/ml could be found on the normal monkey Vero and human Hs68 cells (data not shown), suggesting that their cytotoxicity were preferential against tumor cells only.

3.3. The effect of PTR NSPs on HL-60 cell proliferation

A significant decrease ($p < 0.05$) in the percentage of HL-60 cell proliferation was observed when the HL-60 cells were co-incubated with HWE and CEP (Fig. 2). CEP exerted stronger anti-proliferative activity only at higher concentrations (200 and 400 μg/ml) as compared with the other two NSPs. Besides, all PTR NSPs showed no significant anti-proliferation at a concentration up to 400 μg/ml on normal monkey Vero and human Hs68 cells (data not shown), implying that their anti-proliferative effects seemed to be tumor-specific.

Table 1
The chemical composition and molecular weight of the PTR NSPs

Sample	Monosaccharide composition*					Total carbohydrate content (% dry weight)*	Protein content (% dry weight)*	Molecular weight × 10^4 (Dalton)
	Glc	*Man*	*Gal*	*Rib*	*GlcNAc*			
CEP	7.10 ± 0.75	76.0 ± 1.00	5.37 ± 0.03	n.d.	4.68 ± 3.00	83.4 ± 1.30	15.9 ± 0.19	4.40
EDP	87.3 ± 1.38	4.76 ± 0.12	3.35 ± 0.14	n.d.	2.69 ± 0.85	87.2 ± 4.73	3.24 ± 0.14	50.9
HWE	56.3 ± 0.40	21.8 ± 0.60	15.4 ± 0.05	6.48 ± 0.40	n.d.	45.3 ± 0.70	30.4 ± 0.90	186

n.d., not detected.
* Data are expressed as mean value ± standard deviation.

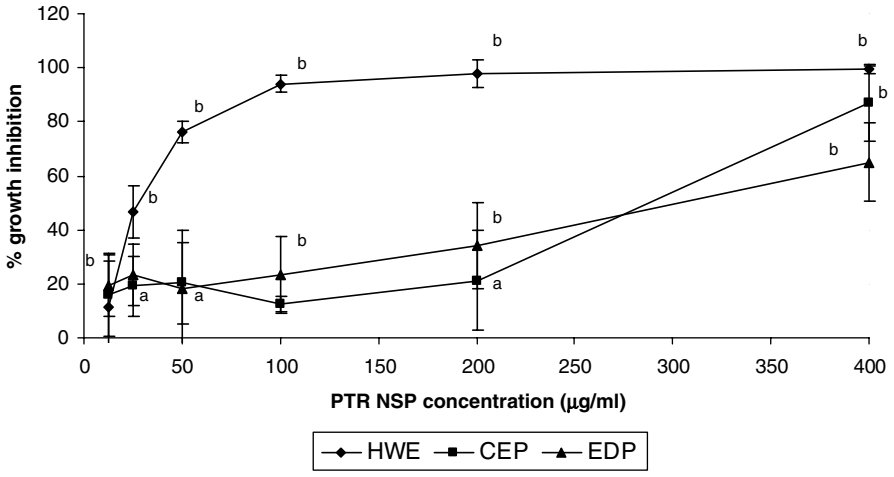

Fig. 1. The effect of PTR NSPs on the viability of HL-60 leukemic cells. The HL-60 cells were incubated with 12.5, 25, 50, 100, 200 and 400 μg/ml of samples respectively for 72 h. Different letters represent the significant difference between the number of cells in control group and treatment group according to Student's *t*-test ($^{a}p < 0.05$ and $^{b}p < 0.01$).

