**A Doctoral Dissertation**

# Protective Effects of 7, 3', 4'-Trihydroxy-dihydroflavone against Oxidative Stress-Induced Cell Damage via Scavenging of Reactive Oxygen Species

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# 활성 산소종의 소거를 통한 산화적 스트레스로 유도되는 세포 손상에 대한 7, 3', 4'-trihydroxydihydroflavone의 보호작용

지도교수 현 진 원

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# Protective Effects of 7, 3', 4'-Trihydroxydihydroflavone against Oxidative Stress-Induced Cell Damage via Scavenging of Reactive Oxygen Species

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# BACKGROUNDS

Flavonoids, which are important constituents of human diet, are a group of naturally occurring polyphenolic compounds ubiquitously found in vegetables and fruits. Recently, to seek for natural antioxidants with superior pharmacological effects and less cytotoxic properties, much attention has been paid on the potential applications of flavonoid-based drugs in the prevention and therapy of free radical-mediated human diseases. 7, 3′, 4′-Trihydroxydihydroflavone, also termed as butin, has been isolated from several medicinal herbs such as *Dalbergia odorifera*, *Adenanthera pavanina*, and *Vernonia anthelmintica Willd*, and reported to possess biological activities such as skin-whitening and anti-implantation properties. Butin was proved to possess antioxidant activity by virtue of its free radical scavenging capacity, however, as far as we know, few studies have reported on the precise mechanisms of butin against oxidative stress-induced cell damage. Therefore, the present study is focused on the underlying mechanisms involved in butin's protective effects.

Initially, we reveal that the protective effects of butin against oxidative stress-induced cell damage might involve dual actions: direct action on oxygen radical scavenging, as shown by free radical scavenging, and indirect action through the induction of antioxidative enzymes.

Mitochondria are the major source of superoxide production and are subjected to direct attack of reactive oxygen species (ROS). Therefore we focused on the role of butin in oxidative stress-induced mitochondrial damage. Consequently, we found that butin decreased mitochondrial ROS accumulation, balanced intracellular $Ca^{2+}$ levels, and improved mitochondrial energy production, thus recovering mitochondrial function.

It is also well known that ROS plays a crucial role in triggering the mitochondria-mediated apoptosis pathway. Thus we demonstrated that the protective effect of butin against oxidative stress-induced apoptosis was exerted via blockage of membrane potential

depolarization, inhibition of the JNK and mitochondria mediated caspase-dependent apoptotic pathways.

Antioxidative enzymes provide a major mechanism by which cells combat the toxicities of ROS, and their induction is also highly effective and sufficient for protecting cells against oxidative stress. Manganese superoxide dismutase (Mn SOD), the primary antioxidant enzyme that scavenges superoxide radicals in mitochondria, has recently been emphasized for its protective functions. Transcriptional regulation of Mn SOD is predominantly mediated by a redox-sensitive transcription factor NF-E2 related factor-2 (Nrf2). In addition, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) are associated with the modulation of antioxidant responsive element (ARE)-driven gene expression via Nrf2 activation. Our study indicated that butin reduced oxidative stress-induced cell damage via an increased activation of PI3K/Akt, which appears to be responsible for nuclear translocation of Nrf2, its subsequent binding to ARE, and the up-regulation of Mn SOD expression.

Keyword: Antioxidant • Butin • Oxidative stress • Antioxidant enzyme • Apoptosis • Cytoprotective activity • Mitochondrial dysfunction • Mitochondrial reactive oxygen species • Succinate dehydrogenase • Mitochondria-dependent apoptotic pathway • Manganese superoxide dismutase • NF-E2-related factor 2 • Phosphatidylinositol 3-kinase/protein kinase B

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# PART I

# Protective Effect of Butin (7, 3', 4'-Trihydroxydihydroflavone) against Hydrogen Peroxide Induced Apoptosis by Scavenging Reactive Oxygen Species and Activating Antioxidant Enzymes

# ABSTRACT

The antioxidant property of butin was investigated for cytoprotective effect against $H_2O_2$ induced cell damage. This compound showed intracellular reactive oxygen species (ROS) scavenging, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, inhibition of lipid peroxidation, and DNA damage. This radical scavenging activity of butin protected cell damage exposed to $H_2O_2$. Also, butin reduced the apoptotic cells induced by $H_2O_2$, as demonstrated by the decreased DNA fragmentation, apoptotic body formation, and caspase 3 activity. In addition, butin restored the activity and protein expression of cellular antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) in $H_2O_2$ treated cells. Taken together, these findings suggest that butin protected cells against $H_2O_2$ induced cell damage via antioxidant property.



Keywords: Antioxidant • Butin • Oxidative stress • Antioxidant enzyme • Apoptosis • Cytoprotective activity

# 1. INTRODUCTION

Flavonoids are a group of naturally occurring polyphenolic compounds ubiquitously found in fruits and vegetables. Flavonoids are benzo-γ-pyrone derivatives that can be grouped according to the presence of different substituents on the rings and to the degree of benzo-γ-pyrone ring saturation (Di Carlo et al., 1999). The various classes of flavonoids differ in the level of oxidation of the C ring of the basic benzo-γ-pyrone structure. Common family members of flavonoids include flavones, flavanes, flavonols, catechins, and anthocyanidins. The structural difference in each flavonoid family results from the variation in the number and substitution pattern of the hydroxyl groups and the extent of glycosylation of these groups (Amic et al., 2003). Flavonoids, important constituents of the human diet, are also found in medicinal plants, herbal remedies containing flavonoids have been used in folk medicine around the world (Di Carlo et al., 1999; Jovanovic et al., 1994). Plant flavonoids are emerging as potent therapeutic drugs for free radical mediated diseases, for which cell membranes generally serve as targets for lipid peroxidation and related deleterious effects. They are known to possess powerful antioxidant properties, which are attributed to the presence of phenolic hydroxyl groups in the B ring of flavonoid structure. They usually contain one or more aromatic hydroxyl groups and this moiety is responsible for the antioxidant activity of the flavonoid (van Acker et al., 2000). Due to its intrinsic feature of radical scavenging activity, flavonoids have shown potential health benefits. Much recent attention has been focused on the potential uses of flavonoid-based drugs for the prevention and therapy of free radical mediated human diseases, such as atherosclerosis, ischemia, inflammation, neuronal degeneration, cardiovascular diseases (Amic et al., 2003; van Acker et al., 2000; Gordon and Roedig-Penman, 1998). Many studies have focused on the biological activities of flavonoids, which are potent antioxidants and free radical scavengers (Rice-Evans et al., 1995; Kahkonen et al., 1999;

Sugihara et al., 1999). Therefore, researchers are looking for natural antioxidants with strong pharmacological action and less cytotoxic properties. Butin was isolated from several medicinal herbs (Jang et al., 2003; Liu et al., 2005; Su et al., 2007; Su et al., 2004) and reported to have biological activities such as skin-whitening and anti-implantation activity (Bhargava, 1986; Lee et al., 2006). To the best of our knowledge, much of studies have not been reported. Therefore, we demonstrate for the first time the intrinsic antioxidant characteristics of the flavonoid, butin (7, 3′, 4′-trihydroxydihydroflavone) as shown in Fig. 1 to explore its antioxidant properties for cytoprotection on oxidative stress induced cell damage.

# 2. MATERIALS AND METHODS

## 2.1. Reagents

Butin was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO did not exceed 0.02%. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2', 7'-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 were purchased from Sigma (St. Louis, USA), and the thiobarbituric acid (TBA) was purchased from BDH laboratories (Dorset, UK). Anti-poly ADP-ribosyl polymerase (PARP), and anti-caspase 3 antibodies were purchased from Cell Signaling Technology (MA, USA). Primary anti-actin was purchased from Santa Cruz Biotechnology (CA, USA). Primary sheep monoclonal Cu/Zn superoxide dismutase and catalase antibodies were purchased from Biodesign International Company (Maine, USA). The other chemicals and reagents used were of analytical grade.

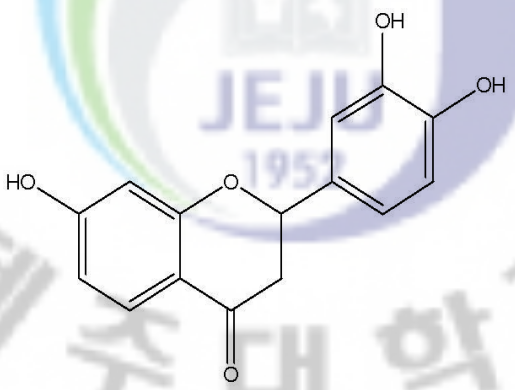



**Fig.1.** Chemical structure of butin (7, 3', 4'-trihydroxydihydroflavone).

## 2.2. Cell culture

It is reported that lung is an organ sensitive to oxidative stress (Murray et al., 2004; Pryor et al., 1998). To study the effect of butin 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% CO2 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).

2.3. Intracellular ROS measurement

To detect intracellular ROS, the DCF-DA method was used. DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2', 7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog is trapped in cells and can be oxidized to the highly fluorescent 2', 7'-dichlorofluorescein by intracellular oxidants (Rosenkranz et al., 1992). The V79-4 cells were seeded in a 96 well plate at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with various concentrations of butin and 30 min later 1 mM $H_2O_2$ was added to the plate. Cells were incubated for an additional 30 min at 37 °C. The fluorescence of 2', 7'-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission, using a PerkinElmer LS-5B spectrofluorometer. The intracellular ROS scavenging activity (%) was calculated as [(optical density of $H_2O_2$)-(optical density of $H_2O_2$ with butin treatment)]/(optical density of $H_2O_2$) × 100.

2.4. DPPH radical scavenging activity

Various concentrations of butin were added to a $1 \times 10^{-4}$ M solution of DPPH in methanol, and the reaction mixture was shaken vigorously. After 5 h, the amount of DPPH remaining was determined at 520 nm (Lo et al., 2004). The DPPH radical scavenging activity (%) was calculated as [(optical density of DPPH radical)-(optical density of DPPH radical with butin treatment)]/(optical density of DPPH radical) × 100.

2.5. Lipid peroxidation detection

Lipid peroxidation was assayed by the measurement of related substances that reacts with thiobarbituric acid (TBARS) (Ohkawa et al., 1979). The V79-4 cells were seeded in a culture dish at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with butin at 10 μg/ml. After 1 h, 1 mM $H_2O_2$ was added to the plate, which was incubated more for a further 1 h. The cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. About 100 μl of cell lysates was combined 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 adjusted 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 a mixture of n-butanol and pyridine (15:1, v/v) was added to each sample, and the mixture shaken vigorously. After centrifugation at 1,000 × g for 10 min, the supernatant fraction was isolated, and the absorbance measured at 532 nm.

2.6. Comet assay

A Comet assay was performed to assess oxidative DNA damage (Rajagopalan et al., 2003; Singh, 2000). The cell pellet ($1.5 \times 10^5$ cells) was mixed with 100 μl of 0.5% low melting agarose (LMA) at 39 °C 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 lysis solution (2.5 M NaCl, 100 mM Na–EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 h at 4 °C. The slides were then placed in a gelelectrophoresis 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 applied (300 mA, 25 V) for 20 min at 4 °C to draw negatively charged DNA toward an anode. After electrophoresis, the slides were washed three times for 5 min at 4 °C in a neutralizing buffer (0.4 M Tris, pH 7.5) and then stained with 75 μl

of ethidium bromide (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.

2.7. SOD activity

The V79-4 cells were seeded in a culture dish at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with butin at 10 μg/ml. After 1 h, 1 mM $H_2O_2$ was added to the plate, which was incubated more for a further 1 h. The cells were then washed with cold PBS, and scraped. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonicating twice for 15 sec. Triton X-100 (1%) was then added to the lysates and incubated for 10 min on ice. The lysates were clarified, by centrifugation at 5,000 × g for 10 min at 4 °C, to remove cellular debris. The protein content of the supernatant was determined by the Bradford method, using bovine serum albumin as the standard. The total SOD activity was used to detect the level of epinephrine auto-oxidation inhibition (Misra and Fridovich, 1972). Fifty microgram of protein was added to 500 mM phosphate buffer (pH 10.2) and 1 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, a pink colored product, which was assayed at 480 nm using a UV/VIS spectrophotometer in the kinetic mode. SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm and the amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity. The total SOD activity was expressed as units/mg protein.

2.8. CAT activity

Fifty microgram of protein was added to 50 mM phosphate buffer (pH 7.0) and 100 mM $H_2O_2$ and then this mixture was incubated for 2 min at 37 °C and the absorbance of the mixture

were monitored for 5 min at 240 nm. The change in absorbance is proportional to the breakdown of $H_2O_2$ (Carrillo et al., 1991). The CAT activity was expressed as units/mg protein.

2.9. Cell viability

The effect of butin on the viability of V79-4 cells was determined by the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells (Carmichael et al., 1987). The V79-4 cells were seeded in a 96 well plate at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with butin at 10 μg/ml, and 1 h later 1 mM $H_2O_2$ was added to the plate and incubated for an additional 24 h at 37 °C. Fifty microliter of MTT stock solution (2 mg/ml) was then added to each well of a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min and the supernatants aspirated. The formazan crystals in each well were dissolved in 150 μl DMSO and the A540 read on a scanning multi-well spectrophotometer. To determine the effect of catalase inhibitor, 3-amino-1, 2, 4-triazole (ATZ) on the cell viability, cells were pre-treated with final 20 mM of ATZ for 1 h, followed by 1 h of incubation with butin and exposure to 1 mM $H_2O_2$ for 24 h and the cell viability was measured using MTT test.

2.10. Nuclear staining with Hoechst 33342

The V79-4 cells were placed in a 24 well plate at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with butin 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. Then stained cells were then observed under a fluorescent microscope, equipped with a CoolSNAP-Pro color digital camera, to examine the degree of nuclear condensation.

2.11. DNA fragmentation

Cellular DNA-fragmentation was assessed by analysis of the cytoplasmic histone-associated DNA fragmentation using a kit from Roche Diagnostics according to the manufacturer's instructions.

2.12. Western blot

The V79-4 cells were placed in a plate at $1 \times 10^5$ cells/ml. Sixteen hours after plating, the cells were treated with 10 μg/ml of butin. 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, CA, USA), which were then incubated with primary antibody. The membranes were further incubated with secondary immunoglobulin G-horseradish peroxidase conjugates (Pierce, IL, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Buckinghamshire, UK).

2.13. Statistical analysis

Results are represented as the mean ± standard error (SE). 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.

# 3. RESULTS

## 3.1. Radical scavenging activity of butin

The radical scavenging effect of butin on the intracellular ROS in V79-4 cells and DPPH radical were measured. The intracellular ROS scavenging activity of butin was 19% at 0.1 μg/ml, 46% at 1 μg/ml, and 62% at 10 μg/ml (Fig. 2A). This compound at 10 μg/ml effectively inhibited the intracellular ROS effectively compared to 86% of N-acetylcystein used as a positive control (data not shown). This ROS scavenging activity of butin is consistent with its DPPH radical scavenging activity (Fig. 2B), and showed dose dependence; 9% in 0.1 μg/ml, 32% in 1 μg/ml, and 59% in 10 μg/ml. Taken together, these results suggest that butin has antioxidant effect.

A

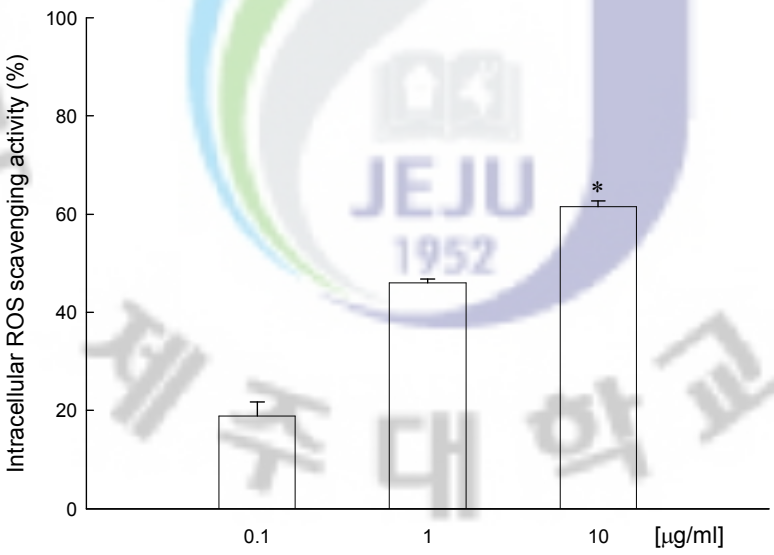



**Fig.2.** Effect of butin on scavenging intracellular ROS in V79-4 cells and DPPH radical. (A) The intracellular ROS was detected by DCF-DA method. The measurements were made in triplicate and values are expressed as the mean ± SE. *Significantly different from control ($p<0.05$).

B

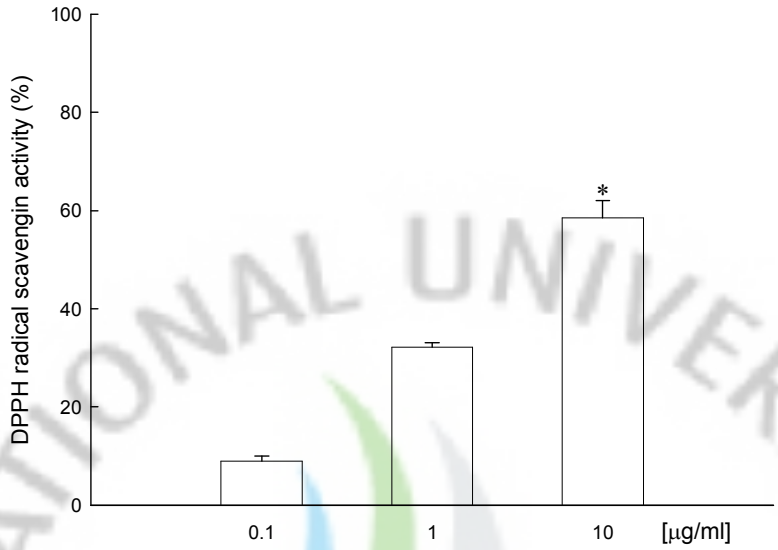



**Fig.2. continued.** (B) The amount of DPPH radicals was determined spectrophotometrically at 520 nm. The measurements were made in triplicate and values are expressed as the mean ± SE. *Significantly different from control ($p<0.05$).

3.2. Effect of butin on lipid peroxidation and cellular DNA damage induced by $H_2O_2$

The abilities of butin to inhibit membrane lipid peroxidation and cellular DNA damage in $H_2O_2$ treated cells were investigated. $H_2O_2$ induced damage to cell membrane, one of the most important lesions, is responsible for the loss of cell viability. The peroxidation of membrane lipids is the major lesion in the membranes. As shown in Fig. 3A, V79-4 cells exposed to $H_2O_2$ showed an increase in the lipid peroxidation, which was monitored by the generation of TBARS. However, butin prevented the $H_2O_2$-induced peroxidation of lipids compared to $H_2O_2$ treated cells. Damage to cellular DNA induced by $H_2O_2$ exposure was detected by using an alkaline comet. The exposure of cells to $H_2O_2$ increased the comet parameters of tail length and percentage of DNA in the tails of the cells. When the cells were exposed to $H_2O_2$, the percentage of DNA in the tail was increased 55% as shown in Figs. 3B and C. Treatment with butin decreased the comet tail length and resulted in a decrease in percentage of DNA in the tail to 33%, indicating a protective effect of butin on $H_2O_2$-induced DNA damage.

