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Protective effect of irisolidone, a metabolite of kakkalide, against hydrogen peroxide induced cell damage via antioxidant effect

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Abstract—The protective properties of irisolidone (a metabolite of kakkalide by intestinal bacteria) against hydrogen peroxide (H_2O_2) induced cell damage were investigated. Irisolidone was found to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and the intracellular reactive oxygen species (ROS), thereby preventing lipid peroxidation and DNA damage. Irisolidone inhibited apoptosis in Chinese hamster lung fibroblast (V79-4) cells induced by H_2O_2 via radical scavenging activity. This was achieved by the activation of the extracellular signal regulated kinase (ERK) and DNA binding activity of activator protein-1 (AP-1) (a downstream transcription factor of ERK) by irisolidone.

1. Introduction

Most traditional medicines are administered orally, and their components inevitably contact the microflora of the gastrointestinal tract. In addition, most of these components are transformed by intestinal bacteria before being absorbed from the gastrointestinal tract.[1–8] Recently, we reported that isoflavone metabolites, tectorigenin and glycitein, showed more biological effects than their precursors.[9,10]

In this study, kakkalide, a biologically active component isolated from the flowers of *Pueraria thunbergiana*, was transformed into irisolidone by human intestinal bacteria. Experimental evidences suggest that irisolidone possesses several properties including anti-*Helicobacter pylori* activity,[11] inhibition of prostaglandin E_2 production,[12] hepatoprotective effect,[13,14] cancer chemopreventive activity,[15] estrogenic effect,[16] inhibitory effect of JC-1 virus gene expression,[17] and anti-inflammatory effect.[18]Recently study has reported that irisolidone had a protective effect against ethanol or *tert*-butyl hyperoxide induced hepatic damage.[13,14] However, the precise mechanisms of the irisolidone effect on the cytoprotective effect have not yet been elucidated.

In our current study, we investigated the protective effect of irisolidone on cell damage induced by H_2O_2 in Chinese hamster lung fibroblast (V79-4) cells and the likely protective mechanism.

2. Results

2.1. Biotransformation of kakkalide by human intestinal bacteria

After kakkalide was incubated with human intestinal bacteria, irisolidone was isolated as metabolite (Fig. 1). The characteristics of irisolidone are: pale

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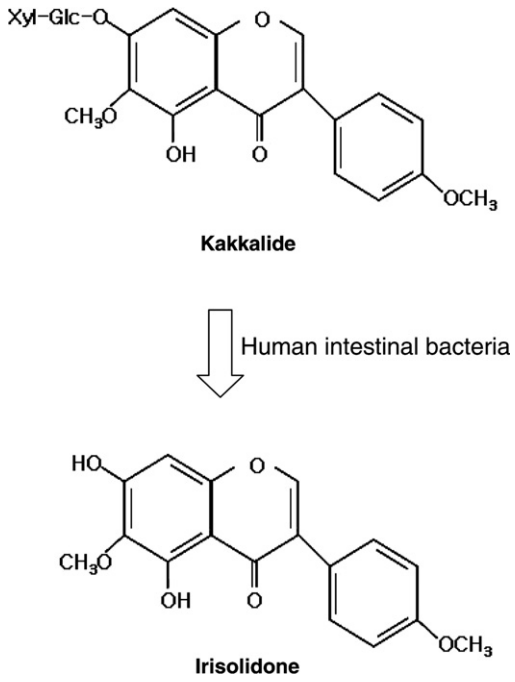

Figure 1. The metabolic formation of irisolidone. Irisolidone is formed by the transformation of kakkalide by human intestinal bacteria.

yellowish amorphous powder; mp 189–190 °C; IR (KBr)υ_{max}: 3447 (OH), 1648 (C=O), and 1023 cm^{-1}; FAB-MS: 315 $[M+H]^+$.

2.2. Radical scavenging activity of kakkalide and irisolidone

The scavenging effects of kakkalide and irisolidone (Fig. 1) on the DPPH free radical and the intracellular ROS were compared. The DPPH radical scavenging activity of kakkalide was 8% at 1 μg/mL, 12% at 10 μg/mL, and 28% at 50 μg/mL, and for irisolidone, it was 10% at 1 μg/mL, 19% at 10 μg/mL, and 44% at 50 μg/mL (Fig. 2A). The intracellular ROS scavenging activity of kakkalide was 29% at 1 μg/mL, 37% at 10 μg/mL, and 51% at 50 μg/mL. In the case of irisolidone, it was 41% at 1 μg/mL, 58% at 10 μg/mL, and 66% at 50 μg/mL (Fig. 2B). As shown in Figure 2C, the fluorescence intensity of DCF-DA staining was enhanced in H_2O_2 treated V79-4 cells. Irisolidone reduced the red fluorescence intensity upon H_2O_2 treatment, thus reflecting a reduction in ROS generation. The radical scavenging effect of irisolidone in both the experiments, however, was more effective when compared to kakkalide. Based on these results, we selected irisolidone as the active compound for further studies on radical scavenging effect.

