



## Antioxidant and anticancer activity of extract from *Betula platyphylla* var. *japonica*

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### Abstract

The antioxidant and anticancer properties of a medicinal plant, *Betula platyphylla* var. *japonica* were investigated. The total methanol extract of *B. platyphylla* var. *japonica* had protective effects against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in the Chinese hamster lung fibroblast (V79-4) cell line and induced apoptotic cell death in human promyelocytic leukemia (HL-60) cells, a cancer cell line. *B. platyphylla* var. *japonica* extract significantly increased cell viability against H<sub>2</sub>O<sub>2</sub>. The extract also showed high 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (IC<sub>50</sub> 2.4 µg/ml) and lipid peroxidation inhibitory activity (IC<sub>50</sub> below 4.0 µg/ml). Furthermore, *B. platyphylla* var. *japonica* extract reduced the number of V79-4 cells arrested in G<sub>2</sub>/M in response to H<sub>2</sub>O<sub>2</sub> treatment and increased the activities of several cellular antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidase. Treatment with *B. platyphylla* var. *japonica* extract induced cytotoxicity and apoptosis in HL-60 cells, as shown by nucleosomal DNA fragmentation, increases in the subdiploid cell population, and fluorescence microscopy. *B. platyphylla* var. *japonica* extract gradually increased the expression of pro-apoptotic Bax and led to the activation of caspase-3 and cleavage of PARP. These findings suggest that *B. platyphylla* var. *japonica* exhibits potential antioxidant and anticancer properties.

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## Introduction

The role of reactive oxygen species (ROS) has been implicated in many human degenerative diseases, including aging, cancer, and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Harman, 1994; Simonian and Coyle, 1996). Hydrogen peroxide ( $H_2O_2$ ), is a prominent ROS that causes lipid peroxidation and DNA damage in cells (Sies, 1985; Halliwell and Aruoma, 1991). In recent years, considerable effort has been directed towards identifying naturally occurring substances that can protect against oxidative stress.

Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential (Finkel and Holbrook, 2000). Antioxidants have been used to inhibit apoptosis because apoptosis was initially thought to be mediated by oxidative stress (Hockenbery et al., 1993). Many antioxidant substances have anticancer or anticarcinogenic properties (Johnson et al., 1994; Dragsted et al., 1993). For example, resveratrol in grapes and other food products has been shown to protect cells from oxidative damage and cell death (Jang et al., 1997; Chanvitayapongs et al., 1997) and to prevent carcinogenesis in a murine model (Clement et al., 1998). Epigallocatechin-3-galate (EGCG) in green tea has been reported to scavenge free radicals (Hannasaki et al., 1994) and to inhibit carcinogen-induced tumors in the skin, lung, forestomach and colon of rodents (Stoner and Mukhtar, 1995). Kim et al. reported that ginsenosides Rb1 and Rg3 isolated from *Panax ginseng* protected cultured rat cortical cells from glutamate-induced neurodegeneration (Kim et al., 1998). The methanol extract of heat-processed neo-ginseng attenuated lipid peroxidation in rat brain homogenates induced by ferric ions or ferric ions plus ascorbic acid (Keum et al., 2000). Curcumin, a yellow coloring ingredient present in turmeric (*Curcuma longa* Linn, Zingiberaceae), has a diarylheptanoid moiety and has anticarcinogenic or antimutagenic effects in diverse animal models and in cultured cells (Rao et al., 1995).

Diarylheptanoids have been isolated from *Betula platyphylla* var. *japonica* together with other aromatic compounds (Matsuda et al., 1998). Earlier studies showed that *B. platyphylla* var. *japonica* could protect mice against  $CCl_4$ - or D-GalN/LPS (D-galactosamine/lipopolysaccharide)-induced liver damage (Soudamini and Kuttan, 1989). These findings suggest that *B. platyphylla* var. *japonica* most likely has both antioxidant and anticancer activity. Considerable attention has been focused on role of apoptosis or programmed cell death in the pathogenesis and treatment of human cancer (Thompson, 1995). Indeed, a variety of cytotoxic drugs have been reported to induce apoptosis of malignant cells in vitro (Planchon et al., 1995; Muller et al., 1997). The present study was therefore designed to investigate whether *B. platyphylla* var. *japonica* inhibits  $H_2O_2$ -induced oxidative stress in Chinese hamster lung fibroblast (V79-4) cells and to characterize the mechanism of its anticancer effects in human promyelocytic leukemia (HL-60) cells.

## Methods

### *Plant material*

Dried bark from *Betula platyphylla* var. *japonica* (100 g) was extracted at 80 °C in 70% methanol for 3 hr. The extract was then filtered and the filtrate was concentrated under low pressure using a vacuum rotary evaporator (Eyela, Japan). The remaining residue was lyophilized in a freezing-dryer (Ilsin,

Korea) and stored at  $-70\text{ }^{\circ}\text{C}$ . Approximately 10 g of powdered extract was recovered. The powder was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give final concentrations of total extract ranging from 0.8 to 500  $\mu\text{g/ml}$ .

### Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA): dimethyl sulfoxide (DMSO), propidium iodide (PI), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride (NBT), nicotinamide adenine phosphate (NADPH), xanthine, xanthine oxidase, ethylenediaminetetraacetic acid sodium salt (Na-EDTA), pyridine, sodium azide, glutathione, glutathione reductase, ethidium bromide. Ethanol was obtained from Hayman Chemical Co. (Witham, Essex, UK). Hydrogen peroxide was purchased from Fluka Chemical Co. (Buchs, SG, Swiss). Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA). All other chemicals were of the highest analytical grade and were purchased from common sources.

### DPPH free radical scavenging activity

In order to measure antioxidant activity, the DPPH free radical scavenging assays were carried out according to the previously described procedures (Blois, 1958; Lee et al., 2002).

### Cell culture

The Chinese hamster lung cell line V79-4 (ATCC CCL-93) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM glutamine, and antibiotics. The human promyelocytic leukemia cell line HL-60 (ATCC CCL-240) was grown in RPMI-1640 supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 unit/ml of penicillin and 100  $\mu\text{g/ml}$  of streptomycin. Cultures were maintained at  $37\text{ }^{\circ}\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ .

### Cell viability and cytotoxicity analysis

Cell viability was estimated by the MTT assay (Hansen et al., 1989; Lee et al., 2002). V79-4 cells were treated with *Betula platyphylla* var. *japonica* extract for 1 hr prior to treatment with 100  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ . The data are expressed as a mean percentage of viable cells as compared to the respective control cultures. Cytotoxicity of *Betula platyphylla* var. *japonica* on HL-60 cells was also evaluated by the MTT assay.

### Lipid peroxidation inhibitory activity

Lipid peroxidation was determined by measuring malondialdehyde (MDA) (Ohkawa et al., 1979). V79-4 cells in culture were incubated with total extract of *Betula platyphylla* var. *japonica* at various concentrations (4, 20 and 100  $\mu\text{g/ml}$ ) for 60 min, followed by 100  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  for 60 min. Inhibitory activity towards lipid peroxidation was expressed as  $\text{IC}_{50}$ .

### *Analysis of nuclear morphology*

V79-4 cells were seeded on sterilized cover glasses and treated with 100 µg/ml of *Betula platyphylla* var. *japonica* extract for 24 hr. Morphology of cellular nuclei were observed as described previously (Lee et al., 2003).

### *Flow cytometry*

V79-4 cells were treated with 100 µg/ml of extract for 1 hr followed by the addition of 100 µM of H<sub>2</sub>O<sub>2</sub> for 7 or 24 hr. HL-60 cells were treated with 500 µg/ml of extract for 0, 3, 7, 16, 24 or 48 hr. Cells were harvested and analyzed as described in Piao et al. (2001).

### *Assays for antioxidant enzymes*

V79-4 cells were treated with 4, 20 and 100 µg/ml of *Betula platyphylla* var. *japonica* extract for 60 min. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay. Results are expressed as enzyme activity per mg protein, as compared to the corresponding control cultures. Superoxide dismutase activity was assayed by the nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich, 1971). Catalase and glutathione peroxidase activity were assayed as previously described (Carrillo et al., 1991; Paglia and Valentine, 1967).

### *DNA fragmentation analysis*

HL-60 cells were treated for different periods with various concentrations of *Betula platyphylla* var. *japonica* extract. Cells were then harvested and DNA fragmentation was analyzed as described (Hyun et al., 1997).

### *Western blot analysis*

HL60 cells were treated with *Betula platyphlla* var. *japonica* extract and subjected to western blot analysis, as described previously (Piao et al., 2001). Blots were probed with mouse monoclonal anti-human anti-Bcl-2 (Oncogene Science, Cambridge, MA, USA), anti-caspase-3 (Transduction Laboratory, Lexington, KY, USA), and rabbit monoclonal anti-human anti-Bax and anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Immunoreactivity was detected using either an anti-mouse (Santa Cruz Biotechnology) or anti-rabbit (Amersham Biosciences, Buckinghamshire, UK) peroxidase-conjugated secondary IgG antibody and an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

## **Results**

### *Antioxidant activity of Betula platyphylla var. japonica extract*

The antioxidant activity of *B. platyphlla* var. *japonica* extract was evaluated on the basis of its DPPH free radical scavenging activity, by its protective effects on cell viability, and by the inhibition

of lipid peroxidation. The DPPH radical scavenging activity of the extract is shown in Fig. 1A. *B. platyphylla* var. *japonica* extract showed relatively high DPPH radical scavenging activity, with an average  $IC_{50}$  value of 2.4  $\mu\text{g/ml}$ . As a control, resveratrol was used and  $IC_{50}$  value obtained was 4.8  $\mu\text{g/ml}$  (data not shown).

