



Antioxidant activity and cell protective effect of loliolide isolated from *Sargassum ringgoldianum* subsp. *coreanum*

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A monoterpene lactone, loliolide was successively isolated from brown seaweed, *Sargassum ringgoldianum* subsp. *coreanum* using column chromatography and high-performance liquid chromatography. The structure of the isolated compound was determined by comparison of EIMS and NMR spectral data with the previously published data. The antioxidant activities of loliolide were also evaluated by DPPH and H₂O₂ radical and intracellular reactive oxygen species scavenging assays, and it showed moderate activities in all antioxidant assays. Furthermore, loliolide was found to exert positive dose-dependent effects in the protective effects against H₂O₂-induced cell damage which were determined via MTT, Hoechst staining assays and cell cycle analysis. This is the first study on chemical constituent of this seaweed according to our best knowledge. These results clearly indicated that loliolide isolated from *Sargassum ringgoldianum* subsp. *coreanum* can protect the cells against H₂O₂-induced cell damage or apoptosis.

Key Words: antioxidant; cell cycle; cell protective; Loliolide; *Sargassum ringgoldianum*

INTRODUCTION

Recently, natural products derived from marine resources have attracted great attention of researchers in drug discovery. Marine brown algae (division Phaeophyta) produce a wide variety of secondary metabolites, such as terpenoids, oxylipins, phlorotannins, volatile hydrocarbons, and steroids. Several of these compounds have been reported to exhibit a wide spectrum of biological activities such as cytotoxic, antimicrobial, and antioxidant activities as well as inhibitory activity against various enzymes (Chang et al. 2008).

Sargassum ringgoldianum subsp. *coreanum* belongs to family of Sargassaceae, is a member of *Sargassum* genus. It widely grows on the coast of Jeju island, Korea. It is often used as animal feed. Ko et al. (2010) and Heo et al. (2005b) reported that the Celluclast and Neutrase

extracts of *S. coreanum* exhibited higher DPPH and hydrogen peroxide (H₂O₂) scavenging activities compared to those of the other enzymatic extracts. The hot water extract of *S. coreanum* was reported to have the moderate anticoagulant activity (Athukorala et al. 2007). The water extract of *S. coreanum* also had the anti-hypertension activity (Athukorala and Jeon 2005). The seasonal and portional variations in the composition of alginic acid from *S. coreanum* were investigated by Kim and Park (1985). To our best knowledge, there is no report on the chemical constituents of *S. coreanum*.

In the course of the phytochemical study of the marine seaweeds from Jeju Island, we have now investigated the constituents of *S. coreanum*. In the present study, a monoterpene lactone, loliolide was isolated from the

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EtOAc fraction of *S. coreanum*. Its antioxidant activities were evaluated by DPPH, H₂O₂ radicals and intracellular reactive oxygen species (ROS) scavenging activities, and the protective effects against H₂O₂-induced cell damage or apoptosis via MTT, Hoechst nuclear staining assays and cell cycle analysis.

MATERIALS AND METHODS

General materials

¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan), using dimethylsulfoxide (DMSO)-*d*₆ solvent peak (2.50 ppm in ¹H and 39.5 ppm in ¹³C NMR) as an internal reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer (JEOL). Column chromatography was carried out by Silica Gel 60 (230-400 mesh, Merck, Darmstadt, Germany), ODS (12 nm, YMC, Kyoto, Japan). Thin-layer chromatography was run on pre-coated Merck Kieselgel 60 F254 plates (0.25 mm). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342 and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

Algal materials

The marine brown seaweed, *S. coreanum* was collected from the coast of Jeju Island, Korea, in July, 2008. The fresh seaweed were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and maintained in a medical refrigerator at -20°C. The lyophilized *S. coreanum* was ground into powder before extraction.

