Antiproliferative Effects of Sclerotial Polysaccharides from *Polyporus rhinocerus* Cooke (Aphyllophoromycetideae) on Different Kinds of Leukemic Cells

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ABSTRACT: Mushroom polysaccharides isolated from fruiting bodies are known for their antitumor activities, whereas those from mushroom sclerotia are not. A hot water- (PR-HW) and a cold alkaline (PR-CA)-soluble polysaccharide isolated from a novel mushroom sclerotium, *Polyporus rhinocerus* Cooke, were characterized to be a polysaccharide-protein complex and a glucan, respectively. Their *in vitro* antitumor activities against different leukemic cell lines were investigated. PR-HW demonstrated significant growth inhibition of human acute promyelocytic leukemic cells (HL-60), chronic myelogenous leukemia cells (K562), and human acute monocytic leukemia cells (THP-1) *in vitro*, whereas PR-CA showed no such inhibition. Flow cytometric analysis has shown that the antiproliferative effect of PR-HW on HL-60 was mediated by cell cycle arrest at the G_1 phase, which subsequently led to apoptosis. This suggests that structural differences in PR-HW and PR-CA greatly affects their antitumor activities.

KEY WORDS: medicinal mushrooms, antiproliferation, cancer, leukemic cells, polysaccharides, *Polyporus rhinocerus*, sclerotium

I. INTRODUCTION

Mushrooms are renowned for their abilities to mediate a wide range of biological activities due to their varied chemical components.[1] Among these biological activities brought about by various kinds of mushroom components, the antitumor activities of mushroom polysaccharides have drawn the most attention in recent years. It is very common for cancer patients to consume mushroom extracts for treatment in China and Japan. It was first reported in 1968 that the hot water extract from the fruiting body of some edible fungi from the Polyporaceae family had a remarkable host-mediated antitumor activity against Sarcoma S-180.[2] In particular, mushrooms extracted with hot water usually give rise to compounds not comprising a pure glucan but, instead, a polysaccharide–protein complex

ABBREVIATIONS

BCR: breakpoint cluster region; **DMEM:** Dulbecco's Modified Eagle's Medium; **FBS:** fetal bovine serum; **G_1 phase:** Gap 1 phase; **G_2 phase:** Gap 2 phase; **GC:** gas chromatography; **HL-60:** human acute promyelocytic leukemia; **K562:** Human chronic myelogenous leukemia; **M phase:** mitotic phase; **MTT:** (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **PBS:** phosphate-buffered saline; **PI:** propidium iodide; **PR:** *Polyporus rhinocerus*; **PR-CA:** *Polyporus rhinocerus* cold alkaline-soluble polysaccharides; **PR-HW:** *Polyporus rhinocerus* hot water-soluble polysaccharides; **PSPC:** polysaccharide-protein complex; **S phase:** synthesis phase; **THP-1:** human acute monocytic leukemia; **VERO:** monkey normal kidney cells.

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(PSPC).[3] The antitumor effects of polysaccharides isolated from mushroom fruiting bodies have long been investigated, but very few reports are available on mushroom polysaccharides obtained from other morphological forms, such as mycelia and, especially, sclerotia, in this area.[4]

Mushroom sclerotia are masses of fungal hyphae that resemble a hardened tuber, wood-like structure.[5] Sclerotia, such as *Poria cocos*, have been known for their pharmaceutical effect in traditional Chinese medicine.[6] In our laboratory, the sclerotia of *Pleurotus tuber-regium* (Fr.) Singer has been found to be rich in bioactive β-glucans (more than 60% dry weight).[7] The β-glucans extracted by hot water from both the mycelia and sclerotia of this mushroom have shown significantly stronger direct cytotoxic effects on HL-60 cells *in vitro* (83% and 34%, respectively) than their cold alkaline-soluble counterparts (58% and 27%, respectively) at 200 μg mL^{-1}.[8] In addition, the novel carboxymethylated β-glucans from *P. tuber-regium*, with improved water solubility, have been found to inhibit the proliferation of HepG2 cells *in vitro* 30% at 200 μg mL^{-1}.[9] Moreover, this carboxymethylated β-glucan was reported to have a significant antiproliferative effect on MCF-7 cells (with IC_{50} at 204 μg mL^{-1}) *in vitro* by cell cycle arrest at G_1 phase and induction of apoptosis.[10]