A

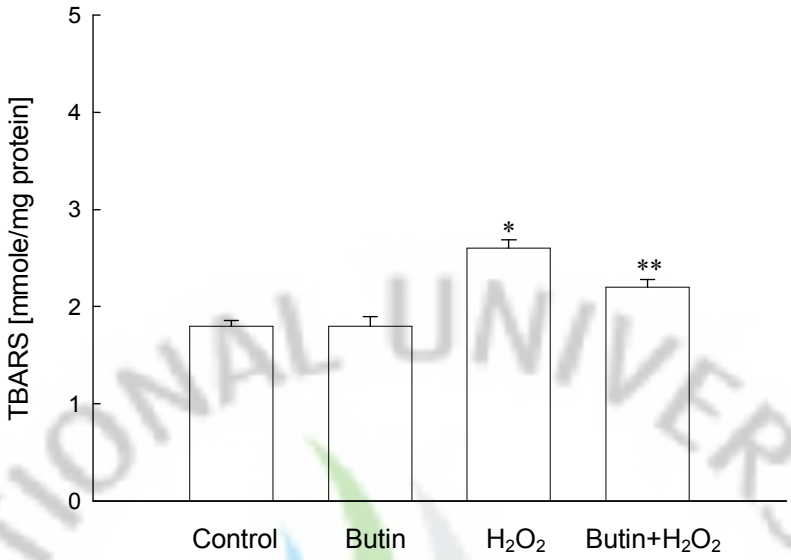
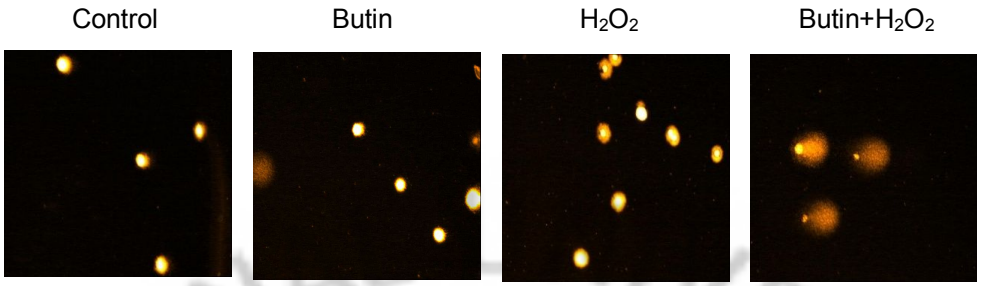



**Fig.3.** Effect of butin on inhibition of lipid peroxidation and cellular DNA damage induced by $H_2O_2$ in V79-4 cells (A) Lipid peroxidation was assayed by measuring the amount of TBARS. *Significantly different from control ($p<0.05$). **Significantly different from $H_2O_2$ treatment ($p<0.05$).

B

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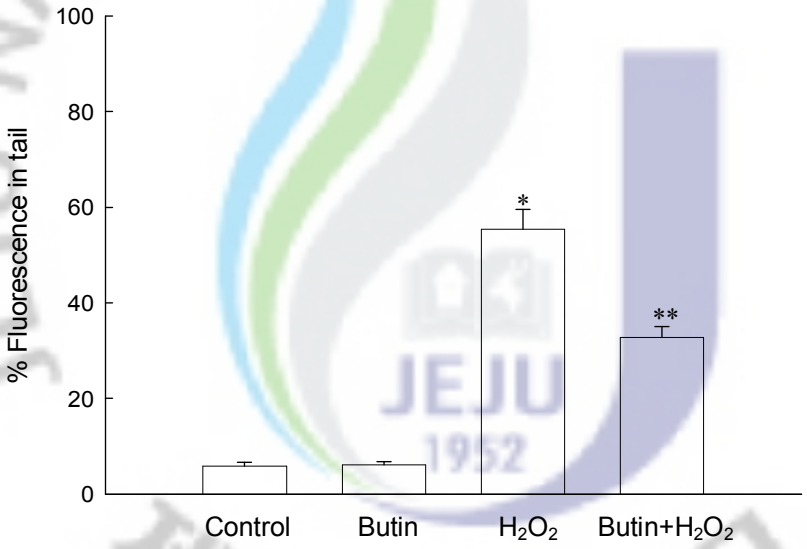
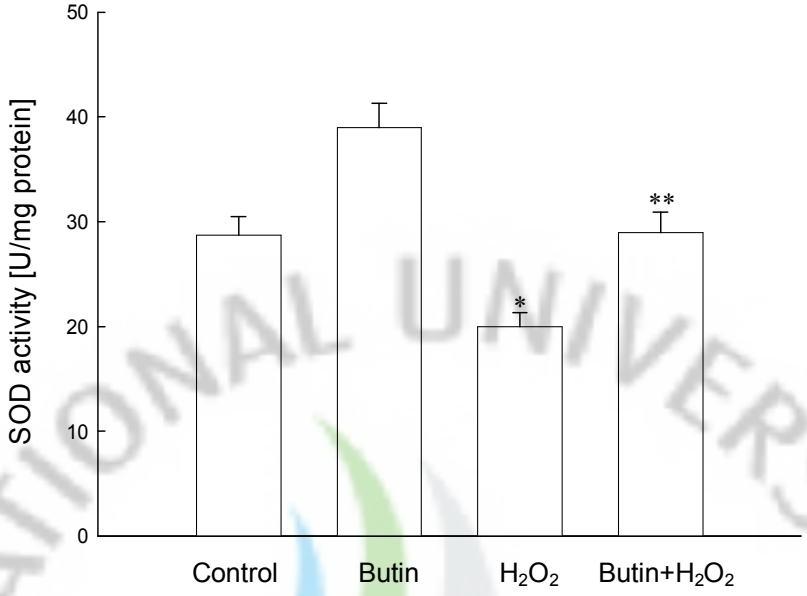



**Fig.3. continued.** (B) Representative images and (C) percentage of cellular DNA damage were detected by an alkaline comet assay. *Significantly different from control ($p<0.05$). **Significantly different from by $H_2O_2$ treatment ($p<0.05$).

3.3. Effect of butin on SOD and CAT

To investigate whether the radical scavenging activity of butin was mediated by the activity of an antioxidant enzyme, the activity of SOD in butin treated V79-4 cells was measured. Butin increased the activity of SOD, showing 39 U/mg protein at 10 μg/ml of butin compared to 29 U/mg protein of the control (Fig. 4A). The exposure of cells to $H_2O_2$ decreased the SOD activity to 20 U/mg protein, however, treatment with butin resulted in an increase to 29 U/mg protein (Fig. 4A). In the case of CAT activity, butin showed same pattern of SOD. The activity of CAT was increased by butin, showing 51 U/mg protein at 10 μg/ml of butin compared to 30 U/mg protein of the control (Fig. 4B). $H_2O_2$ treated V79-4 cells resulted in a decrease of CAT activity to 17 U/mg protein, however, treatment with butin resulted in an increase to 35 U/mg protein (Fig. 4B). To confirm the activation of SOD and CAT by butin in terms of protein expression, the western blot analysis was performed. As shown in Fig. 4C, the protein expressions of Cu/Zn SOD (16 kDa) and CAT (42 kDa) by butin were found to increase. $H_2O_2$ treated V79-4 cells resulted in decreased protein expressions of Cu/Zn SOD and CAT, however, treatment with butin resulted in increased protein expressions of Cu/Zn SOD and CAT. The protein levels of the enzymes (Fig. 4C) were consistent with the enzyme activity (Fig. 4D). The results show that the enhancement in antioxidant enzyme activities by butin may be associated with the inhibition of the production of ROS. 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 butin induced cytoprotection from $H_2O_2$ induced damage, V79-4 cells were pretreated with 20 mM of ATZ for 1 h, followed by 1 h of incubation with butin and exposure to 1 mM $H_2O_2$ for 24 h. As shown in Fig. 4D, ATZ treatment abolished the protective capacity of butin in $H_2O_2$ damaged cells.

A

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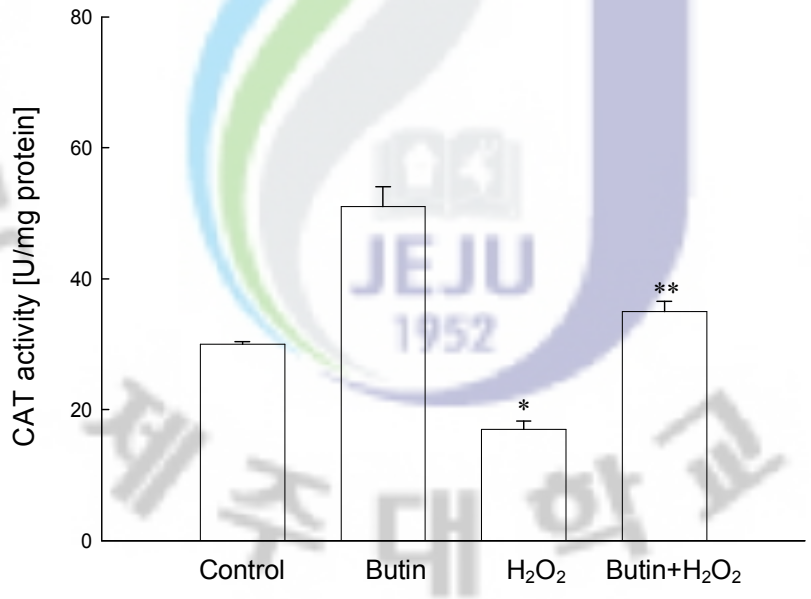
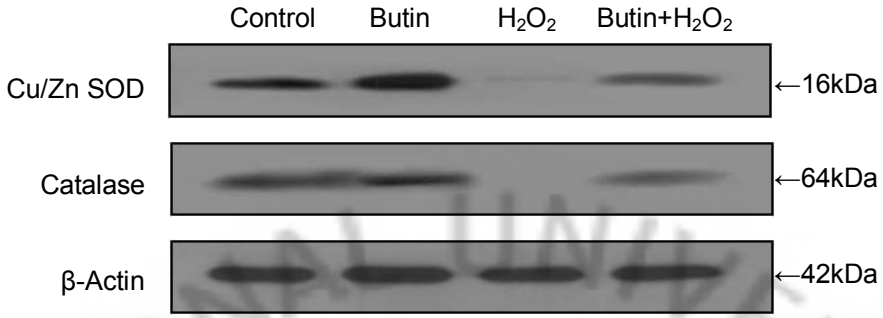



**Fig.4**. Effects of butin on SOD and CAT activity in V79-4 cells. (A and B) The enzyme activities are expressed as average enzyme unit per mg protein ± SE. *Significantly different from control ($p<0.05$). **Significantly different from by $H_2O_2$ treatment ($p<0.05$).

C




D

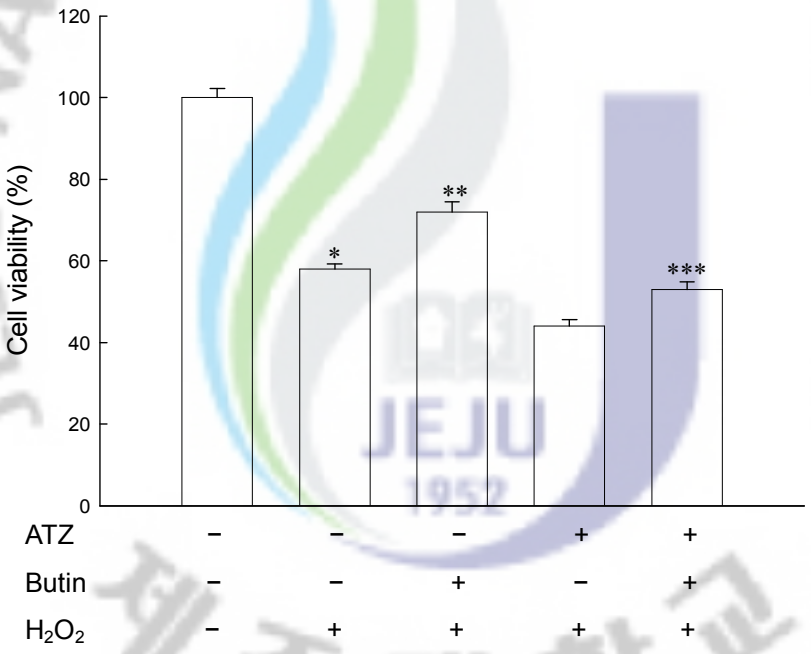
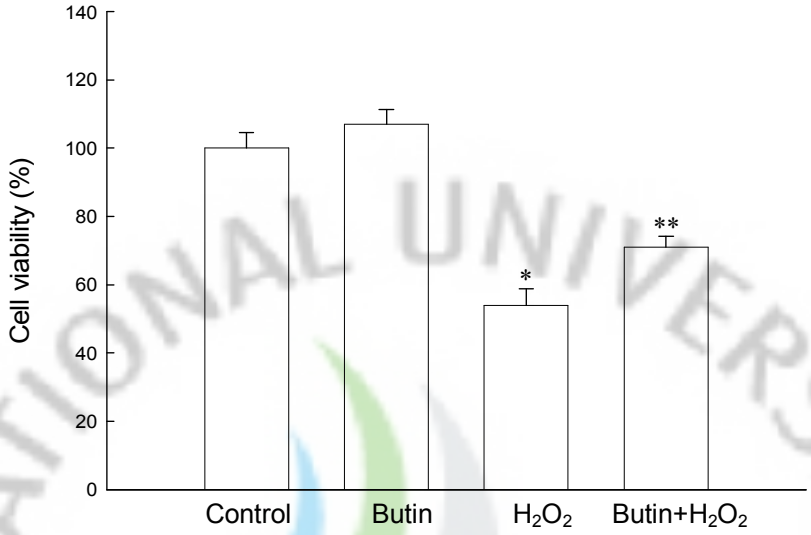



**Fig.4. continued.** (C) Western blot analysis was performed using anti-Cu/Zn SOD and catalase antibodies. (D) After treatment of ATZ, butin or/and $H_2O_2$, the viability of V79-4 cells was determined by MTT assay. *Significantly different from control ($p<0.05$). **Significantly different from by $H_2O_2$ treatment ($p<0.05$). ***Significantly different from $H_2O_2$ treated cells with butin ($p<0.05$).

3.4. Protective effect of butin on cell damage induced by $H_2O_2$

The protective effect of butin on cell survival in $H_2O_2$ treated V79-4 cells was also assessed. Cells were treated with butin at 10 μg/ml for 1 h, prior to the addition of $H_2O_2$. The cell viability was determined 24 h later by MTT assay. As shown in Fig. 5A, butin treated V79-4 cells enhanced cell survival rate of 107% compared to 100% of control, indicating butin at 10 μg/ml did not show cytotoxicity to V79-4 cells. Combination of butin at 10 μg/ml and $H_2O_2$ increased 71% in the cell survival rate compared to 54% in $H_2O_2$ treated cells. To study the cytoprotective effect of butin on apoptosis induced by $H_2O_2$, nuclei of V79-4 cells were stained with Hoechst 33342 for microscopic examination. The microscopic pictures in Fig. 5B 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 butin for 1 h prior to $H_2O_2$ treatment, a dramatic decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of butin against apoptosis was also confirmed by ELISA based quantification of cytoplasmic histone associated DNA fragmentation. Treatment of cells with $H_2O_2$ increased the levels of cytoplasmic histone-associated DNA fragmentations compared to control group, however, treatment with 10 μg/ml of butin decreased the level of DNA fragmentation (Fig. 5C). Next, we examined the caspase 3 activity by western blot since it is known as the major effecter caspase of the apoptotic process. Butin inhibited the $H_2O_2$ induced active form of caspase 3 (17 kDa), which is further demonstrated by the cleavage of poly ADP-ribosyl polymerase (PARP) (89 kDa). These results suggest that butin protects cell death by inhibiting apoptosis induced by $H_2O_2$ treatment.

A

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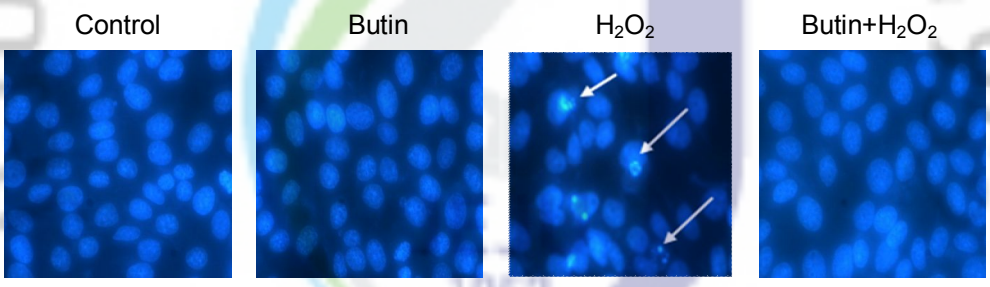
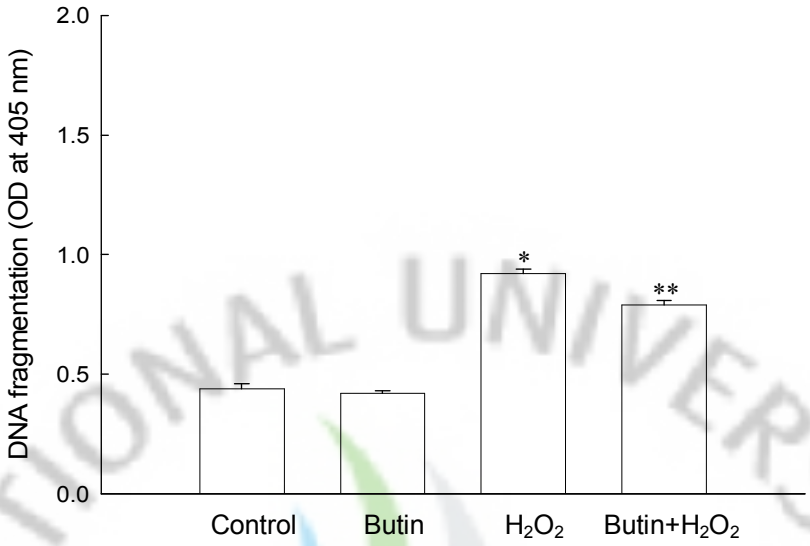



**Fig.5**. Protective effect of butin on $H_2O_2$ induced cell damage. (A) The viability of V79-4 cells on $H_2O_2$ treatment was determined by MTT assay. *Significantly different from control ($p<0.05$). **Significantly different from by $H_2O_2$ treatment ($p<0.05$). (B) Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining Apoptotic bodies are indicated by arrows.

C

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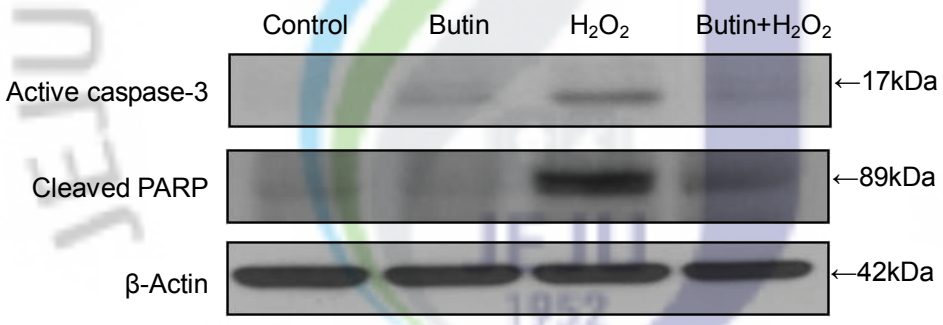



**Fig.5. continued.** (C) DNA fragmentation was quantified by ELISA kit. The measurements were made in triplicate and values are expressed as the mean ± SE. *Significantly different from control ($p<0.05$). **Significantly different from by $H_2O_2$ treatment ($p<0.05$). (D) Western blot analysis was performed using anti-caspase 3 and PARP antibodies.

