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Cytoprotective Activity of *Carpinus tschonoskii* against H_2O_2 Induced Oxidative Stress

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Abstract – We have studied the cytoprotective effect on H_2O_2 induced oxidative stress from leaves of *Carpinus tschonoskii*. The methanol extract of *Carpinus tschonoskii* was found to scavenge intracellular reactive oxygen species (ROS) using flow cytometry and confocal microscope. This extract prevented lipid peroxidation and thus reduced cell death of Chinese hamster lung fibroblast (V79-4) induced by H_2O_2 treatment. The extract increased catalase activity and phosphorylation of extracellular signal regulated kinase (ERK). Taken together, the results suggest that *Carpinus tschonoskii* protects V79-4 cells against oxidative damage by H_2O_2 through scavenging ROS.
Keywords – oxidative stress, reactive oxygen species, Chinese hamster lung fibroblast, *Carpinus tschonoskii*

Introduction

Genus *Carpinus* consists of 40 species and distributed in the temperate area of the northern hemisphere. Only 5 species of *C. tschonoskii*, *C. laxiflora*, *C. cordata*, *C. turczaninowi*, and *C. coreana* grow in Korean peninsula (Lee *et al.*, 1989). And there has been a report on the isolation of flavonoids from Genus *Carpinus*, which contains flavonols myricetin, kaempferol and quercetin, and the flavones, which are apigenin and luteolin (Chang *et al.*, 2004). Reactive oxygen species (ROS) are associated with tissue damage and are the contributing factors for inflammation, aging, cancer, arteriosclerosis, hypertension and diabetes (Laurindo *et al.*, 1991; Nakazono *et al.*, 1991; Parthasarathy *et al.*, 1992; Palinski *et al.*, 1995; Darley-Usmar and Halliwell, 1996; Cooke *et al.*, 1997; Farinati *et al.*, 1998). For cytoprotection against ROS, cells have developed a variety of antioxidant defense mechanisms. Catalase is located at the peroxisome and converts hydrogen peroxide into molecular oxygen and water. Catalase plays important roles in cellular protection by oxidative stress induced cell damages (Pieterinen *et al.*, 1995; Doctrow *et al.*, 2002; Cui *et al.*, 2003; Sun *et al.*, 2005). In addition, catalase regulates the cell growth via activation of the extracellular signal regulated kinase (ERK) pathway, leading to the acceleration of the cell growth inhibited by oxidative stress (Hachiya and Akashi, 2005).

In the present study, we have investigated the protective effect of *C. tschonoskii* on cell damage induced by oxidative stress.

Experimental

Materials – *C. tschonoskii* leaf extract was purchased from Korea Research Institute of Bioscience and Biotechnology (KRIBB). Voucher specimens were deposited in the KRIBB (No. KRIB 0001289). The leaves were dried well in the shade, and then made a course powder by a grinder. Dry powder leaves of *C. tschonoskii* (35 g) were extracted with HPLC grade MeOH (200 mL) under an automatic extractor (ASE300 accelerated solvent extracor, Dionex Corporation) for 20 min. at 50 °C, 1500 psi using N_2 gas. A part of the filtrate which was separated from solid

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substances through defatted cotton-plugged funnel was completely concentrated under reduced pressure at 45 °C.

Cell culture – To study the effect of *C. tschonoskii* on oxidative stress, we used Chinese hamster lung fibroblasts (V79-4) cells. The V79-4 cells from the American type culture collection were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/mL) and penicillin (100 units/mL).

Intracellular reactive oxygen species measurement and image analysis – The DCF-DA method was used to detect the intracellular ROS level (Rosenkranz *et al.*, 1992). DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2',7'-dichlorofluorescein. The V79-4 cells were seeded at 1×10^5 cells/mL and after sixteen hours, the cells were treated with extract at 10 μg/mL and 30 min later, 1 mM H_2O_2 was added to the plate. The cells were incubated for an additional 30 min at 37 °C. After addition of 25 μM of DCF-DA solution, the fluorescence of 2',7'-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission using flowcytometer (Becton Dickinson, Mountain View, CA, USA). For image analysis for production of intracellular ROS, the V79-4 cells were seeded in coverslip loaded 6 well plate at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with extract and 30 min later, 1 mM H_2O_2 was added to the plate. After changing media, 100 μM of DCF-DA was added in the well and was incubated for an additional 30 min at 37 °C. After washing with PBS, stained cells were mounted onto microscope slide in the mounting medium (DAKO, Carpinteria, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