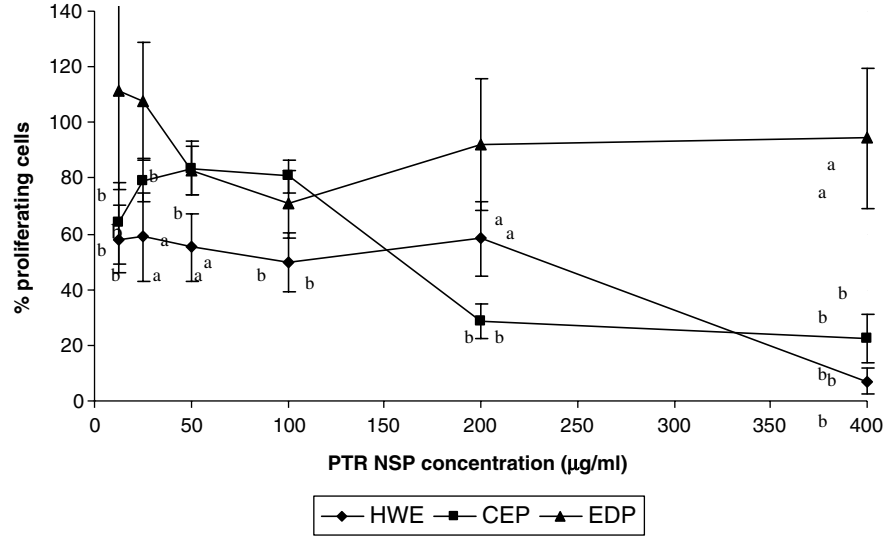

Fig. 2. The effect of PTR NSP samples CEP, EDP and HWE on the proliferation of HL-60 leukemic cells. The HL-60 cells were incubated with 12.5, 25, 50, 100, 200 and 400 μg/ml of samples respectively for 72 h. Different letters represent the significant difference between the number of cells in control group and treatment group according to *t*-test ($^{a}p < 0.05$ and $^{b}p < 0.01$).

3.4. Cell-cycle arrest and apoptosis induction by PTR NSPs

The effective concentration of CEP, EDP and HWE which could exert significant effect on cell cycle and apoptosis was found to be 300, 400 and 200 μg/ml, respectively after several preliminary trials (data not shown). When CEP at 300 μg/ml was incubated with HL-60 cells, an approximate threefold increment of apoptotic peak (Sub-G_1) was observed in a time-dependent manner when compared with the control (Fig. 3). However, there was no significant change in the other phases of the cell cycle of the cells treated with CEP. EDP at 400 μg/ml induced more than 40% of apoptotic cells at three time points, i.e. 24, 48 and 72 h (Fig. 4). Besides, an accumulation of cells at the G_2/M phase which accounted for 16.3% of the total cell numbers was observed after 24 h-incubation. Both apoptosis together with the accumulation of S and G_0/G_1 phase were observed when the HL-60 cells were treated with HWE (Fig. 5). The sub-G_1 peak of the cells treated with HWE was increased from 15.7% to 68.1%, indicating that most cells after 72-h treatment had undergone apoptosis.

3.5. The expression of cyclins, cdk, Bcl-2 and Bax by PTR NSPs

Comparing to the control, the expression of Bcl-2 protein in HL-60 cells was lowered in a time-dependent manner by CEP, whereas that of Bax protein was increased greatly after 72-h incubation (Fig. 6a). EDP

