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Cytotoxic activities of *Coriolus versicolor* (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis

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Abstract

Coriolus versicolor (CV), also known as Yunzhi, is one of the commonly used Chinese medicinal herbs. Although recent studies have demonstrated its antitumour activities on cancer cells in vitro and in vivo, the exact mechanism is not fully elucidated. Hence, the objective of this study was to examine the in vitro cytotoxic activities of a standardized aqueous ethanol extract prepared from *Coriolus versicolor* on a B-cell lymphoma (Raji) and two human promyelocytic leukemia (HL-60, NB-4) cell lines using a MTT cytotoxicity assay, and to test whether the mechanism involves induction of apoptosis. Cell death ELISA was employed to quantify the nucleosome production resulting from nuclear DNA fragmentation during apoptosis. The present results demonstrated that CV extract at 50 to 800 μg/ml dose-dependently suppressed the proliferation of Raji, NB-4, and HL-60 cells by more than 90% ($p < 0.01$), with ascending order of IC_{50} values: HL-60 (147.3 ± 15.2 μg/ml), Raji (253.8 ± 60.7 μg/ml) and NB-4 (269.3 ± 12.4 μg/ml). The extract however did not exert any significant cytotoxic effect on normal liver cell line WRL ($IC_{50} > 800$ μg/ml) when compared with a chemotherapeutic anticancer drug, mitomycin C (MMC), confirming the tumour-selective cytotoxicity. Nucleosome productions in HL-60, NB-4 and Raji cells were significantly increased by 3.6-, 3.6- and 5.6-fold respectively upon the treatment of CV extract, while no significant nucleosome production was detected in

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extract-treated WRL cells. The CV extract was found to selectively and dose-dependently inhibit the proliferation of lymphoma and leukemic cells possibly via an apoptosis-dependent pathway.

Keywords: *Coriolus versicolor*; Yunzhi; Leukemia; Lymphoma; Cytotoxic; Antitumour; Apoptosis; Cancer

Introduction

It is well known that leukemia is divided into lymphocytic and myelocytic types, each of which can be acute or chronic; whereas lymphoma can be categorized into non-Hodgkin's and Hodgkin's lymphoma (with the presence of Reed-Sternberg cells) (The Leukemia and Lymphoma Society, 2003). Global epidemiologic studies over the past few years have demonstrated that the incidence (3 to 5%) and mortality (4 to 5%) rates of leukemia and lymphoma still rank high in the worldwide population (Jemal et al., 2003). Significant geographical and racial differences in age-adjusted incidence and mortality rates of cancers are believed to be influenced by diverse genetic, environmental and dietary factors.

Historically, plant-derived extracts have been considered as important remedies for maintaining health, enhancing overall immune status, and prevention and treatment of chronic diseases (Spencer, 1999). Recent studies have demonstrated that mushroom extracts possess a plethora of biological activities including antibacterial, antiviral, antifungal, antitumour and immuno-potentiating activities (Borchers et al., 1999; Kidd, 2000; Ng, 1998; Ooi and Liu, 1999; Wasser and Weis, 1999). *Coriolus versicolor* (CV), known as Yunzhi in China, is a mushroom belonging to species of the Basidiomycetes class of fungi. Its medicinal value was recorded in the *Compendium of Chinese Materia Medica* and *Shen Non Compendium Medica* thousands of years ago in China. Nowadays, its therapeutic potential has been gaining acceptance among patients worldwide (Kidd, 2000; Wasser and Weis, 1999).

Among various bioactive components derived from CV, polysaccharopeptide (PSP) is found to be the most prominent in antitumour and immunomodulatory efficacy. PSP is a protein-bound polysaccharide isolated from the deep-layer cultivated mycelia of *Coriolus versicolor* COV-1 strain (Yang et al., 1992). The polysaccharide moiety of PSP is a β (1 → 3)-glucan branching at 4′ and 6′ positions, and consists of five different sugars including arabinose, glucose, galactose, mannose and xylose. Its polysaccharide moiety is rich in aspartic and glutamic acids. The substance has a molecular weight of about 100,000 and is highly water-soluble (Ng, 1998; Wang et al., 1996). Previous in vitro studies have reported that PSP and PSK (protein-bound polysaccharide Krestin) (Sakagami et al., 1991) can directly inhibit the proliferation of leukemia, lymphoma, hepatoma, breast and lung tumour cell lines in a dose-dependent manner (Chow et al., 2003; Dong et al., 1996, 1997; Hsieh et al., 2002; Hsieh and Wu, 2001; Kidd, 2000; Wang et al., 1995). PSP was also found to stimulate both humoral and cell-mediated immune responses. It can induce the production of interferon-gamma and interleukin-2, increase T-cell proliferation and counteract the immunosuppression induced by cytotoxic drugs such as cyclophosphamide (Qian et al., 1997; Wang et al., 1995, 1996). In addition, in vivo studies have illustrated that administration of PSP or PSK to nude mice could effectively inhibit the growth of inoculated human leukemia, hepatoma, lung and prostate adenocarcinoma, and extend the survival time (Dong et al., 1996; Kidd, 2000; Mickey et al., 1989; Ng, 1998). In recent clinical trials, PSP administration to

patients with oesophageal cancer, gastric cancer and lung cancer who are undergoing radiotherapy or chemotherapy, can help alleviate symptoms and prevent the decline in immune status (Kidd, 2000; Ng, 1998; Tsang et al., 2003).

