A Thesis

for the Degree of Master of Science in Medicine

Rhapontigenin from *Rheum undulatum* Protects against Oxidative-Stress Induced Cell Damage through Antioxidant Activity

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GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

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Rheum undulatum 에서 추출한 rhapontigenin의 항산화 활성을 통한 세포손상에 대한 보호 작용

지도교수 현 진 원

장 예

이 논문을 의학 석사학위 논문으로 제출함

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2006 . 12 .

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ABSTRACT

The antioxidant properties of rhapontigenin and rhaponticin isolated from *Rheum undulatum* were investigated. Rhapontigenin was found to scavenge intracellular reactive oxygen species (ROS), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and hydrogen peroxide (H_2O_2) by 2',7'-dichlorofluorescin dictate (DCF-DA) method, DPPH radical scavenging activity assay, and H_2O_2 scavenging activity assay respectively. The radical scavenging effect of rhapontigenin was more effective than rhaponticin. Rhapontigenin protected against H_2O_2 induced membrane lipid peroxidation and cellular DNA damage, the main targets of oxidative stress-induced cellular damage, which were detected by thiobarbituric acid reaction (TBAR) method and comet assay. The radical scavenging activity of rhapontigenin protected Chinese hamster lung fibroblast (V79-4) cells exposed to H_2O_2 by inhibiting apoptosis in MTT assay, Hoechst 33342 staining assay, and flow cytometry analysis. Rhapontigenin inhibited cell damage induced by serum starvation and also increased the activity of catalase and its protein expression. Further, western blot analysis of cell lysates indicated that rhapontigenin increased phosphorylation of extracellular signal regulated kinase (ERK) and the result of electrophoretic mobility shift assay (EMSA) showed that rhapontigenin inhibited the activity of activator protein 1 (AP-1), a redox sensitive transcription factor. In summary, these results suggest that rhapontigenin protects V79-4 cells against oxidative damage by enhancing the cellular antioxidant activity and modulating cellular signal pathways.

Key words: Rhapontigenin; Oxidative stress; Reactive oxygen species; Antioxidant activity

CONTENTS

LIST OF FIGURES

Ⅰ. INTRODUCTION

Rhaponticin and rhapontigenin, which are stilbene derivatives isolated from the rhizome of *Rheum undulatum* (Polygonaceae), possess antiallergic, purgative, anticoagulative, antithrombotic, anticomplementary, tyrosinase inhibitory, and hypoglycemic effects (Matsuda et al., 2001; Kim et al., 2000, 2002; Oshino et al., 1978; Ko et al., 1999; Park et al., 2002; Oh et al., 1998; Choi et al., 2006). It was reported that rhaponticin is converted into rhapontigenin by human intestinal microflora before being absorbed into the blood and rhapontigenin showed more potent pharmacological effects than rhaponticin (Park et al., 2002).

Balance between oxidants and antioxidants minimize molecular, cellular, and tissue damage. However, if the balance is upset in favor of the oxidants, oxidative stress occurs and often results in oxidative damage. Especially, reactive oxygen species (ROS) are known to cause oxidative modification of DNA, proteins, lipids and small cellular molecules and are associated with tissue damage and are the prime 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 et al., 1996; Cooke et al., 1997; Farinati et al., 1998). Cells have developed a variety of antioxidant defense mechanisms, for protecting themselves against the detrimental effects of reactive oxygen species. Enzymatic defense mechanisms involve superoxide dismutase, which catalyzes dismutation of superoxide anion to hydrogen peroxide; glutathione peroxidase, which destroys toxic peroxides; catalase, which is located at the peroxisome and converts

hydrogen peroxide into molecular oxygen and water. Catalase plays an important role in cellular protection against oxidative stress induced cell damage (Pietarinen et al., 1995; Doctrow et al., 2002; Cui et al., 2003; Banmeyer et al., 2004; Sun et al., 2005). In addition, catalase regulates cell growth through activation of the extracellular signal regulated kinase (ERK) pathway, which leads to the acceleration of the cell growth inhibited by oxidative stress (Hachiya and Ahashi, 2005).

The aim of the present study was to investigate the protective effects of rhapontigenin on cell damage induced by oxidative stress and the underlying possible mechanism of cytoprotection.

Ⅱ. MATERIALS AND METHODS

Preparation of Rhaponticin and Rhapontigenin

The dried rhizome of *Rheum undulatum* (1 kg, cultivated in Korea) was extracted into methanol for three days at room temperature. The extract was concentrated to dryness yielding 240 g of crude material. The crude material was suspended in 10 L of water followed by extraction with an equal volume of dichloromethane, ethylacetate and butanol, successively, which yielded 10 g of dichloromethane fraction, 110 g of ethylacetate fraction and 60 g of butanol fraction, respectively. 10 g of the ethylacetate fraction was applied to a silica-gel column chromatography using dichloromethane in MeOH [CH_2Cl_2 : MeOH = 20 : 1] as eluent, which resulted in 120 mg of 3,3',5-trihydroxy-4'-methoxystilbene (rhapontigenin, Figure 1) and to silica-gel column with dichloromethane in MeOH [CH_2Cl_2 : MeOH = 5 : 1] as eluent resulting in 280 mg of 3,3',5-trihydroxy-4'-methoxystilbene-3-O-β-D-glucoside (rhaponticin, Figure 1). Each of the two compounds was identified by the direct comparison of ^{1}H-NMR and ^{13}C-NMR data with those of authentic compounds, which was deposited in Phytochemistry Laboratory, Korea Research Institute of Chemical Technology.

Reagents

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 were purchased from the Sigma Chemical Company (St. Louis, MO, USA) and the 2,2-azino-bis-(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) from the Fluka Company (Buchs, Switzerland). Primary rabbit polyclonal anti-ERK 2 (42kDa ERK) and -phospho-ERK1/2 (phosphorylated 44kDa/42kDa ERK) antibodies were purchased

from the Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary sheep monoclonal catalase antibody was purchased from the Biodesign International Company (Saco, Maine, USA).

Cell Culture

Lung is one of the organs suffered from high concentration of oxygen and exposed high level of oxidative stress. So lung has good defense systems against oxidative stresses (Pryor et al., 1998; Kelly et al., 2003). To study the effect of rhaponticin and rhapontigenin on oxidative stress that is induced by hydrogen peroxide, the Chinese hamster lung fibroblasts (V79-4 cells) were used. The V79-4 cells from the American Type Culture Collection (Rockville, MD, USA), were maintained at 37℃ 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 (ROS) Measurement and Image Analysis

The DCF-DA method was used to detect the intracellular ROS levels (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. V79-4 cells were seeded in a 96-well plate at 1×10^5 cells/ml (2×10^4 cells/well). At 16 hr after plating, the cells were treated with rhaponticin or rhapontigenin at final concentration of 5, 10, 20 and 40 μM and 30 min later, final concentration of 1 mM H_2O_2 was added to the plate. The cells were incubated for an additional 30 min at 37℃. After addition of final 25 μM concentration of DCF-DA solution, the fluorescence of 2',7'-dichlorofluorescein

was detected at 485 nm excitation and at 535 nm emission using a Perkin Elmer LS-5B spectrofluorometer. For image analysis for production of intracellular ROS, the V79-4 cells were seeded in a coverslip loaded 6-well plate at 1×10^5 cells/ml (5×10^4 cells/well). At 16 hr after plating, the cells were treated with rhaponticin or rhapontigenin and 30 min later, 1 mM H_2O_2 was added to the plate. After an exchange of media, final 100 μM concentration of DCF-DA solution was added to the well and was incubated for an additional 30 min at 37℃. After washing with PBS, the stained cells were mounted onto microscope slide in the mounting medium (DAKO, Carpinteria, CA, USA). Images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a Zeiss confocal microscope.