# 4. Discussion

Natural flavonoids are emerging as potent therapeutic drugs for free radical mediated diseases. For example, isoliquiritigenin, baicalein, naringin, and catechin showed neuroprotection through inhibition of ROS, suggesting pharmacologic interest agent for the treatment of Parkinson's disease (Guo et al., 2007; Lee et al., 2005; Singh and Chopra, 2004; Zhan and Yang, 2006). Therefore, researchers have made numerous efforts to find antioxidants. Although many studies have reported that antioxidant activity of flavonoids, there is no report on the antioxidant activity of flavonoid, butin. Therefore, in this study we evaluated antioxidant properties and cytoprotective effect of butin against oxidative stress. Butin increased the intracellular ROS and DPPH radical scavenging activities and then enhanced the viability of V79-4 cells exposed to $H_2O_2$. The cells exposed to $H_2O_2$ exhibited distinct morphological features of apoptosis, such as nuclear fragmentation and an increase in the percentage of DNA fragmentation. However, cells that were pretreated with butin had significantly reduced the apoptotic phenomenon (Figs. 3B, 3C, 5B, and 5C). It is well known that the generation of reactive oxygen species plays an important role in mitochondrial apoptosis occurrence through the disruption of redox homeostasis (Brunelle and Chandel, 2002). In general, the radical scavenging activity of flavonoids depends on the molecular structure and the substitution pattern of hydroxyl groups, on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxyl radicals via hydrogen bonding or by expanded electron delocalization (Amic et al., 2003). The structural requirement considered to be essential for effective radical scavenging criteria is the presence of a 3′, 4′-orthodihydroxy group (catechol structure) in the B ring, possessing electron donating properties and being a radical target. Also, the 3-OH moiety of the C ring is also beneficial for the antioxidant activity of flavonoids. The C2-C3 double bond conjugated with a 4-keto group,

which is responsible for electron delocalization from the B ring, enhances further the radical-scavenging capacity and saturation of the 2, 3-double bond is believed to cause a loss of activity potential (Rice-Evans et al., 1996). Also, the presence of both 3-OH and 5-OH groups in combination with a 4-carbonyl function and C2-C3 double bond increases the radical scavenging activity.

For summary, the required structural criteria for high antioxidant activity of the flavonoids included the 3′, 4′-orthodihydroxy group (catechol structure) in the B-ring or in the A-ring, the 3-hydroxyl group or the 3-galloyl group (catechol structure) in the C-ring, and the 2, 3-double bond in conjugation with 4-oxo function (carbonyl group) in the C-ring. There was a report comparing the structural criteria with radical scavenging activity among flavonoids, resulting in decreasing order of flavanols (flavan-3-ols), flavonols, flavones, and flavanones (Cai et al., 2006). Flavanols contain more hydroxyl groups (five to eight OH groups), especially with 3′, 4′-orthodihydroxy group in the B-ring and 3-hydroxyl group and/or 3-galloyl group in the C-ring, as compared to flavonols (one to six OH groups) and other flavonoids (one to four OH). (-)-Epigallocatechin gallate was found to be the strongest radical scavenger among all of the tested flavonoids, because of its structural advantage possessing eight OH groups and 3′, 4′-orthodihydroxy group in the B-ring and 3-galloyl group in the C-ring (Cai et al., 2006). Flavonols display three structural requirements by having 3-hydroxyl group in the C-ring and 3′, 4′-orthodihydroxy group (catechol structure) in the B-ring, as well as possessing the 2, 3-double bond conjugated with 4-oxo function in the C-ring. Some individual compounds within classes of the flavonols did not completely follow the order, for example quercetin (3, 5, 7, 3′, 4′-pentahydroxyflavone), one of the most widely distributed flavonoids in plants, was more active than flavan-3-ols (Cai et al., 2006; Salter et al., 2004). Flavones possess the basic structure of the flavonoids. Some flavones showed moderate antiradical activity, such as baicalein, luteolin, and baicalin, but other flavones exhibited far lower activity, such as

apigenin and chrysin which were almost inactive (Cai et al., 2006). Flavanone has a 4-oxo group without 2, 3-double bond in the C-ring. As compared with the flavan-3-ols and flavonols with very higher scavenging activity, most of the the flavanones lack the most important structural groups for scavenging radicals (especially ortho-dihydroxy group) in the B-ring, which leads to the much lower activity of the flavanones (Rice-Evans et al., 1996). Regardless of the criteria for determining for antioxidant activity, somewhat controversial are the reports on the comparison of a range of flavanone and flavone in their antioxidant capacity, suggesting that the 2, 3-double bond is deemed less important because flavanone is more effective than its unsaturated flavones (Rice-Evans et al., 1996).

Butin, one of flavanones, which has three hydroxyl groups in the A- and B-rings but lack the 2, 3-double bond in the C-ring, showed antioxidant properties. According to our results and previous reports regarding structure and antioxidant activity relationship of flavan-3-ols, flavonols, and flavones, antioxidant activity of butin might be related with this kind of structure which support electron delocalization between the A- and B-rings and stabilize the aryloxyl radicals after hydrogen donation. This indicated that the presence of the 2, 3-double bond in combination with the 4-oxo function (carbonyl group) in the C-ring could be beneficial, not essential, role in radical delocalization from B- to C-rings, and strengthens the activity of the flavone and flavonol. Thus, the effectiveness of antioxidants is dependent on the ortho-dihydroxy groups and is the most important structural feature for the high activity. Also, previous structure-activity relationship studies of 29 flavonoid derivatives corresponds to the reports and have pointed to the importance of the number and location of the phenolic OH groups present for the antiradical efficacy, indicating that highly active flavonoids possess a 3′, 4′-orthodihydroxy group (catechol structure) in the B-ring and/or 3-OH group (Amic et al., 2003). Other approaches for determinants of radical scavenging potential of 5, 7-OH in the A-ring showed it has little influence on antioxidant efficacy (Cos et al., 2001).

Our results on the antioxidant activity of butin are partially consistent with these criteria as mentioned above. Butin, one of the flavanone types, which seems to have weak antioxidant activity in terms of chemical structure itself, however butin increased antioxidant activity as shown by intracellular ROS, DPPH radical scavenging activities, and lipid peroxidation inhibition, further promoted cell viability on $H_2O_2$-induced apoptosis, and enhanced the effects of antioxidant enzymes. As seen from the results, it can be stated that the protective effect of butin does not depend simply on the direct radical quenching due to catechol structure in the B-ring but also indirect induction of antioxidant enzymes such as SOD and CAT. Antioxidant activity of butin might be expected to promote a state of well-being and reduce the risk of ROS related diseases, however our results are initial step for the development of raw materials of functional food and medicine. Therefore, stepwise process of fundamental and challenging research for the target of specific disease, molecular events relevant signaling pathways, in vivo study, bioavailability, and metabolism are remained to guarantee the success of this goal.

In summary, many biochemical and clinical studies suggest that natural and synthetic antioxidant compounds help treat diseases medicated by oxidative stress (Lee et al., 2002). The results presented in this report indicate that butin efficiently attenuated oxidative stress induced cell damage through antioxidant properties.

# PART II

## Butin (7, 3′, 4′-Trihydroxydihydroflavone) Reduces Oxidative Stress-Induced Mitochondrial Dysfunction via Scavenging of Reactive Oxygen Species

# ABSTRACT

This study investigated the cytoprotective effect of butin, a flavonoid, on hydrogen peroxide ($H_2O_2$)-induced mitochondrial dysfunction. Electron spin resonance (ESR) spectrometry revealed butin's significant scavenging effects on superoxide radicals and hydroxyl radicals. When $H_2O_2$ was used to induce an increase in mitochondrial reactive oxygen species (ROS) in Chinese hamster lung fibroblast (V79-4) cells, butin treatment decreased high level of ROS. Butin also attenuated intracellular $Ca^{2+}$ levels that have been induced by $H_2O_2$. Furthermore, butin recovered ATP levels and succinate dehydrogenase activity that had been decreased by $H_2O_2$ treatment. We conclude these results suggest butin decreased mitochondrial ROS accumulation, balanced intracellular $Ca^{2+}$ levels, and improved mitochondrial energy production, thus recovering mitochondrial function.



Keywords: Butin • Mitochondrial dysfunction • Mitochondrial reactive oxygen species • Succinate dehydrogenase

# 1. INTRODUCTION

The primary mitochondrial ROS is superoxide radical ($O_2^{\cdot-}$), which is converted to $H_2O_2$, either by spontaneous dismutation or by the enzyme superoxide dismutase (SOD). $H_2O_2$ can be further transformed to hydroxyl ($OH\cdot$) in the presence of metal ions by the Fenton reaction, although metal chaperone proteins in the mitochondrial matrix (Craig and Marszalek, 2002) likely prevent this from occurring in the organelle.

High concentrations of ROS can alter the balance of endogenous protective systems and attack mitochondria. Calcium ions and other apoptosis-related factors may be released from damaged mitochondria into the cytosol following dysfunction of the mitochondrial membrane that regulates cell apoptosis (Richter, 1993). Mitochondrial dysfunction has been correlated with diabetes mellitus (Lowell and Shulman, 2005), nonalcoholic fatty liver disease (Wei et al., 2008), and neurodegenerative diseases like Alzheimer's disease (Castellani et al., 2002; Mattson et al., 2008).

Flavonoids, which are important constituents of human diet, are a group of naturally occurring polyphenolic compounds ubiquitously found in vegetables and fruits. Flavonoids are also found in medicinal plants; herbal remedies containing flavonoids have been used worldwide in folk medicines (Di Carlo et al., 1999; Jovanovic et al., 1994). Recently, much attention has been focused on the potential uses of flavonoid-based drugs for the prevention and therapy of free radical-mediated human diseases, such as atherosclerosis, ischemia, inflammation, neuronal degeneration, and cardiovascular diseases (Amic et al., 2003; Crespo et al., 2008; Gordon and Roedig-Penman, 1998; van Acker et al., 2000). In view of flavonoids' antioxidant and free radical scavenging properties, their biological activities have been studied intensively (Kahkonen et al., 1999; Montoro et al., 2005; Saric et al., 2009; Sugihara et al., 1999). Researchers are seeking for natural antioxidants with superior pharmacological effects

and less cytotoxic properties. Butin (7, 3′, 4′-trihydroxydihydroflavone, Fig. 1) has been isolated from several medicinal herbs (Liu et al., 2005; Su et al., 2007; Tian et al., 2004) and reported to possess biological properties such as skin-whitening and anti-implantation activity (Lee et al., 2006). Recently, we demonstrated that butin provided protective effects against $H_2O_2$-induced apoptosis by scavenging ROS and activating antioxidant enzymes (Zhang et al., 2008), and protected against oxidative DNA damage via activation of PI3K/Akt/OGG1 pathway (Kang et al., 2009). However, as far as we know, few studies have reported on butin's protective properties against oxidative stress-induced mitochondrial dysfunction, therefore, the present study is focused on this effect.

# 2. MATERIALS AND METHODS

## 2.1. Reagents

Butin was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO did not exceed 0.02%. The 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from the Sigma Chemical Company (St. Louis, MO). Dihydrorhodamin 123 (DHR 123) and fluo-4-acetoxymethyl (AM) ester were purchased from Molecular Probes (Eugene, OR). The other chemicals and reagents used were of analytical grade.

## 2.2. Cell culture

Chinese hamster lung fibroblasts (V79-4) cells from the American type culture collection (Rockville, MD) 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).

## 2.3. Detection of superoxide radical

Superoxide radicals were produced via the xanthine/xanthine oxidase system and then reacted with the spin trap DMPO. The DMPO-OOH adducts were detected using an ESR spectrometer. ESR signaling was detected after 20 μl of xanthine oxidase (0.25 U/ml) was mixed with 20 μl of xanthine (5 mM), 20 μl of DMPO (1.5 M), and 20 μl of butin at 10 μg/ml. The ESR spectrometer parameters were set at a magnetic field of 336 mT, power of 5.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 500, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec, and temperature of 25 °C.

2.4. Detection of hydroxyl radical

The hydroxyl radicals were generated by the Fenton reaction ($H_2O_2$ + $FeSO_4$), and then reacted with a nitrone spin trap, DMPO. The resultant DMPO-OH adducts was detected using an ESR spectrometer. The ESR spectrum was recorded using a JES-FA ESR spectrometer (JEOL, Tokyo, Japan), at 2.5 min after mixing with phosphate buffer solution (pH 7.4) with 20 μl of 0.3 M DMPO, 20 μl of 10 mM $FeSO_4$, 20 μl of 10 mM $H_2O_2$, and 20 μl of butin at 10 μg/ml. The ESR spectrometer parameters were set at a magnetic field of 336 mT, power of 1.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 200, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec, and a temperature of 25 ℃.

2.5. Mitochondrial ROS measurement

For image analysis of mitochondrial ROS generation, cells were seeded onto a coverslip loaded six well plate at $1 \times 10^5$ cells/well. Sixteen hours after plating, cells were treated with butin at 10 μg/ml and 30 min later, 1 mM $H_2O_2$ was added to the plate. After changing the media, 20 μM of DHR 123 was added to each well and the plate was incubated for an additional 30 min at 37 °C. After washing with PBS, the stained cells were mounted onto a microscope slide in mounting medium (DAKO, Carpinteria, CA). Images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a confocal microscope. The level of mitochondrial ROS was also detected by flow cytometry. Cells were loaded for 30 min at 37 °C with 10 μM DHR 123 at indicated time and supernatant was removed by suction and after trypsin treatment, cells were washed with PBS. Fluorescence of DHR 123 loaded cells was measured using a flow cytometer. In addition, cells were seeded in a 96 well plate at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with 10 μg/ml butin and 30 min later 1 mM $H_2O_2$ was added to the plate. Cells were incubated for an additional 30 min at 37 °C. After addition of 20 μM of DHR 123 solution for 10 min, the

fluorescence was detected using a Perkin Elmer LS-5B spectrofluorometer.

2.6. Intracelluar $Ca^{2+}$ measurement

Intracellular $Ca^{2+}$ was detected with the fluorescent probe fluo-4-AM. Cells were loaded for 30 min at 37 °C with 10 μM fluo-4-AM and supernatant was removed by suction and after trypsin treatment, cells were washed with PBS. Fluorescence of fluo-4-AM loaded cells was measured using a flow cytometer.

2.7. Quantification of cellular ATP levels

Mitochondrial function was evaluated by measuring the cellular adenosine triphosphate (ATP) production in cells. Cells were harvested and washed twice with PBS. Harvested cells were then lysed on ice for 30 min in 200 μl of lysis buffer [25 mM Tris (pH 7.8), 270 mM sucrose, 1 mM EDTA] by sonicating three times for 15 sec and centrifuged at 4 °C for 10 min at 16,000 × g. Supernatants were collected from the lysates and ATP content was assayed using a luciferase/luciferin ATP determination kit (Molecular Probes, Eugene, OR).

2.8. Succinate dehydrogenase activity measurement

The effect of butin on mitochondrial succinate dehydrogenase activity was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (Carmichael et al., 1987). Cells were seeded in a 96 well plate at a concentration of $1 \times 10^5$ cells/ml, and 16 h after plating, were treated with butin at 10 μg/ml, and 1 h later 1 mM $H_2O_2$ was added to the plate and incubated for an additional 24 h at 37 °C. Fifty microliter of MTT stock solution (2 mg/ml) was then added to each well of a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min and the supernatants aspirated. The formazan crystals in each well were dissolved in 150 μl DMSO and the A540 read on a scanning multi-

well spectrophotometer.

2.9. Statistical analysis

Results are represented as the mean ± standard error of the mean (SEM). The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. $p<0.05$ were considered significant.

# 3. RESULTS

## 3.1. Radical scavenging activity of butin in a cell-free system

Butin's scavenging effects on the superoxide radicals and the hydroxyl radicals were measured by ESR spectrometry. The ESR data showed no clearly detectable, specific signal in the control or in the 10 μg/ml of butin; however, the superoxide radical signal increased up to a value of 1077 in the xanthine/xanthine oxidase system. Butin treatment decreased the superoxide radical signal to a value of 445 (Fig. 6A). Consistent with its superoxide radical scavenging activity, butin treatment was also reduced hydroxyl radical generation by the Fenton reaction ($H_2O_2$ + $FeSO_4$) (Fig. 6B). Butin treatment reduced hydroxyl radicals to a level of 3573 as compared to a level of 4893 of in $FeSO_4$ + $H_2O_2$ system. These results suggest that butin is an effective free radical scavenger.

A

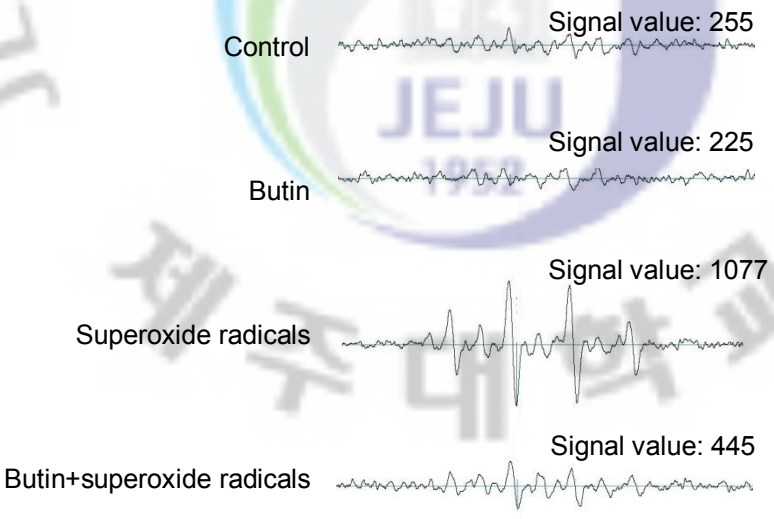



**Fig.6.** The scavenging activity of butin on superoxide and hydroxyl radicals. (A) Superoxide radicals generated by the xanthine and xanthine oxidase were spin-trapped with DMPO, and the resultant DMPO-·OOH adducts were detected by ESR spectrometry.

B

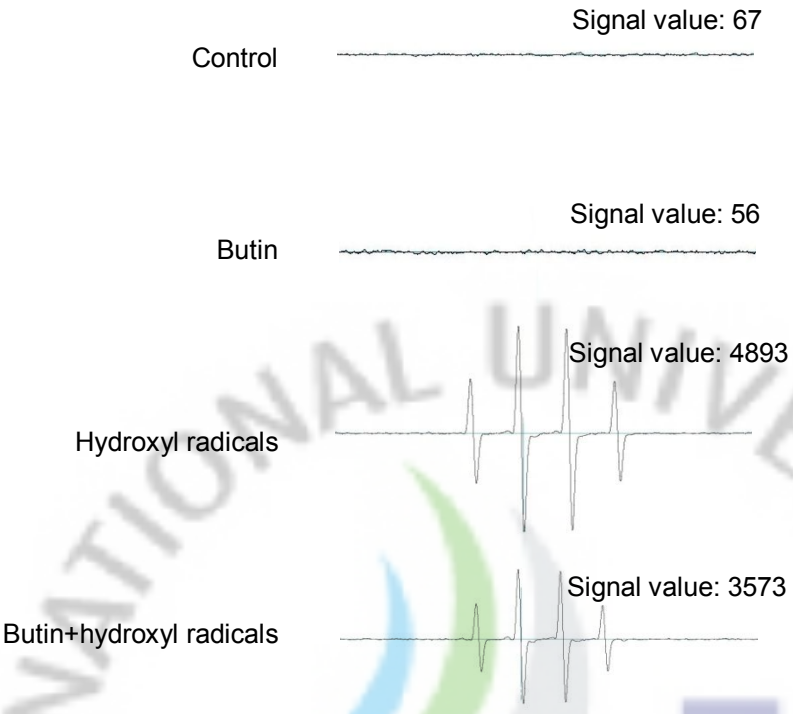



**Fig.6. continued.** (B) Hydroxyl radicals generated by the Fenton reaction ($H_2O_2$ + $FeSO_4$) were spin-trapped with DMPO, and the resultant DMPO-OH adducts were detected by ESR spectrometry.

3.2. The effect of butin on mitochondrial ROS scavenging activity

Butin's free radical-scavenging effect on the mitochondrial ROS in V79-4 cells was detected by means of DHR 123 fluorescence dye. Analysis of confocal microscope revealed that butin at 10 μg/ml reduced the red fluorescence intensity of $H_2O_2$-induced mitochondrial ROS as shown in Fig. 7A. In addition, the level of mitochondrial ROS detected by flow cytometry revealed a fluorescence intensity value of 41 in $H_2O_2$-treated cells with butin at 10 μg/ml, compared to a fluorescence intensity value of 120 in $H_2O_2$-treated cells (Fig. 7B), thus reflecting a reduction in ROS generation. The fluorescence spectrometric data revealed that $H_2O_2$ treatment increased the level of mitochondrial ROS compared to control. However, butin at 10 μg/ml treatment attenuated the $H_2O_2$-induced ROS increase (Fig. 7C). These data suggest that butin is a scavenger of mitochondrial ROS.