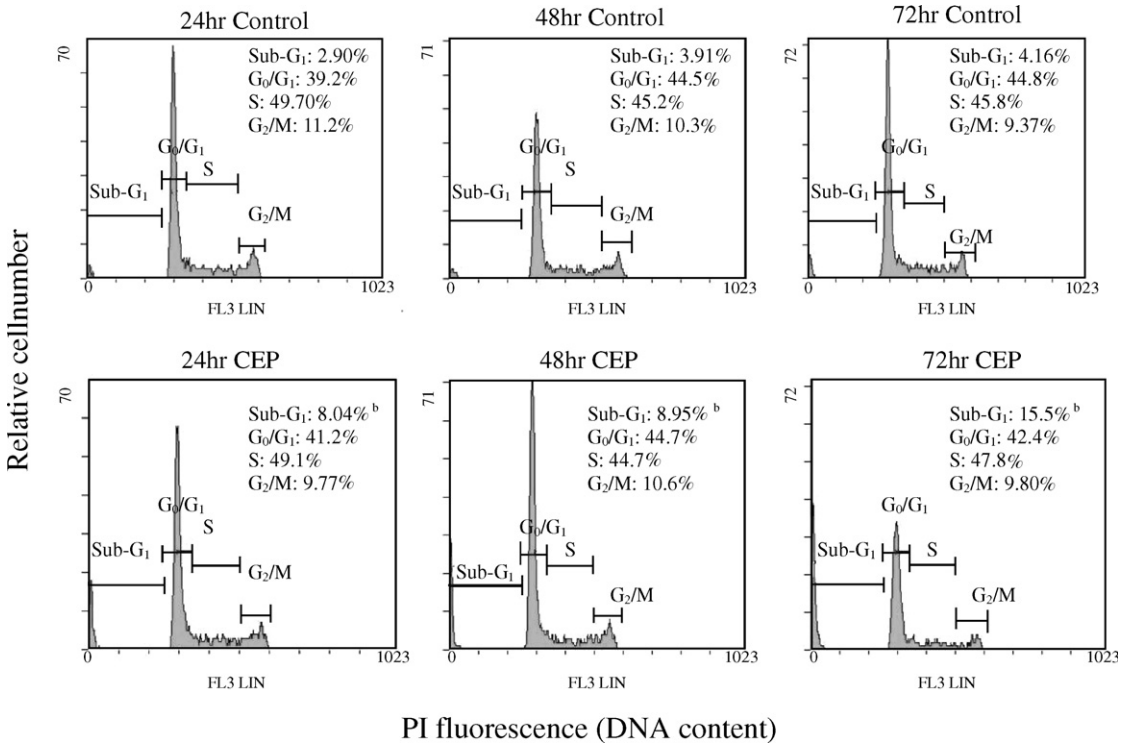

Fig. 3. Representative cytograms showing the effect of CEP at 300 μg/ml on the cell-cycle phases (G_1, S, and G_2/M) and apoptosis (Sub-G_1) of HL-60 leukemic cells. The HL-60 cells were incubated for 24, 48 and 72 h in the absence or presence of CEP. Different letters represent the significant difference between the relative number of cells in control group and treatment group according to *t*-test ($^a p < 0.05$, $^b p < 0.01$ and $^c p < 0.001$).

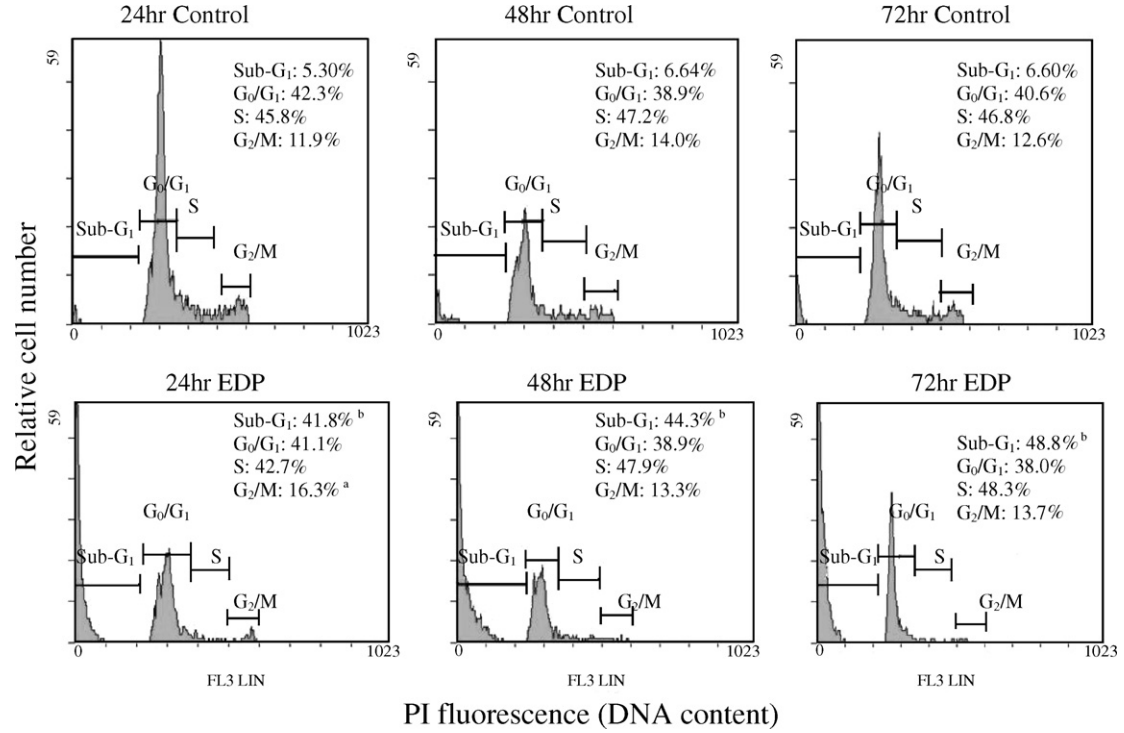

Fig. 4. Representative cytograms showing the effect of EDP at 400 μg/ml on the cell-cycle phases (G_0/G_1, S, and G_2/M) and apoptosis (Sub-G_1) of HL-60 leukemic cells. The HL-60 cells were incubated for 24, 48 and 72 h in the absence or presence of EDP. Different letters represent the significant difference between the relative number of cells in control group and treatment group according to *t*-test ($^a p < 0.05$, $^b p < 0.01$ and $^c p < 0.001$).

also upregulated the expression of Bax protein in a time dependent manner and Bcl-2 protein expression was reduced prominently at all three time points when compared with the control (Fig. 7a). The change of Bax/

