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Protective effects of *Betula platyphylla* var. *japonica* extracts against the cellular damage induced by reactive oxygen species

Sang Jin Ji and Jeong Hee Kim*

Department of Oral Biochemistry and Institute of Oral Biology, College of Dentistry, Kyung Hee University, 130-701, Korea

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In our present study, total methanol extracts prepared from *B. platyphylla* var. *japonica* showed a significant increase in cell proliferation upon the induction of oxidative stress by hydrogen peroxide or γ-ray irradiation. Total methanol extracts were fractionated into five separate preparations i.e. n-hexane, dichloromethane, ethylacetate, n-butanol and water fractions. Among these, the ethylacetate and butanol fractions of *B. platyphylla* var. *japonica* showed the highest protective effects against oxidative stress induced by hydrogen peroxide. These fractions also showed strong protective effects against γ-ray irradiation. When we evaluated the cytotoxicity of these fractions, the butanol fraction showed no effects in a colony formation assay. In addition, the butanol fraction showed a cell proliferation activation effect evidenced by significant increase in the colony formation of γ-ray irradiated cells. Both a radical scavenging activity and clonogenic activity assay suggested that the mechanism behind this protective effect against reactive oxygen species may be due to the radical scavenging and cell proliferation activity of *B. platyphylla* var. *japonica* extracts.

Key words: medicinal plant extract/protection/reactive oxygen species

Introduction

The univalent reduction of molecular oxygen results in reactive oxygen species (ROS). ROS include free radicals such as superoxide ions ($O_2^{\cdot-}$) and hydroxy radicals ($OH^{\cdot}$) as well as non free radical species such as hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1998). Although the generation of ROS is an essential defense mechanism in some instances, in excessive concentrations or in the wrong location it can cause tissue degeneration and a wide range of common diseases. These include neurodegenerative disease, heart diseases, diabetes, and cancer (Fatokun *et al.*, 2008; Forbes *et al.*, 2008; Beevi *et al.*, 2007; Son and Chun, 2007; Seddon *et al.*, 2006). When ionizing radiation is absorbed in biological materials, the radiation may interact with water molecules which compose 80% of a cell. As a result of this interaction, the highly reactive hydroxy radicals are formed. It is estimated that about two third of γ-ray damage to DNA in mammalian cells is due to hydroxy radicals.

Natural plants have been a good source to find a candidate for potential medical benefits. Various antioxidants have been detected in natural products. Borek (1991) reported on the antioxidative effects of aged garlic extract. Aged garlic extract exerts its antioxidant action by scavenging ROS, by enhancing the activities of the cellular antioxidant enzymes and by increasing glutathione in cells. Proanthrocyanidin from persimmon peel showed protective effect against H_2O_2-induced oxidative damage (Lee *et al.*, 2008). Extracts of *Verona amygdalin* and *Hibiscus sabdariffa* exhibited protective effect against radiation-induced liver damage in rats (Adaramoye *et al.*, 2008). Hawthorn fruit extract were radioprotective against gamma irradiation (Hosseinimehr *et al.*, 2007).

Betula platyphylla var. *japonica* (white birch, Betulaceae) is distributed in northern Europe and northern Asia. The bark of this plant has been used for the treatment of various inflammatory diseases including chronic bronchitis, dermatitis, rheumatism and periodontitis. The major

*Corresponding author: Jeong Hee Kim, PhD, Department of Biochemistry and Institute of Oral Biology College of Dentistry, Kyung Hee University 1 Hoeki-Dong, DongDaeMoon-Ku, Seoul, 130-701, Korea. Tel.: +82-2-961-0915, Fax.: +82-2-960-1457; E-mail: jhkimh@khu.ac.kr

constituents of bark of this plant, betulin and several other terpens are reported (Matsuda *et al.*, 1998). Earlier studies showed that *B. platyphylla* var. *japonica* could protect mice against CCl_4^- or D-galactosamine/lipopolysaccharide-induced liver damage (Soudamini and Kuttan, 1989). Recently, total extract of *B. platyphylla* var. *japonica* showed anti-dermitis and anti-osteoarthritis activities (Huh *et al.*, 2008; Kim *et al.*, 2008). The present study was therefore designed to investigate whether *B. platyphylla* var. *japonica* has protective effect against celluar damage induced by H_2O_2 and γ-ray treatment.