DPPH Radical Scavenging Activity

Rhaponticin and rhapontigenin at 5, 10, 20 and 40 μM were added to a final 1×10^{-4} M concentration of DPPH in methanol, and the reaction mixture was shaken vigorously. After 1 hr, the amount of residual DPPH was determined at 520 nm using a spectrophotometer (Lo et al., 2004).

Hydrogen Peroxide Scavenging Activity

This assay was based on the ability of rhaponticin or rhapontigenin to scavenge the H_2O_2 in ABTS-peroxide medium (Muller, 1985). 40 μM of rhaponticin or rhapontigenin and 1 mM H_2O_2 were mixed with 20 μl of 0.1 M phosphate buffer (pH 5) in a 96-well plate and incubated at 37℃ for 5 min. Then 30 μl of ABTS and 30 μl of peroxidase (1 U/ml) were mixed and incubated at 37℃ for 10 min and the absorbance was determined at 405 nm using a spectrophotometer.

Detection of Lipid Peroxidation

Lipid peroxidation was assayed by thiobarbituric acid reaction (Ohkawa et al., 1979). V79-4 cells were seeded in a culture dish at 1×10^5 cells/ml (1×10^6 cells/dish). At 16 hr after plating, the cells were treated with 40 μM of rhapontigenin. At 1 hr later, 1 mM H_2O_2 was added to the plate, and incubated for further 1 hr. The cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. 100 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 (TBA). The mixture was made up to a final volume of 4 ml with distilled water and heated to 95℃ for 2 hr. 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. After centrifugation at 3000 × g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm. Amount of thiobarbituric acid reactive substance (TBARS) was determined using standard curve with 1,1,3,3,-tetrahydroxypropane.

Single Cell Gel Electrophoresis (Comet Assay)

The single cell gel electrophoresis assay (also known as the comet assay) was performed to assess the oxidative DNA damage (Singh, 2000; Rajagopalan et al., 2003). The cell pellet (1.5×10^5 cells) was mixed with 100 μl of 0.5% low melting agarose (LMA) at 39℃ and spread on a fully frosted microscopic slide that was pre-coated with 200 μl of 1% normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 75 μl of 0.5% LMA and then immersed in a lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 hr at 4℃. The slides were then placed in a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA

(pH 13) for 40 min to allow DNA unwinding and the expression of the alkali labile damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4℃ to draw negatively charged DNA toward an anode. After electrophoresis, the slides were washed three times for 5 min at 4℃ in a neutralizing buffer (0.4 M Tris, pH 7.5) and then stained with 75 μl of propidium iodide (20 μg/ml). The slides were observed using a fluorescence microscope and image analysis (Kinetic Imaging, Komet 5.5, UK). The percentage of total fluorescence in the tail and the tail length of the 50 cells per slide were recorded.

Cell Viability

The effect of rhapontigenin 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). To determine the cytoprotective effect of rhapontigenin in H_2O_2 treated V79-4 cells, cells were seeded in a 96-well plate at 1×10^5 cells/ml. 16 hr after plating, the cells were treated with 40 μM of rhapontigenin. 1 hr later, 1 mM H_2O_2 was added to the plate and the mixture was incubated at 37℃ for an additional 24 hr. 50 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 hr, the plate was centrifuged at 800 × g for 5 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide and the A_{540} was read on a scanning multi-well spectrophotometer. To determine the effect of rhapontigenin on the cell viability of V79-4 cells during serum starvation, cells in 10% fetal calf serum were seeded in a 96-well plate at 1×10^5 cells/ml. 16 hr after plating, cells were in serum starved condition (0.1% fetal calf serum), and then treated with rhapontigenin for 1 hr. The

plate was incubated at 37℃ for further 6 hr and the cell viability was measured using MTT test. To determine the effect of catalase inhibitor on the cell viability, cells were pre-treated with final 20 mM of ATZ for 1 hr, followed by 1 hr of incubation with rhapontigenin and exposure to 1 mM H_2O_2 for 24 hr and the cell viability was measured using MTT test. To determine the effect of ERK inhibitor on the cell viability, cells were pre-treated for 30 min with final 10 nM of U0126, followed for 1 hr with rhapontigenin and exposed to 1 mM H_2O_2 for 24 hr and the cell viability was measured using MTT test.

Nuclear Staining with Hoechst 33342

The V79-4 cells were placed in a 24-well plate at 1×10^5 cells/ml (2×10^5 cells/well). At 16 hr after plating, the cells were treated with 40 μM of rhapontigenin and after further incubation for 1 hr, 1 mM H_2O_2 was added to the culture. After 24 hr, 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℃. The stained cells were then observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera in order to assess the degree of nuclear condensation.

Flow Cytometry Analysis

Flow cytometry was performed to assess the content of apoptotic sub-G_1 hypo-diploid cells and cell distributions at each phase of a cell cycle (Nicoletti et al., 1991). The V79-4 cells were placed in a 6-well plate at 1×10^5 cells/ml (5×10^5 cells/well). At 16 hr after plating, the cells were treated with 40 μM of rhapontigenin. After a further incubation of 1 hr, 1 mM H_2O_2 was added to the culture. After 24 hr, the cells were harvested, and fixed in 1 ml of 70% ethanol for

30 min at 4℃. The cells were washed twice with PBS, and then incubated for 30 min in the dark at 37℃ in 1 ml of PBS containing 100 μg propidium iodide and 100 μg RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The proportion of sub-G_1 hypo-diploid cells and cell distributions at each phase of cell cycle was assessed by the histograms generated using the computer program, Cell Quest and Mod-Fit.

Catalase Activity

The V79-4 cells were seeded at 1×10^5 cells/ml (1×10^6 cells/dish), and at 16 hr after plating, the cells were treated with 40 μM of rhapontigenin for 6 hr. 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 incubated for 10 min on ice. The lysates were centrifuged at 5000 × g for 30 min at 4℃ to remove the cellular debris. The protein content of the supernatant was determined by Bradford method (Bradford, 1976), with bovine serum albumin as the standard. 50 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℃ 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 (Carrillo et al., 1991). 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 (1×10^6 cells/dish). At 16 hr after plating, the cells were treated with 40 μM of rhapontigenin. The cells were harvested at 3, 6, 12, and 24 hr, 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 antibody. The membranes were further incubated with secondary immunoglobulin G-horseradish peroxidase conjugates (Pierce, Rockland, IL, USA). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK) and then exposed to X-ray film.