Apoptosis is a fundamental mechanism of cell death that can be engaged by a variety of cellular insults. During apoptosis, there is a rapid reduction in the cellular volume followed by chromatin condensation, associated with characteristic internucleosomal DNA cleavage. This results in the production of nucleosomes of DNA fragments complexed with core histones, which are discrete multiples of an 180 bp subunit (Kerr et al., 1972). One of the major modes of action of chemotherapeutic anti-cancer drugs on malignant cells is via the induction of apoptosis (Makin and Hickman, 2000; Sellers and Fisher, 1999). In some cases, the level of drug-induced apoptosis was shown to act as a parameter for predicting the tumour response in vivo (Frankfurt and Krishan, 2003; Johnson et al., 2001). Although the mechanism for direct inhibition of tumour cell proliferation induced by CV extracts has not yet been completely delineated, some recent studies have shown that extracts of CV and other mushrooms like *Ganoderma lucidum* can induce apoptosis of leukemia, breast and cervical tumor cell lines (Chow et al., 2003; Han et al., 1999; Hsieh et al., 2002; Hu et al., 2002). Other herbal medicines, such as sophorane from *Sophora subprostrata* and mistletoe lectin from *Viscum album* can induce apoptosis of HL-60 and U937 leukemic cell lines (Kajimoto et al., 2002; Kim et al., 2003; Lyu et al., 2001). The determination of whether it is through apoptosis or other factors that CV exerts its direct antitumour effects requires further investigation.

The objective of this study was to examine the in vitro cytotoxic activities of a wildly grown *Coriolus versicolor* ethanol-water standardized extract, supplied by Vita Green Health Products Company Ltd., on a non-Hodgkin's B-cell lymphoma cell line (Raji) and two human promyelocytic leukemia cell lines (HL-60, NB-4), of which NB-4 possesses typical cytogenetic features of promyelocytic leukemia, using a MTT cytotoxicity assay. The study also tested whether the mechanism of action involves induction of apoptosis. Cell death ELISA was employed to quantify the nucleosome production resulting from nuclear DNA fragmentation during apoptosis.

Materials and methods

Preparation of CV extract

An ethanol-water extract from *Coriolus versicolor* L. of Polyporaceae family was provided by Vita Green Health Products Company Limited, Hong Kong, in compliance with Good Manufacturing Practice (GMP) standard. The extract was obtained from the fruiting bodies of wildly grown *Coriolus versicolor* in the Guangxi province of mainland China. The *Coriolus versicolor* was authenticated by Prof Zhang Xiao-qing (Mycology expert, Institute of Microbiology Chinese Academy of Sciences, Beijing, China). Herbarium voucher specimen (no. 2003–2510) is deposited at the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong. The fruiting bodies were cleaned, crushed and extracted with ethanol-water at proprietary proportions and temperatures, followed by differential precipitation with ethanol. Further processing of specific ethanol fractions led to isolation of the extraction product in powder form (Batch no.: Yz03; shelf time: 3 years). The major components of the resulting CV extract are polysaccharides and triterpenoids, which were approximately 30% by weight). Plain RPMI medium 1640 (Invitrogen GIBCO, NY, U.S.A.) and Minimum Essential Medium

(MEM) (Invitrogen GIBCO) were used to dissolve the CV extract as stock solutions of 3.0 mg/ml and 2.2 mg/ml respectively (due to different extent in solubility), for 48 h at room temperature with continuous shaking. Insoluble material was removed by centrifugation and the soluble supernatant was sterilized using a 0.22 μm filter, and further diluted with plain culture medium to the defined concentrations as indicated.

Cell cultures

Human acute promyelocytic leukemia cell lines (HL-60 and NB-4), Burkitt's lymphoma B-cell line (Raji), and normal liver cell line (WRL) were purchased from American Type Culture Collection (ATCC, MD, U.S.A.). The cell lines were grown and maintained in a humidified incubator at 37 °C and in 5% CO_2 atmosphere. RPMI medium 1640 supplemented with fetal bovine serum (FBS, Invitrogen GIBCO; 20% for HL-60 and NB-4, 10% for Raji), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen GIBCO) was used for cell cultures of HL-60, NB-4 and Raji. MEM medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mM MEM sodium pyruvate was used as the culture medium of WRL cells.

After being harvested from culture flasks, the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. For HL-60, NB-4 and Raji, 10^4 cells from log phase cultures were seeded in 100 μl of RPMI medium supplemented with fetal bovine serum (40% for HL-60 and NB-4; 20% for Raji) per well of 96-well flat-bottom Costar culture plates (Corning Inc., MA, U.S.A.); while for WRL cells, 5000 cells were seeded per well. One hundred microlitre of solutions containing 2 × final concentrations of CV extract in plain culture medium were added per well. Control wells were added with 100 μl of plain medium alone. A chemotherapeutic antitumour drug, mitomycin C (MMC, Sigma Chemical Co., MO, U.S.A.) at a final concentration of 20 μg/ml was added as the positive control. Cells were then incubated with the CV extract for a defined time (see below). Proliferative response and cell death of the CV extract-treated cells were determined using MTT assay and cell death ELISA, respectively.