Recently, the antitumor activities of a novel mushroom sclerotia, namely, *Polyporus rhinocerus* Cooke, has been our research interest. *P. rhinocerus* belongs to the Polyporaceae family, and no scientific report has been made on the structure and biological activities of its polysaccharides to date. The structure of the polysaccharides in the soluble extracts from *P. rhinocerus*, as well as their *in vitro* antitumor activity, are reported in this study. The structure–activity relationship of these novel sclerotial polysaccharides are also discussed in order to provide greater insight into the antitumor mechanism of mushroom sclerotial polysaccharides.

II. MATERIALS AND METHODS

A. Materials

Sclerotia of *Polyporus rhinocerus* were supplied by the Sanming Mycological Institute in Fujian, China. The sclerotia were peeled and milled into powder by a mechanical grinder (MF10, IKA) through a 0.5-mm sieve. Suspension cell lines, including human acute promyelocytic leukemia cells—HL-60 (CCL-240, ATCC); chronic myelogenous leukemia cells—K562 (CCL-243, ATCC); and acute monocytic leukemia—THP-1 (TIB-202, ATCC), were grown in 25 cm^2 tissue culture flasks (Sarstedt, Germany) in RPMI 1640 medium (Sigma, St. Louis, MO, USA), Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Invitrogen, CA, USA) and high-glucose RPMI 1640 medium supplemented with 0.05-mM 2-mercaptoethanol (GIBCO), respectively. All media were supplemented with 10% fetal bovine serum (FBS) (GIBCO), 1% penicillin-streptomycin (GIBCO), and 0.1% fungizome (GIBCO). The adherent cell line VERO (CCL-81, ATCC) was grown in monolayer in 25 cm^2 tissue culture flasks (Sarstedt) using the same medium as the suspension cells. All of the above cell cultures were seeded at a concentration of 1×10^5 to 1×10^6 cells mL^{-1} and incubated at 37°C in a humidified atmosphere of 5% of CO_2.

B. Extraction Methods

1. Hot Water Extraction

Two hundred grams of the powdered mushroom sclerotia were extracted with hot water at 95°C–100°C for 2 hours at a sample-to-solvent ratio of 1:25 (w/v). After the mixture was cooled down, the supernatant was separated out by centrifugation at 8000× *g* for 30 minutes, followed by membrane ultrafiltration (MWCO at 10,000), and lyophilized to become the hot water extract designated as PR-HW.

2. Cold Alkaline Extraction Coupled with Ultrasonication

The procedures were modified from our previous report.[8] The powdered mushroom sclerotia were first swollen in 1 M sodium hydroxide for 48 hours at a sample-to-solvent ratio of 1:25 (w/v). This mixture was then transferred into a beaker and neutralized

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with hydrochloric acid. An ultrasonic probe (Sonics, VCX600) together with a mechanical stirrer was placed inside the beaker surrounded by an ice bath, and ultrasonication was carried out for 30 minutes. The mixture was centrifuged at 8000× *g* for 30 minutes, and the supernatant was collected, dialyzed (MWCO at 6000–8000), and lyophilized to yield a sample designated as PR-CA.

C. Chemical Composition Analysis

A monosaccharide profile of the extracts was determined by gas chromatography, in which samples were hydrolyzed with 12 M concentrated sulfuric acid and derivatized as alditol acetates.[11] The resulting derivatives of the neutral sugars were then quantified by gas chromatography (Hewlett-Packard 6890, USA) using an Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 µm film) and an oven temperature program of initial temperature 170°C, followed by a temperature rise of 2°C min^{-1} to 220°C, with a final hold of 10 minutes. The injector and detector were at 270°C. The carrier gas was helium, and detection was by flame ionization. Monosaccharide quantification was made with a standard sugar mixture, and allose (Fluka, Buchs, Switzerland) was used as the internal standard.