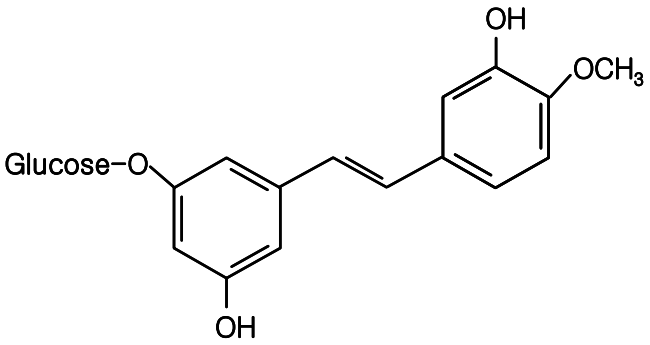
Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

The V79-4 cells were placed in plate at 1×10^5 cells/ml (1×10^6 cells/dish). At 16 hr after plating, the cells were treated with 40 μM of rhapontigenin. The cells were harvested at 3, 6, 12, and 24 hr, and then lysed on ice with 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) for 4 min. After 10 min of centrifugation at 3,000 × g, the pellets were resuspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF), incubated on ice for 30 min, and centrifuged at 13,000 × g for 5 min. The supernatant was then harvested as nuclear protein extracts and stored at -70℃ after determination of protein concentration. Oligonucleotide containing transcription factor activator protein-1 (AP-1) consensus sequence (5'- CGC TTG ATG ACT CAG CCG GAA - 3') was annealed, labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and used as probes. The probes (50,000 cpm) were incubated with 6 μg of the nuclear extracts at 4℃ for 30 min in

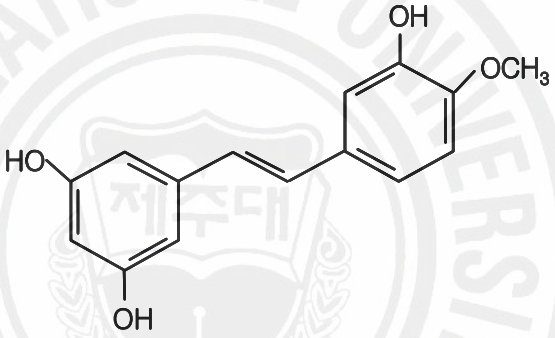
a final volume of 20 μl containing 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly (dI-dC). Binding products were resolved on 5% polyacrylamide gel and the bands were visualized by autoradiography (Kim et al., 1998).

Statistical Analysis

Results are represented as the mean standard error of three separate experiments in triplicate. 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.



Rhaponticin



Rhapontigenin

Figure 1. Chemical structure of rhaponticin and rhapontigenin

Ⅲ. RESULTS

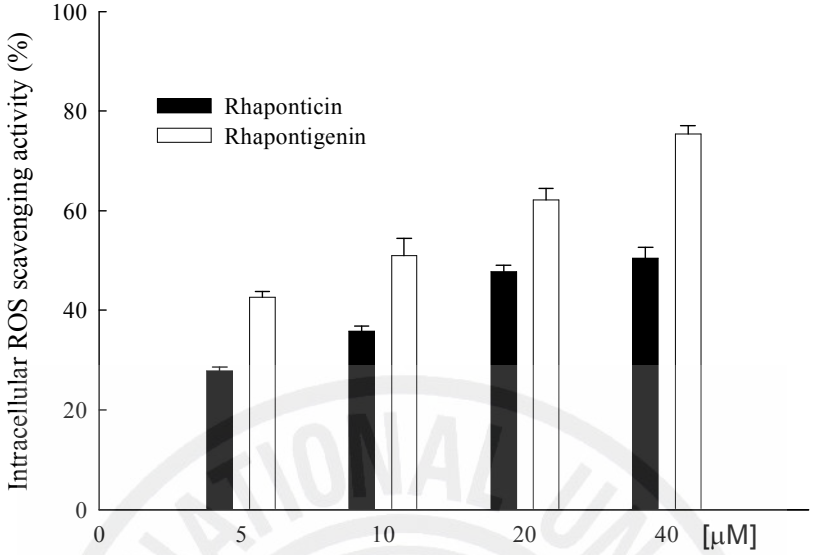
Radical Scavenging Activity of Rhaponticin and Rhapontigenin

The radical scavenging effect of rhaponticin and rhapontigenin on the intracellular ROS, DPPH free radical, and H_2O_2 scavenging activities were compared. The intracellular ROS scavenging activity of rhaponticin and rhapontigenin are shown in Figure 2A. As shown in Figure 2B, the fluorescence intensity of DCF-DA staining was enhanced in H_2O_2 treated V79-4 cells. Rhaponticin and rhapontigenin at 40 μM reduced the red fluorescence intensity upon H_2O_2 treatment, thus reflecting a reduction in ROS generation. The ROS scavenging activity of both the compounds was consistent with its DPPH radical and H_2O_2 scavenging activity. As shown in Figure 2C and D, the radical scavenging effect of rhapontigenin in these experiments was more effective when compared to rhaponticin. Based on these results, rhapontigenin was selected as active compound for further studies on radical scavenging effect.

Effect of Rhapontigenin on Lipid Peroxidation and Cellular DNA Damage Induced by H_2O_2

The ability of rhapontigenin to inhibit lipid peroxidation in H_2O_2 treated V79-4 cells was also investigated. The generation of TBARS was decreased in the presence of rhapontigenin. Rhapontigenin in H_2O_2 treated cells was measured as 2.1±0.3 μM/mg protein of TBARS at 40 μM (n=3/group) when compared to 5.2±0.2 μM/mg protein of TBARS in only H_2O_2 treatment (n=3/group) (Figure 3A). Damage to cellular DNA induced by H_2O_2 exposure was detected using an alkaline comet assay. The exposure of cells to H_2O_2 increased comet parameters like tail length and percentage of DNA in the tails of cells. Rhapontigenin in H_2O_2 treated

A



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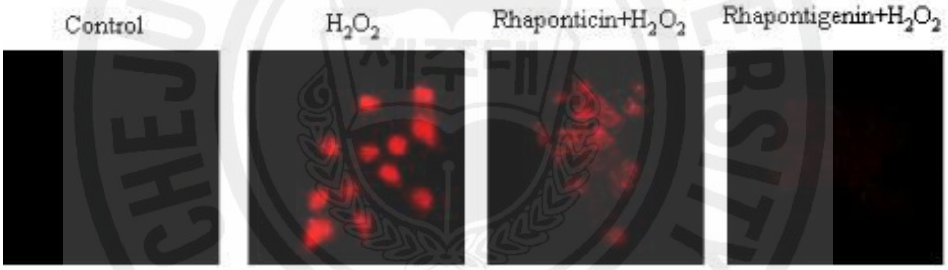
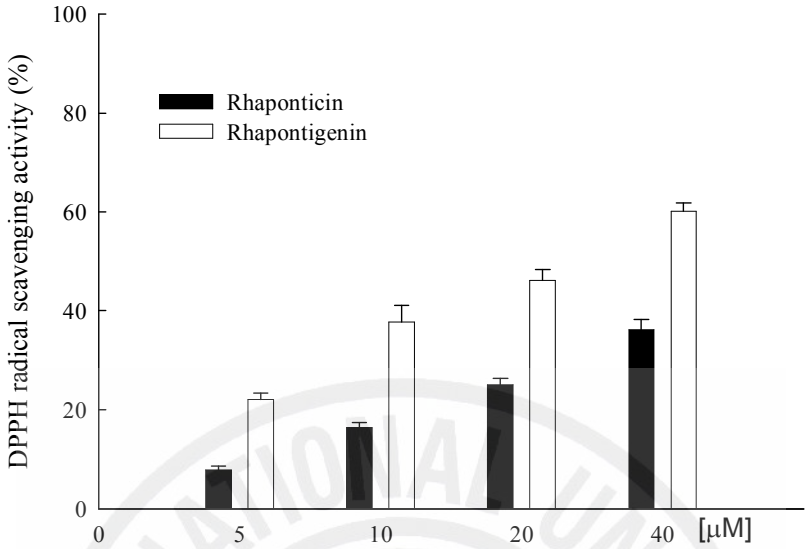