MTT assay

The assay detects the reduction of MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide, Sigma] by mitochondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitochondria and hence cell viability. Following incubation of cells with the CV extract for 48 h (WRL) or 72 h (HL-60, NB-4 and Raji) as previously stated, 30 μl of 5 mg/ml MTT in phosphate buffered saline (PBS, Invitrogen GIBCO) was added to each well and the plate was incubated at 37 °C for 2 h. The plate was then centrifuged and followed by removal of medium. One hundred microliter of dimethylsulfoxide (DMSO, Sigma) was then added. After incubation at 37 °C for 5 min, absorbance in control and extract-treated cells was measured spectrophotometrically at 540 nm using a Benchmark microtiter plate reader (Bio-Rad Laboratories, CA, U.S.A.).

Cell death detection

Prior to the detection of cell death, the cell viability after incubation with CV extract (at concentrations derived from MTT assay) for 16, 24 and 48 h was determined using trypan blue exclusion test. Cell

Death Detection ELISA ^{PLUS} (Roche Applied Science, Basel, Switzerland) is used to quantify histone-complexed DNA fragments (nucleosomes) out of the cytoplasm of cells after induction of apoptosis. The assay is based on a one-step sandwich enzyme-immunoassay principle as described elsewhere (Frankfurt and Krishan, 2001, 2003). Briefly, after incubation with the CV extract for 16, 24 and 48 h, the cells were pelleted and lysed to produce nucleosomes. Mouse monoclonal antibodies against single-strand DNA and histones (H1, H2a, H2b, H3 and H4) specifically detected and bound mononuclesomes and oligonucleosomes derived from cells undergoing apoptosis. Biotinylated anti-histone antibodies then fixed the antibody-nucleosome complexes to the streptavidin-coated microtiter plate. The anti-DNA antibodies were conjugated with a peroxidase that reacted with the substrate ABTS [2,2′-azino-di (3-ethylbenzthiazolin-sulfonate)] to form a coloured product. The resulting colour development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured spectrophotometrically at 405 nm (with reference wavelength at 490 nm) using a Benchmark microtiter plate reader (Bio-Rad).

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Statistical differences were assessed by the Student's unpaired *t*-test, with $p < 0.05$ as statistically significant.

Results

Effects of CV extract on proliferation of leukemia and lymphoma cells

CV extract at 50 to 800 μg/ml exhibited significant dose-dependent inhibitory effects on the proliferation of Raji (Fig. 1A), NB-4 (Fig. 1B) and HL-60 cells (Fig. 1C), with more than 90% suppression. However, the extract induced no significant suppression on the proliferation of normal WRL cells (Fig. 1D). Table 1 shows the concentrations producing 50% growth inhibition (IC_{50}) of the CV extract on the 4 cell lines, of which HL-60 proliferation was most potently suppressed with the lowest IC_{50} value (147.3 μg/ml). Similar inhibitory effects were found in NB-4 (IC_{50} = 269.3 μg/ml) and Raji (IC_{50} = 253.8 μg/ml) cells after incubation with the CV extract. Proliferation of HL-60 cells was most significantly reduced by CV extract at 100 to 200 μg/ml, resulting in a lower IC_{50} value; while NB-4 and Raji cell growth was most significantly reduced from 200 to 400 μg/ml with higher IC_{50} values. Table 2 illustrates that Raji, NB-4 and HL-60 cells were similarly susceptible to the cytotoxicity of both CV extract and MMC with more than 90% growth suppression; however, WRL cells were much less susceptible to the cytotoxic effect of CV extract (7.9 ± 5.4%) when compared with MMC (89.9 ± 3.3%).

Effects of CV extract on cell death of leukemia and lymphoma

As determined by MTT assay, CV extract at 200 and 400 μg/ml were chosen for each cell line in cell death detection ELISA. Before determination of CV extract-induced cell death, cell viability of the 4 cell lines was evaluated. As shown in Table 3, the proportion of dead Raji cells increased sharply (from 13.5 ± 3.2% to 26.9 ± 0.5%) upon 48 h incubation with the CV extract at 400 μg/ml when compared