Total carbohydrate content in the acid hydrolysate of the samples from the monosaccharide profile determination was quantified according to the phenol-sulfuric acid method.[12] In brief, 0.5 mL of appropriately diluted sample solution was vortex-mixed with 0.5 mL of 5% phenol in water. After the addition of 2.5 mL of 18 M concentrated sulfuric acid, the mixture was vortex mixed, stood for 30 minutes at room temperature, and its absorbance measured at 490 nm. The total sugar content of the sample was estimated by reference to a glucose standard. The uronic acid content was determined colorimetrically according to the Official Methods of Analysis (45.4.11)[13] using D-galacturonic acid monohydrate as the standard.[14] The Lowry-Folin method[15] was used to determine the protein content in the samples, with bovine serum albumin as the standard for quantification. All chemical analyses on the extracts were done in duplicate.

D. *In Vitro* Antitumor Assay

The dye exclusion assay was carried out for suspension cells HL-60, K562, and THP-1. In brief, 100 µL of these tumor cells (cell density of 2.5×10^4 cells mL^{-1}) were incubated with different concentrations of the two sclerotial polysaccharides ranging from 12.5 to 400 µg mL^{-1} for 72 hours. The cells were then mixed with equal volumes of trypan blue dye (0.4% in phosphate-buffered saline, PBS; BDH Chemicals Ltd., Poole, UK), and the living cells that excluded the dye were counted by a hemacytometer.

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT assay[16] was carried out to assess the viability of adhesive cells. The VERO cells were incubated with the two sclerotial polysaccharides in the same way as that of the suspension cells. The inhibition ratios of these two assays were compared with the control and calculated as $[(A-B)/A] \times 100\%$, where A and B were the average numbers of viable tumor cells of the control and samples, respectively. All tested samples were carried out in five replicates.

E. Cell Cycle Analysis by Flow Cytometry

The tumor cells were incubated with the sclerotial polysaccharides at a concentration that exerted greater than or equal to 50% inhibition on the cells (IC_{50}) for 24, 48, and 72 hours. After treatment, the cells were washed twice with PBS, fixed in ice-cold 70% ethanol, and stained with propidium iodide (PI). The PI-stained cells were transferred to flow tubes by passing through a 40-µm nylon mesh and were then was kept at 4°C before flow cytometry analysis was carried out.[17] The cells were analyzed for DNA content using a flow cytometer (Beckman Coulter, XL-MCL, CA, USA). The distribution of the cells in sub-G_1, G_1, S, and G_2-M was determined using MultiCycle Analysis AV software (Phoenix Flow Systems, San Diego, CA, USA).

F. Statistical Analyses

All chemical analyses were performed in triplicate. The mean ± SD of both *in vitro* antitumor activities

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between sample and control were analyzed by Student's *t* test (Version 13.0 for Windows XP, SPSS Inc.). Differences between means at 5% ($p < 0.05$) level were considered to be significant.

III. RESULTS

A. Chemical Composition

Hot water extraction (PR-HW) gave a yield of only 8.62% dry weight (DW), whereas cold alkaline coupled with ultrasonication (PR-CA) resulted in a remarkably higher yield of 73.1% DW. The total carbohydrate content of PR-CA was doubled that of PR-HW, whereas the protein content of PR-HW was as high as 41.3% compared to trace amount found in PR-CA (Table 1). The monosaccharide profile showed that glucose was the major component (> 80%) in the two extracts with PR-HW having a substantial amount of mannose (12.1%) and PR-CA having almost only glucose (98.6%) (Table 2).

B. *In Vitro* Antitumor Assay

PR-HW showed a pronounced dose-dependent inhibitory effect on the growth of HL-60 cells (Table 3) as compared to PR-CA, which showed no significant growth inhibition on all three kinds of leukemic cells (data not shown). PR-HW also had a significant antiproliferative effect on K562 and THP-1 cells, although not in a strong dose-dependent manner (Table 3). In addition, neither PR-HW nor PR-CA exerted a cytotoxic effect on normal monkey kidney VERO cells (data not shown).