2.3. Effect of irisolidone on lipid peroxidation and cellular DNA damage induced by H_2O_2

The abilities of irisolidone to inhibit membrane lipid peroxidation and cellular DNA damage in H_2O_2 treated cells were investigated. H_2O_2 induced damage to the cell membrane, one of the most important lesions to cells, responsible for the loss of cell viability. As shown in Figure 3, V79-4 cells exposed to H_2O_2 showed an increase to 1.3 μmole/mg protein of TBARS. However, irisolidone prevented the H_2O_2-induced peroxidation of lipids to 1.2, 1.0, and 0.8 μmole/mg protein at 1, 10, and 50 μg/mL, respectively. Damage to cellular DNA induced by H_2O_2 exposure was detected by assessing the phospho histone-H2A.X expression and the amount of 8-OHdG. The phosphorylation of nuclear histone H2A.X, a sensitive marker for breaks of double stranded DNA,[19] increased in the H_2O_2 treated cells as shown by Western blot (Fig. 4A). However, irisolidone decreased the expression of phospho H2A.X in H_2O_2 treated cells. Furthermore, 8-OHdG adduct in DNA has been most extensively used as a biomarker of oxidative stress.[20]As shown in Figure 4B, H_2O_2 treatment increased the amount of 8-OHdG to 10,013 pg/mL compared to 3033 pg/mL in control cells, and irisolidone treatment decreased 8-OHdG to 9280, 7956, and 6409 pg/mL at 1, 10, and 50 μg/mL, respectively, which suggests that irisolidone provides protection against H_2O_2 induced DNA damage.

2.4. Effect of irisolidone on cell death induced by H_2O_2

The protective effect of irisolidone on the cell survival of H_2O_2 treated cells was measured. As shown in Figure 5A, treatment with irisolidone increased the cell survival by 48% as compared to 35% of H_2O_2 treatment. To evaluate a cytoprotective effect of irisolidone on apoptosis induced by H_2O_2, the nuclei of V79-4 cells were stained with Hoechst 33342 and assessed by microscopy. The microscopic pictures in Figure 5B showed that the control cells had intact nuclei, while H_2O_2 treated cells showed significant nuclear fragmentation, which is characteristic of apoptosis. However, when the cells were treated with irisolidone for 1 h prior to H_2O_2 treatment, a decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of irisolidone against apoptosis was also confirmed by flow cytometric analysis and by ELISA based quantification of cytoplasmic histone-associated DNA fragmentation. As shown in Figure 5C, an analysis of the DNA content in the H_2O_2 treated cells revealed a 36% increase of the apoptotic sub-G_1 DNA content. In addition, treatment with 50 μg/mL of irisolidone decreased the apoptotic sub-G_1 DNA content to 24%. Furthermore, the treatment of cells with H_2O_2 increased the levels of cytoplasmic histone-associated DNA fragmentations compared to control group, however, treatment with 50 μg/mL of irisolidone decreased the level of DNA fragmentation (Fig. 5D). These results suggest that irisolidone protects cell viability by inhibiting H_2O_2 induced apoptosis.

2.5. Effect of irisolidone on ERK and AP-1 activation

To better understand the protective mechanism of irisolidone on V79-4 cells, we examined the activation of the ERK protein by Western blot analysis with the phospho-ERK specific antibody. As shown in Figure 6A, irisolidone dramatically activated phosphorylated ERK within 6 h. However, there was no change in the total ERK protein level. To determine the effect of ERK