Figure 2. Effect of rhaponticin and rhapontigenin on scavenging intracellular ROS, DPPH radicals, and H_2O_2. The intracellular ROS generated was detected by DCF-DA method (A) and by confocal microscopy (B) in H_2O_2 treated cells with rhaponticin or rhapontigenin at 5, 10, 20 and 40 μM. Representative confocal images illustrate the increase in red fluorescence intensity of DCF produced by ROS in H_2O_2 treated cells as compared to control and the lowered fluorescence intensity in H_2O_2 treated cells in the presence of rhaponticin and rhapontigenin at 40 μM (original magnification × 400).

C



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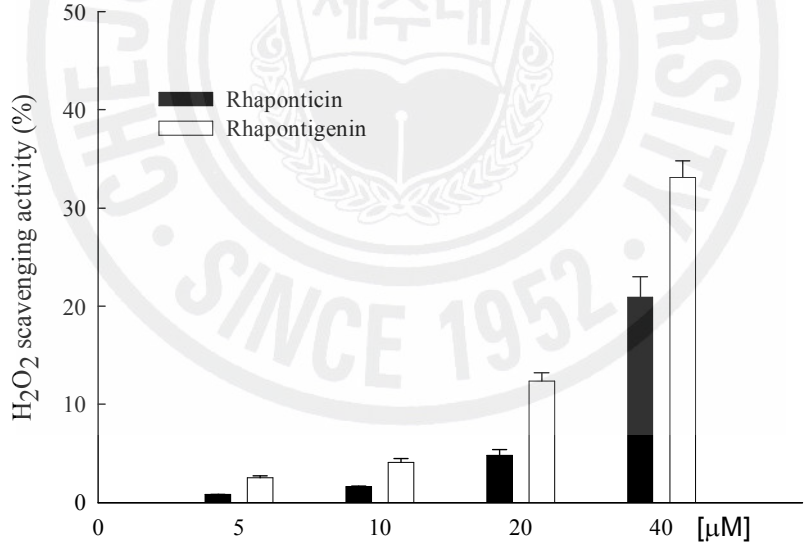


Figure 2 continued. The amount of DPPH (C) and H_2O_2 (D) in rhaponticin or rhapontigenin treatment at 5, 10, 20 and 40μM were determined spectrophotometrically at 520 nm and at 405 nm, respectively.

cells resulted in a marked decrease of tail length (Figure 3B). H_2O_2 increased fluorescence in the tail by 6.1 fold compared to control (*n*=3/group), while rhapontigenin decreased to H_2O_2 effect by 40% (Figure 3C), thereby indicating a protective effect of rhapontigenin on H_2O_2 induced DNA damage.

Effect of Rhapontigenin on Cell Damage Induced by H_2O_2

The protective effect of rhapontigenin on cell survival in H_2O_2 treated V79-4 cells was also assessed. Cells were treated with rhapontigenin at 40 μM for 1 hr, prior to the addition of H_2O_2. The cell viability was determined 24 hr later by MTT assay. As shown in Figure 4A, H_2O_2 decreased viability by 38%, while rhapontigenin increased viability from 62±1.4% to 87±5.1% (*n*=3/group). To study the cytoprotective effect of rhapontigenin on apoptosis induced by H_2O_2, nuclei of V79-4 cells were stained with Hoechst 33342 for microscopic examination and with propidium iodide for flow cytometric analysis. The microscopic pictures in Figure 4B indicated 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 rhapontigenin for 1 hr prior to H_2O_2 treatment, a decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of rhapontigenin against apoptosis was confirmed by flow cytometry. As shown in Figure 4C, an analysis of the DNA content revealed that percentage of DNA with an apoptotic sub-G_1 profile increased from 1% in control to 44% in the H_2O_2 treated cells. Treatment with 40 μM of rhapontigenin decreased the apoptotic sub-G_1 DNA content to 17%. These results suggest that rhapontigenin protects cell viability by inhibiting H_2O_2 induced apoptosis.

A

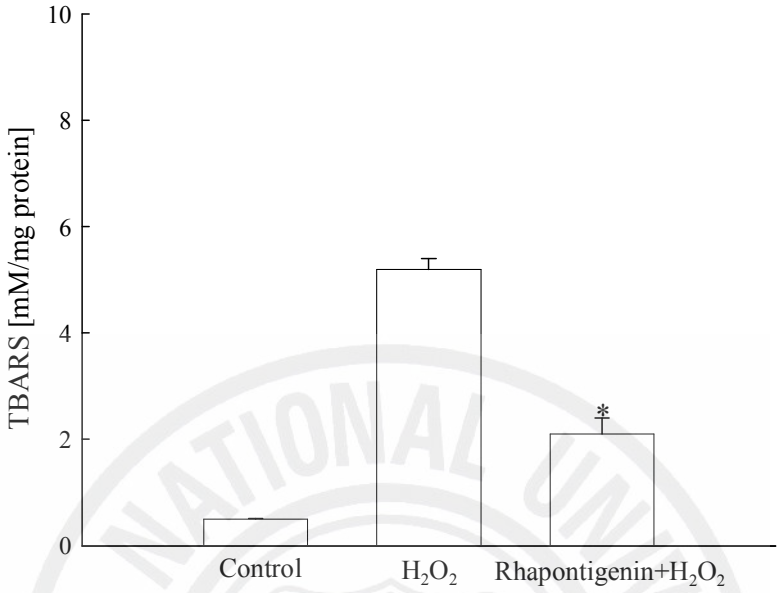
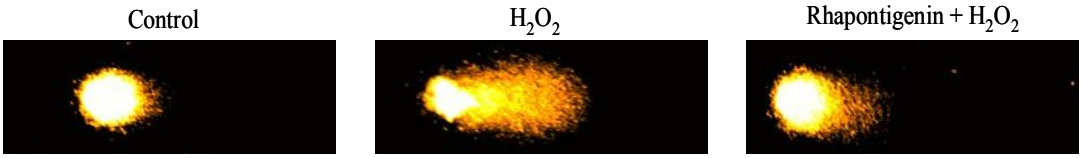


Figure 3. Effect of rhapontigenin on inhibition of lipid peroxidation and cellular DNA damage induced by H_2O_2. (A) Lipid peroxidation was assayed by measuring the amount of TBARS.