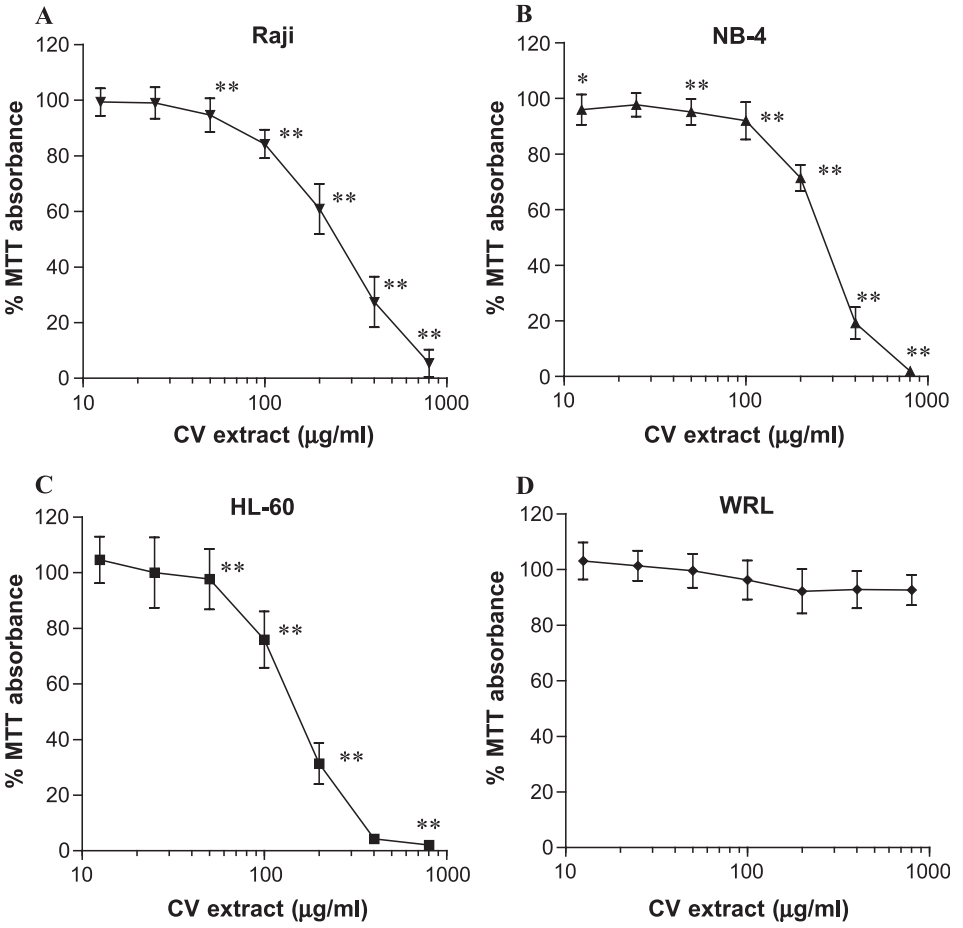

Fig. 1. Effects of CV extract on proliferation of (A) Raji, (B) NB-4, (C) HL-60, and (D) WRL. Cells were incubated with increasing concentrations (12.5–800 μg/ml with 2-fold increase) of CV extract in culture medium for 48 h (WRL) or 72 h (HL-60, NB-4 and Raji) and the proliferative response was then assessed by MTT assay. Results are expressed as the mean % of MTT absorbance (ratio of absorbance in extract-treated and control wells) ± SD of 3 independent experiments with 6 wells each. Differences between the extract-treated and untreated control (100%) wells were determined by Student's unpaired *t*-test. * $p < 0.05$, ** $p < 0.01$.

with 16 and 24 h. However, the percentage of death of HL-60 (15.6 ± 1.5%) and NB-4 cells (15.2 ± 2.2%) peaked at 16 and 24 h, respectively, when incubated with the CV extract of same concentration. These results suggested that the apoptotic response of these 4 cell lines should be evaluated at different time points. As shown in Fig. 2A, the CV extract at 400 μg/ml could strongly induce apoptosis of Raji cells after 48 h in a dose-dependent manner, with maximal enrichment factor of 5.6 ± 0.8 (versus 4.3 ± 1.1 at 16 h and 4.2 ± 0.1 at 24 h). For NB-4, the nucleosome production had no significant difference upon incubation with increasing concentrations of the CV extract (200 and 400 μg/ml) at different time points (Fig. 2B). Only a slight reduction in enrichment factor could be found at 48 h (3.6 ± 1.3 at 24 h versus 2.9 ± 1.3 at 48 h). However, nucleosome production of apoptotic HL-60 cells was notably decreased to an insignificant level upon treatment with the extract after 48 h (3.6 ± 2.0 at 24 h versus

Table 1
Concentration producing 50% growth inhibition (IC_{50})[a] of CV extract on the 4 cell lines

Cell lines	IC_{50} (μg/ml)
Raji	253.8 ± 60.7
NB-4	269.3 ± 12.4
HL-60	147.3 ± 15.2
WRL	>800

[a] IC_{50} values were expressed as the mean ± SD, determined from the results of MTT assay in triplicate experiments.

1.8 ± 0.1 at 48 h, Fig. 2C). As shown in Fig. 2D, no significant nucleosome production was found in WRL cells after incubation with the CV extract at all 3 time points.