C. Flow Cytometric Analysis

The concentration of PR-HW chosen for the flow cytometric study of HL-60 cells was 200 μg mL^{-1} (approximate IC_{50} value). After 24 hours of incubation of PR-HW with HL-60 cells, there was already a significant increase of sub-G_1 apoptotic peak (33.9%) compared with the control group (Figs. 1A and 1B). After a 48-hour incubation period, the percent of PR-HW-treated HL-60 cells at both sub-G_1 (56.0%) and G_1 (70.1%) peaks was significantly ($p < 0.05$) higher than those of the control group (Figs. 1C and 1D). On the other hand, the percent of PR-HW-treated HL-60 cells at the S (21.3%) and G_2 (8.53%) phases was significantly ($p < 0.05$) lower than those of the control after 48 hours (Fig. 1). After 72 hours of incubation, the majority of HL-60 cells present in the sub-G_1 peak (77.8%) and G_1 peak (85.3%) was significantly ($p < 0.001$) reduced (Figs. 1E and 1F). Overall, the percentage of PR-HW-treated HL-60 cells at both sub-G_1 and G_1 phases gradually increased during incubation from 24 hours to 72 hours, indicating that apoptosis had taken place.

The concentration of PR-HW chosen for the flow cytometric study of K562 cells was 400 μg mL^{-1} (approximate IC_{50} value). The percentage of PR-HW-treated K562 cells in all the phases of the cell cycle did not show a significant change when compared

TABLE 1
Yield and Chemical Composition of Hot Water- and Cold Alkaline-Soluble Polysaccharides of *Polyporus rhinocerus*[a]

Extract	Yield[b]	Total carbohydrate[c]	Uronic acids[c]	Protein[b]
Hot water	8.62	37.4	0.501	41.3
Cold alkaline	73.1	82.3	0.933	1.34

[a] Data are mean value of duplicate measurement.
[b] Percent dry weight of mushroom sample.
[c] Percent dry weight of polysaccharide extract.

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TABLE 2
Monosaccharide Profile of Hot Water- and Cold Alkaline-Soluble Polysaccharides of *Polyporus rhinocerus*[a]

	Rhamnose	Ribose	Xylose	Mannose	Galactose	Glucose	Glucosamine
Hot water	1.04[b]	1.84	—	12.1	2.31	82.7	—
Cold alkaline	—[c]	—	0.09	0.19	—	98.6	1.17

[a] Data are mean value of duplicate measurement.
[b] Normalized percentage of total monosaccharides found by the GC method.
[c] Not detected.

with the control at 24, 48, and 72-hour incubations except that of sub-G_1 peak, which only showed a trend of increase (Fig. 2).

IV. DISCUSSION

The use of hot water and cold alkaline coupled with ultrasonication had isolated two polysaccharides from the sclerotia of *P. rhinocerus* with very different chemical composition (Table 1). Active antitumor components, including polysaccharide-protein complex (PSPC), from most medicinal mushrooms are extracted with hot water.[18] PR-HW resembled PSPC, with an equal proportion of polysaccharide and protein.[19] Mushroom sclerotia, when swollen in cold dilute alkali and subjected to ultrasonic treatment, resulted in a very high yield of PR-CA, which was almost a pure glucan (98.6% glucose derived from glucan and 1.17% glucosamine derived from chitin). This was probably due to the cleavage of the noncovalent bonds in the β-glucan-chitin complexes.[20] Such bonding in the fungal cell wall is present in large amounts as chitinous materials because cell walls of Basidiomycetes have cellulose and chitin coexisting as structural components.[21] The use of a cold alkaline solution for extraction increased the yield of soluble polysaccharides from the mushroom sclerotia.[8] High temperature has to be avoided during the alkaline extraction because

TABLE 3
Growth Inhibition of HL-60, K562, and THP-1 Cells by Hot Water-Soluble Polysaccharide from *Polyporus rhinocerus* (PR-HW)[a]

	Growth Inhibition (%)		
PR-HW	HL-60	K562	THP-1
400 μg mL^{-1}	96.6 ± 4.66**	50.7 ± 10.9**	28.6 ± 9.05*
200 μg mL^{-1}	67.7 ± 11.0**	36.7 ± 10.7*	24.8 ± 14.6*
100 μg mL^{-1}	50.2 ± 21.6*	16.5 ± 10.2	—
50 μg mL^{-1}	11.5 ± 5.75	21.2 ± 8.66*	—
25 μg mL^{-1}	10.2 ± 13.0	5.76 ± 9.57	—
12.5 μg mL^{-1}	8.09 ± 19.2	—	—

[a] Data were analyzed by Student's *t* test.
Note: "—" represents no significant growth inhibition on cells; * and ** represent significant differences of the treatment compared with control at the same concentration at $p < 0.05$ and $p < 0.001$, respectively.