B



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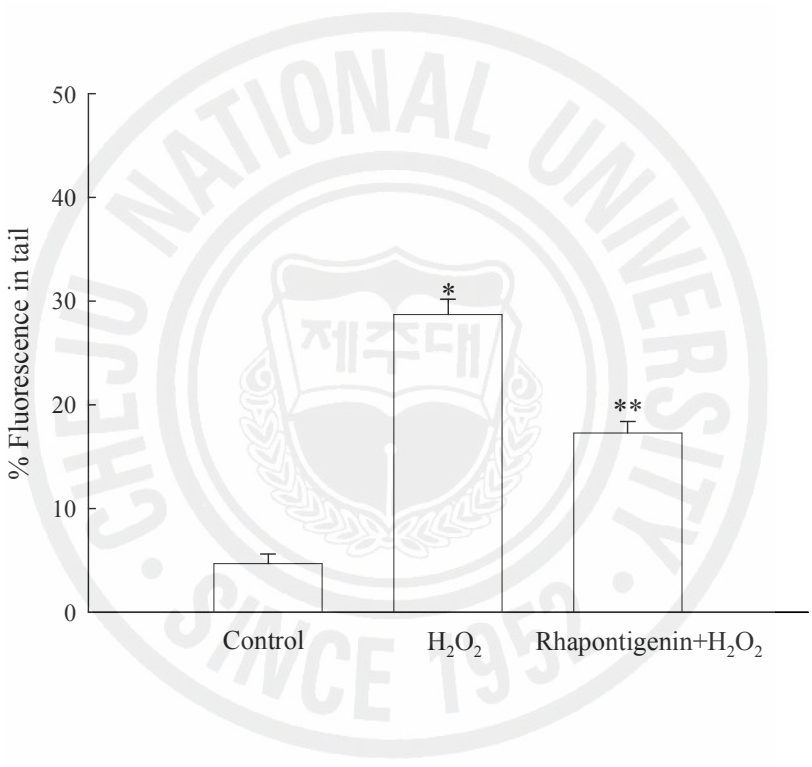


Figure 3 continued. (B) Representative images and (C) percentage of cellular DNA damage were detected by an alkaline comet assay. *Significantly different from control ($p<0.05$). **Significantly different from H_2O_2 treated cells ($p<0.05$).

A

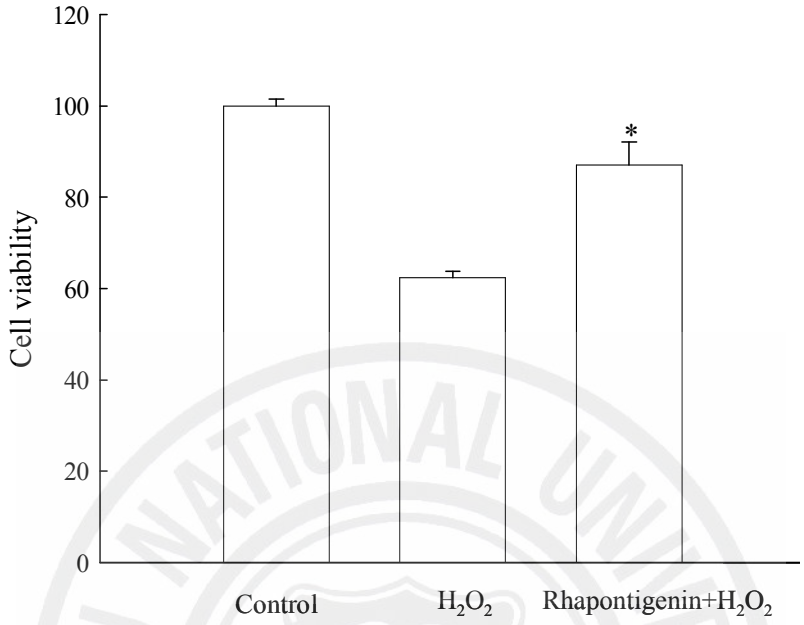
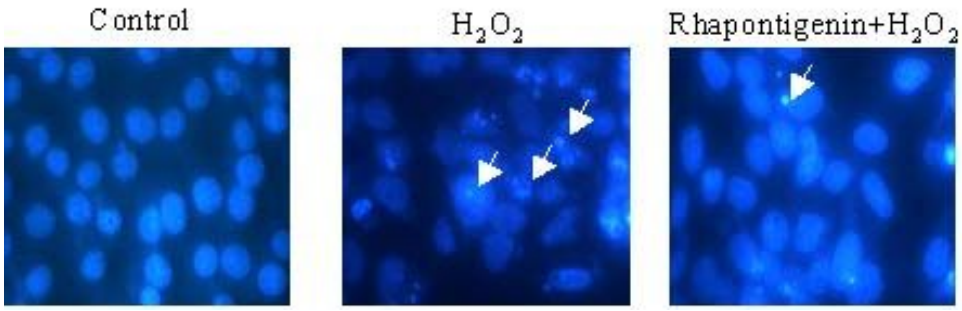


Figure 4. Protective effect of rhapontigenin on H_2O_2 induced oxidative cell damage. (A) The viability of V79-4 cells was determined by MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$).

B



C

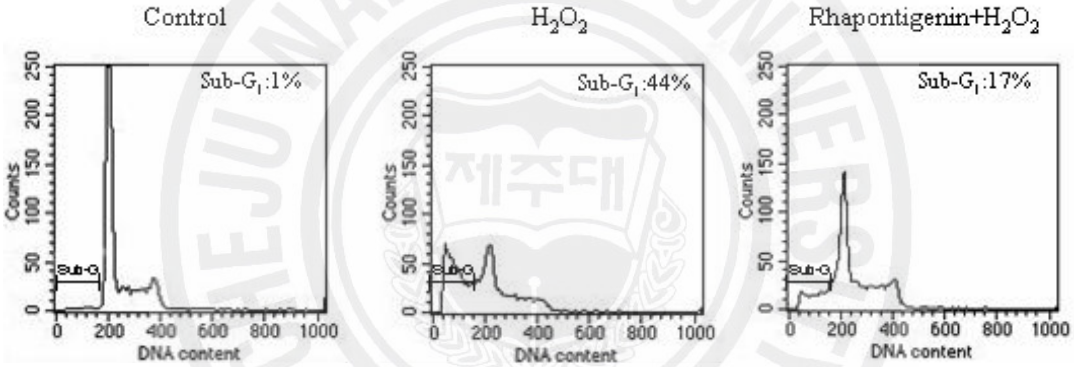


Figure 4 continued. (B) Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows. (C) Apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining.

Effect of Rhapontigenin on Serum Starvation

It was reported that serum starvation produces a marked accumulation of ROS and results in cell death (Kang et al., 2003). Hence, experiments examined whether rhapontigenin exhibits ROS scavenging effect and protective effect upon serum starvation. The ROS scavenging effect by rhapontigenin was determined after 6 hr of serum starvation. As shown in Figure 5A, serum starvation increased ROS generation by 2.5 fold compared to control, while rhapontigenin decreased to ROS generation by 56%. Cell survival was determined after 6 hr of serum starvation. As shown in Figure 5B, rhapontigenin increased cell survival rate 17% upon serum starvation. These results suggest that rhapontigenin prevented cell death caused by serum starvation.