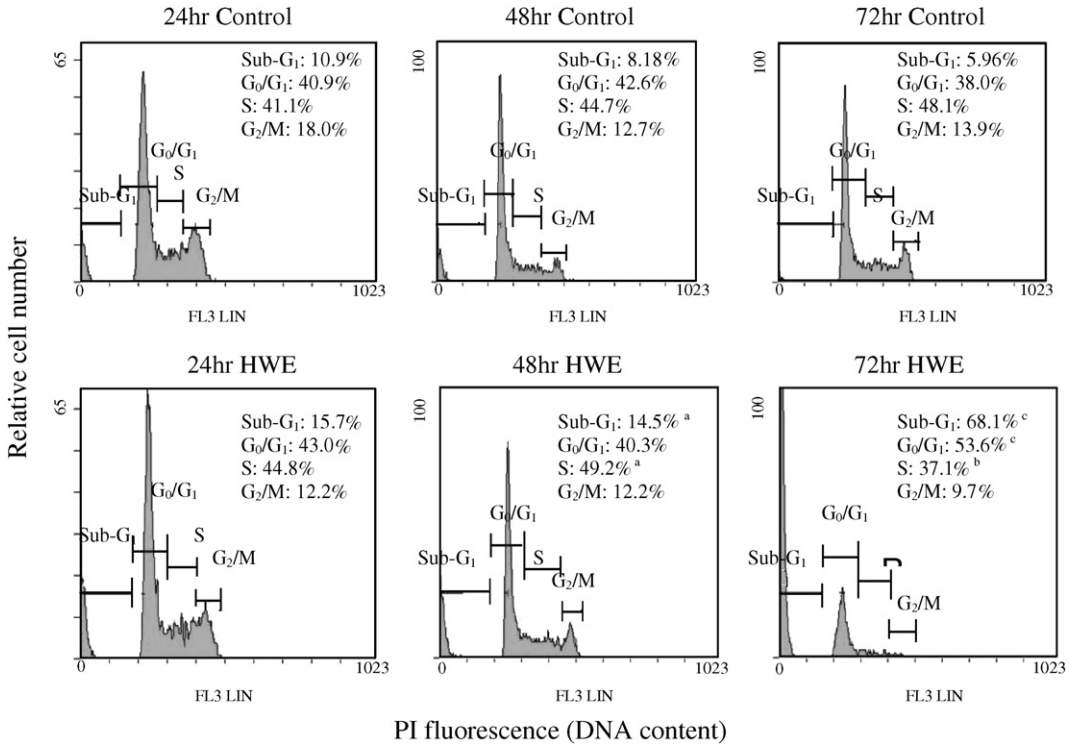

Fig. 5. Representative cytograms showing the effect of HWE at 200 μg/ml on the cell-cycle phases (G_0/G_1, S, and G_2/M) and apoptosis (Sub-G_1) of HL-60 leukemic cells. The HL-60 cells were incubated for 24, 48 and 72 h in the absence or presence of HWE. Different letters represent the significant difference between the relative number of cells in control group and treatment group according to *t*-test ($^a p < 0.05$, $^b p < 0.01$ and $^c p < 0.001$).

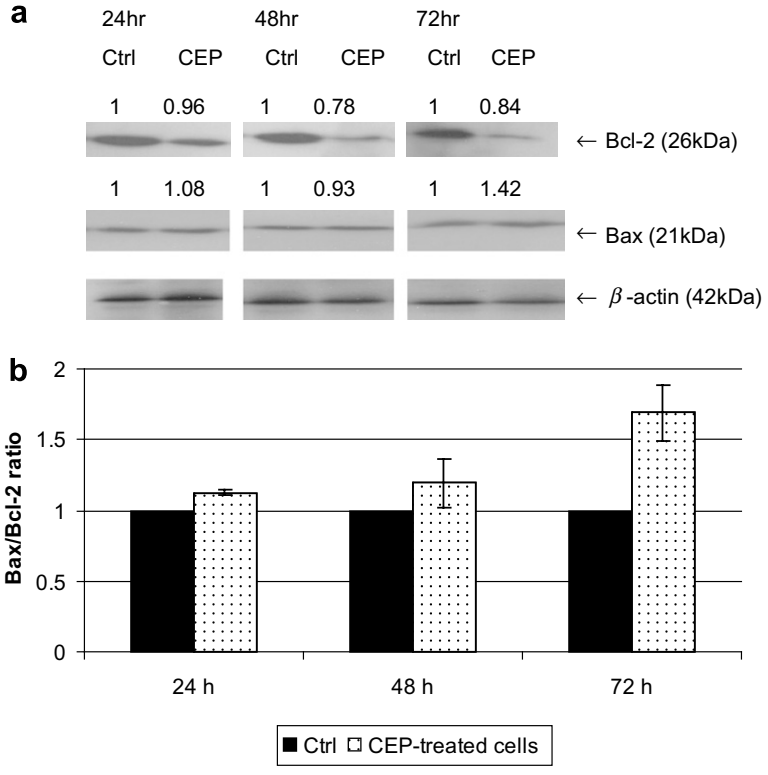

Fig. 6. (a) The Bcl-2 and Bax expressions in HL-60 cells incubated with CEP at 300 μg/ml for 24, 48 and 72 h, respectively were shown with the measured relative density by densitometer. β-actin was used as the loading control. (b) The corresponding Bax/Bcl-2 ratio of CEP-treated HL-60 cells was shown. It was calculated by dividing the relative density of the Bax with that of the Bcl-2 band.