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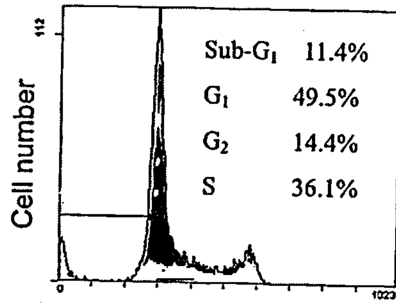
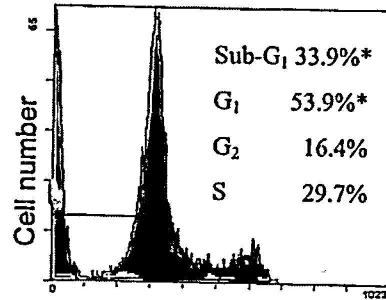
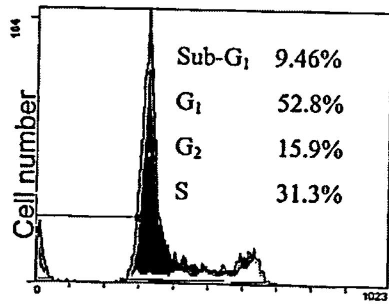


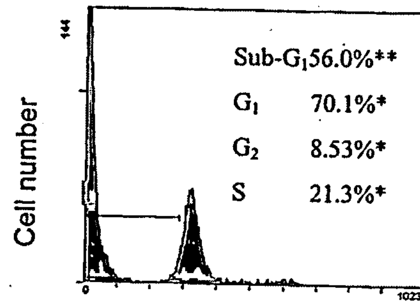
(A) 24-hour control



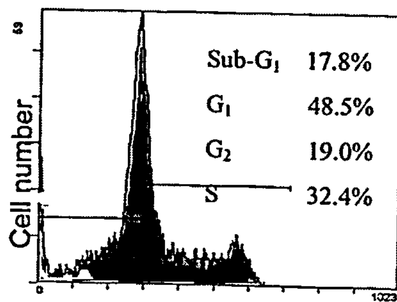
(B) 24-hour PR-HW



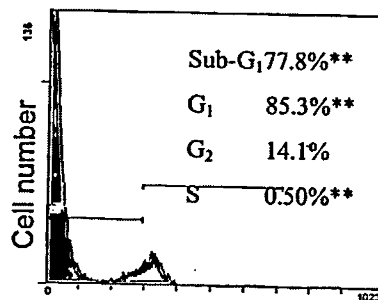
(C) 48-hour control



(D) 48-hour PR-HW



(E) 72-hour control



(F) 72-hour PR-HW

FIGURE 1. DNA histograms showing the effect of PR-HW of *Polyporus rhinocerus* on the cell cycle of HL-60 cells. The HL-60 cells were incubated with 200 μg mL^{-1} PR-HW for (B) 24 hours, (D) 48 hours, and (F) 72 hours and compared to their respective controls (A, C, and E). Data were analyzed by Student's *t* test; * and ** represented significant differences of the treatment compared with the control at the same time point at $p < 0.05$ and $p < 0.001$, respectively.