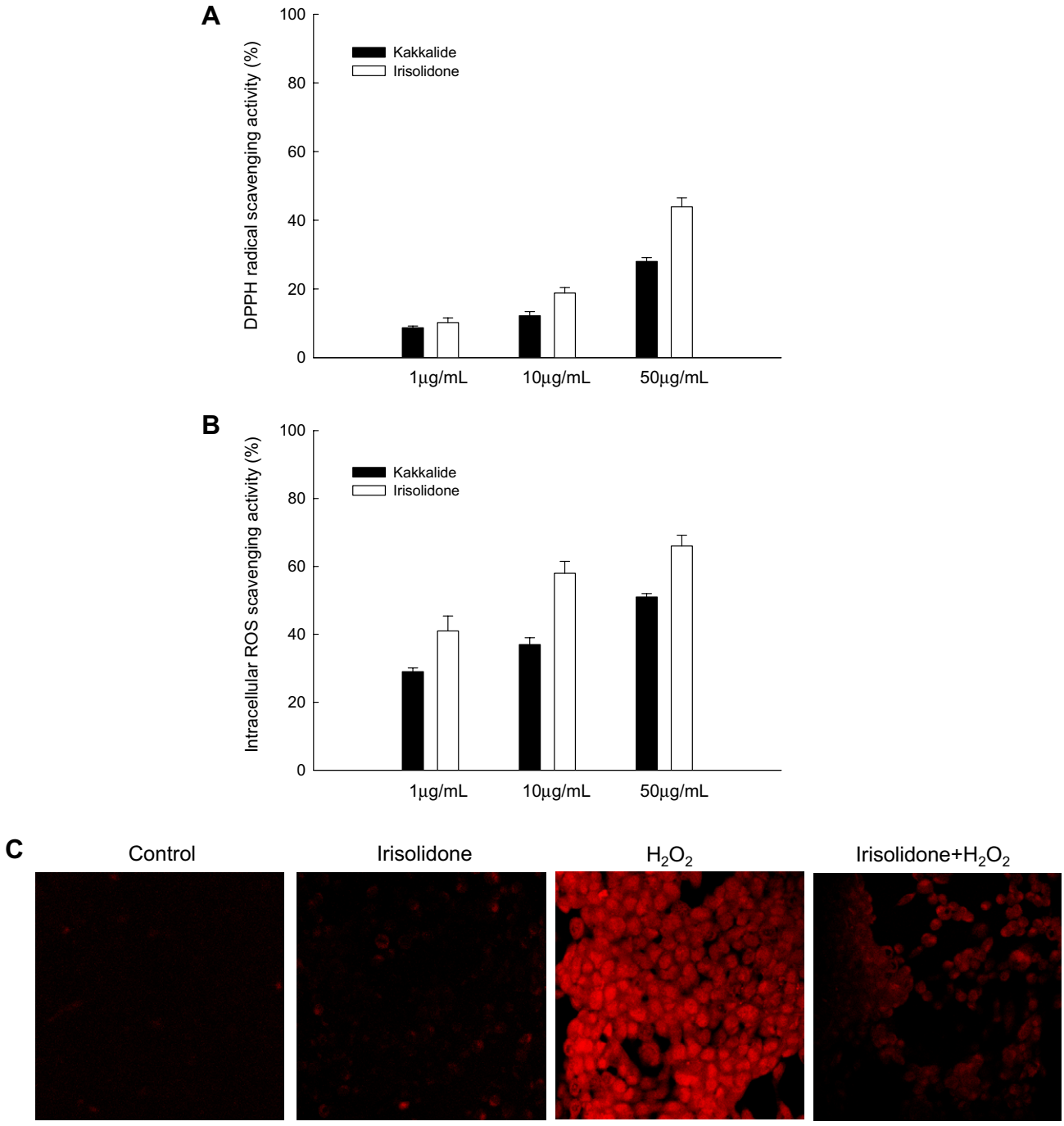

Figure 2. The effect of kakkalide and irisolidone on scavenging DPPH radicals and intracellular ROS. (A) The amount of DPPH radicals was determined spectrophotometrically at 520 nm. The measurements were made in triplicate and values were expressed as means ± SE. (B) The intracellular ROS generated was detected by the DCF-DA method. (C) Representative confocal images illustrate the increase in red fluorescence intensity of DCF 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 irisolidone (original magnification 400×).

inhibitor on protection of irisolidone from H_2O_2 induced damage, V79-4 cells were pre-treated for 30 min with U0126 (10 nM), a specific inhibitor of ERK kinase, followed by 30 min incubation with irisolidone and exposure to 1 mM of H_2O_2 for 24 h. As shown in Figure 6B, the U0126 treatment abolished the protective activity of irisolidone in H_2O_2 damaged cells. Subsequently, we examined the effect of irisolidone on AP-1, which is a downstream target of phospho-ERK pathway. As shown in Figure 6C, AP-1 activation in irisolidone treated cells was assessed by the EMSA with an oligonucleotide harboring a consensus AP-1 binding element. Irisolidone treated cells exhibited a high level of AP-1 activation. The transcriptional activity of AP-1 was also assessed using an AP-1 reporter plasmid containing the AP-1 binding DNA consensus site linked to a luciferase

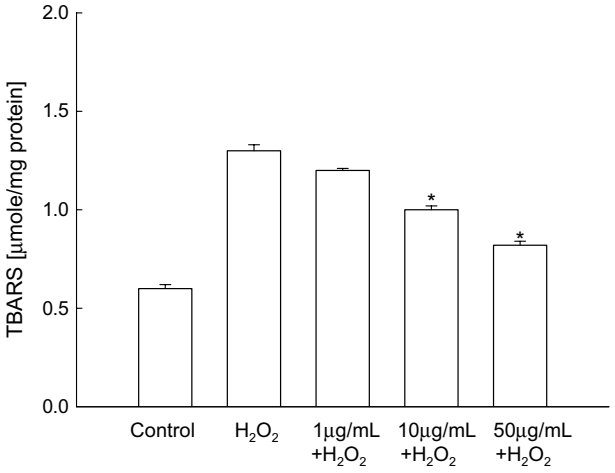

Figure 3. The effect of irisolidone on the inhibition of lipid peroxidation. Lipid peroxidation was assayed by measuring the amount of TBARS formation. The measurements were made in triplicate and the values expressed as means ± SE. *Significantly different from H_2O_2 treated cells ($p < 0.05$).