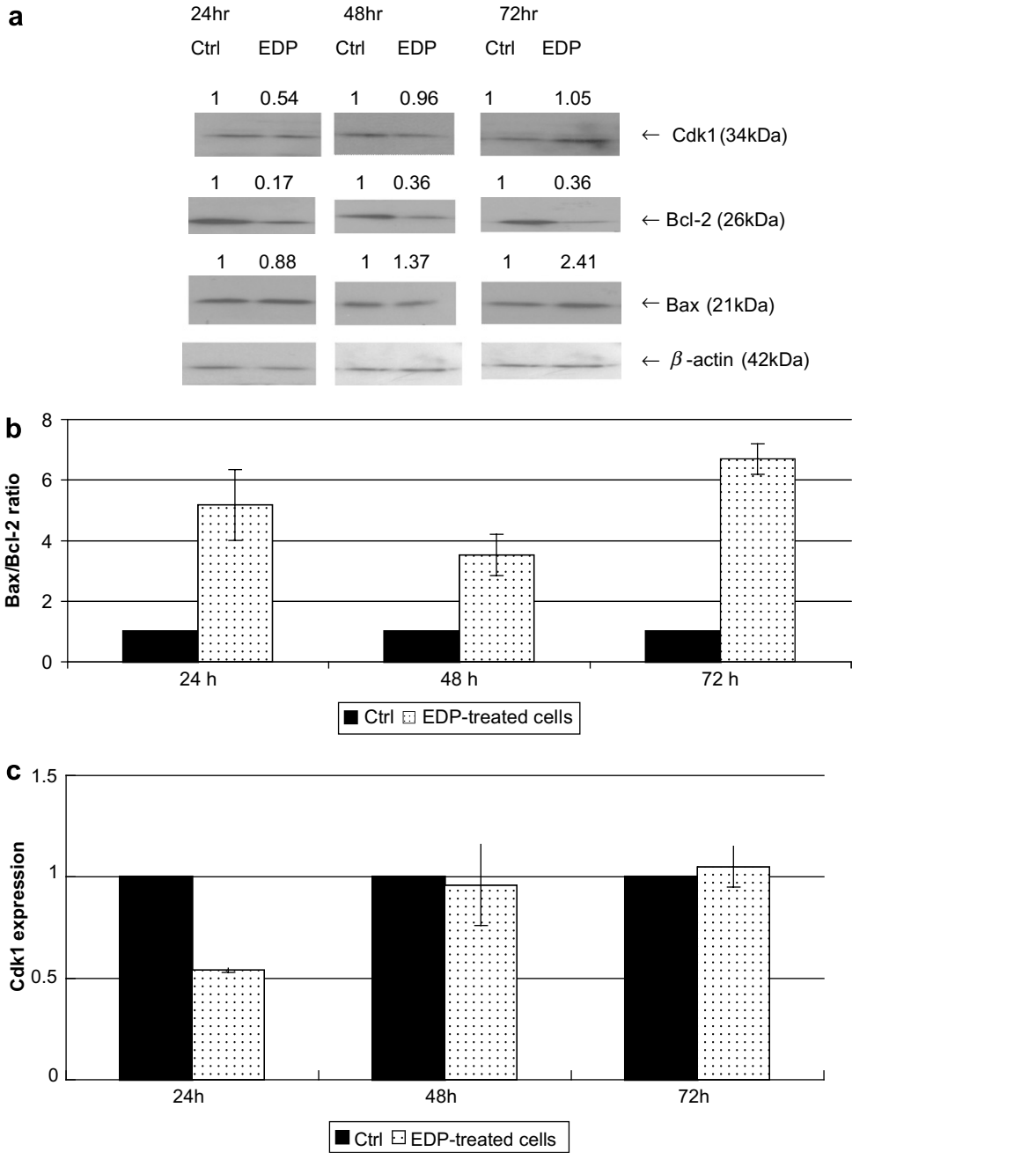

Fig. 7. (a) The cdk1, Bcl-2 and Bax expressions in HL-60 cells incubated with EDP at 400 μg/ml for 24, 48 and 72 h, respectively were shown with the measured relative density by densitometer. β-actin was used as the loading control. (b) The corresponding Bax/bBcl-2 ratio of EDP-treated HL-60 cells was shown. It was calculated by dividing the relative density of the Bax with that of the Bcl-2 band. (c) The relative Cdk1 expression of EDP-treated HL-60 cells was shown.