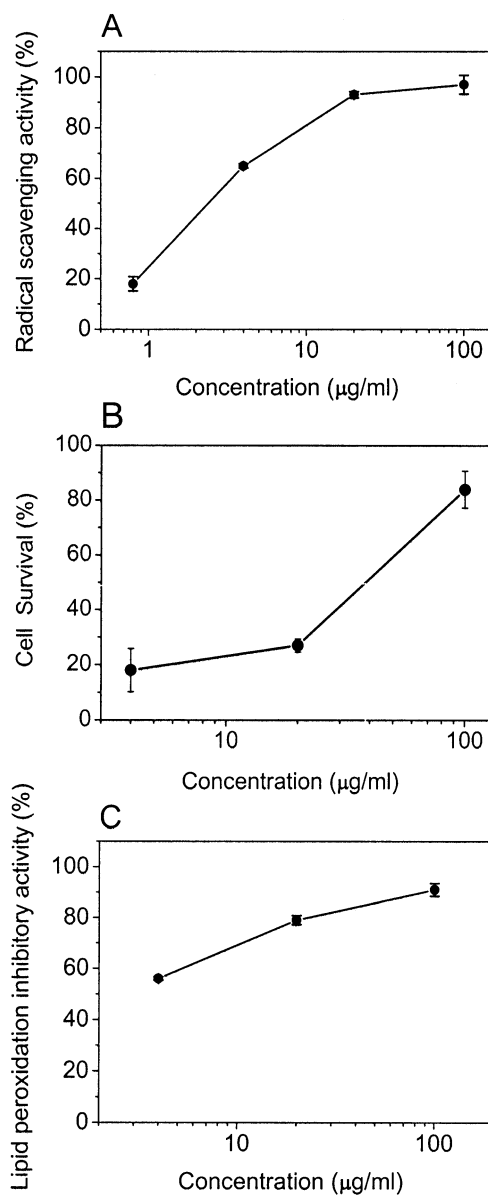


Fig. 1. Effects of *Betula platyphylla* var. *japonica* extract on (A) DPPH radical scavenging activity, (B) cell survival, (C) and lipid peroxidation inhibitory activity in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as average percent change from control  $\pm$  S.D.

We next measured the protective effect of *B. platyphylla* var. *japonica* extract on the survival in H<sub>2</sub>O<sub>2</sub>-treated cells. Cells were treated with extract for 1 hr prior to the addition of H<sub>2</sub>O<sub>2</sub>. Control cells were treated with H<sub>2</sub>O<sub>2</sub> in the presence of vehicle (DMSO) only. There was no cytoprotective effect of DMSO at the concentrations used in this study (below 0.1%). The relative cell survival of V79-4 cells was decreased in response to treatment with various concentrations of H<sub>2</sub>O<sub>2</sub> for 24 hr, with an average IC<sub>50</sub> value of about 100 μM (data not shown). Treatment with *B. platyphylla* var. *japonica* extract for 1 hr prior to the addition of 100 μM of H<sub>2</sub>O<sub>2</sub> induced a dose-dependent increase in cell survival (Fig. 1B). At a dose of 100 μg/ml, the extract increased cell viability by 85%, as compared to control levels. The extract itself showed little cytotoxicity in V79-4 cells (IC<sub>50</sub>>500 μg/ml). We also tested whether *B. platyphylla* var. *japonica* extract could inhibit lipid peroxidation in H<sub>2</sub>O<sub>2</sub>-treated V79-4 cells. As shown in Fig. 1C, cells pretreated with the extract exhibited a dose-dependent reduction in lipid peroxidation. Treatment of 4, 20 and 100 μg/ml of extract inhibited lipid peroxidation by 91%, 79% and 56%, respectively (IC<sub>50</sub> < 4.0 μg/ml). These values are similar to the values obtained from resveratrol. At concentrations of 4, 20 and 100 μg/ml of resveratrol, lipid peroxidation was inhibited by 74%, 72% and 65%, respectively (data not shown).

#### Reduction of H<sub>2</sub>O<sub>2</sub>-induced nuclear fragmentation

In order to analyze the protective effect of *Betula platyphylla* var. *japonica* extract on H<sub>2</sub>O<sub>2</sub>-induced apoptosis, we used propidium iodide to stain nuclei of V79-4 cells treated with either H<sub>2</sub>O<sub>2</sub> alone or with both *B. platyphylla* var. *japonica* extract and H<sub>2</sub>O<sub>2</sub>. As shown in Fig. 2A, control cells exhibited intact nuclei, but cells treated with 100 μM of H<sub>2</sub>O<sub>2</sub> showed significant nuclear fragmentation (Fig. 2B). However, when cells were treated with *B. platyphylla* var. *japonica* extract for 1 hr prior to H<sub>2</sub>O<sub>2</sub> treatment, a marked reduction in nuclear fragmentation was observed (Fig. 2C). In addition to these morphological observations, a protective effect of *B. platyphylla* var. *japonica* extract was confirmed using flow cytometry. DNA content was analyzed in V79-4 cells that were treated with H<sub>2</sub>O<sub>2</sub> for 7 and 24 hr, with or without pretreatment with *B. platyphylla* var.