reporter gene. As shown in Figure 6D, irisolidone was found to increase the transcriptional activity of AP-1. These results suggest that irisolidone inhibits H_2O_2 induced cell death via the activation of the ERK and AP-1 protein.

3. Discussion

In this study, irisolidone, an isoflavone metabolite formed from the transformation of kakkalide (glycoside) by intestinal bacteria, demonstrated a more potent antioxidant effect than did kakkalide, which suggests that the aglycon form of this metabolite may possess active biological activity. In general, hydrophilic compounds with sugar moieties are not easily permeable into the cell membrane,[21] and hence glycosides are not easily absorbed from the gastrointestinal tract. Some reports suggest that the irisolidone form has more biologically active properties compared to the kakkalide form.[11,12,14,16,18] In our study, irisolidone was found to decrease the intracellular ROS and DPPH radical levels. The cells exposed to H_2O_2 exhibited the distinct morphological features of apoptosis, including nuclear fragmentation, sub-G_1 hypo-diploid cells, and DNA fragmentation. Cells that were pre-treated with irisolidone, however, had significantly reduced proportion of apoptotic cells. These findings suggest that irisolidone inhibited H_2O_2 induced apoptosis through its ROS scavenging effect. To further analyze the molecular mechanism underlying inhibitory effect of apoptosis by irisolidone, we investigated the ERK and AP-1 signaling pathway. ERK plays a major role in cell proliferation and differentiation as well as survival by various growth factors.[22] The activation of the ERK pathway induces cell proliferation, which leads to the induction of AP-1 activity, one of the transcription factors.[23,24] Irisolidone induced the activation of ERK and AP-1 pathway. The AP-1 transcription factor is mainly composed of either heterodimers of c-Jun/c-Fos or homodimers of c-Jun.[25] It has been reported that ERK signaling pathway leads to activation of c-Fos,[26] while JNK leads to c-Jun activation.[27] ERK pathway induces proliferation and cell growth of differentiation[28] and JNK pathway mediates inhibition of proliferation or cell death.[29] Recently, we reported that rhapontigenin compound protects cell death against oxidative stress and increased phosphory-

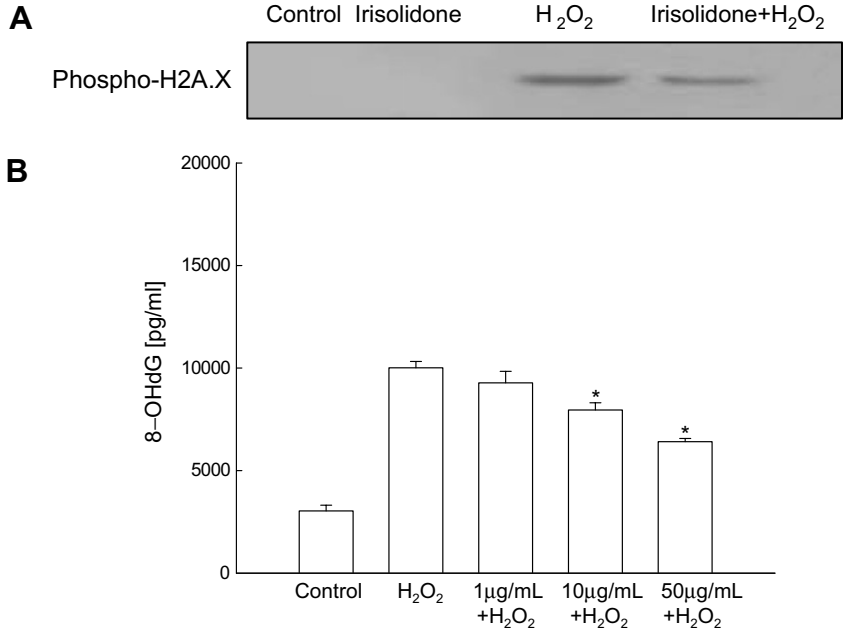

Figure 4. The effect of irisolidone on the inhibition of DNA damage. (A) The cell lysates were electrophoresed and phospho H2A.X protein was detected by specific antibody. (B) 8-OHdG content in cellular DNA was measured by an ELISA kit. The measurements were made in triplicate and the values expressed as means ± SE. *Significantly different from H_2O_2 treated cells ($p < 0.05$).