Bcl-2 ratio obtained in CEP- and EDP-treated HL-60 cells at three time points (Fig. 6b and Fig. 7b) agreed with their changes in the % of apoptosis found from flow cytometry (Figs. 3 and 4). In HWE-treated cells, the expression level of Bax protein was markedly increased at three time points while that of Bcl-2 protein was reduced in a time-dependent manner (Fig. 8a). The Bax/Bcl-2 ratio was not elevated in a time-dependent fashion as expected from the changes in the % of apoptosis (Fig. 5). However, it seemed that the expression level of Bcl-2 protein might be a determining factor for triggering of apoptosis as the percentage of apoptosis (68.1%) was sharply increased (Fig. 5) when the Bcl-2 protein expression was the lowest at 72 h (Fig. 8b). Besides, the expression of cdk1 was downregulated by EDP at 24 h only, consistent with the accumulation of cells in G_2/M peak found in flow cytometry (Figs. 7a and c). The depletion of Cdk2 and an upregulation of cyclin E were observed in HWE-treated cells, suggesting that S arrest might be occurred (Figs. 8a, c and d).

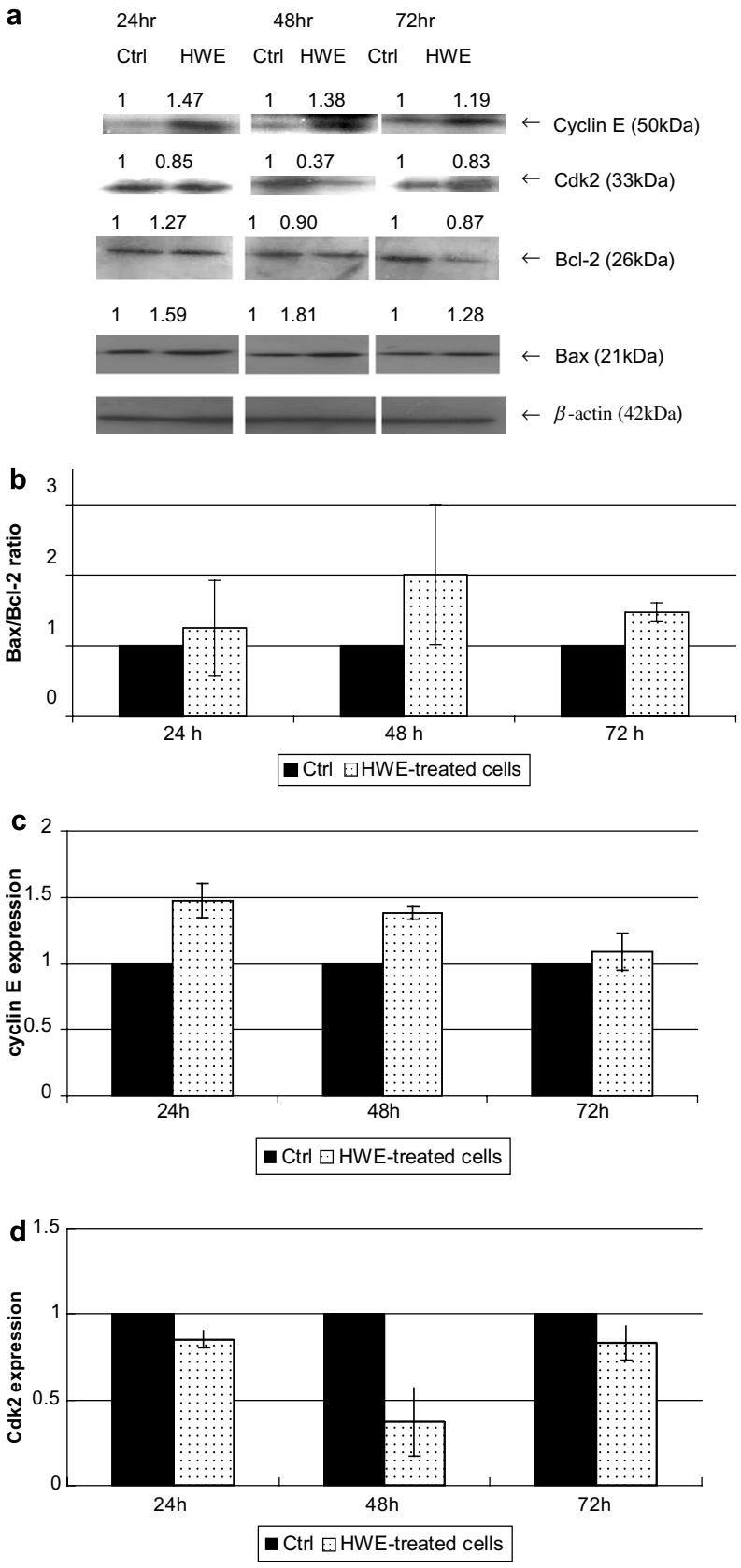

Fig. 8. (a) The cyclin E, cdk2, Bcl-2 and bax expressions in HL-60 cells incubated with HWE at 200 μg/ml for 24, 48 and 72 h, respectively were shown with the measured relative density by densitometer. β-actin was used as the loading control. (b) The corresponding Bax/Bcl-2 ratio of EDP-treated HL-60 cells was shown. It was calculated by dividing the relative density of the Bax with that of the Bcl-2 band. (c) The relative cyclin E expression of HWE-treated HL-60 cells was shown. (d) The relative Cdk2 expression of HWE-treated HL-60 cells was shown.

4. Discussion

PTR consists of three morphological forms, including mycelium, sclerotium and fruiting body, among which sclerotium has been studied most extensively (Zhang et al., 2004a). In this project, a hot-water extraction was used since hot water extracts obtained from other edible mushrooms usually exhibited strong anti-tumor activities (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969; Ikekawa, Nakanishi, Uehara, Chihara, & Fukuoka, 1968; Ikekawa, Uehara, Maeda, Nakanishi, & Fukuoka, 1969), particularly those extracted from fruiting body and mycelium (Reshetnikov, Wasser, & Tan, 2001). Moreover, the water solubility of the NSPs could facilitate their medicinal applications.