Lipid peroxidation inhibitory activity – Lipid peroxidation was assayed by thiobarbituric acid reaction (Ohkawa *et al.*, 1979). The V79-4 cells were seeded in a culture dish at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with extract at 10 μg/mL. One hour later, 1 mM H_2O_2 was added to the plate, and was incubated for further 1 h. The cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. One hundred μl of the cell lysates was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (adjusted to pH 3.5) and 1.5 mL of 0.8% thiobarbituric acid. The mixture was made up to a final volume of 4 mL with distilled water and heated to 95 °C for 2 h. After cooling to room temperature, 5 mL of *n*-butanol and pyridine mixture (15 : 1, v/v) was added to each sample, and the mixture was shaken well. After centrifugation at 1000 × g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

Cell viability – The effect of extract on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in the viable cells (Carmichael *et al.*, 1987). The V79-4 cells were seeded in a 96 well plate at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with extract at 10 μg/mL. One hour later, 1 mM H_2O_2 was added to the plate and incubated at 37 °C for an additional 24 h. Fifty μl of the MTT stock solution (2 mg/mL) was then added to each well to attain a total reaction volume of 200 μl. After incubating for 4 hours, the plate was centrifuged at 800 × g for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide (DMSO) and the A_{540} was read on a scanning multi-well spectrophotometer.

Nuclear staining with Hoechst 33342 – The V79-4 cells were placed in a 24 well plate at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with extract at 10 μg/mL and after further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, 1.5 μl of Hoechst 33342 (stock 10 mg/mL, a DNA specific fluorescent dye) was added to each well (1.5 mL) and incubated for 10 min at 37 °C. The stained cells were then observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

Catalase activity – The V79-4 cells were seeded at 1×10^5 cells/mL, and sixteen hours after plating, the cells were treated with extract for 1 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonication twice for 15 sec. Triton X-100 (1%) was then added to the lysates and was incubated for 10 min on ice. The lysates were centrifugated at 5000 × g for 30 min at 4 °C to remove the cellular debris. Fifty μg of protein was added to 50 mM phosphate buffer (pH 7) containing 100 mM (v/v) H_2O_2. The reaction mixture was incubated for 2 min at 37 °C and the absorbance was monitored at 240 nm for 5 min. The change in absorbance with time was proportional to the breakdown of H_2O_2 (Misra and Fridovich, 1972). The catalase activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required to breakdown of 1 μM H_2O_2.

Western blot – The V79-4 cells were placed in a plate at 1×10^5 cells/mL. Sixteen hours after plating, the cells were treated with extract at 10 μg/mL. The cells were harvested at the indicated times, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μL of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1 % NP 40] and centrifuged at 13,000 × g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with primary rabbit monoclonal anti ERK2, anti phospho ERK1/2 antibodies. The membranes were further incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugates (Pierce, Rockland, IL, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistical analysis – All the measurements were made in triplicate and all values were represented as means ± S.E. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. $p < 0.05$ were considered significantly.

Results and Discussion

Oxidative stress refers to the mismatched redox equilibrium between the production of ROS and ability of the cells to defend against ROS. ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide are unwanted and toxic by-products formed during aerobic metabolism. ROS can cause cell death via apoptosis and/or necrosis in many cell types, which can be blocked or delayed by various antioxidants and antioxidative proteins/enzymes (Kim *et al.*, 2001; Jang *et al.*, 2003). Although there is a report on the isolation of flavonoids from Genus *Carpinus*, which contains flavonols, and flavones (Chang *et al.*, 2004), there are no reports on the cytoprotective effect against oxidative stress induced cell damage and its