A

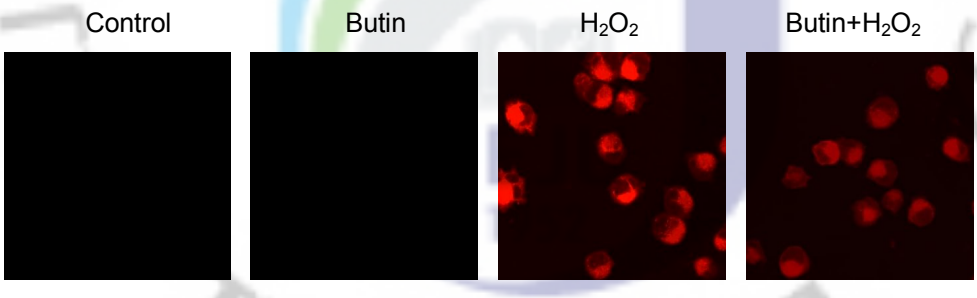
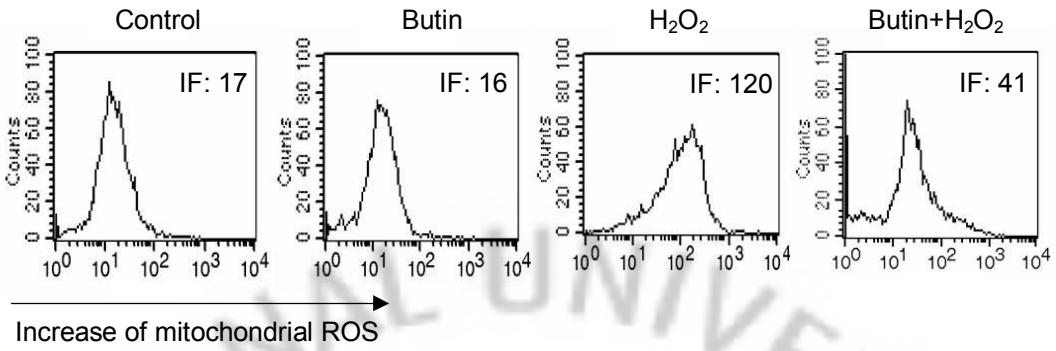



**Fig.7.** The effect of butin on $H_2O_2$-induced mitochondrial ROS generation. (A) Cells were treated with butin at 10 μg/ml. After 1 h, 1 mM $H_2O_2$ was added to the plate. After changing the media, 20 μM of DHR 123 was added to each well and the plate was incubated for an additional 30 min at 37°C. The representative confocal images illustrate the increase in red fluorescence intensity of DHR 123 produced by ROS in $H_2O_2$-treated cells as compared to the control and the lowered fluorescence intensity in $H_2O_2$-treated cells with butin (original magnification × 400).

B




C

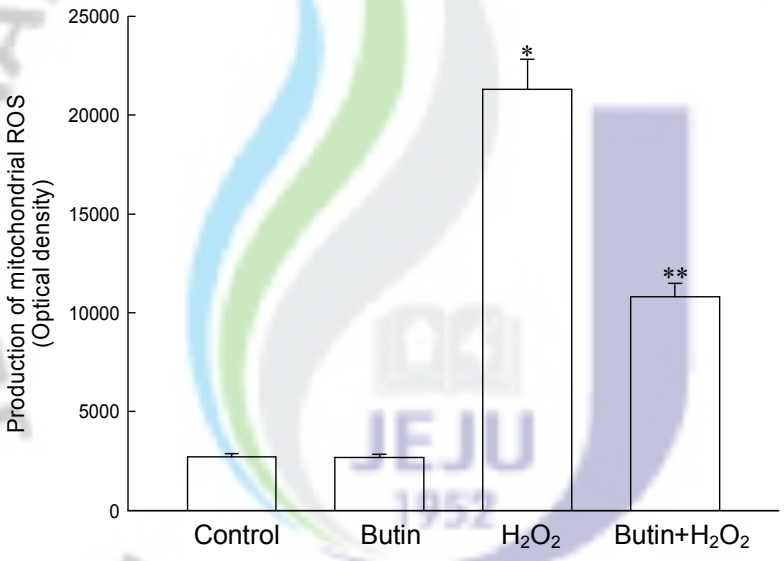



**Fig.7. continued.** The mitochondrial ROS generated was detected by flow cytometry (B), and spectrofluorometry (C) after the DHR 123 treatment. FI indicates the fluorescence intensity of DHR 123. The measurements were made in triplicate and the values were expressed as means ± SEM. *Significantly different from control cells ($p<0.05$). **Significantly different from $H_2O_2$ treated cells ($p<0.05$).

3.3. The effect of butin on intracellular $Ca^{2+}$

ROS can induce $Ca^{2+}$ release from the mitochondria and result in an increase of cytosolic $Ca^{2+}$ levels, which may target to cell apoptotic death. As shown in Fig 8, $H_2O_2$-treated cells with butin revealed a fluorescence intensity value of 58, as compared to a fluorescence intensity value of 92 in $H_2O_2$-treated cells. These data suggest that butin reduces intracellular $Ca^{2+}$ level induced by $H_2O_2$ treatment.

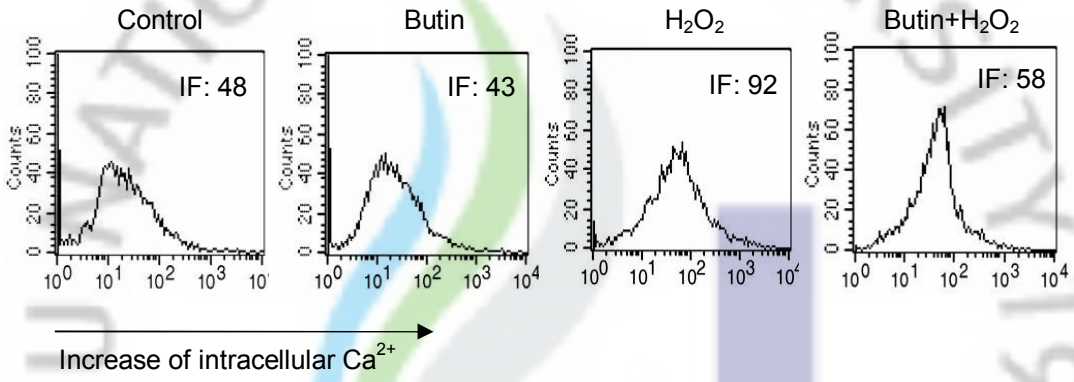



**Fig.8.** The effect of butin on intracellular $Ca^{2+}$ levels. Cells pretreated with butin at 10 μg/ml, and 1 h later, 1 mM $H_2O_2$ was treated. And then cells were harvested and treated with fluorescent probe fluo-4-AM (10 μM). $Ca^{2+}$ levels were detected by flow cytometry. FI indicates the fluorescence intensity of fluo-4-AM.

3.4. The effect of butin on intracellular ATP levels and succinate dehydrogenase activity

Mitochondrial injury is followed by the depletion of intracellular ATP levels. As shown in Fig. 9A, $H_2O_2$ treatment reduced the ATP levels as compared to control, however, $H_2O_2$-treated cells with butin treatment recovered the ATP levels, suggesting butin has a protective effect against $H_2O_2$-induced loss of intracellular ATP levels. These data suggest that butin restores mitochondrial function by preventing the loss of mitochondrial membrane integrity. Succinate dehydrogenase is an enzyme existing in the mitochondrial respiratory chain, and its activity was assessed by MTT assay. As shown in Fig. 9B, a combination of butin at 10 μg/ml and $H_2O_2$ enhanced succinate dehydrogenase activity to 52%, as compared to 32% in $H_2O_2$ treated cells. Taken together, these results indicate butin recovers mitochondrial function damaged by $H_2O_2$ treatment through preventing loss of ATP levels and of succinate dehydrogenase activity.

A

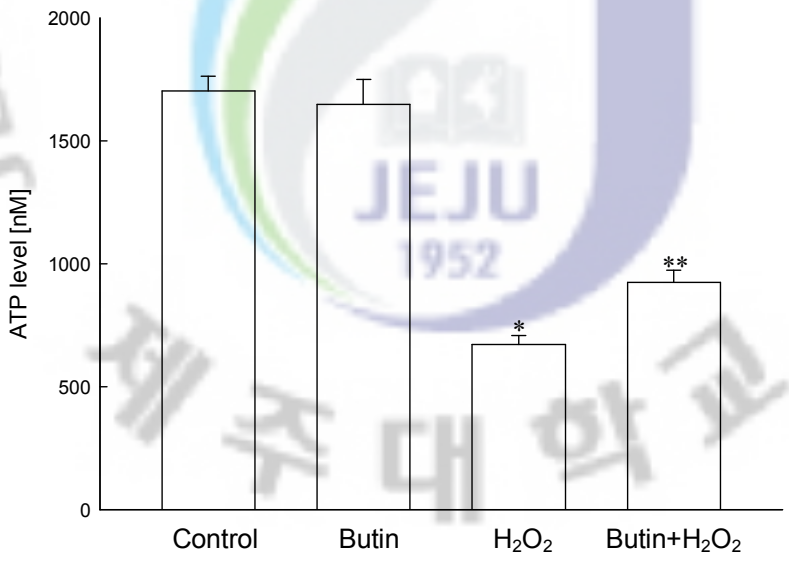



**Fig.9.** The effects of butin on intracellular ATP levels and succinate dehydrogenase activity. (A) ATP content was assayed using a luciferase/luciferin ATP determination kit. *Significantly different from control cells ($p<0.05$). **Significantly different from $H_2O_2$ treated cells ($p<0.05$).

B

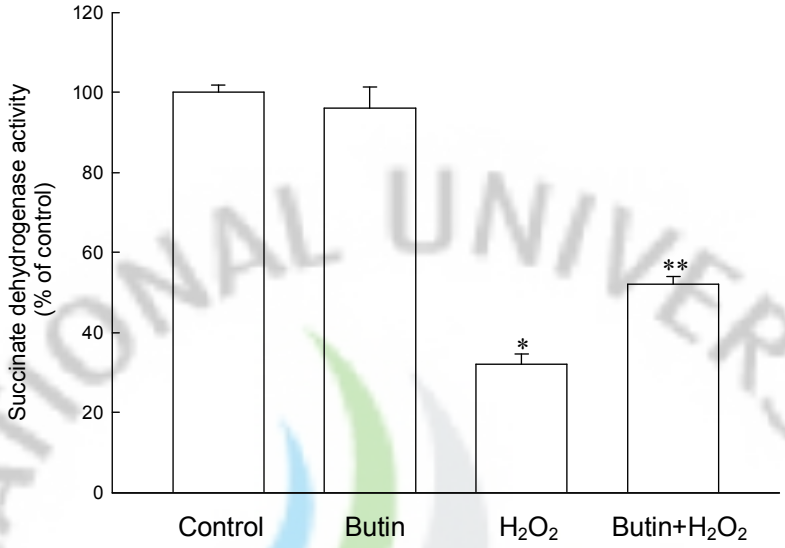



**Fig.9. continued.** (B) Succinate dehydrogenase activity was detected by MTT assay. *Significantly different from control cells ($p<0.05$). **Significantly different from $H_2O_2$ treated cells ($p<0.05$).

# 4. DISCUSSION

Mitochondria contributes to a number of different processes in living cells, such as ATP synthesis by oxidative phosphorylation, the production of ROS, and $Ca^{2+}$ uptake and release (Pedersen, 1999), of which the most important is ATP synthesis by oxidative phosphorylation (Saraste, 1999). Oxidative phosphorylation is a major source of endogenous, toxic free radicals, including $H_2O_2$, $OH\cdot$, and $O_2^{\cdot-}$, which are products of normal cellular respiration (Wallace, 1999). With inhibition of electron transport, electrons accumulate in the early stages of the electron transport chain, where they may be donated directly to molecular oxygen, to give the superoxide radical $O_2^{\cdot-}$. Superoxide anions are detoxified to $H_2O_2$ by the mitochondrial Mn superoxide dismutase (Mn SOD). $H_2O_2$ is converted to water by catalase and glutathione peroxidase. However, $H_2O_2$ can convert to toxic hydroxyl radicals in the presence of reduced transition metals, via the Fenton and/or Haber-Weiss reactions. Multiple classes of macromolecules are altered by free radicals, including proteins, lipids, and nucleic acids. ROS generated as a consequence of ATP production in the mitochondria are important for cellular signaling, and therefore they contribute to oxidative stress and cellular damage. ROS regulate the activity of redox-sensitive enzymes and ion channels within the cell, including $Ca^{2+}$ channels (Feissner et al., 2009). Calcium is a key regulator of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis (Feissner et al., 2009). $Ca^{2+}$ released from the endoplasmic reticulum (ER) enters mitochondria leading to depolarization of the inner membrane, cytochrome c release, and activation of Apaf-1 (apoptosis protease-activating factor 1)/procaspase-9-regulated apoptosis (Crompton 1999; Boya et al., 2002). Numerous data have shown that the elevation of cytosolic $Ca^{2+}$ is sufficient to induce apoptosis in many different cell types (Takadera and Ohyashiki, 1997; Nakamura et al., 2000). In our result, $H_2O_2$ increased the $Ca^{2+}$ levels,

and butin inhibited the release of $Ca^{2+}$ from mitochondria. For $Ca^{2+}$ and ROS, a delicate balance exists between beneficial and detrimental effects on mitochondria. Mitochondrial succinate dehydrogenase, which catalyses the oxidation of succinate to fumarate in the Krebs cycle, is a crucial mitochondrial antioxidant enzyme, controlling the superoxide-scavenging activity in the respiratory chain (Rustin et al., 2002). In our result, the $Ca^{2+}$ level in cells treated by oxidative stress inducer, $H_2O_2$ showed higher than control cells, and accordingly, induced a mitochondrial calcium influx, and mitochondrial degradation. The ATP production and succinate dehydrogenase activity in the respiratory chain was decreased by $H_2O_2$ treatment. Impaired mitochondrial function exposed to $H_2O_2$ was restored by butin.

Flavonoids like butin are known to possess powerful antioxidant properties, which are attributed to the presence of phenolic hydroxyl groups in the flavonoid structure. They unually contain one or more aromatic hydroxyl groups, and this moiety is responsible for the antioxidant activity of the flavonoid (van Acker et al., 2000). The results of radical scavenging activity of butin were consistent with its intrinsic feature of chemical structure, meaning potential application for health. Antioxidants are generally categorized by primary and secondary antioxidant. Primary antioxidants are capable of interrupting and terminating the free radical propagation step via proton transfer to radical species. Secondary antioxidants are chemicals that can prevent free radical formation. The main secondary antioxidants are metal chelators (Shao et al., 2008). It is known that flavonids have antioxidant activity by a free radical scavenging mechanism with the formation of less reactive phenoxyl radicals by donation of electron or hydrogen (Arora et al., 1998; Seyoum et al., 2006), meaning primary prevention of oxidative stress. Another antioxidant mechanism is based on the ability to chelate transition metal like iron, thereby suppressing the hydrogen peroxide-driven Fenton reaction. For example, one of the well known radical scavengers, quercetin showed highly effective chelating activity on transition metals (Soczynska-Kordala et al., 2001). The antioxidant of

butin might be related with combination of metal chelation and free radical-scavenging activities. To our understanding, butin might be ambidextrous antioxidant for bearing primary and secondary feature of antioxidant. Thus, butin might have potential application for either foods or pharmaceutical supplements. Further investigation on the contribution of metal chelation to the antioxidant activity of butin is remained for further study. The influence of metal chelation on the antioxidant activity of butin needs to be compared in biological relevant assay system between dependent and independent of metal individually.

Natural flavonoids are emerging as potent therapeutic drugs for free radical-mediated diseases. For example, isoliquiritigenin, baicalein, naringin, and catechin have demonstrated neuroprotective effects through the inhibition of ROS, suggesting that these are potential pharmacological agents for the treatment of Parkinson's disease (Guo et al., 2007; Zhan and Yang, 2006). Also, it is well known that the generation of mitochondrial ROS plays an important role in mitochondrial dysfunction through the disruption of redox homeostasis (Brunelle and Chandel, 2002). Recently, we demonstrated that butin possesses antioxidant properties and cytoprotective effects against oxidative stress (Zhang et al., 2008; Kang et al., 2009). Therefore, in the present study, we focused on butin's effect on mitochondrial dysfunction induced by oxidative stress. Many of different clinical mechanisms of flavonoids have been related with their antioxidant properties, either through their reducing capacities or through influencing on intracellular components. The precise mechanisms by which flavonoids exert their beneficial or toxic actions remain unclear (Arora et al., 2000; Williams et al., 2004). However there is increasing interest in the research of flavonids, due to growing evidence of the health benefits of flavonoids through epidemiological studies. Many flavonoids are shown to have antioxidant activity, coronary heart desease prevention, anti-inflammation, oestrogenic activity, anticancer activity, and other biological activities (Harborne and Williams, 2000; Yao et al., 2004). As such research progresses, potential application of flavonoids in either foods or

pharmaceutical supplements will expand. Considering butin's reduction of mitochondrial dysfunction, it might give a hopeful picture for the mitochondria related diseases such as aging, diabetes, and neurological diseases for the initial step of clinical trial and development of raw materials of medicine. Accrodingly, an appropriate system for assessment of intake of butin needs to be developed for further study.

In the present study, the antioxidant activity of butin in V79-4 cells was evaluated in two categories: direct action on superoxide and hydroxyl radical scavenging in a cell-free system, as shown by ESR spectrometry, and indirect action through induction of mitochondrial antioxidant enzyme. Butin balanced intracellular $Ca^{2+}$ levels, recovered the decreased ATP levels, and enhanced succinate dehydrogenase activity via superoxide and hydroxyl radical-scavenging activities.

# PART III

## Butin (7, 3′, 4′-Trihydroxydihydroflavone) Reduced Oxidative Stress-Induced Cell Death via Inhibition of the Mitochondria-Dependent Apoptotic Pathway

# ABSTRACT

The objective of this study was to investigate the cytoprotective effects of butin (7, 3′, 4′-trihydroxydihydroflavone) on oxidative stress-induced apoptosis, and the possible mechanisms involved. Butin significantly reduced hydrogen peroxide ($H_2O_2$)-induced loss of the mitochondrial membrane potential ($\Delta\psi_m$), the alteration of Bcl-2 family proteins, the release of cytochrome c from mitochondria into the cytosol and the activation of caspase 9 and caspase 3. Furthermore, the anti-apoptotic effect of butin was exerted via inhibition of mitogen-activated protein kinase kinase-4 (MKK4/SEK1), c-Jun $NH_2$-terminal kinase (JNK) and activator protein-1 (AP-1) cascades induced by $H_2O_2$ treatment. Finally, butin exhibited protective effects against $H_2O_2$-induced apoptosis, as demonstrated by decreased apoptotic bodies, sub-$G_1$ levels and DNA fragmentation. In conclusion, our results suggest that butin exerts its cytoprotective effects via inhibiting the mitochondria-dependent apoptotic pathway.