Discussion

The present study has demonstrated that ethanol-water extract of a widely used Chinese medicinal herb, *Coriolus versicolor* in its natural form, could significantly suppress the proliferation of human promyelocytic leukemia HL-60 and NB-4, and B-cell lymphoma Raji cells in vitro using the MTT assay. Such antiproliferative activity of CV extract was characterized by the dose-dependent and tumour-selective manner, as reflected by the comparatively low IC_{50} values and the absence of significant effects on normal liver WRL cells respectively (Fig. 1, Tables 1, 2 and 3). On the contrary, MMC at an optimal in vitro concentration was found to non-selectively induce at least 90% growth suppression on all the studied cell lines including WRL (Table 2). These results suggested that when compared with the commonly used chemotherapeutic antitumour drug (e.g., MMC), the CV extract, albeit at higher concentration, could induce comparable antitumour activity with much less cytotoxic effects on normal cells.

Previous studies have revealed that PSP and PSK extracted from cultured CV possess cytotoxic effects of antitumour activity. PSP was found to dose-dependently inhibit the proliferation of HL-60 and Hep-G2 cells, without significant effect on normal human peripheral blood lymphocytes and fetal hepatocytes respectively (Dong et al., 1996, 1997). These results were consistent with our present findings that ethanol-water extract of CV selectively inhibited the tumour cell growth in a dose-dependent manner. Our preliminary results demonstrated that CV extract exhibited no inhibitory effect on proliferation of mouse

Table 2
Comparison of cytotoxic activity of CV extract (800 μg/ml) and mitomycin C (MMC, 20 μg/ml) on the 4 cell lines

Cell lines	% Growth suppression	
	CV extract[a]	MMC
Raji	94.6 ± 4.9	98.4 ± 0.4
NB-4	98.1 ± 0.4	99.1 ± 0.6
HL-60	98.0 ± 0.5	98.2 ± 1.1
WRL	7.9 ± 5.4	89.9 ± 3.3

Results are expressed as the mean % growth suppression (100%−% MTT absorbance from ratio of absorbance in extract-treated and control wells) ± SD of 3 independent experiments with 6 wells each.

[a] Maximal % growth suppression on the cell lines was chosen for CV extract, as calculated from the results of MTT assay.

Table 3
Percentage of dead cells induced by CV extract on the 4 cell lines

Cell lines	Time (h)	CV (μg/ml)		
		0	200	400
Raji	16	1.2 ± 2.1	5.6 ± 2.1	12.4 ± 2.0
	24	1.2 ± 0.1	4.5 ± 0.2	13.5 ± 3.2
	48	0.5 ± 0.9	7.3 ± 1.2	26.9 ± 0.5*
NB-4	16	2.6 ± 2.6	4.5 ± 1.6	6.7 ± 2.8
	24	2.2 ± 2.1	8.1 ± 0.9	15.2 ± 2.2*
	48	1.0 ± 0.8	8.4 ± 1.2	12.4 ± 1.6
HL-60	16	2.7 ± 2.6	6.8 ± 0.4	15.6 ± 1.5*
	24	2.8 ± 1.2	4.4 ± 3.1	9.3 ± 2.2
	48	1.8 ± 1.6	7.3 ± 0.6	8.6 ± 0.5
WRL	16	0.0 ± 0.0	5.9 ± 2.0	1.5 ± 1.6
	24	1.0 ± 0.8	0.6 ± 1.1	1.5 ± 1.6
	48	0.0 ± 0.0	1.2 ± 2.1	0.8 ± 1.3

Results are expressed as the mean percentage of dead cells ± SD in duplicate experiments with 3 wells each. Percentage of dead cells was calculated from the ratio of dead cells to total number of cells using trypan blue exclusion test.

*Values represent the maximum percentage of dead cells.

splenic lymphocytes after 72 h incubation (data not shown), proposing that the antiproliferative effect of the extract was tumour-specific.

In order to determine whether the antiproliferative activity of CV extract is manifested by induction of apoptosis, cell death detection ELISA was employed to quantify the nucleosome production during nuclear DNA denaturation of apoptotic cells. Before evaluating the CV extract-induced apoptosis, the percentages of death of the 4 cell lines at 3 different time points were determined since the incubation time necessary to induce apoptosis can show broad variation. For Raji cells, the sharp increase in percentage of dead cells paralleled the significant nucleosome production upregulated by the CV extract from 24 to 48 h (Table 3 and Fig. 2A), suggesting the dose- and time-dependent induction of apoptosis. Similar results were seen in NB-4 cells, but the percentage of death and the induced nucleosome production concurrently peaked at 24 h and decreased at 48 h incubation with the CV extract (Table 3 and Fig. 2B). These results suggested that the extract exerts its antitumour effect on these leukemia and lymphoma cells possibly via an apoptosis-dependent pathway. Meanwhile, the antiproliferative mechanism of CV extract may be related to the inhibition of DNA synthesis in Raji and NB-4 cells, according to our preliminary results using tritiated thymidine incorporation assay (data not shown). To our knowledge, there was no previous mechanistic study on antitumour effect of CV extract, PSP or PSK against myelocytic leukemia (NB-4) and B-cell lymphoma (Raji) cells. Being a model cell line of acute promyelocytic leukemia, NB-4 is suggested to be one of the genuine cell lines with typical cytogenetic and molecular characterisitics of promyelocytic leukemia, including expression of t(15;17) gene and myeloperoxidase (Drexler et al., 1995). Therefore, the antiproliferative effect of our CV extract on NB-4 cells reflects its potential therapeutic value in cancer treatment. On the other hand, Raji cells of Burkitt's B-cell lymphoma have been previously used for in vitro screening of potential chemopreventive drugs with antitumour-promoting activities, as assayed by the Epstein-Barr virus early antigen activation (Kapadia et al., 2002; Ukiya et al., 2002). It is suggested that CV extract may be a possible candidate of anti-cancer drug due to its apoptosis-dependent cytotoxic effects on Raji cells.