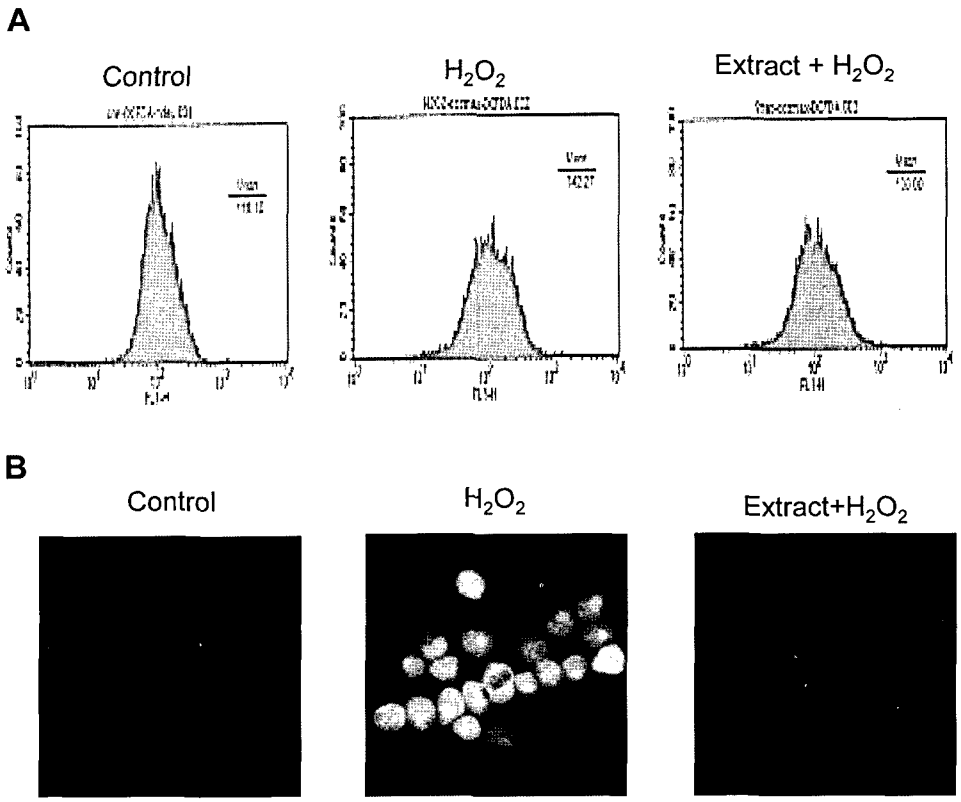

Fig. 1. Effect of methanol extract from *Carpinus tschonoskii* on scavenging intracellular ROS generated by H_2O_2 treatment. The intracellular ROS generated was detected by flow cytometry (A) and by confocal microscopy (B) after staining DCF-DA dye. Representative confocal images illustrate the increase in red fluorescence intensity of DCF produced by ROS in H_2O_2 treated V79-4 cells as compared to control and the lowered fluorescence intensity in H_2O_2 treated V79-4 cells in the presence of extract (original magnification × 400).

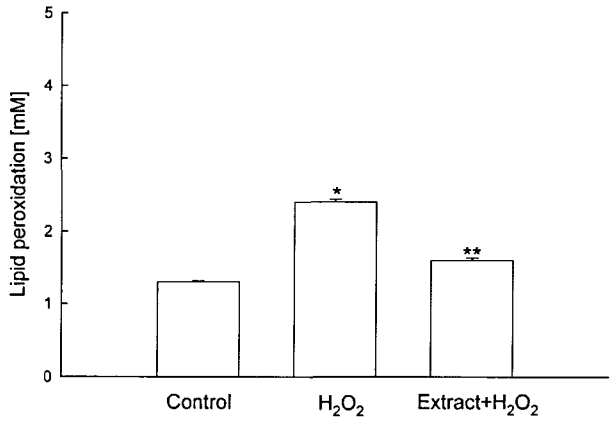

Fig. 2. Effect of methanol extract on inhibition of lipid peroxidation. Lipid peroxidation was assayed by measuring the amount of TBARS. *Significantly different from control and ** significantly different from H_2O_2 treatment ($p < 0.05$).

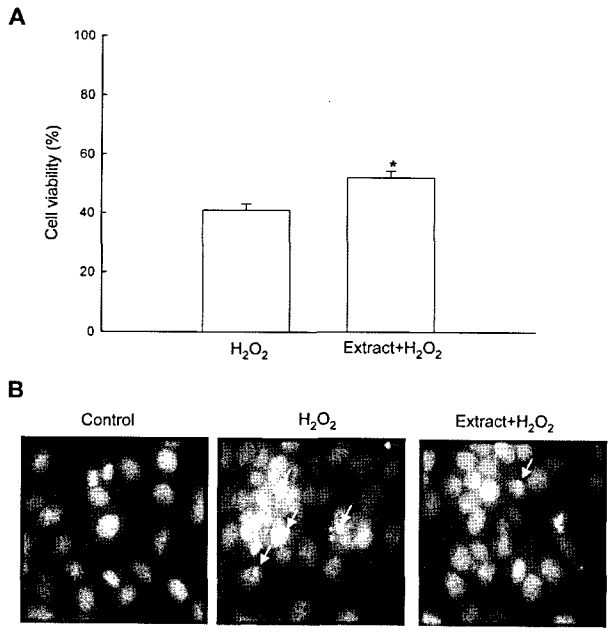

Fig. 3. Protective effect of methanol extract on H_2O_2 induced oxidative damage of V79-4 cells. The viability of V79-4 cells (A) was determined by MTT assay. Apoptotic body formation (B) was observed under a fluorescent microscope after Hoechst 33342 staining and is indicated by arrows. *Significantly different from H_2O_2 treatment ($p < 0.05$).