Materials and Methods

Preparation of total methanol extract and fraction samples

Dried bark from *B. 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°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 100 μg/ml.

Fraction samples were prepared as the followings. Frozen-dried methanol extract sample was dissolved in d-H_2O and the equal volume of n-hexane was added and extracted twice. Then the equal volume of dichloromethane (CH_2Cl_2), ethylacetate (EtOAc) and n-butanol (BuOH) were added to the water fraction one by one and the extraction procedure was performed twice. Each fraction samples were dried in a vacuum rotary evaporator (Eyela, Japan) and the water fraction was frozen-dried using a freezing-drier (Ilsin, Korea).

Cell culture and treatment

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. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For hydrogen peroxide treatment, V79-4 cells were seeded in a 96-well plate and incubated for 16 hr. Then 100 μM H_2O_2 was added to the culture and cells were incubated for 24 hr at 37°C For g-ray treatment, cells were irradiated with indicated doses of ^{60}Co γ-rays (Picker, USA) at a dose rate of 0.9 Gy/min at room temperature.

Cell viability assay

Cell viability was estimated by the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (Hansen *et al.*, 1989). V79-4 cells were treated with various concentrations of extract or fraction samples (4, 20, or 100 μg/ml, respectively) for 1 hr where stated. Cells were incubated for an additional 24 hr at 37°C. During the last 4 hr, cells were incubated with 20 μl of MTT stock solution (5 mg/ml) in 200 μl medium at 37°C. Samples were then extracted with acidic isopropanol and the absorbance was measured with the ELISA reader (Bio-Rad, USA) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of formazan formed in control cells was taken as 100% of viability. The data are expressed as mean percentage of viable cells as compared to the respective control cultures.

Clonogenic assay

Cells were irradiated as above with 0, 1, 2, 3, 5, 7, and 9 Gy of ^{60}Co γ-rays (Picker, USA). After 7 days of incubation, the cells were fixed in methanol:acetic acid (3:1) and stained with trypan blue. Colonies of more than 50 cells were scored. Plating efficiencies were about 55~60% for the cell line used.

Statistics

All data represent means ± S.E. Statistical analysis was performed using analysis of variance followed by the Student's *t*-test.

Results

Protective effect of methanol extract on H_2O_2-induced oxidative damage

In order to study cell damage protective effect of a medicinal plant, B. *platyphylla* var. *japonica* total methanol extract, we treated cells with chemical and physical stress which cause cellular damage. First of all, in order to determine appropriate H_2O_2 concentration, H_2O_2 dose-dependent cell proliferation was measured. Cell proliferation was gradually decreased as the concentration of H_2O_2 increases (Fig. 1A). Approximately 50% of cell proliferation was observed at 70 μM of H_2O_2. When cells were pre-treated with total extract before treatment of 100 μM of H_2O_2, the cell proliferation of H_2O_2 treated cells was increased as dose-dependent manner of total extract treated (Fig. 1B); 106.9 %, 122.1% and 173.7% of cell proliferation at 4, 20 and 100 μg/ml of total extract, respectively.

Protective effect of total extract against γ-ray irradiation

Before observing the protective effect of total extract against γ-ray irradiation, we measured cell proliferation after γ-ray irradiation without any treatment. Cells were seeded in 96-well plates and irradiated with 5 or 10 Gy of γ-ray, and the relative cell proliferation was measured using