Keywords: Butin • Oxidative stress • Mitochondria-dependent apoptotic pathway

# 1. INTRODUCTION

Oxidative stress mediated by reactive oxygen species (ROS) has been implicated as a major cause of cellular damage and contributed to inflammation, aging, cancer, arteriosclerosis, hypertension and diabetes (Koblyakov, 2001; Loeb et al., 2005; Schumacker, 2006; Lubos et al., 2008; Harrison et al., 2009; Pennathre and Heinecke, 2007). Persistent ROS elevation is a result of an imbalance between ROS production and scavenging by endogenous antioxidants that directly or indirectly disturb physiological functions of many cellular macromolecules, such as DNA, proteins and lipids (Droge, 2002) that ultimately induce cell death either by apoptosis or necrosis (Kannan and Jain, 2000). Mitochondrial dysfunction results in increased ROS production that enhances oxidative stress if the cellular defense systems are overwhelmed (Jezek and Hlavata, 2005). Previous studies have indicated that ROS can alter intracellular redox state, change the inner mitochondrial membrane potential ($\Delta\psi_m$) and release soluble inter-membrane proteins from the mitochondria into the cytosol, including cytochrome c (Green and Reed, 1998; Li et al., 2000). It is also well known that ROS plays a crucial role in triggering the mitochondria-mediated apoptosis pathway, which is associated with activation of caspase cascades and the family of Bcl-2 proteins (Park et al., 2003; Nicholson and Thornberry, 1997; Adams and Cory, 1998).

Flavonoids are a group of naturally occurring polyphenolic compounds ubiquitously found in fruits and vegetables, and represent substantial constituents of the non-energetic part of the human diet. Butin (7, 3′, 4′-trihydroxydihydroflavone) is one of the most widely distributed flavonoids, reported to be potent antioxidants beneficial against oxidative stress-related diseases, such as cancer, aging, liver diseases and diabetes (Brusselmans et al., 2005; Patil et al., 2003; Kuzu et al., 2008; Shu et al., 2009). Butin was isolated from several medicinal herbs (Jang et al., 2003; Liu et al., 2005; Su et al., 2007; Tian et al., 2004) and reported to have skin-

whitening and antioxidant activity (Bhargava, 1986; Lee et al., 2006). In a previous report, we demonstrated that butin protected cells against hydrogen peroxide-induced apoptosis by scavenging ROS and activating antioxidant enzymes (Zhang et al., 2008), decreased oxidative stress-induced 8-hydroxy-2'-deoxyguanosine levels via activation of oxoguanine glycosylase 1 (Kang et al., 2009), and reduced oxidative stress-induced mitochondrial dysfunction via ROS scavenging (Zhang et al., 2010). To extend our previous investigations on butin's effect on mitochondria mediated cell damage induced by oxidative stress (Zhang et al., 2010), we focused on the protective effect of butin against oxidative stress-induced mitochondrial mediated apoptosis, and the possible underlying mechanisms involved in this present study.

# 2. MATERIALS AND METHODS

## 2.1. Reagents

Butin (Fig. 1) was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). The 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) was purchased from the Invitrogen (Carlsbad, CA). The primary anti-Bcl-2, -Bax, -phospho Bcl-2, and -cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary anti-caspase 9, -caspase 3, -JNK, -phospho JNK, -SEK1, and -phospho SEK1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). A plasmid containing AP-1 binding site-luciferase construct was a generous gift from Professor Young Joon Surh of Seoul National University (Seoul, Korea). Propidium iodide and Hoechst 33342 were purchased from the Sigma Chemical Company (St. Louis, MO).

## 2.2. Cell culture

Chinese hamster lung fibroblasts (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).

## 2.3. Mitochondrial membrane potential ($\Delta\psi_m$) analysis

$\Delta\psi_m$ analysis was determined by confocal image analysis and flow cytometer. Cells were treated with butin at 10 μg/ml, and after 1 h, 1 mM of $H_2O_2$ was added to the plate, and the mixture was incubated for 24 h. Cells were then harvested, and after changing the media, JC-1 was added to each well and was incubated for an additional 30 min at 37 ℃. After washing with PBS, the stained cells were mounted onto microscope slide in mounting medium (DAKO,

Carpinteria, CA). Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on confocal microscope (Cossarizza et al., 1993). In addition, $\Delta\psi_m$ analysis was also determined by flow cytometer. The cells were harvested, washed and suspended in phosphate buffered saline (PBS) containing JC-1 (10 μg/ml). After incubation for 15 min at 37 ℃, the cells were washed and were suspended in PBS and were analyzed by flow cytometer (Troiano et al., 2007).

2.4. Western blot analysis

Cells were harvested, washed twice with PBS, lysed on ice for 30 min in 100 μl of a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and then centrifuged at 13,000 × g for 15 min. The supernatants were collected from the lysates and the protein concentrations determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% sodium dodecysulfate-polyacrylamide gel. The blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were then incubated with the primary antibodies. The membranes were further incubated with the secondary immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK), and then exposed onto X-ray film.

2.5. Preparation of the nuclear extract and electrophoretic mobility shift assay

Cells were harvested, and subsequently 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 (nuclear

protein) was stored at -70 ºC after determining the protein concentration. Oligonucleotides containing transcription factor AP-1 consensus sequence (5'- CGC TTG ATG ACT CAG CCG GAA - 3') were annealed, labeled with [$\gamma$-$^{32}$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 ºC for 30 min, to reach 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). The binding products were resolved on 5% polyacrylamide gel and the bands were visualized by autoradiography.

2.6. Transient transfection and AP-1 luciferase assay

Cells were transiently transfected with plasmid harboring the AP-1 promoter using DOTAP as the transfection reagent, according to the manufacturer's protocol (Roche Diagnostics). Following overnight transfection, cells were treated with 10 μg/ml of butin for 24 h. Cells were washed twice with PBS and lysed with reporter lysis buffer (Promega, Madison, WI). Following vortex mixing and centrifugation at 12,000 × g for 1 min at 4 °C, the supernatant was stored at -70 °C for the luciferase assay. After mixing 20 μl of cell extract with 100 μl of luciferase assay reagent at room temperature, the mixture was placed in an illuminometer to measure the light produced.

2.7. Nuclear staining with Hoechst 33342

Cells were treated with butin at 10 μg/ml. After 1 h, 1 mM of $H_2O_2$ was added to the plate and the mixture was incubated for 24 h. 1.5 μl of Hoechst 33342 (stock 10 mg/ml), a DNA specific fluorescent dye, was added to each well 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, in order to examine the degree of nuclear condensation.

2.8. Detection of apoptotic sub-$G_1$ hypodiploid cells

The amount of apoptotic sub-$G_1$ hypodiploid cells was determined using flow cytometer (Nicoletti et al., 1991). Cells were harvested and fixed in 1 ml of 70% ethanol for 30 min at 4 °C. The cells were then washed twice with PBS, and incubated for 30 min in the dark at 37 °C in 1 ml of PBS containing 100 μg of propidium iodide and 100 μg of RNase A. A flow cytometric analysis was performed using a FACS Calibur flow cytometer. Sub-$G_1$ hypodiploid cells were assessed based on histograms generated by the Cell Quest and Mod-Fit computer programs.

2.9. DNA fragmentation

Cellular DNA-fragmentation was assessed by analyzing cytoplasmic histone-associated DNA fragmentation, using a kit from Roche Diagnostics (Portland, OR) according to the manufacturer's protocol.

2.10. Statistical analysis

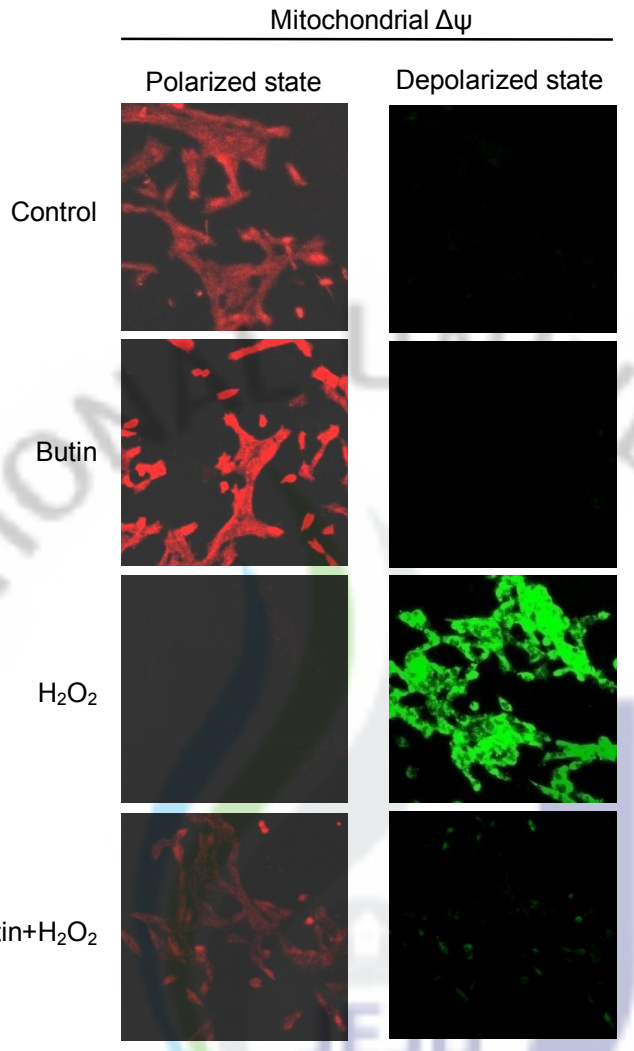
All measurements were performed in triplicate and all values were represented as the mean ± standard error of the mean (SEM). The results were subjected to an analysis of variance (ANOVA) using the Tukey's test to analyze difference. $P<0.05$ were considered statistically significant.

# 3. RESULTS

## 3.1. Effect of butin on $H_2O_2$-induced $\Delta\psi_m$ depolarization

In a previous report, we have indicated that butin protected against $H_2O_2$-induced apoptosis (Zhang et al., 2008). Change in $\Delta\psi_m$ was examined to improve understanding on butin's protection mechanism for $H_2O_2$-induced apoptotic process in terms of mitochondrial involvement. The mitochondria are instrumental in oxidative phosphorylation, cell death regulation and ROS production (Orrenius et al., 2007; Ott et al., 2007). JC-1 is a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green (~525 nm) to red (~590 nm). As shown in Fig. 10A, control cells and butin-treated cells exhibited strong red fluorescence (JC-1 aggregated form, indicative of mitochondrial polarization) in the mitochondria. However, $H_2O_2$ resulted in reducing red fluorescence and increasing green fluorescence (JC-1 monomer form, indicative of mitochondrial depolarization) in the mitochondria. Butin treatment blocked reducing red fluorescence and increasing green fluorescence in $H_2O_2$-treated cells. Image analysis data was consistent with flow cytometric data; the level of $\Delta\psi_m$ loss was increased in $H_2O_2$-treated cells, as substantiated by an increase in fluorescence with JC-1 dye. However, butin recovered the level of $\Delta\psi_m$ loss (Fig. 10B), suggesting that butin inhibited loss of $\Delta\psi_m$ in response to $H_2O_2$ treatment.

A




B

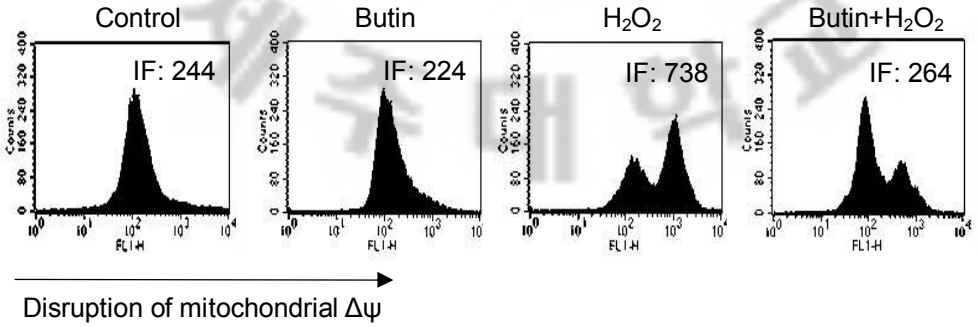
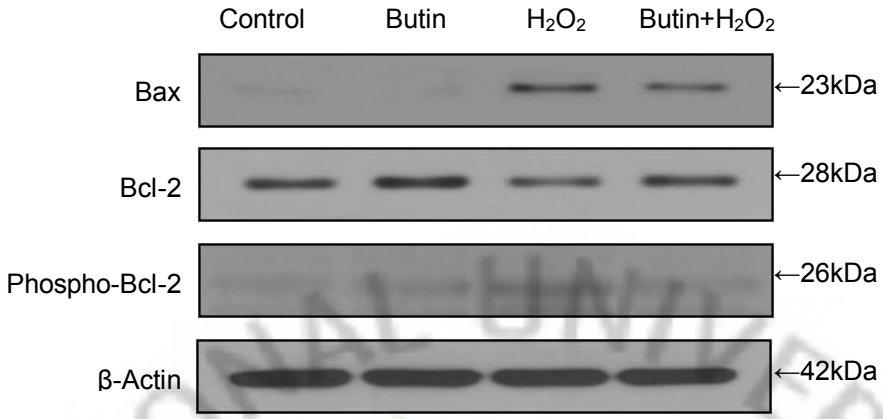



**Fig.10.** Effects of butin on $H_2O_2$-induced $\Delta\psi_m$ depolarization. $\Delta\psi_m$ was analyzed by (A) confocal microscope and (B) flow cytometer after staining cells with JC-1.

3.2. Effect of butin on apoptosis related proteins

To further understand the protection mechanism of butin on $H_2O_2$-induced apoptotic process, we detected the protein expressions involved in mitochondria related apoptosis. Beforehand, changes in Bcl-2 expression, an anti-apoptotic protein, and Bax expression, a pro-apoptotic protein, were examined. As shown in Fig. 11A, butin showed an increase in Bcl-2 expression and a decrease in Bax expression in $H_2O_2$-treated cells. It has been reported that Bcl-2 fails to inhibit cell apoptosis when inactivated via phosphorylation (Adams and Cory, 1998; Shimada et al., 2003). We noticed that butin also decreased phosphorylation of Bcl-2 (Ser 87) induced by $H_2O_2$ treatment. During the apoptotic process, Bcl-2 prevented the opening of the mitochondrial membrane pore, whereas Bax induced the opening of membrane pore (Zamzami et al., 1995). Pore opening induces loss of $\Delta\psi_m$, which in turn induces the release of cytochrome c from the mitochondria (Zamzami et al., 1996; Cai et al., 1998). As shown in Fig. 11B, butin inhibited the release of mitochondrial cytochrome c. Next, caspase 9 activity was examined by Western blot since it is known that this enzyme is activated due to mitochondrial membrane disruption (Perkins et al., 2000). As shown in Fig. 11C, treatment of cells with butin inhibited $H_2O_2$-induced active form of caspase 9 (39 and 37 kDa) and caspase 3 (19 and 17 kDa), a target of caspase 9. These results suggest that butin protects cells from apoptosis by inhibiting the caspase dependent pathway via mitochondria.

A

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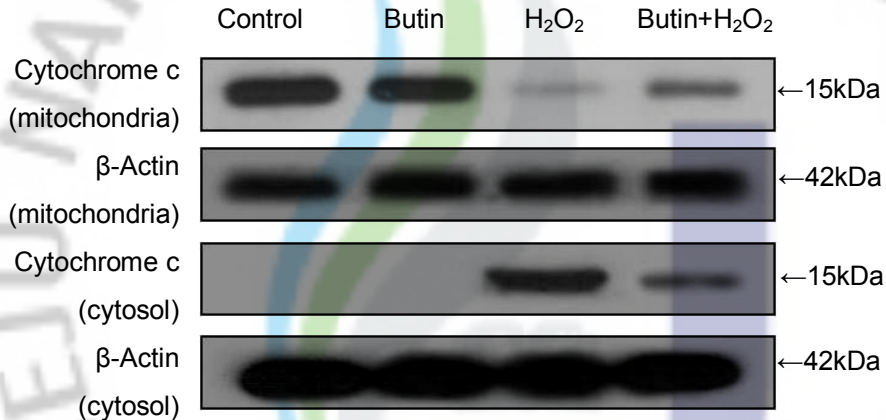


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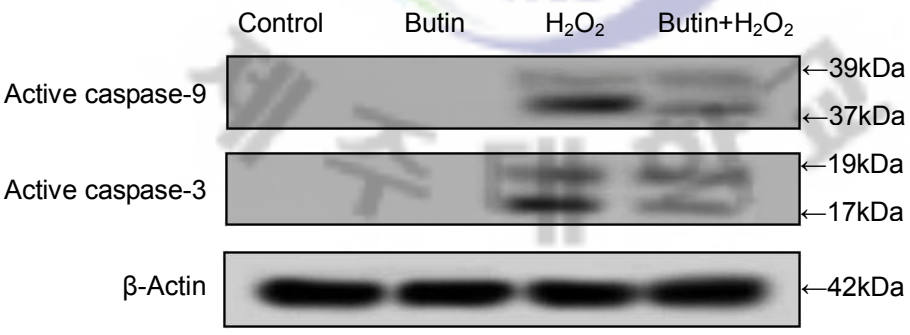
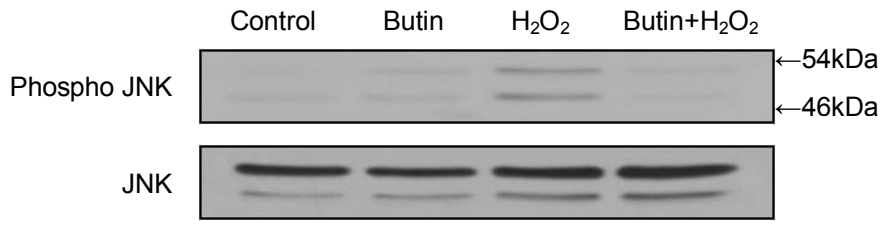



**Fig.11.** Effects of butin on mitochondrial apoptosis related proteins. Western blot analysis was performed. Cell lysates were electrophoresed and (A) Bax, Bcl-2, phospho Bcl-2, (B) cytochrome c, (C) active caspase 9, and active caspase 3 proteins were detected by their specific antibodies.

3.3. Effect of butin on the SEK1-JNK-AP-1 signaling pathway

As the JNK signal pathway plays an important role in oxidative stress-induced apoptosis (Inanami et al., 1999), we tested whether butin regulates this signaling pathway. As shown in Fig. 12A, treatment with butin inhibited JNK activation in $H_2O_2$-treated cells. Moreover, SEK1 is known to be an upstream component in the JNK signaling pathway (Mann et al., 2006). To investigate whether this upstream kinase plays a role in $H_2O_2$-induced JNK activation, SEK1 phosphorylation was determined by Western blot analysis. As shown in Fig. 12B, SEK1 phosphorylation levels were increased in $H_2O_2$-treated cells. However, treatment of cells with butin inhibited $H_2O_2$-induced SEK1 phosphorylation. AP-1 is a downstream target of the phospho-JNK pathway, and activated AP-1 is involved in cell death including apoptosis (Whitmarshm and Davis, 1996). Subsequently, we examined the effect of butin treatment on the DNA binding activity of AP-1 after $H_2O_2$ treatment. As shown in Fig. 12C, AP-1 DNA binding activity was increased in $H_2O_2$ treated cells, whereas treatment of cells with butin inhibited AP-1 activity. The transcriptional activity of AP-1 was also assessed using a promoter construct containing AP-1 binding DNA consensus sequences, which were linked to a luciferase reporter gene. As shown in Fig. 12D, treatment of cells with butin inhibited the transcriptional activity of AP-1 induced by $H_2O_2$. These results suggest that butin inhibits $H_2O_2$-induced apoptosis via suppression of the SEK1-JNK-AP-1 pathway.