Effect of Rhapontigenin on Catalase Activity

To investigate whether the radical scavenging activity of rhapontigenin was mediated by the activity of an antioxidant enzyme, the activity of catalase in rhapontigenin treated V79-4 cells was measured. Rhapontigenin increased the activity of catalase, showing 44±2.5 U/mg protein at 40 μM of rhapontigenin compared to 12±0.3 U/mg protein of the control (n=3/group) (Figure 6A). In addition, the protein expression of catalase by rhapontigenin at 40 μM increased in a time dependent pattern (Figure 6B). 3-Amino-1,2,4 triazol (ATZ) is known as a specific inhibitor of catalase activity (Margoliash et al., 1960). To determine the effect of catalase inhibitor on rhapontigenin induced cytoprotection from H_2O_2 induced damage, V79-4 cells were pre-treated with 20 mM of ATZ for 1 hr, followed by 1 hr of incubation with rhapontigenin and exposure to 1 mM H_2O_2 for 24 hr. As shown in Figure 6C, ATZ treatment abolished the protective capacity of rhapontigenin in H_2O_2 damaged cells.

A

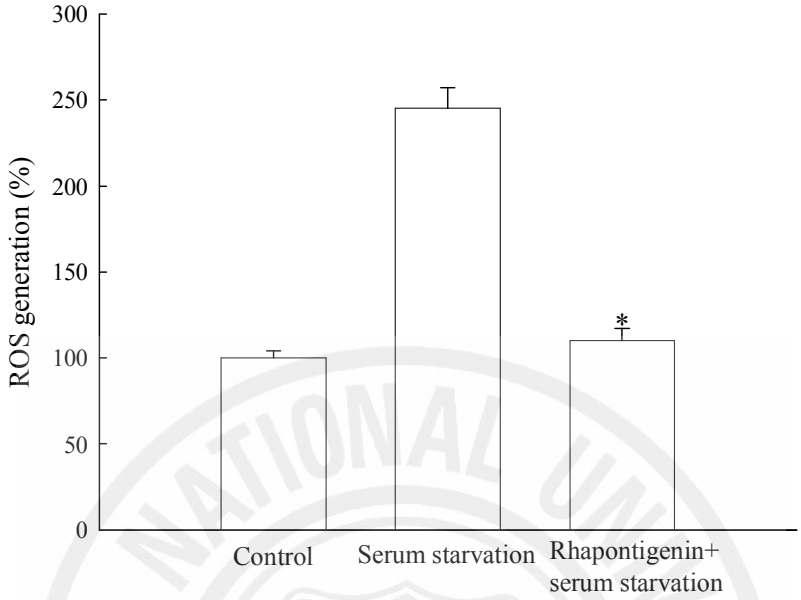


Figure 5. Protective effect of rhapontigenin on serum starvation induced oxidative cell damage. (A) The intracellular ROS generated by serum starvation was detected by DCF-DA method.

B

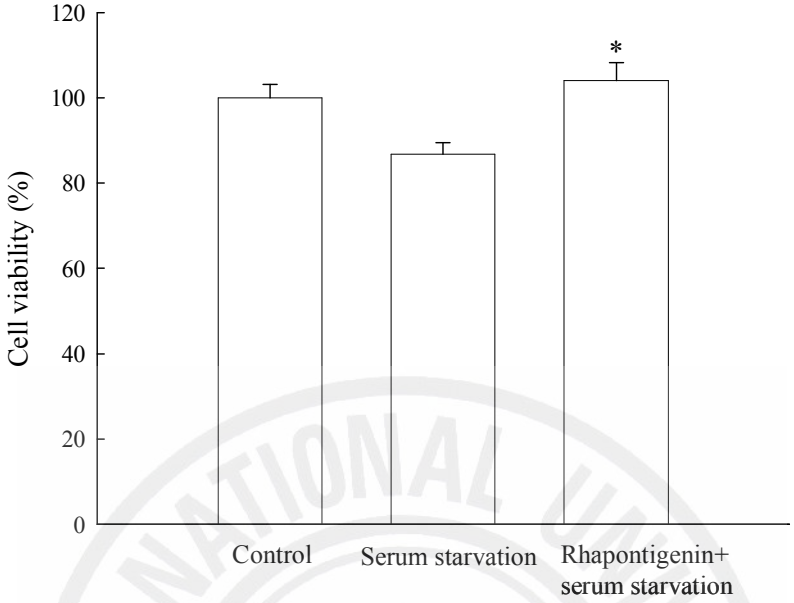


Figure 5 continued. (B) The viability of V79-4 cells upon serum starvation was determined by MTT assay. *Significantly different from serum starved cells ($p<0.05$).

A

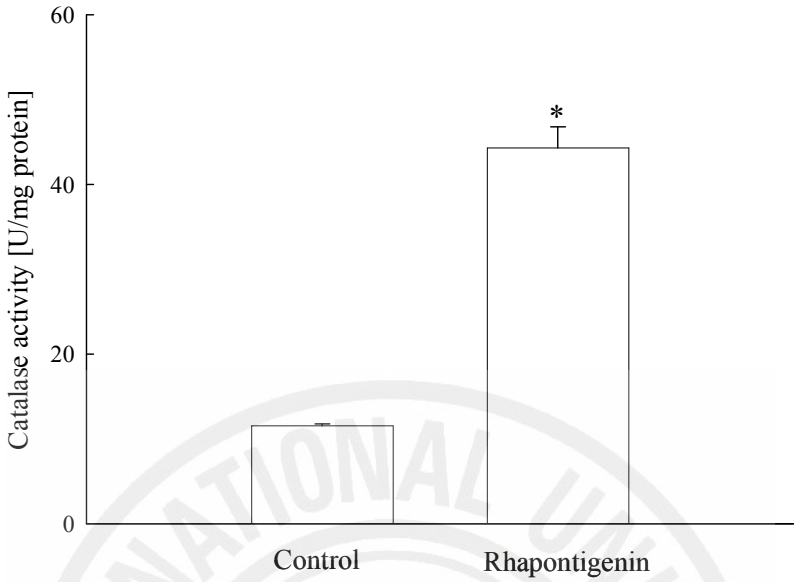
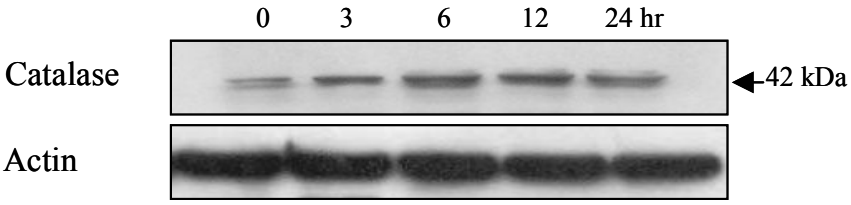


Figure 6. Effect of rhapontigenin on catalase activity. (A) The enzyme activities are expressed as average enzyme unit per mg protein ± S.E. *Significantly different from control ($p<0.05$).