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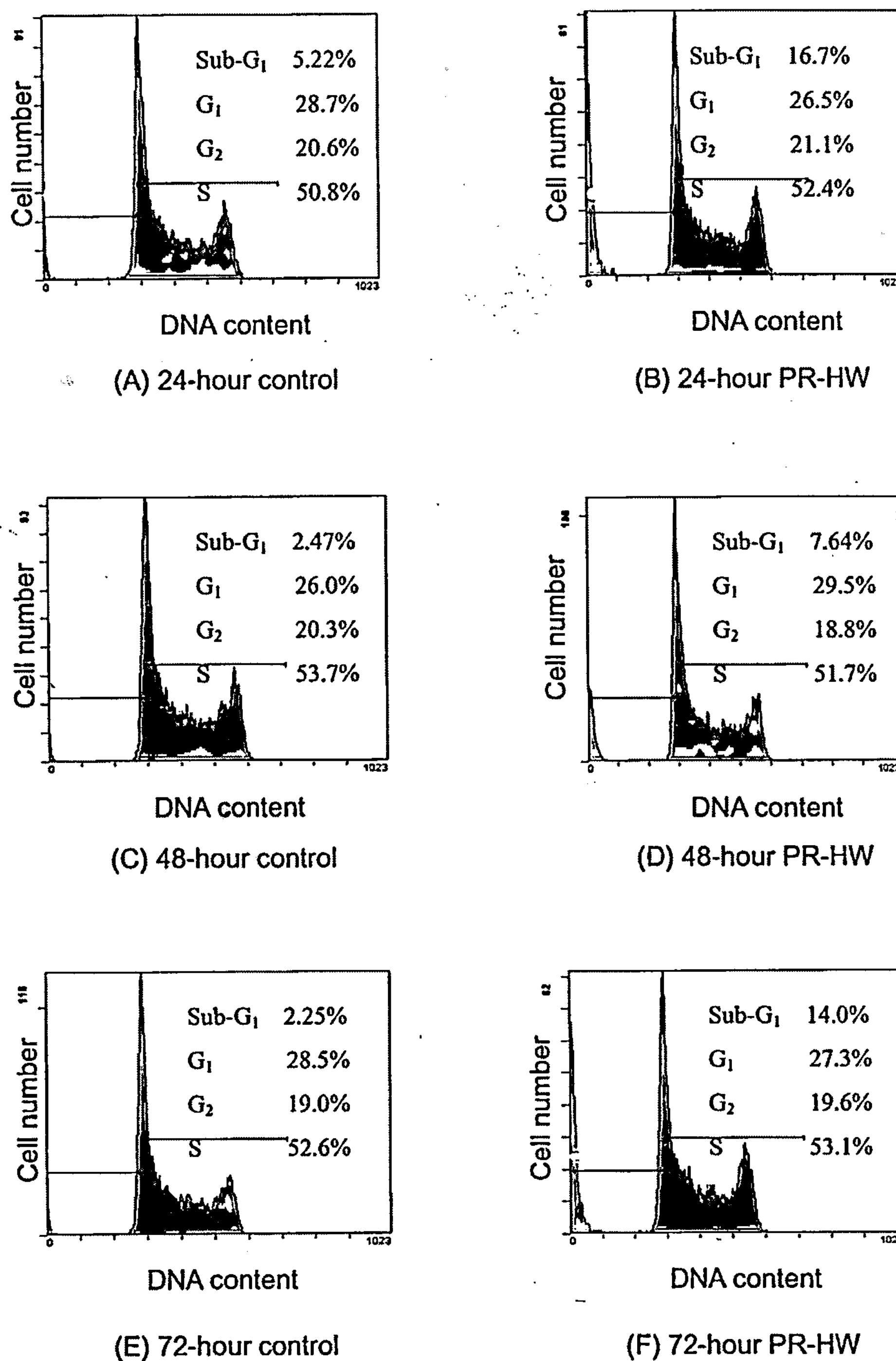

FIGURE 2. DNA histograms showing the effect of PR-HW of *Polyporus rhinocerus* on the cell cycle of K562 cells. The K562 cells were incubated with 400 μg mL^{-1} PR-HW for (B) 24 hours, (D) 48 hours, and (F) 72 hours and compared to their respective controls (A, C, and E). Data were analyzed by Student's *t* test.

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of the extensive chemical degradation that it causes to the polysaccharides.[22]

The observations that chronic leukemic K562 cells were more resistant to PR-HW treatment in terms of antiproliferation than the acute HL-60 cells might be related to the differences in their genetic makeup. K562 possessed the Philadelphia chromosome (Ph), which was originated from a reciprocal translocation between chromosomes 9 and 22.[23,24] This caused a head-to-tail fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22 with the cellular homologue of the Abelson (*c-ABL*) viral oncogene on chromosome 9, which resulted in the fused *BCR-ABL* oncogenes.[25] This kind of gene translocation was found to be one of the causes of the drug resistance found in K562 because this cell line was resistant to many kinds of anticancer drugs.[26] The present findings of a more effective inhibition on the growth of HL-60 by PR-HW than that of K562 was consistent with the previous findings in which many kinds of anticancer drugs were found to be effective in inducing apoptosis in HL-60 but not in K562 in terms of internucleosomal DNA cleavage.[26]