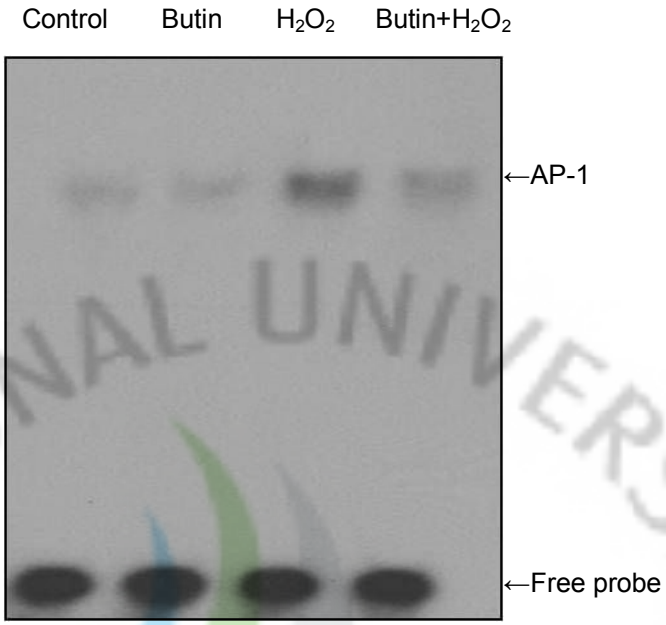
A




B


**Fig.12.** Effects of butin on $H_2O_2$-induced SEK1-JNK-AP-1 activation. Cell lysates were electrophoresed and the cell lysates were immunoblotted using (A) anti-JNK, phospho JNK and (B) -phospho SEK1 and -SEK1 antibodies.

C

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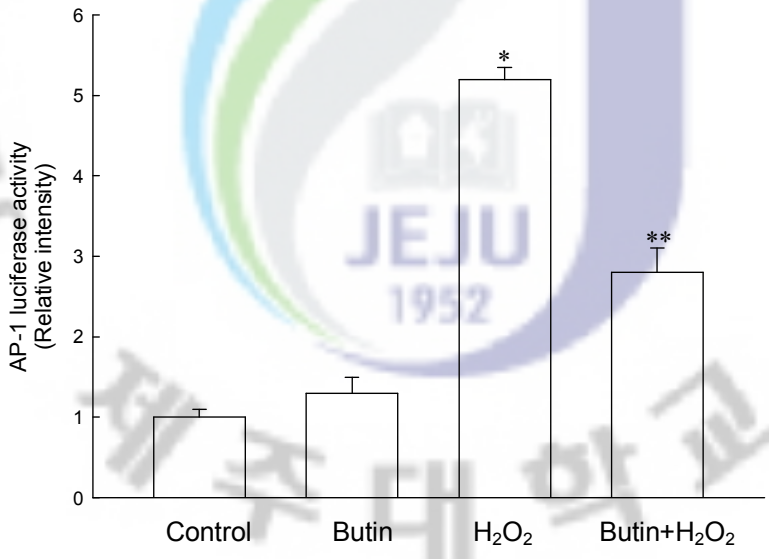



**Fig.12. continued.** (C) AP-1 specific oligonucleotide-protein complexes were detected by the electrophoresis mobility shift assay. (D) The transcriptional activity of AP-1 was assessed using plasmid containing an AP-1 binding site-luciferase construct. *Significantly different from control ($p<0.05$). **Significantly different from $H_2O_2$ treated cells ($p<0.05$).

3.4. Effect of butin against $H_2O_2$-induced apoptosis

In order to confirm the cytoprotective impact of butin on $H_2O_2$-induced apoptosis, cell nuclei were stained with Hoechst 33342 for visualization by microscopy. The microscopic images in Fig. 13A demonstrate that the control cells had intact nuclei, whereas $H_2O_2$-treated cells showed significant nuclear fragmentation, a characteristic of apoptosis. However, butin-pretreated cells exhibited a dramatic decrease in nuclear fragmentation. In addition to morphological evaluation, the protective effect of butin against apoptosis was also confirmed by apoptotic sub-$G_1$ DNA analysis. As shown in Fig. 13B, an analysis of DNA content in $H_2O_2$-treated cells revealed a 36% increase in the apoptotic sub-$G_1$ DNA content. However, butin decreased the apoptotic sub-$G_1$ DNA content to 16%. Furthermore, $H_2O_2$-treated cells increased the levels of cytoplasmic histone-associated DNA fragmentations as compared to control, and butin significantly decreased the level of DNA fragmentation (Fig. 13C).

A

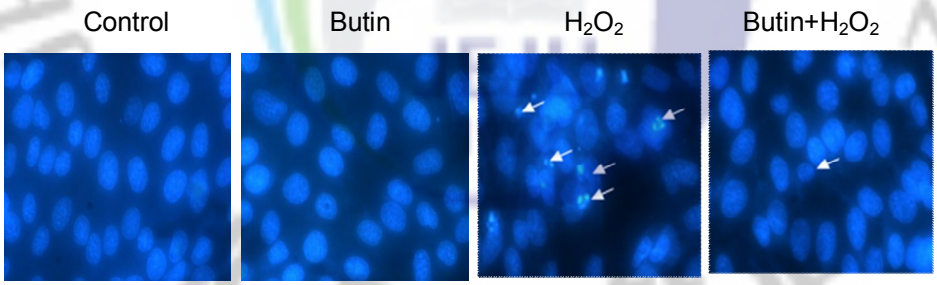
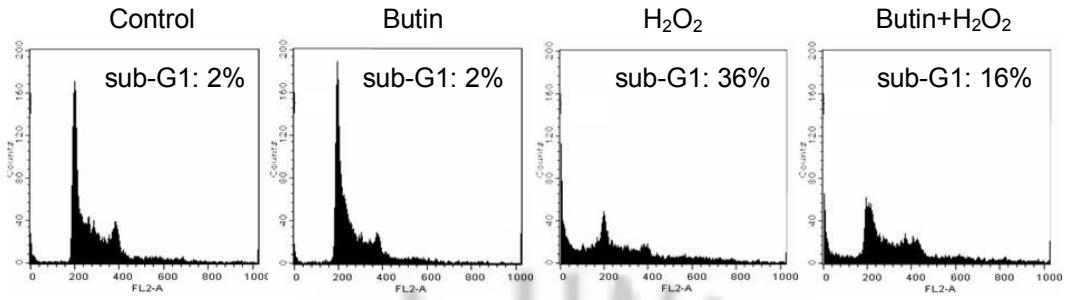



**Fig.13.** Effects of butin on $H_2O_2$-induced apoptosis. (A) Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and arrows indicate apoptotic bodies.

B




C

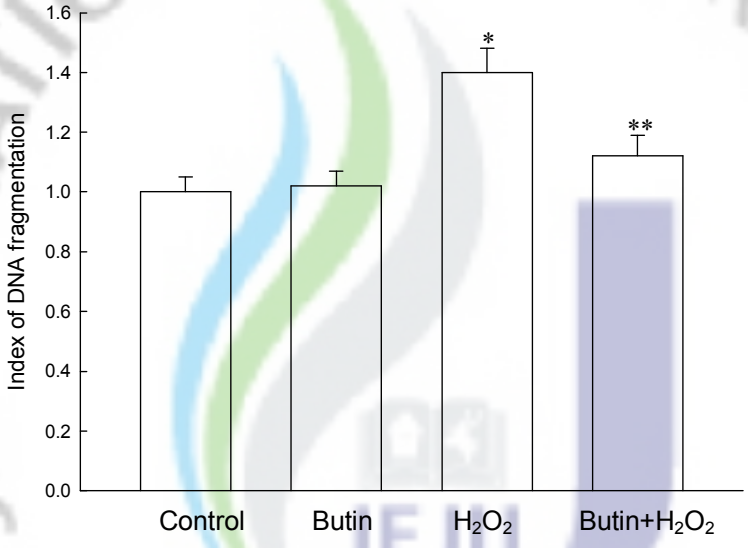



**Fig.13. continued.** (B) The apoptotic sub-$G_1$ DNA content was detected by a flow cytometry after propidium iodide staining. (C) DNA fragmentation was quantified by ELISA kit. The measurements were made in triplicate and values are expressed as means ± SE. *Significantly different from control cells ($p<0.05$). **Significantly different from $H_2O_2$-treated cells ($p<0.05$).

# 4. DISCUSSION

In general, apoptosis is triggered through two distinct pathways: one is the extrinsic pathway involved death receptor signaling, the other is intrinsic pathway involving the mitochondrial cascades (Henry-Mowatt, 2004). Mitochondria have been shown to play a pivotal role in the apoptotic process because both intrinsic and extrinsic pathways can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization (Green and Reed, 1998). Bcl-2 family proteins are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability required for the release of cytochrome c (Green and Reed, 1998; Gross et al., 1999). The release of mitochondrial cytochrome c is a signal event in the intrinsic apoptotic activation pathway. Cytochrome c binds to Apaf-1 and pro-caspase 9 to form the apoptosome, activating caspase 9, the primary caspase involved in the mitochondrial apoptotic pathway. Various studies have suggested possible mechanisms for the JNK pathway also related to mitochondrial depolarization and apoptosis induction. It has been reported that JNK translocates to the mitochondrial, then phosphorylates Bcl-2 and Bcl-XL, anti-apoptotic members of Bcl-2 family, and presumably inactivates them (Fan et al., 2000). In addition, JNK induces the mitochondrial pathway of apoptosis by activating Bim and Bax, pro-apoptotic members of the Bcl-2 family (Malhi et al., 2006).

The depolarization of $\Delta\psi_m$ and the loss of cytochrome c from the mitochondrial inter-membrane space have been proposed as the early events during apoptotic cell death (Kroemer and Reed, 2000). In our case, treatment of cells with $H_2O_2$ resulted in significant collapse of $\Delta\psi_m$, however, treatment with butin recovered $H_2O_2$-induced depolarization of $\Delta\psi_m$. In addition, $H_2O_2$ treatment dramatically induced increase in Bax expression and decrease in Bcl-2 expression, suggesting that changes in the pro-apoptotic and anti-apoptotic Bcl-2 family

proteins may contribute to apoptosis. Moreover, elevation of phosphor Bcl-2 by $H_2O_2$ treatment further helps to reduce its ability to bind with Bax and enhance translocation of Bax from the cytosol to mitochondria, leading to an enhanced susceptibility of the cells to apoptosis (Ishikawa et al., 2003; Zu et al., 2005). Butin significantly restored these changes induced by $H_2O_2$. These results confirmed that butin inhibited $H_2O_2$-induced apoptosis associated with regulation of Bcl-2 family proteins. Changes in caspase 9 and caspase 3 protein expressions were evaluated for the underlying mechanisms, as the Bcl-2 protein can prevent activation of caspases during apoptosis (Ling et al., 2002), and cleaved caspase 9 and caspase 3 represent downstream signals of apoptosis (Budihardjo et al., 1999). We confirmed butin inhibited $H_2O_2$-induced activation of caspase 9 and caspase 3.

SEK1-JNK-AP-1 activation has been suggested as a critical component in the oxidative stress induced apoptosis process (Karin et al., 1997). Butin inhibited $H_2O_2$-induced JNK phosphorylation, resulting in a decrease of AP-1 activity. $H_2O_2$-induced phosphorylation of SEK1, an upstream regulator of JNK, was also attenuated by butin treatment. These results demonstrated that butin attenuated $H_2O_2$-induced apoptosis through the SEK1-JNK-AP-1 pathway. Treatment of cells with butin showed anti-apoptotic activity in cells exposed to $H_2O_2$, as shown by reduction of the distinct morphological features of apoptosis, such as apoptotic bodies formation, sub $G_1$-hypodiploid cells levels and nuclear fragmentation.

Taken together, the protective effect of butin against $H_2O_2$-induced apoptosis was exerted via blockage of membrane potential depolarization, inhibition of the JNK and mitochondria involved caspase-dependent apoptosis pathways. Therefore, we suggest that inhibition of these pathways by butin may provide oxidative stress protection (Fig. 14).

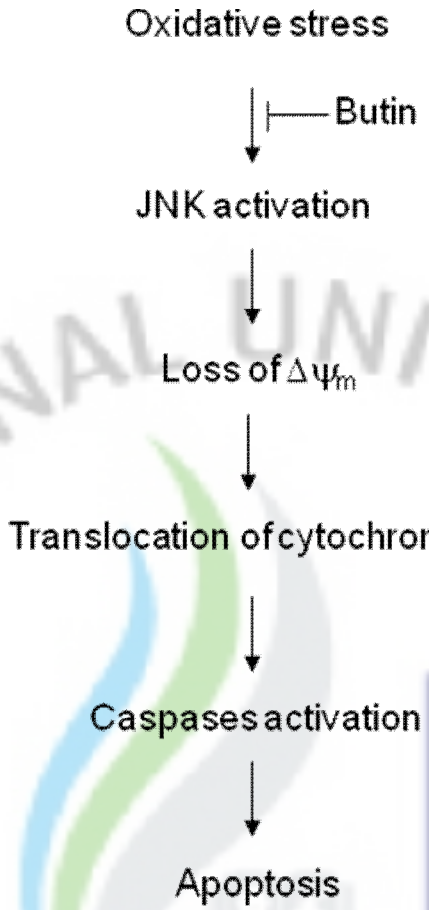



**Fig.14.** A proposed cyto-protective pathway of butin, which explains its properties against oxidative stress-induced mitochondrial involved apoptosis.

# PART IV

## Cytoprotective Effect of Butin (7, 3', 4'-Trihydroxy-dihydroflavone) against Hydrogen Peroxide-Induced Oxidative Stress Is Mediated by Up-Regulation of Manganese Superoxide Dismutase Expression through PI3K/Akt/Nrf2 Dependent Pathway

# ABSTRACT

Butin is a common flavonoid with antioxidant activity. The objective of the present study is to elucidate the cytoprotective effect of butin and its underlying mechanisms. Manganese superoxide dismutase (Mn SOD) is an important antioxidant enzyme against oxidative stress. Butin recovered Mn SOD expression both at the level of mRNA and protein in Chinese hamster lung fibroblast (V79-4) cells which were abolished by hydrogen peroxide ($H_2O_2$) treatment, thus resulted in the increase of Mn SOD activity. The transcription factor NF-E2-related factor 2 (Nrf2) is a critical regulator of Mn SOD, achieved by binding to the antioxidant response element (ARE). Butin enhanced the level of nuclear translocation and ARE binding activity of Nrf2 which was decreased by $H_2O_2$. Specific Nrf2 siRNA attenuated Mn SOD expression, while butin recovered it. Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) contributed to ARE-driven Mn SOD expression. Butin activated PI3K/Akt, and treatment with LY294002 (a PI3K inhibitor), Akt inhibitor IV (an Akt specific inhibitor), and specific Akt siRNA suppressed the butin-induced activation of Nrf2, resulting in a decrease of Mn SOD expression. Butin treatment enhanced the Mn SOD protein expression which was knockdown by Mn SOD siRNA. DEDTC (a Mn SOD inhibitor) and Mn SOD siRNA markedly abolished the cytoprotective effect of butin against $H_2O_2$-induced cell damage. These studies demonstrate that butin attenuates oxidative stress by activating Nrf2-mediated Mn SOD induction via PI3K/Akt signaling pathway.



Keywords: Butin • Manganese superoxide dismutase • NF-E2-related factor 2 • Cytoprotection • Phosphatidylinositol 3-kinase/protein kinase B

# 1. INTRODUCTION

Oxidative stress resulting from the increase of reactive oxygen species (ROS) and/or concomitant decline in antioxidant scavenging capacity may damage proteins, lipids, nucleic acids and other cellular components (Valko et al., 2006). Because ROS formation is a naturally occurring process, mammalian cells have developed several adaptive mechanisms to prevent ROS formation or to detoxify ROS. These mechanisms employ molecules called antioxidants as well as protective enzymes (Chen and Kunsch, 2004). Cytoprotective enzymes provide a major mechanism by which cells combat the toxicities of ROS and their induction is also highly effective and sufficient for protecting cells against oxidative stress as well as the toxic and neoplastic effects of many toxicants and carcinogens (Rushmore and Kong, 2002; Droge, 2002). Among the various cytoprotective enzymes, the protective functions of manganese superoxide dismutase (Mn SOD) have recently been emphasized (Macmillan-Crow and Cruthirds, 2001; Belikova et al., 2009).

Mn SOD, the primary antioxidant enzyme that scavenges superoxide radicals in mitochondria, is essential for the survival of aerobic life (Weisiger and Fridovich, 1973). Lack of Mn SOD expression results in dilated ventricular cardiomyopathy, neonatal lethality, and neurodegeneration (Li et al., 1995; Lebovitz et al., 1996). Overexpression of Mn SOD has been shown to protect against oxidative stress-induced cell death and tissue injury (Yen et al., 1999; Kiningham et al., 1999). Transcriptional regulation of Mn SOD is predominantly mediated by a redox-sensitive transcription factor NF-E2 related factor-2 (Nrf2) (Na et al., 2008). The function of Nrf2 is regulated by its dissociation from the cytoskeleton-associated protein Keap1, which acts as a cytoplasmic repressor of Nrf2. When Nrf2 dissociates from Keap1, Nrf2 can translocate to the nucleus, where it interacts with other transcription factors, binds to the antioxidant responsive element (ARE) (Nguyen et al., 2003). The ARE is a *cis*-

acting enhancer sequence that mediates transcriptional activation of Nrf2 in response to oxidative stress (Rushmore et al., 1991). It is found in the promoter region of genes encoding many antioxidant enzymes, such as superoxide dismutase (SOD), γ-glutamylcysteine synthetase (γ-GCS) and heme oxygenase-1 (HO-1) (Dreger et al., 2009; Li et al., 2006; Wild et al., 1999; Alam et al., 1999). Therefore, genes regulated by the ARE encode proteins maintain the cellular redox status and protect the cells from oxidative damage (Hayes and McLellan, 1999). Recent results have demonstrated that Nrf2 nuclear translocation requires the activation of several signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) (Nakaso et al., 2003). PI3K/Akt are important signaling enzymes involved in transduction of various signals from the cell surface to the nucleus. Both of their pathways are associated with the modulation of ARE-driven gene expression via Nrf2 activation (Li et al., 2007; Yu et al., 2000).

A variety of edible phytochemicals are able to activate Nrf2 signaling thereby up-regulating a set of enzymes including NADP(H): quinone oxidoreductase-1 (NQO1), SOD, glutathione S-transferase (GST), HO-1, and γ-glutamyl cysteine ligase (γ-GCL) (Chen and Kong, 2004; Lee and Surh, 2005). Therefore, it has been suggested that the use of antioxidant compounds may help prevent or alleviate diseases, particularly in those for which oxidative stress is the main cause. Flavonoids, important constituents of human diet, are a group of naturally occurring polyphenolic compounds ubiquitously found in vegetables and fruits. Flavonoids are also found in medicinal plants and herbal remedies containing flavonoids have been used worldwide in folk medicines (Di Carlo et al., 1999; Jovanovic et al., 1994). Recently, much attention has been focused on the potential appication of flavonoid-based drugs for the prevention and therapy of free radical-mediated human diseases, such as atherosclerosis, ischemia, inflammation, neuronal degeneration, and cardiovascular diseases (Amic et al., 2003; Crespo et al., 2008; Gordon and Roedig-Penman, 1998; van Acker et al., 2000). In view of

flavonoids' antioxidant and free radical scavenging properties, their biological activities have been studied intensively (Kahkonen et al., 1999; Montoro et al., 2005; Saric et al., 2009; Sugihara et al., 1999).

Butin (7, 3′, 4′-trihydroxydihydroflavone, Fig. 1) has been isolated from several medicinal herbs, such as *Dalbergia odorifera*, *Adenanthera pavanina*, and *Vernonia anthelmintica Willd* (Liu et al., 2005; Su et al., 2007; Tian et al., 2004), and reported to possess biological properties such as skin-whitening and anti-implantation activity (Lee et al., 2006). Recently, we demonstrated that butin provided protective effects against $H_2O_2$-induced apoptosis by scavenging ROS and activating antioxidant enzymes (Zhang et al., 2008), protected against oxidative DNA damage via activation of PI3K/Akt/OGG1 pathway (Kang et al., 2009), and reduced oxidative stress-induced mitochondrial dysfunction via scavenging of reactive oxygen species (Zhang et al., 2010). However, as far as we know, few studies have been performed on the precise mechanism of butin's protective properties, therefore, in the present study, we investigated the capability of butin to up-regulate Mn SOD expression via activation of the PI3K/Akt/Nrf2 dependent pathway in lung fibroblast cells.