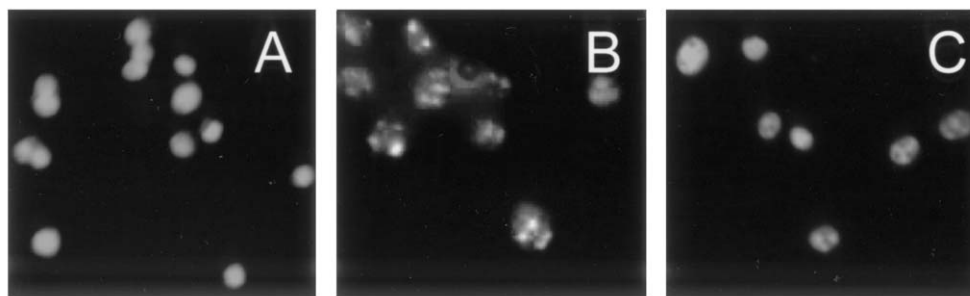


Fig. 2. *Betula platyphlla* var. *japonica* extract reduces H<sub>2</sub>O<sub>2</sub>-induced apoptosis in V79-4 cells. Cells were treated with *B. platyphlla* var. *japonica* extract for 1 hr prior to H<sub>2</sub>O<sub>2</sub> treatment and cellular morphological changes were observed using a fluorescence microscope at the magnitude of 200×. Representative photomicrographs of V79-4 cells treated with (A) vehicle only (B) 100 μM of H<sub>2</sub>O<sub>2</sub> and (C) 100 μg/ml of *B. platyphlla* var. *japonica* extract for 1 hr prior to the addition of H<sub>2</sub>O<sub>2</sub> (100 μM) are shown.

*japonica* extract. As shown in Fig. 3, exposure to H<sub>2</sub>O<sub>2</sub> for 7 and 24 hr induced cell cycle arrest in the G2/M phase in 42.7% and 33.4% of cells, respectively (Fig. 3A). Pretreatment of cells with *B. platyphylla* var. *japonica* extract (100 µg/ml) prior to H<sub>2</sub>O<sub>2</sub> treatment reduced the number of cells in the G2/M phase to 35.6% and 25.9%, respectively (Fig. 3B). H<sub>2</sub>O<sub>2</sub> treatment for 7 and 24 hr induced apoptosis in 6.1% and 4.2% of cells, and this was reduced to 0.4% and 0.8% in cells pretreated with extract.

#### Effect of *Betula platyphylla* var. *japonica* extract on antioxidant enzyme activity

In order to investigate whether these antioxidant properties of *B. platyphylla* var. *japonica* extract were mediated by an increase in antioxidant enzymes, we measured superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities in V79-4 cells treated with the extract (Fig. 4). Treatment with the extract at doses of 4, 20 and 100 µg/ml induced 21%, 31% and 36% increases, respectively, in SOD levels (Fig. 4A). Each sample dose-dependently increased SOD activity over a range of 4 to 100 µg/ml. The activity of SOD in control cells untreated with the extract was  $24.9 \pm 1.5$  U/mg protein. *B. platyphylla* var. *japonica* extract also dose-dependently increased CAT activity, although this effect was not as robust as the effect on SOD activity (Fig. 4B). At doses of 4, 20 and 100 µg/ml, the extract increased CAT activity by 12%, 20% and 24%, respectively. CAT activity in untreated control cells was  $14.9 \pm 1.9$  U/mg protein. GPX activity increased dramatically in response to treatment with extract (Fig. 4C). This occurred in a dose-dependent manner, in that 4, 20 and 100 µg/ml of extract increased GPX activity by 51%, 62% and 67%, respectively. The activity of GPX in control cells untreated with the extract was  $11.7 \pm 1.3$  U/mg protein.

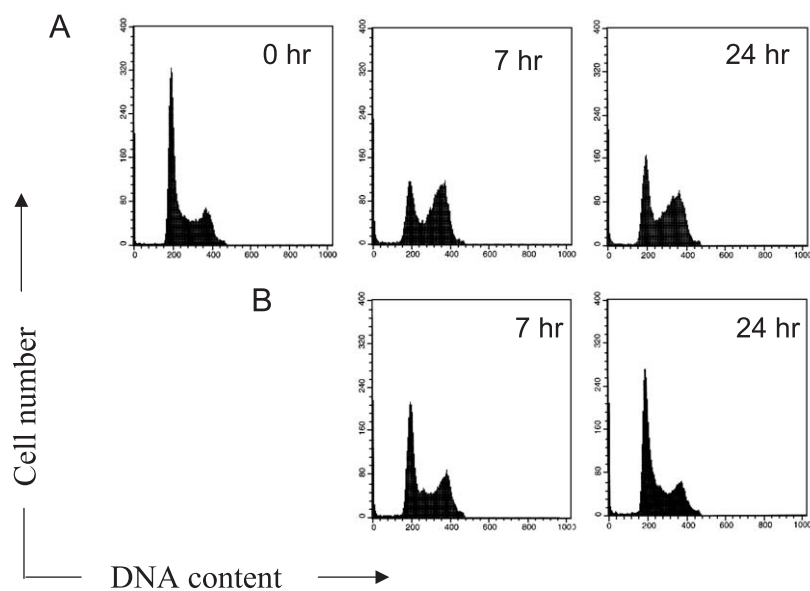


Fig. 3. *Betula platyphylla* var. *japonica* extract protects against H<sub>2</sub>O<sub>2</sub>-induced apoptosis in V79-4 cells. Flow cytometric DNA profiles of V79-4 cells treated with (A) 100 µM of H<sub>2</sub>O<sub>2</sub> alone or with (B) 100 µg/ml of *B. platyphylla* var. *japonica* extract prior to the addition of H<sub>2</sub>O<sub>2</sub> for 0, 7, or 24 hr, as indicated.