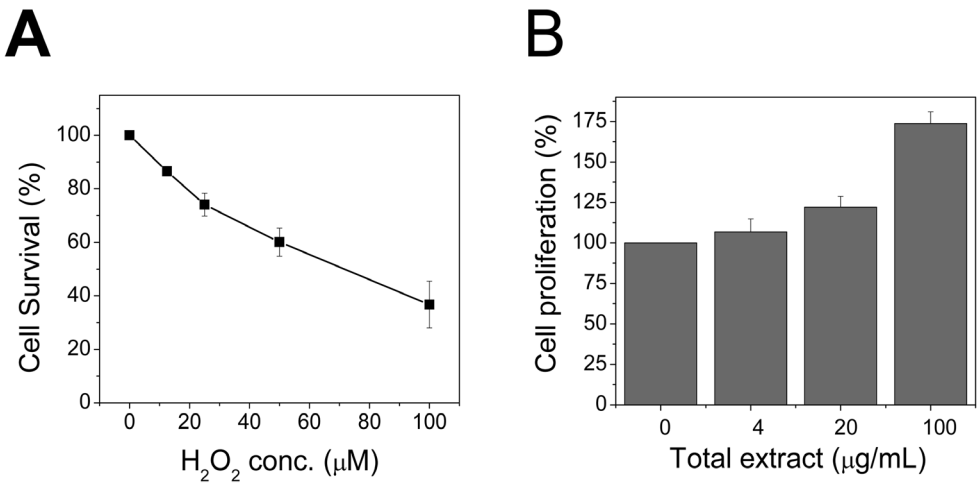

Fig. 1. Effect on cell proliferation of *B. platyphylla* var. *japonica* total methanol extract against H_2O_2-induced oxidative damage in V79-4 cells. (A) Cells were treated with various concentrations of H_2O_2 and the relative cell proliferation was measured and plotted. (B) Cells were treated with various concentrations of total extract 1 hr before 100 μM of H_2O_2 treatment, then the relative cell proliferation was measured. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D.

MTT assay. As shown in Fig. 2A, cell proliferation was decreased as the irradiated dose increased. At dose of 10 Gy, approximately 65.9% of cells were survived. After pre-treatment with total extract, then cells were irradiated with 10 Gy of γ-ray. The relative cell proliferation was measured and plotted as shown in Fig. 2B. At 4, 20 and 100 μg/ml of total extract treatment cell proliferation was increased to 106.1%, 134.0% and 140.6%, respectively.

Protective effect of fraction samples against γ-ray-induced cell damages

In order to narrow down the fractions which contain the protective activity against cell damaging stress, we prepared five fraction samples according to its polarity; hexane, dichloromethane, ethylacetate, butanol and water fractions. Fraction samples were tested for the protective affect against γ-ray irradiation. Among 5 fraction samples, butanol and ethylacetate fractions showed significant increase the relative cell proliferation of γ-ray irradiated cells. At a concentration of 20 μg/ml of ethylacetate and butanol fraction samples, the relative cell proliferation was similar, 152.6% and 152.4%, respectively (Fig. 3). At 4 μg/ml, the relative cell proliferation acquired from butanol fraction (122.7%) was relatively higher than that of ethylacetate (111.3%).

DPPH radical scavenging activity

In order to investigate the possible mechanism involved in the protection activity against cellular damage, DPPH radical scavenging activity of the fraction samples were

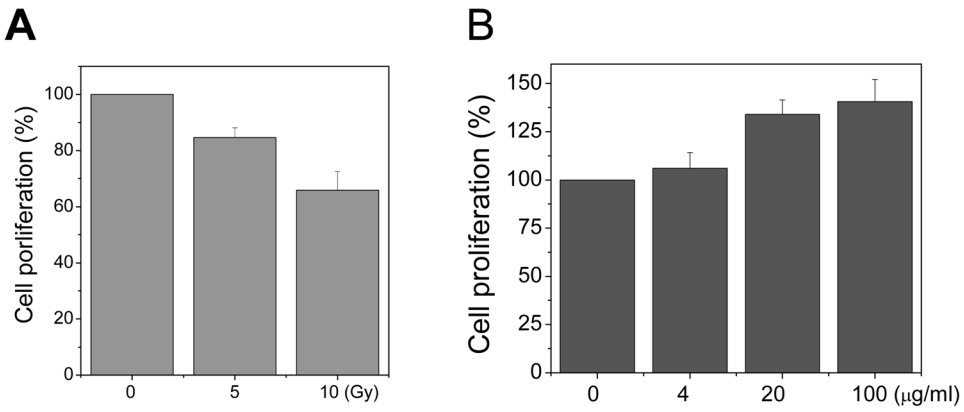

Fig. 2. Effect on cell proliferation of *B. platyphylla* var. *japonica* total methanol extract against γ-ray-induced oxidative damage in V79-4 cells. (A) Cells were treated with 0, 5 and 10 Gy of γ-ray and the relative cell proliferation rates were measured. (B) Cells were treated with various concentrations of total extract 1 hr before 10 Gy of γ-ray treatment, then the relative cell proliferation was measured. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D.