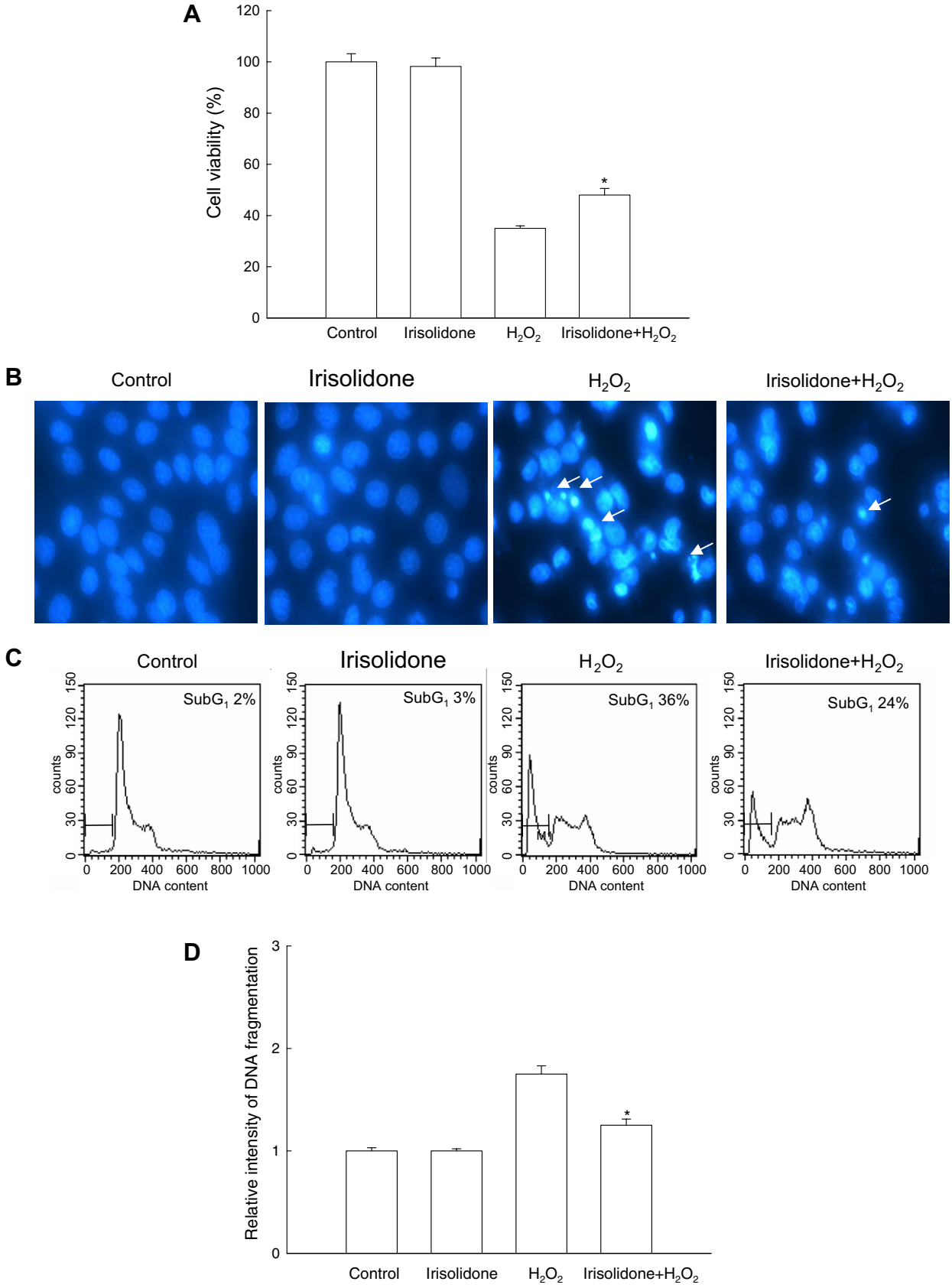

Figure 5. The protective effect of irisolidone on H_2O_2 induced cell damage. (A) The viability of V79-4 cells on H_2O_2 treatment was determined by a MTT assay. (B) The apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining. The apoptotic bodies are indicated by arrows. (C) The apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining. (D) DNA fragmentation was quantified with an ELISA kit. The measurements were made in triplicate and values are expressed as means ± SE. *Significantly different from H_2O_2 treated cells ($p < 0.05$).