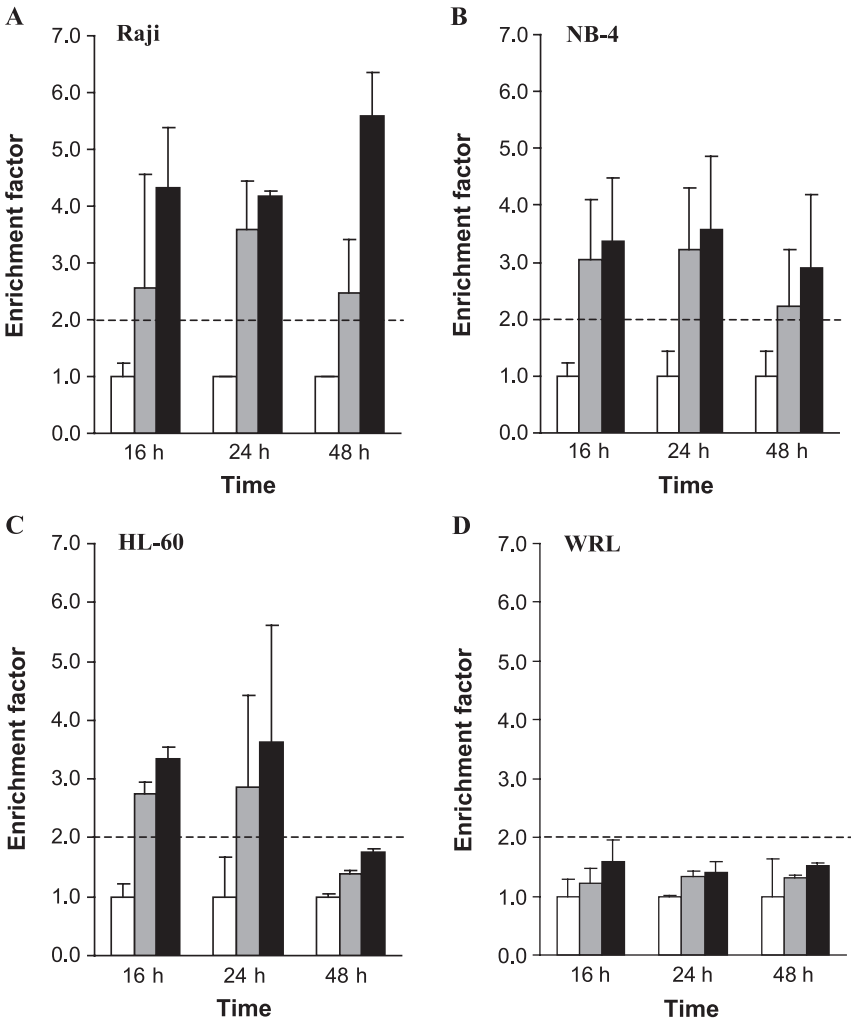

Fig. 2. Effects of CV extract on cell death of (A) Raji, (B) NB-4, (C) HL-60, and (D) WRL. Cells were incubated with the CV extract in culture medium at concentrations derived from IC_{50}, 200 μg/ml (grey bar) and 400 μg/ml (black bar), or culture medium alone (white bar) for 16, 24 and 48 h. The induced apoptosis (internucleosomal DNA fragmentation) was then assessed by cell death detection ELISA. Results are expressed as the enrichment factor (mean ± SD), which was calculated as the ratio of absorbance readings in CV extract-treated and control wells in duplicate experiments with 3 wells each. Enrichment factor greater than or equal to 2 (dashed line) was considered significant when compared with the untreated control.

Our results illustrated that the percentage of HL-60 cell death declined from 16 h to 24 and 48 h when treated with the CV extract (Table 3). Similar results were obtained in apoptosis for HL-60 (Fig. 2). Therefore, the internucleosomal fragmentation of apoptotic HL-60 cells, like NB-4, also paralleled the CV extract-induced cell death in a dose-dependent manner. However, the nucleosome production was notably reduced to an insignificant level after 48 h incubation with the extract. These might suggest the absence or deactivation of apoptosis, as reflected by the low percentage of the extract-induced cell death. When comparing the time course of apoptotic response of HL-60 with NB-4 cells, the mode of