B



C

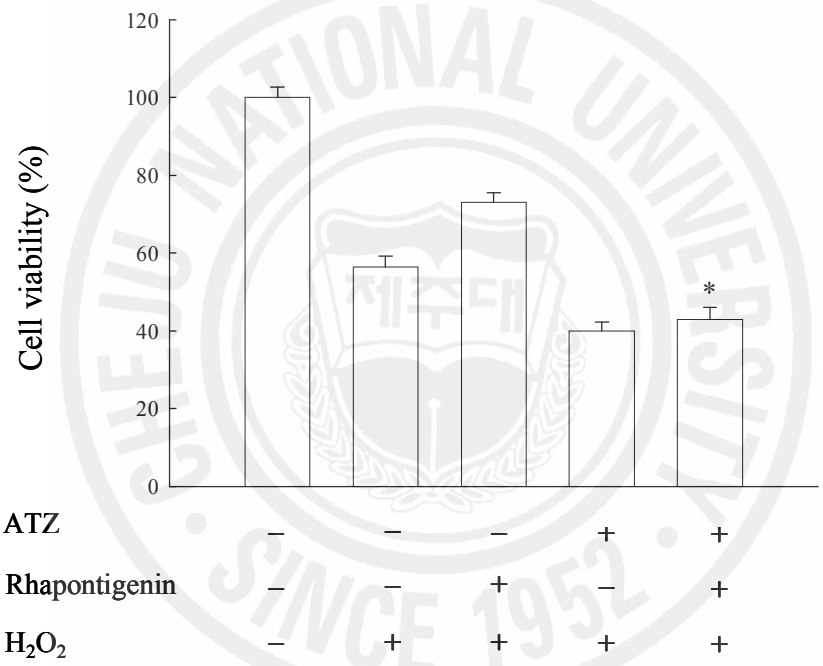
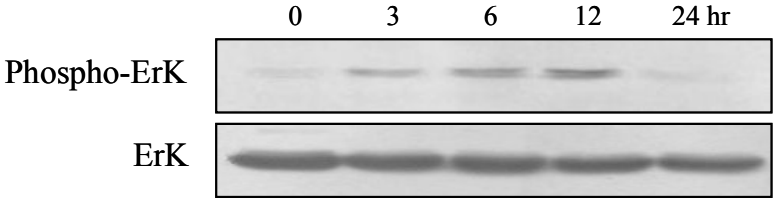


Figure 6 continued. (B) Cell lysates were electrophoresed and the protein expression of catalase was detected by a specific antibody. (C) After treatment of ATZ, rhapontigenin or/and H_2O_2, the viability of V79-4 cells was determined by MTT assay. *Significantly different from H_2O_2 treated cells with rhapontigenin ($p<0.05$).

Effect of Rhapontigenin on ERK and the AP-1 Transcription Factor

The activation of extracellular signal regulated kinase (ERK) is known to induce cell proliferation (Pages et al., 1993). To better understand the protective mechanism of rhapontigenin on V79-4 cells, the activation of the ERK protein was examined by western blot analysis with the phospho-ERK specific antibody. As shown in Figure 7A, within 12 hr rhapontigenin induced markedly phosphorylated ERK. However, there was no change in the total ERK protein level. To determine the effect of ERK inhibitor on protection of rhapontigenin from H_2O_2 induced damage, V79-4 cells were pre-treated for 30 min with 10 nM of U0126, specific inhibitor of ERK kinase, followed for 1 hr with rhapontigenin and exposed to 1 mM H_2O_2 for 24 hr. As shown in Figure 7B, U0126 treatment abolished the protection capacity of rhapontigenin in H_2O_2 damaged cells. The AP-1 transcription factor is activated by oxidative stress or inflammatory stress (Karin et al., 2001). The effect of rhapontigenin on AP-1 transcription factor was examined. Activity of AP-1 was assessed by the EMSA with a consensus AP-1 binding element. As shown in Figure 7C, rhapontigenin treated cells inhibited AP-1 activation.

A



B

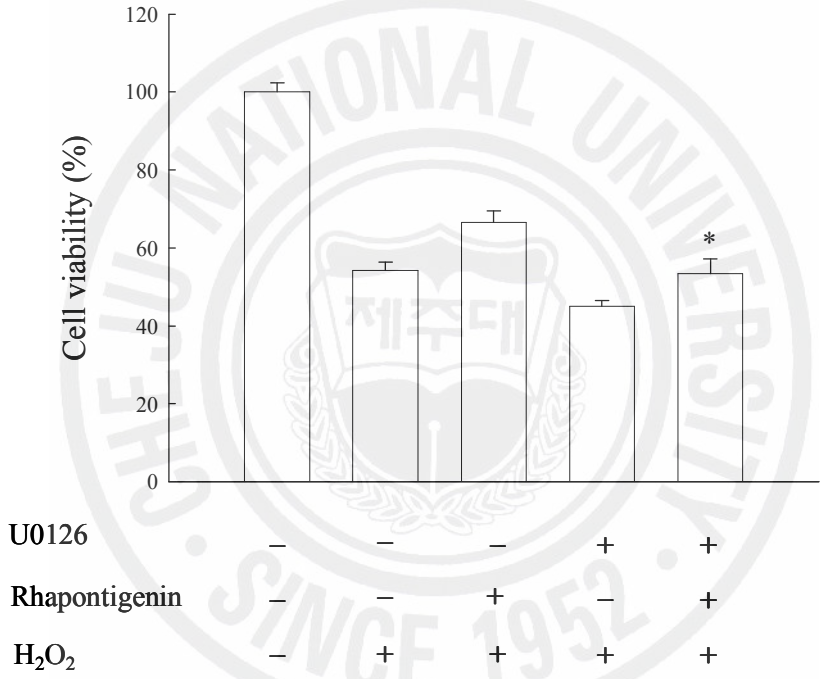


Figure 7. Effect of rhapontigenin on ERK and AP-1 transcription factor. (A) Cell lysates were electrophoresed and proteins of ERK2 and phospho-ERK1/2 were detected by their respective antibodies. (B) After treatment of U0126, rhapontigenin or/and H_2O_2, the viability of V79-4 cells was determined by MTT assay. *Significantly different from H_2O_2 treated cells with rhapontigenin ($p<0.05$).

C

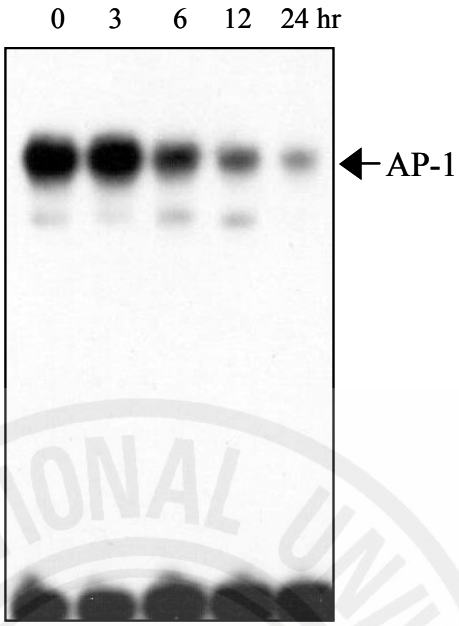


Figure 7 continued. (C) DNA binding activity of AP-1 from rhapontigenin treated cells was detected using EMSA.