# 2. MATERIALS AND METHODS

## 2.1. Reagents

Butin was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO did not exceed 0.02%. The 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from the Sigma Chemical Company (St. Louis, MO). Diethyl dithiocarbamate (DEDTC) and [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium] bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Primary rabbit polyclonal Mn SOD antibody was purchased from Stressgen Corporation (Victoria, Canada) and primary rabbit polyclonal Nrf2, β-actin antibodies and Akt inhibitor IV were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary anti-phospho Akt (Ser 473) and Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). LY294002 was provided by Calbiochem (San Diego, CA, USA). The other chemicals and reagents were of analytical grade.

## 2.2. Cell culture

The Chinese hamster lung fibroblast cells (V79-4) were obtained from the American Type Culture Collection. The V79-4 cells 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).

## 2.3. Reverse transcriptase polymerase chain reaction

Total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described

previously (Kang et al., 2006). PCR conditions for MnSOD and for the housekeeping gene, GAPDH, were as follows: 30 cycles of 94 °C for 15 sec; 60 °C for 30 sec; and 68 °C for 60 sec. The primer pairs (Bionics, Seoul, Korea) were follows (forward and reverse, respectively): MnSOD, 5'-GACCTGCCTTACGACTATGG-3' and 5'-GACCTTGCTCCTTATTGAAG-3'; and GAPDH, 5'-GTGGGCCGCCCTAGGCACCAGG-3'; and 5'-GGAGGAAGAGGATGCG GCAGTG-3'. Amplified products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light.

2.4. Western blotting analysis

The cells were harvested, washed twice with PBS, lysed on ice for 30 min in 100 μl of a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and then centrifuged at 13,000 × g for 15 min. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% sodium dodecysulfate-polyacrylamide gel. The blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were then incubated with the primary antibodies. The membranes were further incubated with the secondary immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK), and then exposed onto X-ray film.

2.5. Measurement of Mn SOD activity

Cells were seeded in a culture dish at a concentration of $1 \times 10^5$ cells/ml, and at 16 h after plating, were treated with butin at 10 μg/ml. After 1 h, 1 mM $H_2O_2$ was added to the plate, which was incubated more for a further 24 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonicating twice for 15 sec. Triton X-100

(1%) was then added to the lysates and incubated for 10 min on ice. The lysates were clarified, by centrifugation at 5,000 × g for 10 min at 4°C, and the protein concentration of the supernatant was determined. Fifty microgram of protein was mixed with 500 mM phosphate buffer (pH 10.2), 1 mM potassium cyanide (inhibitor of Cu Zn SOD) and 1 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, a pink colored product, which was assayed at 480 nm using a UV/VIS spectrophotometer in the kinetic mode. Mn SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm and the amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity, and the Mn SOD activity was expressed as units/mg protein (Casano et al., 1994).

2.6. Immunocytochemistry

Cells plated on coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 1 min. Cells were treated with blocking medium (3% bovine serum albumin in PBS) for 1 h and incubated with Nrf2 antibody diluted in blocking medium for 2 h. Immuno-reacted primary Nrf2 antibody was detected by a 1:500 dilution of FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h. After washing with PBS, stained cells were mounted onto microscope slides in mounting medium with DAPI (Vector, Burlingame, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

2.7. Nuclear extract preparation and electrophoretic mobility shift assay

Cells were harvested at the indicated times, and were 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 re-suspended in 50 μl of extraction

buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 300 mM NaCl, 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 ºC after determination of protein concentration. Synthetic double strand oligonucleotides containing the Nrf2-binding domain (ARE) were labeled with [$\gamma$-$^{32}$P] ATP using T4 polynucleotide kinase, and subsequently used as probes. The double stranded sequence of oligonucleotides was 5'-TTT TCT GCT GAG TCA AGG GTC CG-3' and 3'-AAA AGA CGA CTC AGT TCC AGG C-5'. The probes (50,000 cpm) were incubated with 6 μg of the nuclear extracts at 4 ºC 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 a 5% polyacrylamide gel and the bands were visualized by autoradiography.

2.8. Transient transfection of small RNA interference (siRNA)

V79-4 cells were seeded at 1.5 x $10^5$ cells/well in 24 well plate and allowed to reach approximately 50% confluence on the day of transfection. The siRNA construct used were obtained as mismatched siRNA control (siControl, Santa Cruz Biotechnology, Santa Cruz, CA, USA), siRNA against Nrf2 (siNrf2, Santa Cruz Biotechnology, Santa Cruz, CA, USA ), siRNA against Akt (siAkt, Dharmacon, Lafayette, CO, USA), and siRNA against Mn SOD (siMn SOD, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were transfected with 10-50 nM siRNA using lipofectamineTM 2000 (Invitrogen, Carlsbad, CA) based on the manufacturer's instruction. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 24 h and examined by either western blot analysis or MTT assay.

2.9. Cell viability

The effect of butin on the viability of the V79-4 cells was determined by the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells (Carmichael et al, 1987). The cells were seeded in a 96 well plate at a density of $1 \times 10^5$ cells/ml and treated with 10 μM DEDTC (an inhibitor of Mn SOD), 10 μg/ml of butin, followed 2 h later by 1 mM of $H_2O_2$. After incubating for 24 h at 37 °C, 50 μl of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 250 μl. After incubation for 4 h, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl DMSO, and the $ABS_{540}$ was read on a scanning multi-well spectrophotometer.

2.10. Statistical analysis

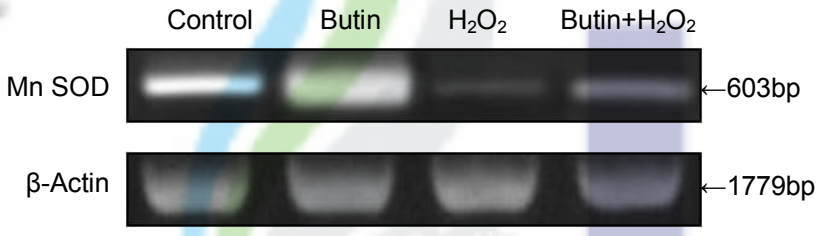
All the measurements were made in triplicate and all values are represented as mean ± standard error of the mean (SEM). The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. A value of $p<0.05$ was considered significant.

# 3. RESULTS

## 3.1. Butin recovered Mn SOD mRNA and protein expression as well as its activity

Treatment of lung fibroblast cells with 1 mM $H_2O_2$ decreased the mRNA and protein expression of Mn SOD. However, butin at 10 μg/ml recovered the mRNA and protein expression of Mn SOD (Fig. 15A, B). Given that Mn SOD requires posttranslational alteration for its activity, its activity was also assessed. A significant increase in Mn SOD activity was observed by butin treatment in $H_2O_2$-treated cells (Fig. 15C). This result suggests that the increase in Mn SOD mRNA and protein is accompanied by enhanced Mn SOD activity.

A




B

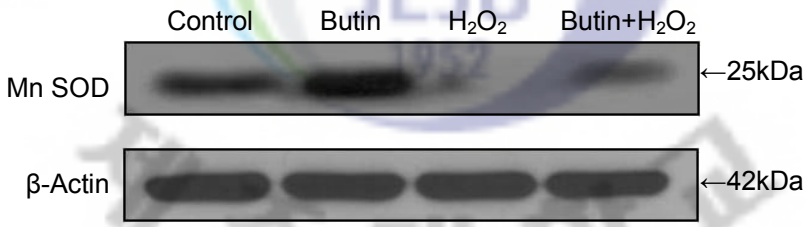



**Fig.15.** Effect of butin on Mn SOD mRNA expression, protein expression, and enzyme activity. (A) Cells were treated with butin at 10 μg/ml, and 1 h later, 1 mM $H_2O_2$ was added for 12 h. Total RNA was extracted, and Mn SOD mRNA expression was analyzed by RT-PCR. (B) Cell lysates were electrophoresed and the expression of Mn SOD protein was detected using a Mn SOD specific antibody.

C

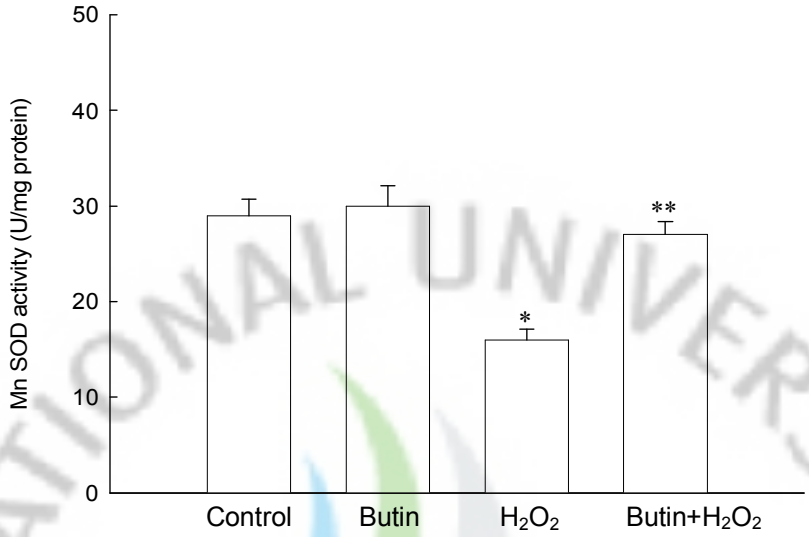



**Fig.15. continued.** (C) Mn SOD activity was expressed as average enzyme unit per mg protein ± SEM. Each column represents the mean ± SEM in triplicate experiments. *Significantly different from control cells ($p<0.05$). **Significantly different from $H_2O_2$-treated cells ($p<0.05$).

3.2. Butin increased the levels of Nrf2 transcription factor

Most of the genes encoding phase II detoxifying and antioxidant enzymes have an ARE sequence in their promoter region. Nrf2 is an important transcription factor that regulates ARE-driven Mn SOD gene expression. We examined whether butin is able to activate Nrf2 in association with Mn SOD up-regulation. Butin treatment restored Nrf2 protein expression which is attenuated by $H_2O_2$ treatment (Fig. 16A) and resulted in a translocalization of Nrf2 protein from the cytosol to the nucleus (Fig. 16B). Moreover, butin treated cells significantly elevated Nrf2 binding to the ARE sequence as assessed by the gel shift assay (Fig. 16C). These results further suggest that butin up-regulated the activation of Nrf2. To confirm the requirement of Nrf2 for butin-induced Mn SOD expression, cells were transfected with siNrf2 for 24 h prior to butin treatment. As shown in Fig. 16D, butin-induced expression of Mn SOD was markedly inhibited by siRNA knock down of Nrf2 gene. These results further suggest that Nrf2 mediates the butin-induced activation of the Mn SOD.

A

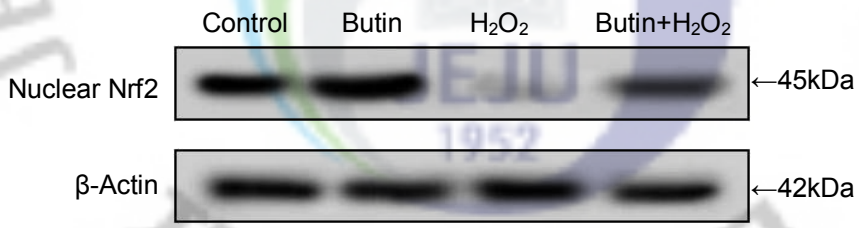



**Fig.16.** Effect of butin on levels of Nrf2 transcription factor. (A) Nuclear extracts from V79-4 cells were prepared after treatment with 10 μg/ml of butin and 1 h later, 1 mM $H_2O_2$ was add for 24 h. Western blot for nuclear lysates were detected with Nrf2-specific antibody.

B

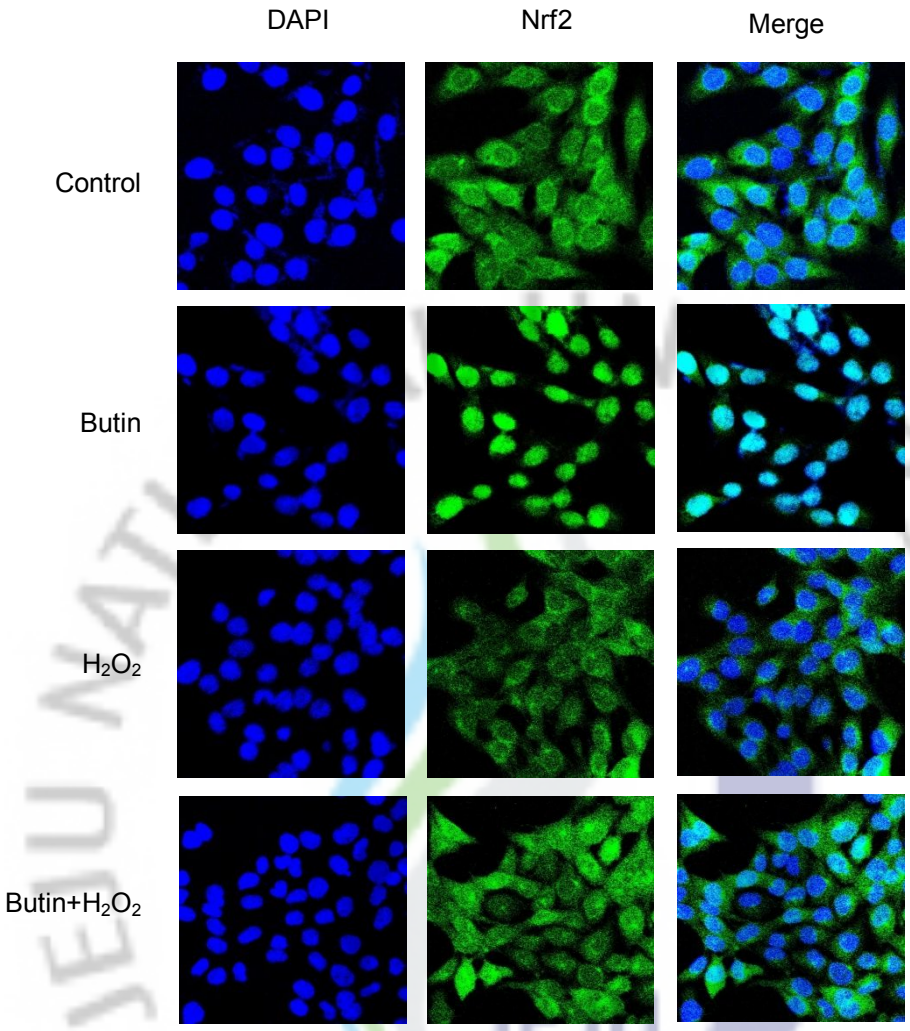
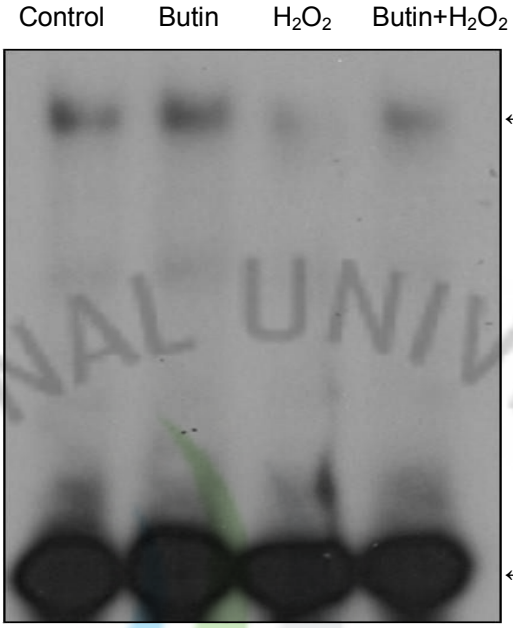



**Fig.16. continued.** Confocal imaging using FITC-conjugated secondary antibody staining indicates the location of Nrf2 (green) by anti-Nrf2 antibody, DAPI staining indicates the location of the nucleus (blue), and the merged image indicates the nuclear location of Nrf2 protein.

C




D

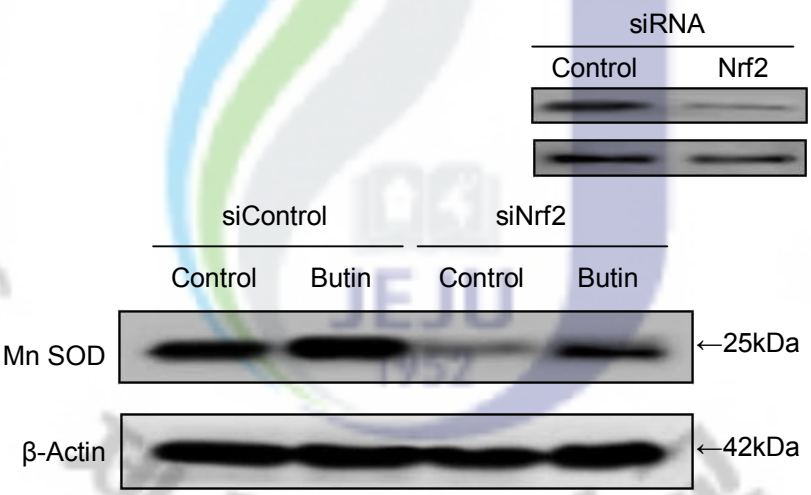



**Fig.16. continued.** (C) Nuclear extracts prepared from cells that were pretreated with 10 μg/ml of butin and 1 mM $H_2O_2$ was added after 1 h. Electrophoretic mobility shift assay was performed for the detection of ARE-binding activity of Nrf2. (D) Cells were transfected with 10-50 nM siControl and siNrf2 using lipofectamineTM 2000 based on the manufacturer's instruction. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 24 h and the expression of Mn SOD protein was examined by western blot analysis.

3.3. Butin activated Nrf2-driven Mn SOD via phosphorylation of PI3K/Akt

To further elucidate the upstream signaling pathway involved in butin-mediated Nrf2 activation and Mn SOD induction, we examined the activation of Akt, which is a major signaling enzyme involved in cellular protection against oxidative stress. Activation of Akt by butin was assessed using Western blot with phospho-specific antibodies against Akt. As shown in Fig. 17A, exposure to butin caused an increase in the phosphorylation of Akt in $H_2O_2$ treated cells. We then analyzed whether Akt pathways is involved in the induction of Nrf2 activation and Mn SOD expression. Cells were pre-incubated for 30 min with inhibitors of LY294002 (a PI3K inhibitor) and Akt inhibitor IV (an Akt inhibitor) and then treated with butin for additional 12 h. The effectiveness of these inhibitors was confirmed in butin-treated cells with antibodies specific for phospho Akt (data not shown). Increase in nuclear Nrf2 accumulation and Mn SOD protein expression occurred following treatment with butin as expected. Inhibition of the PI3K/Akt pathways dramatically reduced the capacity of butin to increase Nrf2 and Mn SOD protein levels (Fig. 17B). To further confirm these observations, cells were transfected with siAkt. As shown in Fig. 17C, the expression of nuclear Nrf2 and Mn SOD were markedly inhibited in siAkt-transfected cells regardless of butin treatment. These results indicate that PI3K/Akt is required to induce Mn SOD expression as well as nuclear accumulation of Nrf2.