antitumour action of the CV extract on HL-60 cells might slightly differ from that of NB-4 cells, since HL-60 may represent a discrete stage of differentiation between the late myeloblast and promyelocyte without expressing the typical genes of cytogenetic hallmark for acute promyelocytic leukemia (Drexler et al., 1995). A previous study has demonstrated that PSP from CV exhibited no effect on morphological features of HL-60 cells and did not cause DNA fragmentation (Dong et al., 1997). Another recent report, however, revealed that ethanol and water extracts of CV could trigger a partial cell cycle arrest in the G1/S checkpoint and induce apoptosis using flow cytometric analysis of cellular DNA content (Hsieh et al., 2002). These contradictory results may be explained by: (1) different compositions of bio-active components in different CV extracts, and (2) the assays employed for measuring different parameters of apoptosis. In our study, the nucleosome concentration of apoptotic cells was directly measured by antibody-antigen interactions using ELISA, which is a quantitative method with higher specificity and sensitivity when compared with agarose gel eletrophoresis for detecting DNA fragmentation (Dong et al., 1997; Frankfurt and Krishan, 2001, 2003). Meanwhile, Hsieh et al. (2002) only examined a relative number of apoptotic cells with subdiploid fragmented DNA at the sub-G_0 peak using flow cytometric analysis of cell cycle phases. Therefore, further investigations are required to evaluate whether cell cycle arrest or apoptosis induction contributes more to the CV extract-induced cytotoxic effect on HL-60 cells.

In order to elucidate the cytotoxic activities of CV extract on the growth of leukemia and lymphoma cells of other sub-types, acute T-cell leukemia cell line (Jurkat) and Hodgkin's lymphoma may become the target cells used in our future studies. In addition, mechanistic studies on cell cycle arrest and early apoptotic events may be conducted to delineate other possible antitumour mechanisms of the CV extract. Besides, future in vivo antitumour studies will be performed in order to confirm these in vitro results.

Conclusion

This study provides the evidence that in vitro antitumour activity of an ethanol-water standardized extract from wild CV was found to tumour-selectively and dose-dependently inhibit the proliferation of lymphoma and leukemic cells possibly via an apoptosis-dependent pathway.

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References

Borchers, A.T., Stern, J.S., Hackman, R.M., Keen, C.L., Gershwin, M.E., 1999. Mushrooms, tumors, and immunity. Proceedings of the Society for Experimental Biology and Medicine 221, 281–293.

Chow, L.W., Lo, C.S., Loo, W.T., Hu, X.C., Sham, J.S., 2003. Polysaccharide peptide mediates apoptosis by up-regulating p21 gene and down-regulating cyclin D1 gene. American Journal of Chinese Medicine 31, 1–9.

Dong, Y., Kwan, C.Y., Chen, Z.N., Yang, M.P., 1996. Antitumour effects of a refined polysaccharide peptide fraction isolated from *Coriolus versicolor* in vitro and in vivo studies. Research Communications in Molecular Pathology and Pharmacology 92, 140–148.

Dong, Y., Yang, M.P., Kwan, C.Y., 1997. In vitro inhibition of proliferation of HL-60 cells by tetrandrine and *Coriolous versicolor* peptide derived from Chinese medicinal herbs. Life Sciences 60, 135–140.

Drexler, H.G., Quentmeier, H., MacLeod, R.A.F., Uphoff, C.C., Hu, Z.-B., 1995. Leukemia cell lines: in vitro models for the study of acute promyelocytic leukemia. Leukemia Research 19, 681–691.

Frankfurt, O.S., Krishan, A., 2003. Apoptosis enzyme-linked immunosorbent assay distinguishes anticancer drugs from toxic chemicals and predicts drug synergism. Chemico-Biological Interactions 145, 89–99.

Frankfurt, O.S., Krishan, A., 2001. Enzyme-linked immunosorbent assay (ELISA) for the specific detection of apoptotic cells and its application to rapid drug screening. Journal of Immunological Methods 253, 133–144.

Han, B., Toyomasu, T., Shinozawa, T., 1999. Induction of apoptosis by *Coprinus disseminatus* mycelial culture broth extract in human cervical carcinoma cells. Cell Structure and Function 24, 209–215.

Hsieh, T.C., Kunicki, J., Darzynkiewicz, Z., Wu, J.M., 2002. Effects of extracts of *Coriolus versicolor* (I'm-Yunity) on cell-cycle progression and expression of interleukins-1 beta,-6, and -8 in promyelocytic HL-60 leukemic cells and mitogenically stimulated and non-stimulated human lymphocytes. Journal of Alternative and Complementary Medicine 8, 591–602.

Hsieh, T.C., Wu, J.M., 2001. Cell growth and gene modulatory activities of Yunzhi (Windsor Wunxi) from mushroom *Trametes versicolor* in androgen-dependent and androgen-insensitive human prostate cancer cells. International Journal of Oncology 18, 81–88.

Hu, H., Ahn, N.S., Yang, X., Lee, Y.S., Kang, K.S., 2002. *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. International Journal of Cancer 102, 250–253.

Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E., Thun, M., 2003. Cancer statistics, 2003. CA: A Cancer Journal for Clinicians 53, 5–26.

Johnson, J.I., Decker, S., Zaharevitz, D., Rubinstein, L.V., Venditti, J.M., Schepartz, S., Kalyandrug, S., Christian, M., Arbuck, S., Hollingshead, M., Sausville, E.A., 2001. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. British Journal of Cancer 84, 1424–1431.