N-acetylglucosamine was found in both EDP and CEP indicating the presence of chitin (Table 1). CEP extracted from the culture medium was high in mannan (Table 1) that is similar to other mushroom extra-cellular NSPs, for example, that from *Ganoderma tsugae* (Peng, Zhang, Zeng, & Xu, 2003). The findings on the chemical composition of EDP were consistent with our previous study on the analysis of PTR mycelial NSPs (Zhang et al., 2004b). EDP consisted of a high level of carbohydrate with glucose as its major sugar but had a low protein content (Table 1), suggesting that PTR mycelial NSPs are mainly glucans, similar with the mycelial NSPs isolated from *Poria cocos* which was β-glucan consisting of mainly $(1 \rightarrow 3)$- and $(1 \rightarrow 4)$-linkages (Jin et al., 2003; Zhang et al., 2006). It is not surprising that the highest protein content (30.4% dry weight) was obtained in HWE (Table 1), since the fruiting body of PTR is protein-rich (Kadiri & Fasidi, 1990). As a result, the carbohydrate amount of HWE was concomitantly lowered. HWE might be a polysaccharide–protein complex according to the above results. Many NSPs extracted from the protein-rich fruiting body of mushrooms such as *Phellinus linteus* (Li et al., 2004) and *Agaricus blazei* (Fujimiya et al., 1998), are also polysaccharide–protein complex or proteoglucan. In contrast, PTR sclerotial NSPs are almost pure glucans (Cheung & Lee, 1998).

Many research findings have indicated that a higher MW can improve the bioactivities of the NSPs (Bohn et al., 1995). The MW of HWE, being the highest among all three PTR NSPs, had the lowest IC_{50} value and was in agreement with previous findings. Molecular weight of sclerotial PTR NSPs had been found to be 43.5×10^4 dalton and had only 40% inhibition on the HL-60 cells at concentrations from 100 μg/ml to 400 μg/ml (Zhang et al., 2004b).

Cell-cycle arrest and apoptosis have been illustrated recently as the mode of actions of cancer treatment since cancer cells, unlike normal ones, have uncontrolled proliferation and defective death machinery. Cell-cycle comprises a DNA synthesizing S phase and a mitotic M phase (Howard & Pelc, 1953). These two phases are separated by Gap1 (G_1) and Gap2 (G_2). Cells must pass through G_1, S, G_2 and M phases sequentially to complete a cycle. The cell-cycle transition is tightly regulated by cyclins, cyclin-dependent kinases (cdk), cyclin-dependent kinase inhibitors (cdki) and tumor suppressor proteins (Murray, 2004). Most cells are non-dividing in human body and stay in a quiescent state classified as G_0 in which cellular growth still occurs (Baguley, 2002). Bcl-2 family proteins are key regulators in apoptosis, particularly the antiapoptotic Bcl-2 and proapoptotic Bax proteins (Oltvai, Milliman, & Korsmeyer, 1993). Bcl-2/Bax ratio determines the susceptibility of cells to apoptosis and the therapeutic response to the chemotherapy (Raisova et al., 2001). Therefore, elimination of cancer cells is achievable if the agents could arresting its cell-cycle and/or induce apoptosis.