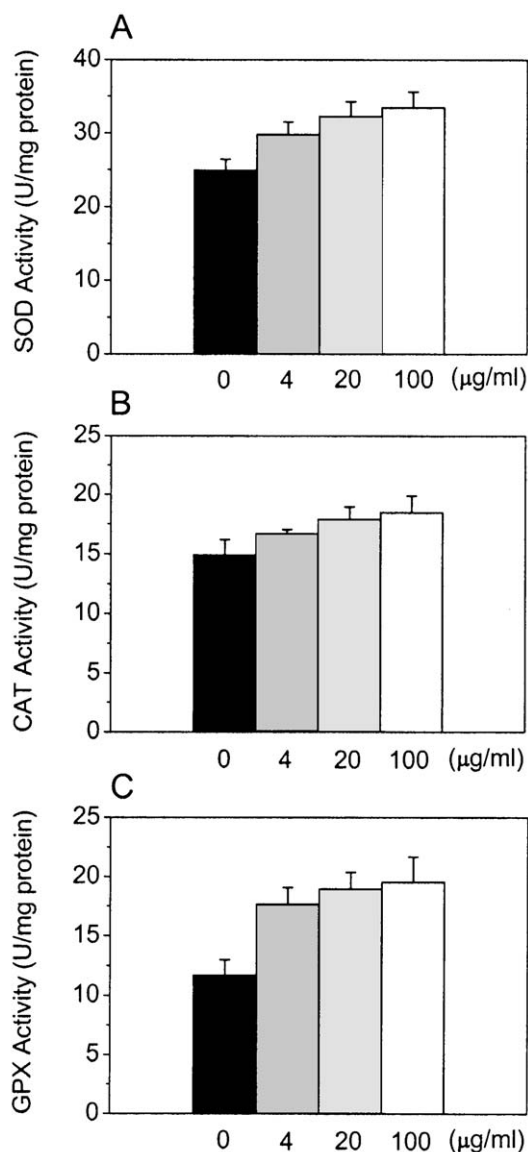


Fig. 4. Effect of *Betula platyphylla* var. *japonica* extract on various antioxidant enzymes. V79-4 cells were treated with 0, 4, 20 and 100 µg/ml of *B. platyphylla* var. *japonica* extract for 1 hr. (A) superoxide dismutase activity, (B) catalase activity, and (C) glutathione peroxidase activity were measured, as described in Methods. Each experiment was performed at least 3 times and the data are expressed as average enzyme units per mg protein from control  $\pm$  S.D.

#### *Cytotoxicity of Betula platyphylla* var. *japonica* extract in cancer cell

We evaluated the cytotoxicity of *B. platyphylla* var. *japonica* extract in human promyelocytic leukemia (HL-60) cells with the MTT assay. When cells were treated for 2 days with 0, 20, 100, and 500 µg/ml of *B. platyphylla* var. *japonica* extract, the relative cell survival progressively decreased in a

dose-dependent manner, as shown in Fig 5. The  $IC_{50}$  value for *B. platyphylla* var. *japonica* extract on HL-60 cells was 159.0  $\mu\text{g/ml}$ .

#### *Effect of Betula platyphylla* var. *japonica* extract on DNA and nuclear fragmentation in HL-60 cells

One of the mechanisms by which cell growth is suppressed is apoptotic cell death. Therefore, the effect of *B. platyphylla* var. *japonica* extract on DNA fragmentation was examined in HL-60 cells (Fig. 6). As shown in Fig. 6A, nucleosomal DNA fragmentation was observed when cells were treated with 100 and 500  $\mu\text{g/ml}$  of *B. platyphylla* var. *japonica* extract for 24 hr. Exposure to the higher dose of *B. platyphylla* var. *japonica* extract (500  $\mu\text{g/ml}$ ) induced DNA fragmentation in as little as 3 hr (Fig. 6B). The profile for *B. platyphylla* var. *japonica* extract-induced apoptosis closely correlated with its growth suppressive effects. Thus, the growth suppression induced by *B. platyphylla* var. *japonica* extract in HL-60 cells may be related to the induction of apoptosis.

The apoptotic effects of *B. platyphylla* var. *japonica* extract were confirmed by flow cytometric analysis. The extract clearly induced apoptosis in a time- and dose-dependent manner, as shown in Fig. 7. HL-60 cells were incubated with increasing concentrations of *B. platyphylla* var. *japonica* extract for 24 hr. The percentages of apoptotic cells observed at 4, 20, 100 and 500  $\mu\text{g/ml}$  of extract were 0, 5.7%, 12.2% and 68.5%, respectively (Fig. 7A). When HL-60 cells were incubated with 500  $\mu\text{g/ml}$  of *B. platyphylla* var. *japonica* extract for 3, 7, 16, and 24 hr, the relative percentage of apoptotic cells observed were 25.5%, 46.3%, 58.2% and 68.5%, respectively (Fig. 7B).