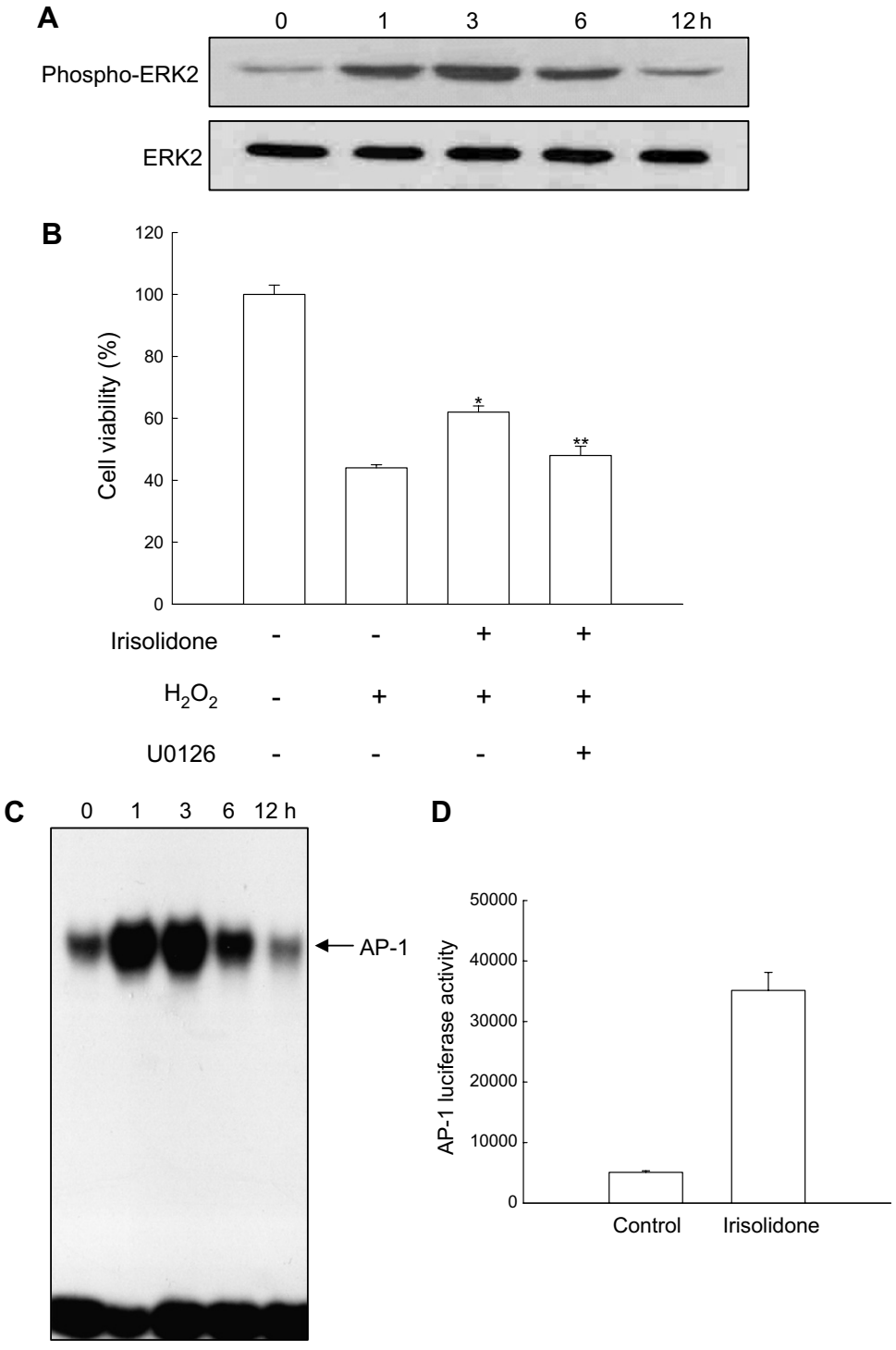

Figure 6. The effect of irisolidone on ERK and AP-1 activation. (A) The cell lysates were electrophoresed and cell lysates were immunoblotted using anti-phospho-ERK and anti-ERK antibodies. (B) After treatment with U0126, irisolidone, and H_2O_2, the viability of V79-4 cells was determined by an MTT assay. The measurements were made in triplicate and values are expressed as means ± SE. *Significantly different from H_2O_2 treated cells ($p < 0.05$), **significantly different from irisolidone plus H_2O_2 treated cells ($p < 0.05$). (C) The AP-1 specific oligonucleotide–protein complexes were detected by electrophoresis mobility shift assay. (D) The transcriptional activity of AP-1 was assessed using the plasmid containing AP-1 binding site-luciferase construct.

lation of ERK and inhibited the activity of AP-1. The difference of the mechanism of irisolidone and rhapontigenin might be that rhapontigenin exerts its effect via other transcription factors of ERK downstream or via inhibiting JNK activation, thus resulting in inhibition of AP-1 activity.

Irisolidone was found to significantly protect V79-4 cells against H_2O_2 induced damage, and in addition, the level of phosphorylated ERK and AP-1 activity was elevated, suggesting that the protective effect of irisolidone on cells may be involved in the activation of the ERK pathway.