In the present *in vitro* study, all the PTR NSPs demonstrated a significant decrease in the HL-60 cell viability dose-dependently, with HWE being the most effective one by having the lowest approximate IC_{50} value of 25 μg/ml. Water-soluble sclerotial NSPs showed only about 40% inhibitions on the HL-60 cells (Zhang et al., 2004b). Besides, our BrdU results indicated that only CEP and HWE could significantly inhibit the proliferation of HL-60 cells while EDP could not. Obvious apoptotic peaks and increase in the Bax/Bcl-2 ratio were observed in all PTR NSP-treated cells. Hence it seemed that apoptosis induced by PTR NSPs was associated with the Bax/Bcl-2 ratio and might be at least partially controlled by the mitochondrial-dependent pathway. This was similar to Grifolan isolated from the fruiting bodies of *Albatrellus confluens* that inhibited the human nasopharyngeal carcinoma CNE1 *in vitro* by apoptosis involving downregulation of Bcl-2 and upregulation of Bax with a concomitant increase of Bax/Bcl-2 ratio (Ye et al., 2005). The HWE-induced apoptosis was suggested to be associated with the increase of the Bax/Bcl-2 ratio. However, the Bax/Bcl-2 ratio in HWE-treated cells was not increased in a time-dependent fashion, so further investigation was required to study this phenomenon.

Cyclin B/Cdk1 complex is important in the G_2/M transition and is only activated when mitosis is ready to begin (Schwartz & Shah, 2005). G_2/M arrested cells and lowered cdk1 expression were only observed after 24 h-incubation with EDP-treated cells, suggesting that the arrest might be due to the limited supply of cdk1 and the late stage of arrest might have already been attained; however, further investigation was required for confirmation. HWE upregulated the cyclin E expression which ruled out the possibility of G_1 arrest in HWE-treated cells as cyclin D and cyclin E govern the G_1/S transition and therefore their expressions should be reduced during the G_1 arrest so that pRb protein could not be phosphorylated and cells were arrested at the G_1 phase. In cytograms of HWE-treated cells (Fig. 5), the highest percentage of cells observed in G_1 phase might be due to the cells in S and G_2/M phases, which shifted to apoptosis after long incubation period. There was relatively smaller amount of cells in these phases, leading to an increase in the numeric expression of cells found in G_1 phase. Besides, inactivation of Cdk2 would block the load-

ing of CDC45 onto DNA. DNA polymerase would not be recruited and so no DNA replication was initiated (Kastan & Bartek, 2004). Therefore, the downregulation of cdk2 expression by HWE might halt cells at S phase. All these results suggested that PTR NSPs could either induce apoptosis or delay the cell-cycle progression.

To conclude, NSPs extracted from the different morphological forms of PTR varied greatly not only in their chemical composition but also their potency and mechanism of anti-tumor activities. Although the anti-proliferative activity of other mushroom extra-cellular NSPs has been studied previously (Peng et al., 2003), the present study was the first one to demonstrate that the mannose-rich CEP obtained from PTR culture medium reduced the viability and rate of proliferation of HL-60 cells significantly ($p < 0.05$) via apoptosis. Moreover, the glucose-rich EDP isolated from PTR mycelium caused a G_2/M arrest and induced apoptosis in the HL-60 cells. This was in contrast to our previous study of a β-glucan obtained from *Poria cocos* mycelium which was found to inhibit the proliferation of MCF-7 cancer cells by G_1 arrest and apoptotic induction via down-regulating anti-apoptotic protein Bcl-2 (Zhang et al., 2006). Furthermore, HWE isolated from PTR fruiting body was probably a polysaccharide–protein complex which arrested HL-60 cells at the S phase via upregulation of cyclin E. This was consistent with a previous study on PSP, showing polysaccharide–protein complex from *Coriolus versicolor* that also caused the S phase arrest in HL-60 cells (Hui, Sit, & Wan, 2005). Another protein-bound polysaccharide isolated from *Phellinus linteus* induced G_2/M arrest and apoptosis in SW 480 human colon cancer cells (Li et al., 2004). Further analysis on the detailed structure of PTR NSPs including their glycosidic linkages and molecular conformation in solution are underway in order to explain their different mode of actions towards the anti-proliferation of cancer cells. It is anticipated that the understanding of such structure–activity relationship in mushroom polysaccharides can provide more insights on cancer treatment.

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