Kajimoto, S., Takanashi, N., Kajimoto, T., Xu, M., Cao, J., Masuda, Y., Aiuchi, T., Nakajo, S., Ida, Y., Nakaya, K., 2002. Sophoranone, extracted from a traditional Chinese medicine Shan Dou Gen, induces apoptosis in human leukemia U937 cells via formation of reactive oxygen species and opening of mitochondrial permeability transition pores. International Journal of Cancer 99, 879–890.

Kapadia, G.J., Azuine, M.A., Tokuda, H., Hang, E., Mukainaka, T., Nishino, H., Sridhar, R., 2002. Inhibitory effect of herbal remedies on 12-*O*-tetradecanoylphorbol-13-acetate-promoted Epstein-Barr virus early antigen activation. Pharmacological Research 45, 213–220.

Kerr, J.F., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer 26, 239–257.

Kidd, P.M., 2000. The use of mushroom glucans and proteoglycans in cancer treatment. Alternative Medicine Review 5, 4–27.

Kim, M.S., Lee, J., Lee, K.M., Yang, S.H., Choi, S., Chung, S.Y., Kim, T.Y., Jeong, W.H., Park, R., 2003. Involvement of hydrogen peroxide in mistletoe lectin-II-induced apoptosis of myeloleukemic U937 cells. Life Sciences 73, 1231–1243.

Lyu, S.Y., Park, W.B., Choi, K.H., Kim, W.H., 2001. Involvement of caspase-3 in apoptosis induced by *Viscum album var. coloratum* agglutinin in HL-60 cells. Bioscience, Biotechnology, and Biochemistry 65, 534–541.

Makin, G., Hickman, J.A., 2000. Apoptosis and cancer therapy. Cell and Tissue Research 301, 143–152.

Mickey, D.D., Carvalho, L., Foulkes, K., 1989. Combined therapeutic effects of conventional agents and an immunomodulator, PSK, on rat prostatic adenocarcinoma. Journal of Urology 142, 1594–1598.

Ng, T.B., 1998. A review of research on the protein-bound polysaccharide (polysaccharopeptide, PSP) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae). General Pharmacology 30, 1–4.

Ooi, V.E., Liu, F., 1999. A review of pharmacological activities of mushroom polysaccharides. International Journal of Medicinal Mushrooms 1, 195–206.

Qian, Z.M., Xu, M.F., Tang, P.L., 1997. Polysaccharide peptide (PSP) restores immunosuppression induced by cyclophosphamide in rats. American Journal of Chinese Medicine 25, 27–35.

Sakagami, H., Aoki, T., Simpson, A., Tanuma, S., 1991. Induction of immunopotentiation activity by a protein-bound polysaccharide, PSK (review). Anticancer Research 11, 993–999.

Sellers, W.R., Fisher, D.E., 1999. Apoptosis and cancer drug targeting. Journal of Clinical Investigation 104, 1655–1661.

Spencer, J.W., 1999. Essential issues in complementary/alternative medicine. In: Spencer, J.W., Jacobs, J.J. (Eds.), Complementary/Alternative Medicine. An Evidence-based Approach. Mosby Inc., St. Louis, pp. 3–36.

The Leukemia and Lymphoma Society, 2003. Leukemia, lymphoma, myeloma facts 2003. The Leukemia and Lymphoma Society, White Plains, NY.

Tsang, K.W., Lam, C.L., Yan, C., Mak, J.C., Ooi, G.C., Ho, J.C., Lam, B., Man, R., Sham, J.S., Lam, W.K., 2003. *Coriolus versicolor* polysaccharide peptide slows progression of advanced non-small cell lung cancer. Respiratory medicine 97, 618–624.

Ukiya, M., Akihisa, T., Tokuda, H., Suzuki, H., Mukainaka, T., Ichiishi, E., Yasukawa, K., Kasahara, Y., Nishino, H., 2002. Constituents of Compositae plants: III. Anti-tumour promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible chrysanthemum flowers. Cancer Letters 177, 7–12.

Wang, H.X., Ng, T.B., Liu, W.K., Ooi, V.E., Chang, S.T., 1996. Polysaccharide-peptide complexes from the cultured mycelia of the mushroom *Coriolus versicolor* and their culture medium activate mouse lymphocytes and macrophages. International Journal of Biochemistry and Cell Biology 28, 601–607.

Wang, H.X., Liu, W.K., Ng, T.B., Ooi, V.E., Chang, S.T., 1995. Immunomodulatory and antitumor activities of a polysaccharide-peptide complex from a mycelial culture of *Tricholoma* sp., a local edible mushroom. Life Science 57, 269–281.

Wasser, S.P., Weis, A.L., 1999. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. Critical Reviews in Immunology 19, 65–96.

Yang, M.M.P., Chen, Z., Kwok, J.S.L., 1992. The anti-tumor effect of a small polypeptide from *Coriolus versicolor* (SPCV). American Journal of Chinese Medicine XX, 221–232.