4. Experimental

4.1. Preparation of kakkalide and irisolidone

Kakkalide and irisolidone were isolated from dried flowers of the *P. thunbergiana* according to the method described in Han et al.[13] The flowers of the *P. thunbergiana* were extracted with boiling water, concentrated in a rotary evaporator, extracted with ethyl acetate, and evaporated. The extract was loaded on a silica gel column chromatograph, eluted with $CHCl_3$/MeOH (20:1–4:1), and kakkalide was isolated. To obtain the metabolite of kakkalide by human intestinal bacteria, a reaction mixture was prepared containing 0.5 g of kakkalide and 0.5 g of fresh human feces in a final volume of 100 mL of anaerobic dilution medium according to the procedure described in Hattori et al.[30] The mixture was anaerobically incubated at 37 °C for 20 h and the reaction mixture was extracted with ethyl acetate. The ethyl acetate-soluble fraction of the reaction mixture was dried on a rotary evaporator and subjected to silica gel column chromatography with $CHCl_3$/MeOH (10:1–10:2). Irisolidone was obtained and identified according to the previously reported method.[11] The characteristics of irisolidone are: pale yellowish amorphous powder; mp 189–190 °C; IR (KBr)υ_{max}: 3447 (OH), 1648 (C=O), and 1023 cm^{-1}; FAB-MS: 315 $[M+H]^+$.

4.2. Reagents

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 were purchased from the Sigma Chemical Company (St. Louis, MO), and thiobarbituric acid from BDH Laboratories (Poole, Dorset, UK). Primary anti-ERK and anti-phospho-ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA), and primary phospho H2A.X antibody from Upstate Biotechnology (Lake Placid, NY). The plasmid containing the AP-1 binding site-luciferase construct was a generous gift of Dr. Young Joon Surh (Seoul National University, Seoul, Korea).

4.3. Cell culture

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

4.4. DPPH radical scavenging activity

Kakkalide and irisolidone at 1, 10, and 50 μg/mL were added to a 1×10^{-4} M solution of DPPH in methanol. The resulting reaction mixture was shaken vigorously. After 30 min, the amount of remaining DPPH was determined at 520 nm using a spectrophotometer.[31]

4.5. Intracellular reactive oxygen species (ROS) measurement

The DCF-DA method was used to detect the levels of intracellular ROS.[32] The V79-4 cells were seeded in a 96-well plate at 2×10^4 cells/well. Sixteen hours after plating, the cells were treated with kakkalide and irisolidone at 1, 10, and 50 μg/mL, and 30 min later, 1 mM of H_2O_2 was added to the plate. The cells were incubated for an additional 30 min at 37 °C. After adding 25 μM of the DCF-DA solution for 10 min, the fluorescence of 2′,7′-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission using a Perkin Elmer LS-5B spectrofluorometer (Foster, CA). For the image analysis for generation of intracellular ROS, the cells were seeded on coverslip loaded six-well plate at 2×10^5 cells/well. Sixteen hours after plating, the cells were treated with irisolidone and 30 min later, 1 mM of H_2O_2 was added to the plate. After changing the media, 100 μM of DCF-DA was added to each well and was incubated for an additional 30 min at 37 °C. Next, after washing with PBS, the stained cells were mounted onto microscope slide in mounting medium (DAKO, Carpinteria, CA). The images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a confocal microscope.

4.6. Lipid peroxidation assay

A lipid peroxidation was assayed by the thiobarbituric acid reaction.[33] The V79-4 cells were seeded in a culture dish at 1×10^6 cells/dish. Sixteen hours after plating, the cells were treated with 50 μg/mL of irisolidone. After 1 h, 1 mM of H_2O_2 was added to the plate and incubated for an additional hour. The cells were then washed with cold phosphate-buffered saline (PBS), scraped, and homogenized in an ice-cold 1.15% KCl. One hundred microliters of the cell lysates was mixed with 0.2 mL of 8.1% sodium dodecylsulfate, 1.5 mL of 20% acetic acid (adjusted to pH 3.5), and 1.5 mL of 0.8% thiobarbituric acid (TBA). The mixture was made up to a final volume of 4 mL with distilled water and heated to 95 °C for 2 h. After cooling to room temperature, 5 mL of an *n*-butanol/pyridine mixture (15:1, v/v) was added to each sample, and the mixture was shaken. After centrifugation at 1000*g* for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm. The amount of thiobarbituric acid reactive substance (TBARS) was determined using standard curve with 1,1,3,3,-tetrahydroxypropane.