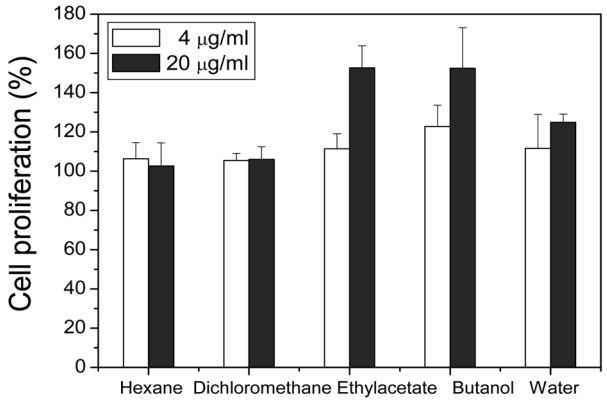

Fig. 3. Effect of fraction samples on the cell proliferation against γ-ray induced oxidative stress. Cells were treated with fraction samples 1 hr prior to 10 Gy of γ-irradiation. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D. White and gray bars indicate 4 and 20 μg/ml of samples, respectively.

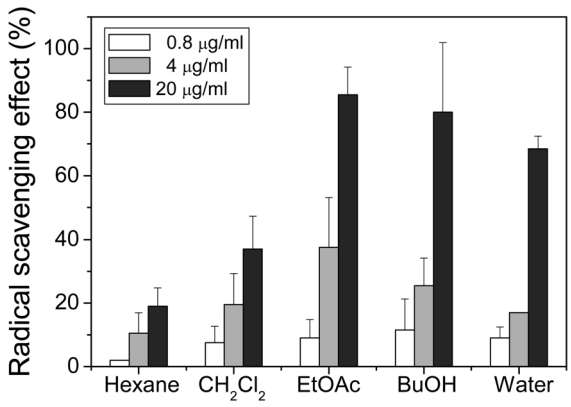

Fig. 4. DPPH radical scavenging activity of fraction samples. Fraction samples were added to DPPH solution and radical scavenging activity was measured at 520 nm. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D. White, gray and black bars indicate 0.8, 4 and 20 μg/ml of fraction samples, respectively.

measured (Fig. 4). Ethylacetate and butanol fractions showed higher than 80% radical scavenging activity at the concentration tested. And the radical scavenging activities of these two fractions were dose-dependent.

Protection effect of butanol fraction against γ-ray irradiation

Before we proceed to perform cellular damage protection activity, we measured the cytotoxicity of the ethylacetate and butanol fraction samples using clonogenic assay (Fig. 5). Most of the samples tested did not show significant cytotoxic effect at the concentration used. Among fractions butanol fraction showed the least cytotoxicity, even this fraction increased cell proliferation slightly. Thus, we tested the butanol fraction for the protection activity against γ-ray

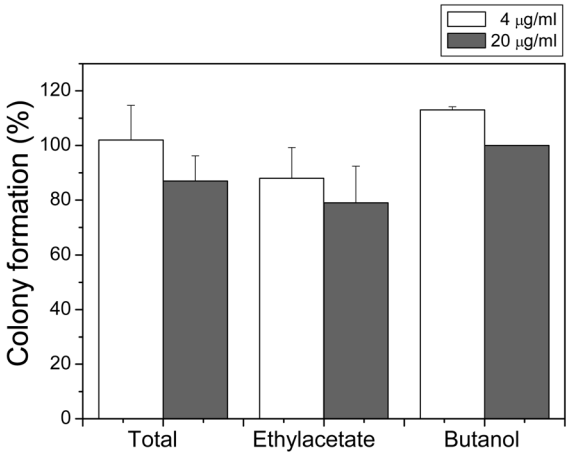

Fig. 5. Effect of fraction samples to clonogenic activity of Chinese hamster lung (V79-4) cells. Cells were treated with 4 and 20 μg/ml of fraction samples and incubated for 7 days. The relative number of colonies formed were observed and plotted.