#### *Effects Betula platyphylla* var. *japonica* extract on apoptosis-related protein levels HL-60 cells

To understand the molecular mechanisms by which the *B. platyphylla* var. *japonica* extract induced apoptosis, we examined various apoptosis-related proteins. HL-60 cells were cultured in media

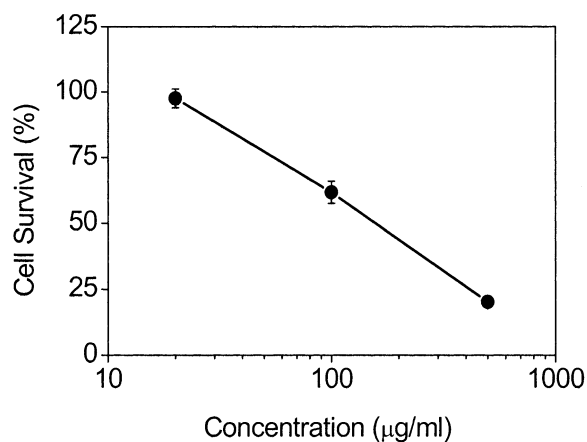


Fig. 5. Effects of *Betula platyphylla* var. *japonica* extract on cell survival in HL-60 cells. Cytotoxicity was measured by the MTT assay. HL-60 cells were precultured in 96-well microplates for 16 hr and then incubated with 4, 20, 100 and 500  $\mu\text{g/ml}$  of *B. platyphylla* var. *japonica* extract for 48 hr. Data are presented as means  $\pm$  S.D. The percentage of cell growth in the control group was designated as 100%.

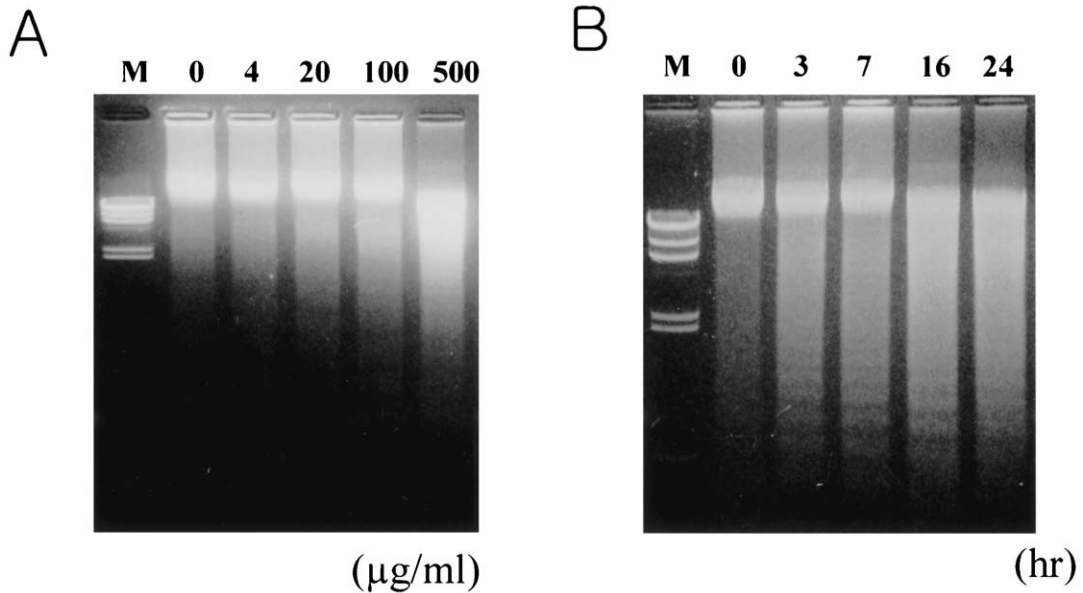


Fig. 6. *Betula platyphlla* var. *japonica* extract induced a time- and dose-dependent induction of DNA fragmentation in HL-60 cells. (A) HL-60 cells were treated with *B. platyphlla* var. *japonica* extract at concentrations of 0, 4, 20, 100 and 500  $\mu\text{g/ml}$  for 24 hr. (B) Cells were treated with *B. platyphlla* var. *japonica* extract at a concentration of 500  $\mu\text{g/ml}$  and harvested at 0, 3, 7, 16, and 24 hr. Nuclear DNA was extracted and subjected to electrophoresis on 1.5% agarose gels followed by ethidium bromide staining.

containing 500  $\mu\text{g/ml}$  of *B. platyphlla* var. *japonica* extract for 0, 1, 3, 7, 16, and 24 hr. At each time point, total protein was isolated and Bcl-2, Bax, caspase-3, and PARP (poly(ADP-ribose)polymerase) immunoreactivity levels were measured by Western blotting (Fig. 8). The pro-apoptotic protein, Bax was