Ⅳ. DISCUSSION

Rhapontigenin is a stilbene derivative, which is major component in *Rheum undulatum* (Kashiwada et al., 1984; Ko et al., 1995; Ko, 2000). Several reports suggest that rhapontigenin exhibits various pharmacological effects (Matsuda et al., 2001a and b; Kim et al., 2000, 2002; Oshino et al., 1978; Ko et al., 1999; Park et al., 2002; Oh et al., 1998; Choi et al., 2006), yet there are no reports on the antioxidant activity of rhapontigenin isolated from *Rheum undulatum* and its cytoprotection effect against oxidative stress. In our present study, rhapontigenin was shown to decrease intracellular ROS generation upon exposure to H_2O_2, the DPPH radical, and H_2O_2. The radical scavenging effect of rhapontigenin was more effective than rhaponticin (the glycoside of rhapontigenin). As most of the traditional herbal medicines are administered orally, their components inevitably come into contact with intestinal microflora in the alimentary tract. Most of the components, therefore, are converted by intestinal bacteria before absorption from the gastrointestinal tract (Akao et al., 1994; Kim et al., 1998). Indeed, rhaponticin isolated from the rhizome of *Rheum undulatum* was metabolized into rhapontigenin by human intestinal bacteria (Park et al., 2002). The transformed rhapontigenin showed more potent antithrombotic and antiallergic activity than rhaponticin (Park et al., 2002). These results suggested that rhaponticin may serve as a prodrug, which may be metabolized into the active compounds by human intestinal bacteria.

Rhapontigenin has a polyphenolic structure and polyphenols are electron-rich compounds that tend to enter into efficient electron-donation reactions with oxidizing agents to produce phenoxyl radical (PhO·) species as intermediates. Phenoxyl radicals are stabilized by resonance delocaliztion of the unpaired electron to the *ortho* and *para* positions of the ring. In addition to the resonance stability, phenoxyl radicals

may also be stabilized by hydrogen bonding with an adjacent hydroxyl group. Phenoxyl radicals also undergo dimerization ("phenol coupling") to produce new C-C or C-O linkage (Larson, 1997). This intrinsic stability of phenolic structures might be relative to antioxidative activity of rhapontigenin. The cells exposed to H_2O_2 exhibited distinct morphological features of apoptosis, such as nuclear fragmentation and an increase in sub-G_1 DNA content. However, cells that were pretreated with rhapontigenin had significantly reduced percentage of apoptotic cells, as shown by morphological changes and reduction in sub-G_1 DNA content. These observations indicate that the inhibition of ROS formation may be important for cytoprotection against oxidative damage. Catalase plays a significant role in effective augmentation of antioxidant defense mechanisms in cells. Rhapontigenin increased catalase activity and its protein expression, suggesting that the scavenging of ROS may be relative to the increased antioxidant activity. It was reported that most of the polyphenolic compounds interact with commonly used cell culture media to generate H_2O_2 (Long et al., 2000). The generated low level of H_2O_2 may trigger a rise in the levels of antioxidant enzymes. To confirm whether rhapontigenin generates H_2O_2 in the media, rhapontigenin was added to cell culture media at a final concentration of 40 μM and the amount of generated H_2O_2 was measured by the ferrous iron oxidation-xylenol orange assay. H_2O_2 was not detectable in rhapontigenin treated media (data not shown), suggesting that the antioxidant activities in rhapontigenin treated cells were not increased by H_2O_2 generated in rhapontigenin treated media. The effects of rhapontigenin on cell viability, therefore, might involve dual actions: direct action on oxygen radical scavenging, as shown by intracellular ROS, DPPH radical, and H_2O_2 scavenging; and indirect action through induction of catalase activity. Antioxidant enzymes would be potential target molecules mediating antiapoptotic function of ERK pathway against oxidative stress. In many cell types, ERK pathway is induced by a

variety of extracellular stimuli (McCrbrey et al., 2000). The phosphorylation of ERK phosphorylates cytoplasmic and nuclear targets, and participates in a wide range of cellular programs including proliferation, differentiation, and movement (Pages et al., 1993; Robinson and Cobb, 1997; Widmann et al., 1999). The level of phosphorylated ERK in rhapontigenin treated cells was elevated, and treatment of U0126, a specific inhibitor of ERK kinase, suppressed the protection capacity of rhapontigenin in H_2O_2 damaged cells, suggesting that the protective effect of rhapontigenin on cells may also be involved in activating ERK pathway. The activator protein 1 (AP-1) transcription factor is activated by oxidative stress or inflammatory stress (Karin et al., 2001) and this redox sensitive transcription factor is one of prime targets in mediating inflammatory response or carcinogenesis (Surh et al., 2005). Rhapontigenin treated cells inhibited the AP-1 activation, suggesting that rhapontigenin regulates negatively the activity of AP-1.

In conclusion, our studies demonstrated that rhapontigenin exerted ROS scavenging activity, promoted cell viability via inhibition of H_2O_2 induced apoptosis, and enhanced the effects of antioxidant enzyme and activation of ERK protein. Thus, this study provides the possibility that rhapontigenin might have a preventive effect on oxidative induced pathological condition.

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Ⅵ. ABSTRACT IN KOREAN

Rheum undulatum 에서 추출한 rhapontigenin의 항산화 활성을 통한 세포손상에 대한 보호 작용

Rheum undulatum 에서 추출한 항산화 활성 물질인 rhapontigenin 과 rhaponticin 에 대해 연구를 진행하였다. Rhapontigenin은 세포 내 ROS 소거활성, DPPH 라디칼 소거활성 및 과산화수소 소거활성을 나타내었으며 rhapontigenin은 rhaponticin보다 더 높은 라디칼 소거활성을 나타내었다. Thiobarbituric acid reaction method 와 comet assay를 통하여 rhapontigenin은 세포손상의 주요 타깃으로 되는 과산화수소에 의한 세포막의 지질과산화 및 DNA 손상으로부터 세포를 보호함을 관찰하였다. Rhapontigenin는 과산화수소에 노출된 Chinese hamster lung fibroblast (V79-4) cells의 apoptosis를 저해함을 MTT assay, Hoechst 33342 staining assay 및 flow cytometry analysis로 측정하였다. Rhapontigenin는 serum starvation에 의해 유도되는 세포손상을 저해하였으며 catalase의 활성을 증가시켰고 상응한 protein의 expression을 증가시켰다. 또한 western blot analysis로부터 rhapontigenin은 extracellular signal regulated kinase (ERK)의 phosphorylation을 증가시킴을 알 수 있었으며 EMSA의 결과로부터 rhapontigenin은 activator protein 1 (AP-1)- a redox sensitive transcription factor 의 활성을 저해하였음을 관찰하였다. 이러한 결과들로부터 rhapontigenin은 세포의 항산화활성을 증가시키고 signal pathways를 조절함으로서 V79-4 세포의 산화적 손상을 저해함을 알 수 있었다.

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