irradiation. Cells were pretreated with 20 μg/ml of butanol fraction and irradiated with 0, 1, 2, 3, 5, 7 and 9 Gy of γ-ray. After 7 days of incubation, colonies with more than 50 cells were counted and the relative colony formation was plotted. As shown in Fig. 6, cell survival fraction of butanol treated cell was increased at all range of γ-ray irradiated in this study.

Discussion

Reactive oxygen species (ROS) are unwanted metabolic by-products of normal aerobic metabolism under high levels of O_2 pressure. High levels of ROS create oxidative stress, which leads to a variety of biochemical and physiological lesions. Such cellular damage often impairs metabolic function and leads to cell death (Finkel and Holbrook, 2000). Cells are protected from ROS-induced damage by a variety of endogeneous ROS scavenging enzymes, chemical compounds and natural products. Recently there has been increasing interest in the therapeutic

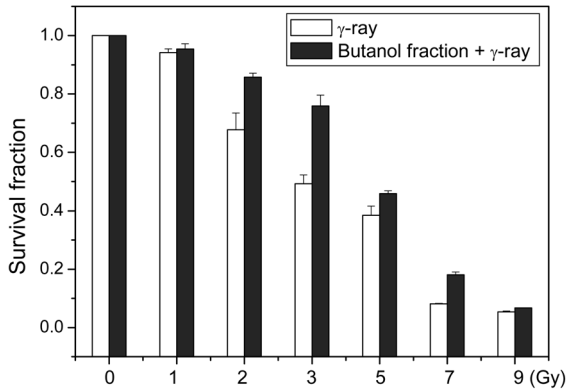

Fig. 6. Relative survival of V79-4 cells after γ-ray irradiation in the absence (white bars) or presence (gray bars) of butanol fraction samples.

potential of natural medicinal plants in reducing such free radical and/or γ-ray induced tissue injury.

In recent years, there has been a worldwide trend towards the use of the phytochemicals present in natural plants. Efforts to find radioprotectors among plants products are also made (Arora *et al*., 2005). Earlier studies showed that extracts from *Areca catechu* var. *dulcissima* showed strong scavenging activity against the superoxide anion radical (Ohsugi *et al.*, 1999). A cyclic phenylacetamide of *Solvia miltiorrhiza* was found to be a scavenger of the DPPH radical (Choi *et al.*, 2001). Methyl gallate from *Toona sienesis* showed protective against H_2O_2–induced stress (Hsieh *et al.*, 2004). The active fraction of *Pilea microphylla* (L.) ethanol extract showed anti-oxidant and radioprotective effect (Prabhakar *et al.*, 2007). The radioprotective effect of *Coleus aromaticus* on Chinese hamster fibroblast cells was reported (Rao *et al.*, 2006).

In previous study, we reported the antioxidant and apoptosis-inducing effect of *B. platyphylla* var. *japonica* extract (Ju *et al.*, 2004). Recently, inhibition of dermatitis development and collagen-induced osteoarthritis of *B. platyphylla* var. *japonica* treatment were reported (Huh *et al.*, 2008; Kim *et al.*, 2008). Here we reported that *B. platyphylla* var. *japonica* total methanol extract showed protective effect against cellular damages induced by H_2O_2 and γ-ray irradiation. Among 5 fraction samples butanol fraction revealed significant protective activity against γ-ray irradiation. The underlying mechanisms involved in the cellular protective effect of butanol fraction of *B. platyphylla* var. *japonica* extract are the radical scavenging activity and cell proliferation increase without having cytotoxicity to the cells. Our data support that the butanol fraction of *B. platyphylla* var. *japonica* can be developed as a protector against radicals and γ-ray irradiation. However, further studies needed to identify the active compound involved in this process.

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