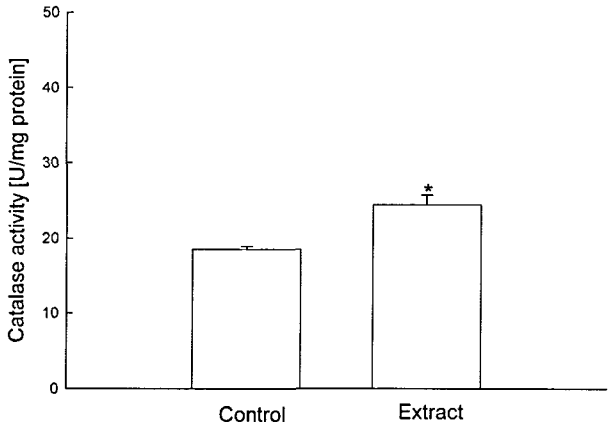

Fig. 4. Effect of methanol extract on the catalase activity. The enzyme activity is expressed as average enzyme unit per mg protein ± S.E. *Significantly different from control ($p < 0.05$).

mechanism of Genus *Carpinus*. In our present study, it was observed that upon exposure to H_2O_2, the methanol extract of *C. tschonoskii* decreased intracellular ROS. The level of ROS detected using flow cytometry (Fig. 1A) showed 130 value of fluorescence intensity which was produced from ROS stained by DCF-DA fluorescence dye in extract at 10 μg/mL treated cells compared to 142 value of fluorescence intensity in H_2O_2 treated cells. And red fluorescence intensity of DCF-DA staining detected by confocal microscope was enhanced in H_2O_2 treated V79-4 cells (Fig. 1B). However, methanol extract at 10 mg/mL reduced the red fluorescence intensity by H_2O_2 treatment, reflecting a reduction of ROS generation. The ability of methanol extract to inhibit lipid peroxidation in H_2O_2 treated V79-4 cells was also investigated. The generation of thiobarbituric acid reactive substance (TBARS) was inhibited in the presence of extract (Fig. 2). The protective effect of extract on cell survival in H_2O_2 treated V79-4 cells was measured. Cells were treated with extract at 10 μg/mL for 1 h prior to the addition to H_2O_2. The cell viability was determined 24 h later by MTT assay. As shown in Fig. 3A, treatment with methanol extract increased 11% of the cell survival rate at 10 μg/mL of extract. In order to study the cytoprotective effect of extract on apoptosis induced by H_2O_2, nuclei of V79-4 cells were stained with Hoechst 33342 for microscopy. The microscopic pictures in Fig. 3B showed that the control cells had intact nuclei, and the H_2O_2 treated cells showed significant nuclear fragmentation, characteristic of apoptosis. However, when the cells were treated with extract for 1 h prior to H_2O_2 treatment, a dramatic decrease in nuclear fragmentation was observed. In order to investigate whether the radical scavenging activity of methanol extract was mediated by antioxidant enzyme, the catalase activity in methanol extract treated V79-4 cells were measured. Methanol extract increased catalase activity (Fig. 4); it was 24 U/mg protein at 10 μg/mL, as compared to 18 U/mg protein of the control. To better understand the protective mechanism of extract on V79-4 cells, we examined the activation of the ERK protein by western blot analysis with the phospho-ERK specific

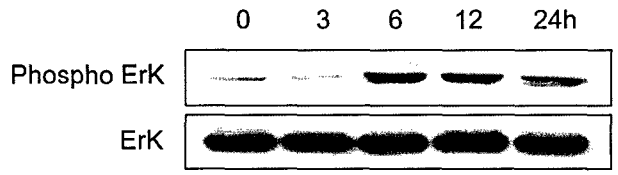

Fig. 5. Effect of methanol extract on phospho-ERK expression. Cell lysates were electrophoresed and proteins of phospho-ERK1/2 and ERK2 were detected by their respective specific antibodies

antibody. As shown in Fig. 5, methanol extract activated phosphorylated ERK dramatically at 6 h. However, there was no change in the total ERK protein level.

In conclusion, the methanol extract of *C. tschonoskii* exerted ROS scavenging activity, promoted cell viability, activated ERK protein and enhanced the catalase activity.

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