HL-60 was known as p53-null because the p53 gene had been deleted.[27] Therefore, the role of p53 was lost in HL-60 cells, resulting in a loss of apoptotic function.[28] Surprisingly, apoptosis took place in HL-60 when PR-HW was added at a concentration of 200 $\mu g\ mL^{-1}$, indicating that apoptosis had occurred that was mediated by other cell cycle proteins. In response to the accumulation of cells in the sub-G_1 and G_1 phases, there was a decrease in the percentage of cells in both the S and G_2 phases (Fig. 1). In particular, there was a sharp decrease in the percentage of cells in the S phase after a 72-hour incubation period, so that almost no cells passed the G_1-phase checkpoints to enter this phase (Fig. 1). Therefore, G_1 arrest was deemed to be induced by PR-HW on HL-60 cells after 24, 48, and 72-hour incubation periods, which eventually caused apoptosis.

Although there was a slight increase in the percentage of K562 cells with sub-G_1 peak, apoptosis did not take place as no specific cell cycle arrest was observed (Fig. 2). This was consistent with previous findings that K562 seemed to be quite apoptosis-resistant toward many stimuli due to the presence of the *bcr-abl* gene[26] and developed resistance, particularly to drug-induced apoptosis.[29] Since cell cycle arrest was related to the drug sensitivity of the cells,[30] a further increase in the concentration of PR-HW added to K562 (>400 $\mu g\ mL^{-1}$) might be able to trigger apoptosis in the K562 cells, but then the solubility problem of the PR-HW would arise.

In comparing the two types of acute myeloid leukemia (AML)—HL-60 and THP-1—it was observed that THP-1 had great resistance to direct cytotoxicity exerted by PR-HW. According to Amico et al.,[31] the addition of gemtuzumab ozogamacin (GO), an anti-CD33 antibody conjugated to the anticancer agent calicheamicin for treating relapsed $CD33^+$-related AML, resulted in a great loss of cell viability of HL-60 with an IC_{50} of 22 ng mL^{-1}; however, there was no significant decrease in cell viability of THP-1 even when the concentration of GO was increased to 400 ng mL^{-1}, as assessed by the Alamar blue exclusive assay.[31] It was suggested that the resistance of THP-1 to apoptosis was mainly due to the higher expression of antiapoptotic proteins and/or the lower expression of proapoptotic proteins belonging to the bcl-2 family.[31,32]

In our previous report, both PR-HW and PR-CA demonstrated significant growth inhibition ($p < 0.001$) of murine solid tumor cells (Sarcoma S-180) on male BALB/c mice.[33] Apart from the significant *in vivo* response, PR-HW had significant *in vitro* antitumor activity, indicating that it might also exert its antitumor effect through direct cytotoxicity. PR-CA only showed a significant *in vivo* antitumor effect, indicating that it only exerted host-mediated antitumor effect.[33] The two polysaccharide fractions from the same mushroom sclerotia seemed to have antitumor activities mediated by different mechanisms; it was observed that only PR-HW exerted apoptotic effect on leukemia cell lines through cell cycle arrest *in vitro*. This might be related to the relatively high amount of protein content present in the PR-HW extract. Most of the mushroom polysaccharides that are biologically active possess the structure of $(1\rightarrow3)$-β-glucan linkages as backbone with $(1\rightarrow6)$-β-linkages as branches.[34] The presence of both glucose and mannose together with substantial amounts of protein in PR-HW suggested the presence of the glucomannan—protein

complex. In fact, mannan peptide in *Lentinus edodes*,[18] glucomannan-protein complex (ATOM), and mannan–protein complex (AB-FP) in *Agaricus blazei*[35] have been shown to have strong antitumor activities. Therefore, the variations in the biological responses of the sclerotial polysaccharides in these two extracts might be due to their structural differences in which the protein residues of PR-HW facilitated a better cell interaction to trigger cell signaling for *in vitro* inhibition of the tumor cell growth, whereas the almost pure homoglycan structure of PR-CA acted systemically on the host immune system.

V. CONCLUSIONS

Further investigation on the detailed structure of the sclerotial polysaccharides in these two extracts in relationship to their effect on cell cycle arrest of leukemic cells is underway. Research on the active cell signaling pathway and various immune responses of mice will also be conducted to explain how the tumors are being eliminated by both PR-HW and PR-CA through immunomodulation.

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