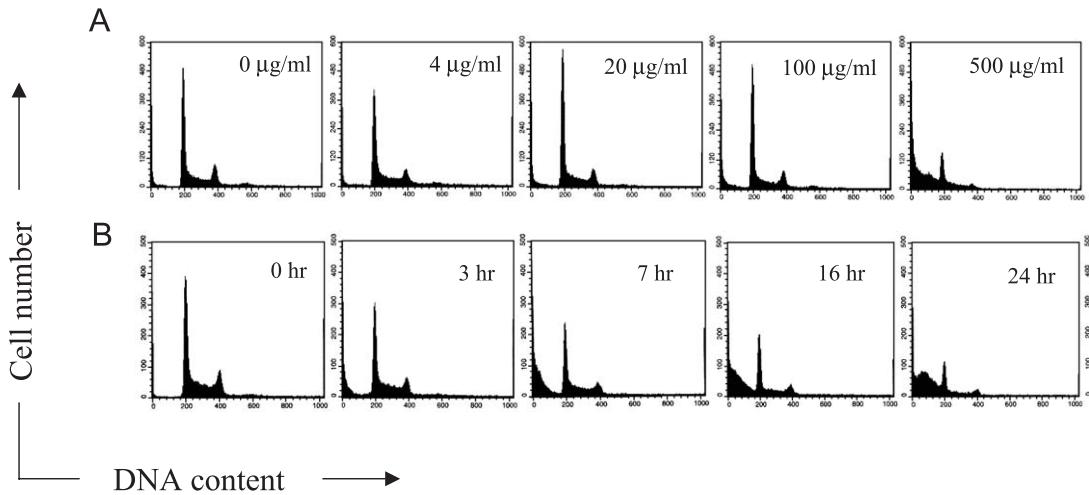


Fig. 7. Flow cytometric analysis of DNA cleavage in *Betula platyphlla* var. *japonica* extract-treated HL-60 cells. Cells were exposed to (A) 0, 4, 20, 100 and 500  $\mu\text{g/ml}$  of *B. platyphlla* var. *japonica* extract for 24 hr or (B) 500  $\mu\text{g/ml}$  of *B. platyphlla* var. *japonica* extract for 0, 3, 7, 16, and 24 hr. Cells were stained with propidium iodide for DNA content analysis.

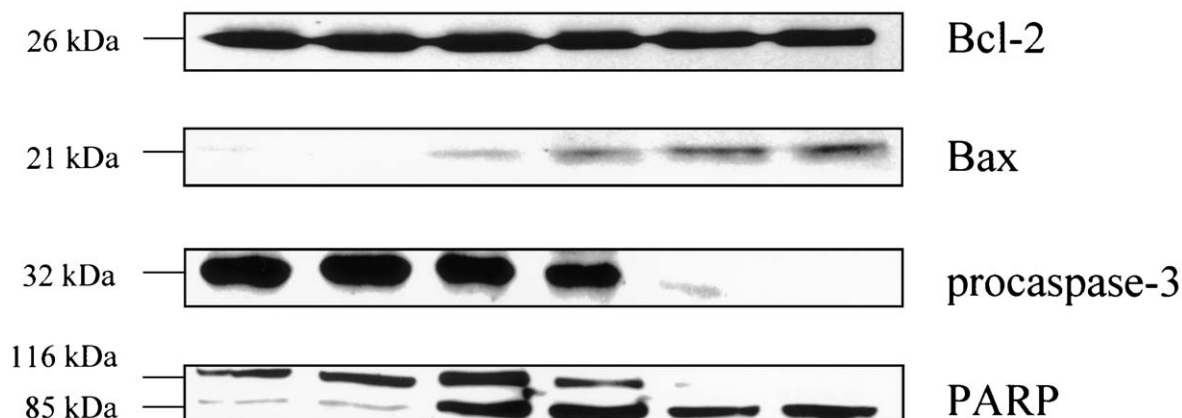


Fig. 8. Changes in the expression of apoptosis-related proteins in response to treatment with *Betula platyphylla* var. *japonica* extract. HL-60 cells were treated with 500  $\mu\text{g/ml}$  of *B. platyphylla* var. *japonica* extract for 0, 1, 3, 7, 16 and 24 hr. Cell extracts were subjected to Western blotting to determine immunoreactivity levels of Bcl-2, Bax, procaspase-3, and PARP, as described in Methods section. Representative western blots are shown.

increased in a time-dependent manner in response to extract, whereas the levels of the anti-apoptotic molecule Bcl-2 were unchanged; thus, the ratio of Bcl-2/Bax progressively decreased. Pro-caspase-3 levels were also decreased, implying that the levels of active caspase-3 were increased. To investigate the enzymatic activation of caspase-3, we measured the cleavage of PARP, which is a caspase-3 substrate. When cells were treated with 500  $\mu\text{g/ml}$  of *B. platyphylla* var. *japonica* extract, a time-dependent increase in the formation of the 85 kDa fragment and a decrease in the formation of the 116 kDa PARP were observed.