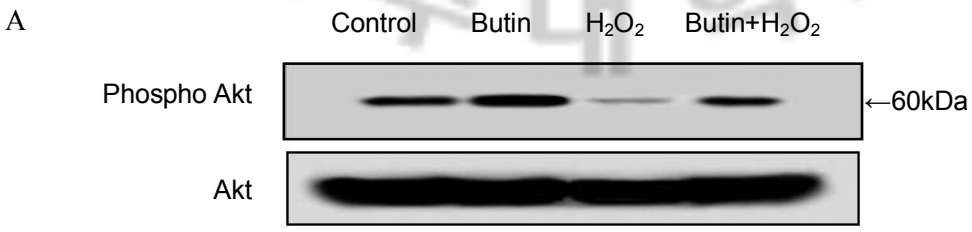
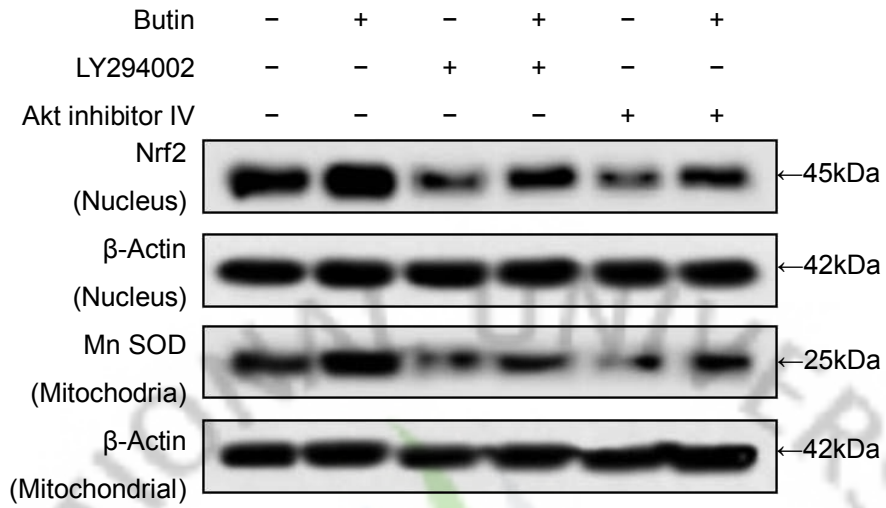



**Fig.17.** Induction of Mn SOD and activation of Nrf2 by butin via phosphorylation of PI3K/Akt. (A) Cell lysates were electrophoresed, and phospho-Akt, and Akt were detected using their respective specific antibodies.

B

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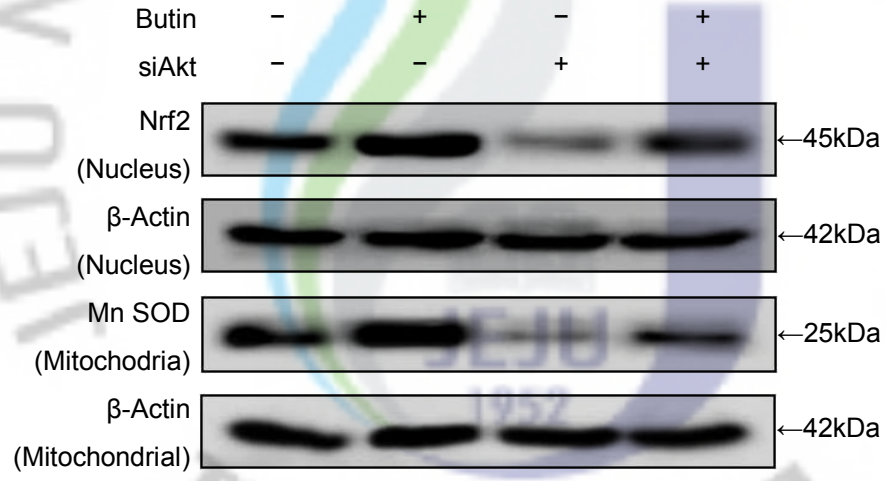


**Fig.17. continued.** (B) After treatment with LY294002 or Akt inhibitor IV, cell lysates were electrophoresed and nuclear Nrf2 and mitochondrial Mn SOD were detected using their respective specific antibodies. (C) Cells were transfected with 10-50 nM siControl and siAkt. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 24 h and the expression of nuclear Nrf2 and Mn SOD protein were examined by western blot analysis.

3.4. Involvement of Mn SOD in cell damage induced by oxidative stress

As shown in Fig. 18A, the protein expression of MnSOD was markedly inhibited in siMn SOD-transfected cells regardless of butin treatment. To determine whether the increased level of Mn SOD activity enhanced by butin confers cytoprotection against oxidative stress, V79-4 cells were pretreated with the Mn SOD inhibitor (DEDTC). DEDTC attenuated the protective effect of butin on $H_2O_2$-induced cytotoxicity and siMn SOD-transfected cells exhibited similar results (Fig. 18B). Therefore, the cytoprotective effect of butin is likely to be mediated through Mn SOD induction.

A

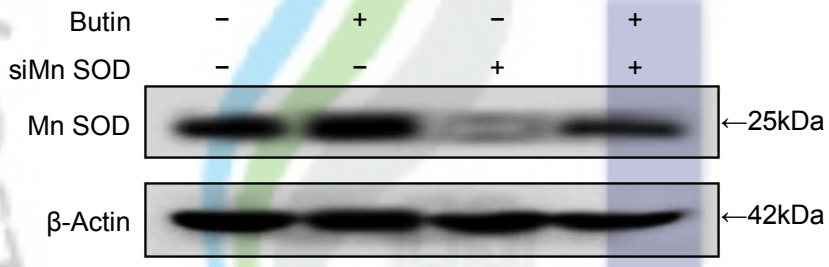


**Fig.18.** The cytoprotective effect of butin against $H_2O_2$-induced cell death via up-regulation the Mn SOD activity. (A) Cells were transfected with 10-50 nM siControl and siMn SOD. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 24 h and the expression of Mn SOD protein was examined by western blot analysis.

B

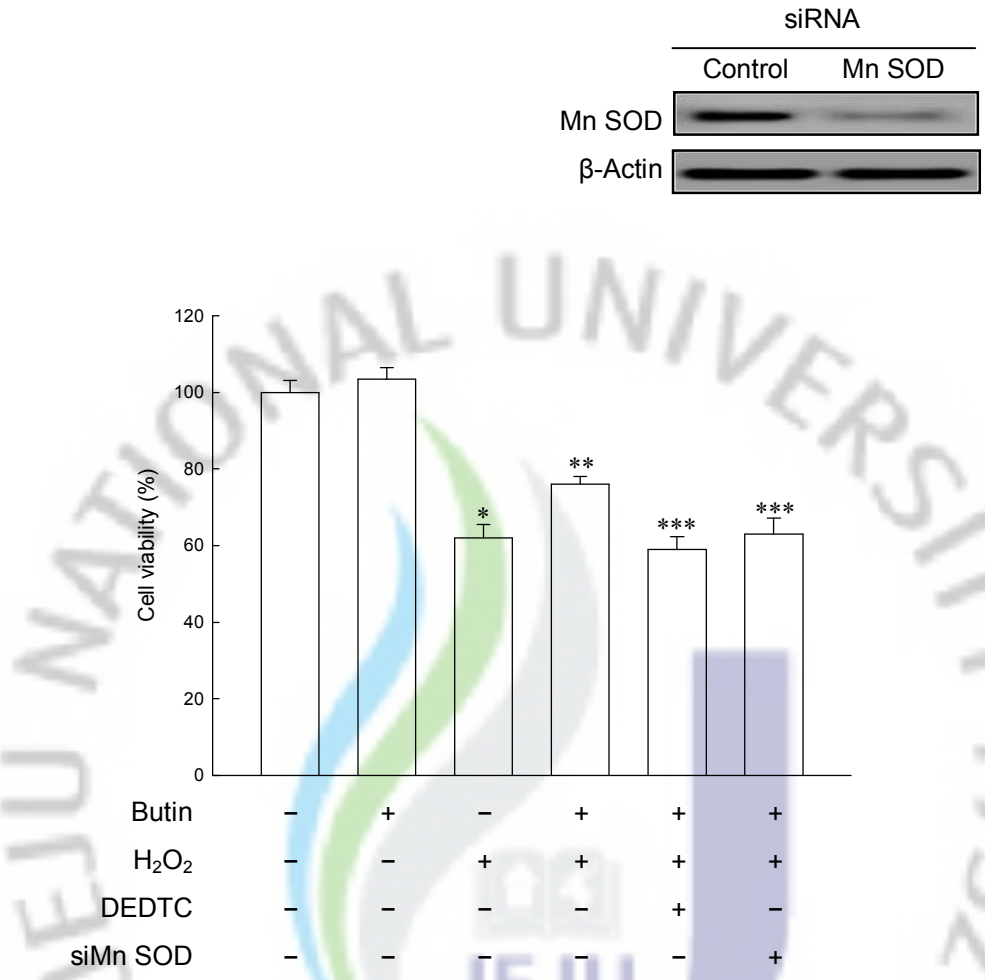


**Fig.18. continued.** (B) Cells were pre-incubated with DEDTC at 10 μM for 1 h or transfected with 10-50 nM siMn SOD, followed by 1 h of incubation with butin and exposure to 1 mM of $H_2O_2$ for 24 h. Cell viability was measured using the MTT assay. *Significantly different from control cells ($p<0.05$). **Significantly different from $H_2O_2$ treated cells ($p<0.05$). ***Significantly different from butin plus $H_2O_2$ treated cells ($p<0.05$).

# 4. DISCUSSION

Flavonoids like butin are known to possess powerful antioxidative properties, which are attributed to the presence of phenolic hydroxyl groups in the flavonoid structure. They usually contain one or more aromatic hydroxyl groups, and this moiety is responsible for the antioxidant activity of the flavonoid (van Acker et al., 2000). Recently, we demonstrated that butin provided protective effects against $H_2O_2$-induced apoptosis by scavenging ROS and activating antioxidant enzymes (Zhang et al., 2008), protected against oxidative DNA damage via activation of PI3K/Akt/OGG1 pathway (Kang et al., 2009) and reduced oxidative stress-induced mitochondrial dysfunction via scavenging of reactive oxygen species (Zhang et al., 2010). In this study, we demonstrate that butin modulates MnSOD induction in a PI3K/Akt/Nrf2 dependent pathway. To our knowledge, this is the first report demonstrating the capacity of butin to regulate the Mn SOD expression.

Oxidative stress refers to the mismatched redox equilibrium between the production of free radicals and the ability of cells to defend against them. One feasible way to alleviate free radical mediated cellular injuries is to augment the oxidative defense capacity through intake of antioxidants. Moreover, the induction of endogenous phase II detoxifying enzymes or antioxidative proteins seems to be a reasonable strategy for remitting disease aggravation. Although superoxide radicals are not considered as highly reactive when compared to other ROS, their toxicity may be, at least in part, due to their location in mitochondria, which makes an attack on critical targets in the respiratory chain highly possible. Manganese superoxide dismutase (Mn SOD) is a mitochondrial matrix enzyme that scavenges reactive oxygen species (ROS) and protects the cell against the insults of oxidative stress (Li et al., 1995). Therefore, many studies suggest that Mn SOD could play a key role in cell survival, and that Mn SOD is necessary for the maintenance of mitochondrial integrity in cells exposed to oxidative stress

(Oberley, 2004). Transcriptional regulation of Mn SOD is predominantly mediated by a redox-sensitive transcription factor NF-E2 related factor-2 (Nrf2) (Na et al., 2008). The ability of Nrf2 to up-regulate the expression of antioxidant genes via ARE suggests that increasing Nrf2 activity may provide a useful system for combating oxidative insults.

Nrf2, a member of the cap'n'collar family of bZIP transcription factors, can act as a master regulator of ARE-driven transactivation of antioxidant genes (Lee et al., 2005). A distinct set of Nrf2-regulated proteins detoxify xenobiotics, reduce oxidized proteins, maintain cellular reducing equivalents, disrupt redox cycling reactions, and counteract the noxious effects of ROS (Owuor and Kong, 2002; Jaiswal, 2004). Nrf2 is sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Kelch-like ECH associated protein 1 (Keap1). Dissociation of Nrf2 from the inhibitory protein Keap1 is a prerequisite for nuclear translocation and subsequent DNA binding of Nrf2. After forming a heterodimer with small Maf protein inside the nucleus, the active Nrf2 binds to cis-acting ARE or EpRE, also alternatively known as Maf recognition element (Juan et al., 2005). Besides the dissociation of the Nrf2-Keap1 complex that is facilitated by upstream kinase mediated signals, covalent modification of multiple cysteine residues on Keap1 by electrophiles or inducers of detoxifying enzymes is also considered to release Nrf2 from the Keap1 repression (Dinkova-Kostova et al., 2002). Multiple mechanisms of Nrf2 activation by signals mediated via one or more of the upstream kinases, including MAPKs, PI3K, PKC, and Akt, were recently reviewed (Chen and Kong, 2004; Lee and Surh, 2005; Kwak et al., 2004).

To determine the mechanism of butin recovering $H_2O_2$-induced the attenuation of MnSOD mRNA level and protein expression, as well as Mn SOD activity (Fig. 15), we examined the effect of butin on Nrf2 expression in V79-4 cells. Our study indicated that butin up-regulated MnSOD expression through regulation the activation of Nrf2 transcription factor. Butin increases the nuclear levels of the Nrf2 and its binding activity to the ARE which were

attenuated by $H_2O_2$ (Fig. 16). The mechanisms leading to nuclear translocation of Nrf2 include its release from Keap1 in the cytosol. However, because the half-life of Nrf2 transcription factor is very short, these mechanisms should necessarily rely on the stabilization of the Nrf2 protein (Jain et al., 2008; Lee et al., 2007). Our results demonstrate that butin increased the level of Nrf2, suggesting that butin may retard Nrf2 degradation. Most studies on the regulation of phase II gene expression have focused on the role of the PI3K/Akt pathways. Activation of the PI3K/Akt pathway is a key step in diverse biological processes, including cell proliferation, growth, and survival (Huang et al., 2004). Our results demonstrated that the increase of Nrf2-mediated Mn SOD protein level induced by butin was dependent on the activation PI3K/Akt, since LY294002 (a PI3K inhibitor), and Akt inhibitor IV as well as Akt siRNA decreased the butin-induced accumulation of Nrf2 and Mn SOD protein through inhibition of Akt phosphorylation. These results show that PI3K/Akt pathways are regulated by butin, and they participate in the induction of Nrf2 and thus regulate the Mn SOD expression. In addition, we showed that DEDTC, a potent inhibitor of MnSOD activity, and MnSOD siRNA can partially reverse the protective effects of butin, thus providing further evidence for MnSOD as a possible cytoprotective pathway for butin (Fig. 18).

In summary, the present results suggest that butin protects V79-4 cells against oxidative stress-induced cell death. This occurs via an elevated activation of PI3K/Akt, which appears to be responsible for nuclear translocation of Nrf2, its subsequent binding to ARE, and the up-regulation of Mn SOD gene expression (Fig. 19).

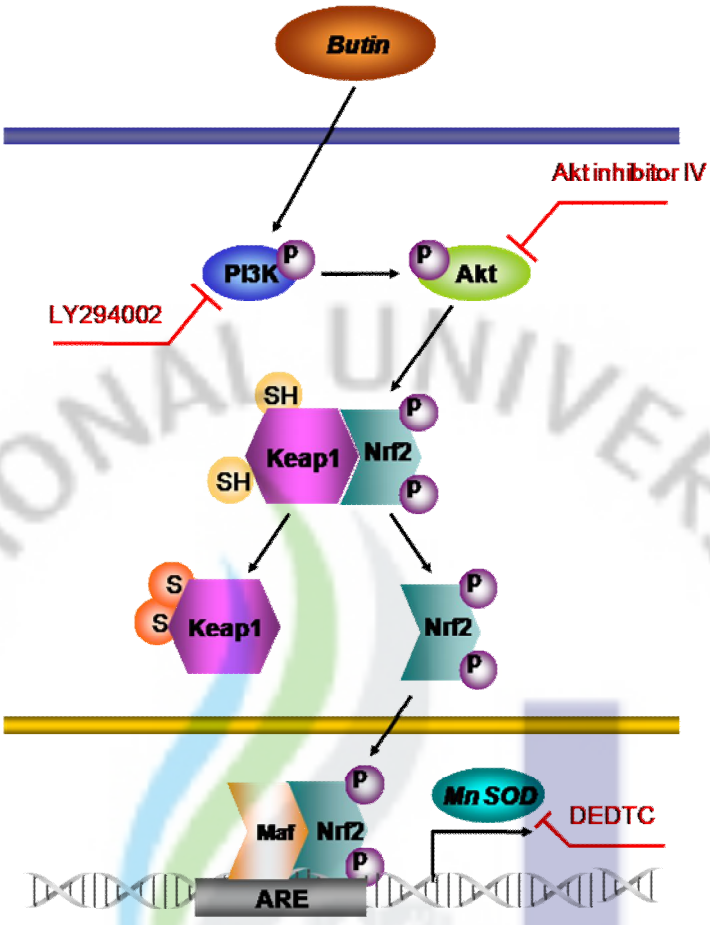


**Fig.19.** A proposed pathway for butin-induced Mn SOD via up-regulation of PI3K/Akt and Nrf2, explaining the cytoprotective effect of butin against oxidative stress in V79-4 cells.

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# Ⅵ. ABSTRACT IN KOREAN


플라보노이드는 다이어트의 중요한 요소로 야채와 과일에 보편적으로 존재하는 폴리페놀릭 화합물이다. 최근에는 우수한 약리작용과 세포독성이 낮은 천연 항산화제를 탐색하고 있으며 더욱이 프리 라디칼로 매개되는 인간 질환의 예방과 치료에 있어서 플라보노이드 약물의 잠재적 응용에 관한 관심이 매우 높다. 7,3',4'-Trihydroxydihydroflavone을 butin이라고도 명명하는데 강진향 (*Dalbergia odorifera*), 해홍두 (*Adenanthera pavanina*), 그리고 *Vernonia anthelmintica Willd* (살충 베르노니아) 와 같은 약초로부터 분리되었고 피부미백, 항재협착 특성과 같은 생물학적 활성을 가진다고 보고되었다. Butin은 프리 라디칼 소거능력에 의한 항산화제 활성을 갖고 있지만 지금까지 알려진 바로는 산화적 스트레스로 유도되는 세포 손상에 대한 확실한 메커니즘을 규명한 연구는 아직도 매우 적은 것으로 알려져 있다. 그러므로 본 논문은 butin의 보호작용과 관련된 메커니즘에 초점을 맞춰서 연구를 진행하였다.

우선, 산화적 스트레스로 유도되는 세포손상에 대한 butin의 보호작용은 가능하게 두 가지 활성과 연관되는바 프리 라디칼 소거작용과 같은 산소 라디칼 소거에 관한 직접적인 작용과 항산화 효소의 유발을 통한 간접적인 작용을 나타내었다.

미토콘드리아는 슈퍼옥사이드 생성의 주요원인이며 활성산소종의 직접적인 공격을 받는다. 그러므로 산화적 스트레스로 유도되는 미토콘드리아의 손상에 있어서 butin의 역할을 중점적으로 연구하였다. 그 결과 butin이 미토콘드리아의 ROS 축적을 감소하고 세포 내 $Ca^{2+}$ 레벨을 조절하고 미토콘드리아의 에너지 생성을 개선시킴으로써 미토콘드리아의 기능을 회복시킨다는 것을 발견하였다.

아시다 싶이 ROS는 미토콘드리아로 매개되는 아폽토시스 경로의 유발에 중요한 역할을 한다. 그러므로 막전위 탈분극의 붕괴 그리고 JNK와 미토콘드리아 관련 caspase 의존적 아폽토시스 경로의 억제작용을 통하여 산화적 스트레스로 유도되는 아폽토시스에 대한 butin의 보호작용을 증명하였다.

항산화 효소는 ROS의 독성에 대처할 수 있을 뿐만 아니라 게다가 항산화 효소의 유발은 효과적이고 충분하게 산화적 스트레스로부터 세포를 보호할 수 있다. Mn SOD는 중요한 항산화 효소로 미토콘드리아에서 슈퍼옥사이드 라디칼을 소거하는데 최근에는 보호 기능을 강조하고 있다. Mn SOD의 전사조절작용은 주요하게 산화환원반응에 민감한 전사인자 Nrf2에 의하여 매개된다. 게다가 PI3K/AKT는 Nrf2 활성의 유도를 통하여 항산화 반응 요소 (ARE)로 유발되는 유전자 발현을 조절한다. 본 연구는 butin이 PI3K/Akt의 활성화의 증가를 통하여 Nrf2의 핵내 이동으로 ARE에 결합함으로써 Mn SOD 발현을 상위 조절하여 산화적 스트레스로 유도되는 세포 손상을 감소시킨다는 것을 나타내었다.

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