4.7. Western blot

The V79-4 cells were placed in a culture dish at 1×10^6 cells/dish. Sixteen hours after plating, the cells were treated with 50 μg/mL of irisolidone. The cells were then harvested and washed twice with PBS. The harvested cells were then 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 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 electropho-

resed in 10% sodium dodecylsulfate–polyacrylamide gel. Moreover, blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were then incubated with primary antibodies. The membranes were further incubated with secondary immunoglobulin-G–horseradish peroxidase conjugates (Pierce, Rockford, IL). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) and then exposed to X-ray film.

4.8. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) assay

The amount of 8-OHdG in DNA was determined using the Bioxytech 8-OHdG-ELISA Kit purchased from OXIS Health Products (Portland, OR) according to the manufacturer's instructions. Cellular DNA was isolated using a DNAzol reagent (Life Technologies, Grand Island, NY) and quantified using a spectrophotometer.

4.9. Cell viability

The effect of irisolidone on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]bromide (MTT) assay.[34] To determine the cytoprotective effect of irisolidone in H_2O_2 treated V79-4 cells, cells were seeded in a 96-well plate at 2×10^4 cells/well. Sixteen hours after plating, the cells were treated with 50 μg/mL of irisolidone. One hour later, 1 mM of H_2O_2 was added to the plate and incubated at 37 °C for an additional 24 h. Fifty microliters of the MTT stock solution (2 mg/mL) was then added into each well to attain 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 were aspirated. The formazan crystals of each well were dissolved in 150 μL of dimethylsulfoxide and were read at A_{540} on a scanning multi-well spectrophotometer.

4.10. Nuclear staining with Hoechst 33342

The V79-4 cells were placed in a 24-well plate at 2×10^5 cells/well. Sixteen hours after plating, the cells were treated with 50 μg/mL of irisolidone and after further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, 1.5 μL of Hoechst 33342 (stock 10 mg/mL), a DNA specific fluorescent dye, was added to each well (1.5 mL) and incubated for 10 min at 37 °C. The stained cells were then observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.

4.11. Flow cytometry analysis

We performed flow cytometry to determine the content of apoptotic sub-G_1 hypo-diploid cells.[35]The V79-4 cells were placed in a six-well plate at 5×10^5 cells/well. At 16 h after plating, the cells were treated with 50 μg/mL of irisolidone. After incubation for an additional hour, 1 mM of H_2O_2 was added to the culture. After 24 h, the cells were harvested and fixed in 1 mL of 70% ethanol for 30 min at 4 °C. The cells were washed twice with PBS, and then incubated for 30 min in dark at 37 °C in 1 mL of PBS containing 100 μg of propidium iodide and 100 μg of RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The proportion of sub-G_1 hypo-diploid cells was assessed by the histograms generated using the computer program, Cell Quest and ModFit.

4.12. DNA fragmentation

The V79-4 cells were placed in a culture dish at 1×10^6 cells/dish. Sixteen hours after plating, the cells were treated with 50 μg/mL of irisolidone. After a further incubation for 1 h, 1 mM of H_2O_2 was added to the culture, and the cells were harvested after 24 h. Cellular DNA fragmentation was assessed by analysis of the cytoplasmic histone-associated DNA fragmentation using a kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions.

4.13. Preparation of nuclear extract and electrophoretic mobility shift assay

The V79-4 cells were placed in a culture dish at 1×10^6 cells/dish. Sixteen hours after plating, the cells were treated with 50 μg/mL of irisolidone. After a further incubation for 1 h, 1 mM of H_2O_2 was added to the culture. After 6 h, the cells were harvested 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 3000*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 °C after determining the protein concentration. The oligonucleotides containing the transcription factor AP-1 consensus sequence (5′-CGC TTG ATG ACT CAG CCG GAA-3′) were annealed, labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and used as probes. The probes (50,000 cpm) were incubated with 6 μg of the nuclear extracts at 4 °C for 30 min in a final volume of 20 μL containing 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris–HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly(-dI–dC). Binding products were resolved on 5% polyacrylamide gel and the bands were visualized by autoradiography.

4.14. Transient transfection and AP-1 luciferase assay

The V79-4 cells were placed in culture dish at 1×10^6 cells/dish and were transiently transfected with the plasmid harboring the AP-1 promoter using DOTAP as the transfection reagent according to the instructions given by the manufacturer's instruction (Roche Diagnostics). After an overnight transfection, the cells were treated with 50 μg/mL of irisolidone. After a further incubation for 1 h, 1 mM of H_2O_2 was added to the culture. After 6 h, the cells were then washed twice

with PBS and lysed with reporter lysis buffer (Promega, Madison, WI). After 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 20 μL of the cell extract was mixed with 100 μL of the luciferase assay reagent at room temperature, the mixture was placed in a illuminometer to measure the light produced.

4.15. Statistical analysis

All the measurements were made in triplicate and all values were expressed as means ± standard error (SE). The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the differences. $p < 0.05$ were considered to be significant.

Acknowledgments

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