## Discussion

Reactive oxygen species generated by mitochondria or from other intracellular or extracellular sites can cause cell damage and initiate various degradation processes (Davies and Hochstein, 1982). Mild amounts of oxidative damage may actually stimulate physiological mitochondrial biogenesis, via the production of superoxide from ubiquinone. However, more severe or more prolonged oxidative damage clearly induces toxic reactions that can contribute significantly to the aging process (Cadenas and Davies, 2000). Oxidative damage induced by the intracellular production of  $\text{H}_2\text{O}_2$  occurs during many physiological and pathological processes. The cytotoxic effects of  $\text{H}_2\text{O}_2$  on Hep G2 cells included a potent inhibition of cell growth, elevated lactate dehydrogenase leakage, and MDA formation (Yang et al., 1999).  $\text{H}_2\text{O}_2$  itself is not highly reactive; the primary mechanism by which  $\text{H}_2\text{O}_2$  induces cytotoxicity during oxidative stress is via the formation of a highly reactive species in the presence of transition metal ions, or through other mechanisms (Halliwell et al., 1992).

In this work, the antioxidant activities of *Betula platyphylla* var. *japonica* extract were determined by measuring its protective effects on cell viability, DPPH radical scavenging activity, inhibition of lipid peroxidation activity, the fluorescent staining of nuclei, and by flow cytometry. The total extract from *B. platyphylla* var. *japonica* exhibited significant DPPH free radical scavenging activity and enhanced cell

viability in V79-4 cells exposed to  $H_2O_2$ . The protective effects of *B. platyphylla* var. *japonica* extract on  $H_2O_2$ -induced apoptosis were observed with the aid of a fluorescence microscope and a flow cytometer. Cells exposed to  $H_2O_2$  exhibit distinct morphological features of programmed cell death, such as nuclear fragmentation and an increase in the percentage of cells with a sub-G1 DNA content. However, cells pretreated with *B. platyphylla* var. *japonica* extract exhibited significantly lower levels of these characteristics of apoptotic cells. The morphological features and DNA profiles of extract-treated cells were very similar to those of control cells. Treatment with *B. platyphylla* var. *japonica* extract also decreased the DNA content of G2/M phase cells. These data suggested that *B. platyphylla* var. *japonica* extract inhibited  $H_2O_2$ -induced apoptosis.

We also found that the *B. platyphylla* var. *japonica* extract increased the activity of three antioxidant enzymes, SOD, CAT and GPX, that are altered in various diseases involving free radical attack (Halliwell and Gutteridge, 1998). Thus, maintaining the balance between the rates of radical generation and radical scavenging is an essential part of biological homeostasis. It is of particular interest to note that SOD catalyzes the breakdown of  $O_2\cdot$  to  $O_2$  and  $H_2O_2$  and prevents the formation of  $OH\cdot$ . Thus, SOD has been implicated as playing an essential defensive role against potential oxygen toxicity. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPX, because the dismutase activity of SOD generates  $H_2O_2$ , which needs to be further scavenged by CAT and GPX. *B. platyphylla* var. *japonica* extract modestly activated SOD and CAT and strongly activated GPX. It appears that *B. platyphylla* var. *japonica* extract can effectively scavenge  $H_2O_2$  generated by SOD.

A vast variety of naturally occurring substances have been shown to protect against experimental carcinogenesis. Thus, it is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, many have important cancer chemopreventive properties (Sanaha et al., 1997). Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis (Bellosillo et al., 1998). Our present results demonstrated that extract from *B. platyphylla* var. *japonica* induced apoptosis in HL-60 cells at relatively higher concentrations than those that mediate its growth inhibitory and antiproliferative activities. The induction of apoptotic cell death was accompanied by characteristic morphological and structural changes. Internucleosomal DNA fragmentation, as determined by agarose gel electrophoretic analysis, were consistent with apoptosis in cells treated with the *B. platyphylla* var. *japonica* extract. The flow cytometry data more clearly confirmed apoptosis in *B. platyphylla* var. *japonica* extract-treated HL-60 cells.

Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes. One of the major genes that regulates apoptosis is the protooncogene *bcl-2*, which encodes a 26 kDa mitochondria-associated protein. The *bcl-2* gene product prolongs cell survival by blocking apoptosis induced by a wide variety of stimuli (Okura et al., 1998; Kuo et al., 1996; Finucane et al., 1999). In our study, there was no change in Bcl-2 protein levels in HL-60 cells treated with *B. platyphylla* var. *japonica* extract, but the levels of Bax, a pro-apoptotic protein, were increased in a time-dependent manner, resulting in a decrease in the ratio of Bcl-2/Bax. Cytoplasmic aspartate-specific cysteine proteases of the ICE/CED-3 family, known as caspases, play an important role in apoptosis (Klaus et al., 1998). Caspase-3, one of the caspase family enzymes, can inactivate PARP by proteolytically cleaving this 116 kDa enzyme into an 85 kDa fragment (Nigata, 2000; Duriez and Shah, 1997; Li and Darzynkiewicz, 2000). When HL-60 cells were treated with *B. platyphylla* var. *japonica* extract, pro-caspase-3 was activated and the caspase substrate PARP was proteolytically cleaved.

Taken together, these findings suggest that *B. platyphylla* var. *japonica* extract exhibits antioxidant activity at lower concentrations and apoptotic effects at higher concentrations, through the down-regulation of Bcl-2/Bax and activation of caspase-3. Further studies will be needed to identify the active compounds that confer the antioxidant and/or anticancer activities of the *B. platyphylla* var. *japonica* extract. Once such compounds are identified, the mechanisms by which they exert their effects can begin to be characterized.

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