Extraction and isolation

The dried *S. coreanum* powder (500 g) was extracted by stirring with 80% methanol (3 × 4 L) at room temperature for 24 h and filtered. The solvent was removed under vacuum at 40°C. The extract was partitioned with *n*-hexane (5.79 g), EtOAc (4.39 g), and *n*-BuOH (40.15 g) in sequence. The EtOAc extract (SCE), which exhibited a most potent antioxidant activity, was subject to a silica gel flash chromatography eluted with *n*-hexane / EtOAc

/ MeOH (gradient) to yield eight sub-fractions (F1-F8). F4 was further purified by ODS column with H₂O-MeOH (gradient) to give ten fractions (F4.1-F4.10). The fraction F4.2 was then separated by preparative reverse-phase high-performance liquid chromatography (HPLC) utilizing a gradient program of 0 to 100% MeOH in water to furnish the compound (15.2 mg). The HPLC was carried out using a Dionex HPLC system equipped with a UV-D170U detector (Sunnyvale, CA, USA). The detector was set at 220 nm.

Loliolide: white powder. EIMS: *m/z* 197 [M+H]⁺ (C₁₁H₁₆O₃); ¹H-NMR (DMSO-*d*₆, 400 MHz): δ 5.80 (1H, s, H-7), 4.84 (1H, s, -OH), 3.97 (1H, m, H-3), 2.33 (1H, dd, J = 12.6; 2.08 Hz, H-4), 1.87 (1H, dd, J = 12.6; 2.32 Hz, H-2), 1.51 (3H, s, H₃-11), 1.16 (1H, t, J = 11.56 Hz, H-4'), 1.27 (1H, t, J = 12.12 Hz, H-2'), 1.22 (3H, s, H₃-9), 1.19 (3H, s, H₃-10); ¹³C NMR (DMSO-*d*₆, 100 MHz): δ 181.7 (C-6), 170.9 (C-8), 112.3 (C-7), 86.4 (C-5), 62.9 (C-3), 49.6 (C-2), 47.8 (C-4), 34.7 (C-1), 29.7 (CH₃-10), 25.2 (CH₃-11), 24.6 (CH₃-9).

DPPH radical scavenging assay

DPPH radical scavenging activity of the isolated compound was determined according to a slightly modified method of Blois (1958). DPPH solution was prepared at the concentration of 4 × 10⁻⁴ M in MeOH. The different concentrations of the test samples (100 μL) and 100 μL of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated at room temperature for 30 min. Thereafter, the absorbance was measured at 517 nm by enzyme-linked immunosorbent assay (ELISA) reader. The scavenging activity was calculated according the following equation:

$$\text{DPPH scavenging activity (\%)} = [1 - (A_{\text{sample}} / A_{\text{control}})] \times 100$$

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Müller (1985). A hundred microliter of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96 microwell plate. A 20 μL of hydrogen peroxide was added to the mixture, and then incubated at 37°C for 5 min. After the incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit mL⁻¹) were added to the mixture. The reaction mixture was incubated at 37°C for 10 min. The absorbance was measured at 405 nm by ELISA reader.

Cell culture

Monkey kidney fibroblast cell line (Vero cell) was purchased from Korean Cell Line Bank (KCLB), maintained at 37°C in an incubator with humidified atmosphere of 5% CO₂. Cells were cultured at a concentration of 1 × 10⁵ cells mL⁻¹ in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 µg mL⁻¹), and penicillin (100 unit mL⁻¹) for further experiments.

Determination of intracellular ROS by DCFH-DA

For the detection of intracellular ROS, the DCFH-DA method as previously described by Rosenkranz et al. (1992) was used. Vero cells were seeded in 96-well plates at a concentration of 1 × 10⁵ cells mL⁻¹. After 16 h, the cells were treated with various concentrations of the test samples, and incubated at 37°C under a humidified atmosphere. After 30 min, 10 µL of 1 mM H₂O₂ was added, and then cells were incubated for an additional 30 min at 37°C. Finally, 2',7'-dichloro-dihydrofluorescein diacetate (DCFH-DA; 5 µg mL⁻¹) was introduced to the cells, and 2',7'-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Waltham, MA, USA). The percentage of intracellular ROS scavenging activity was calculated according to the following equation:

$$\text{Intracellular ROS scavenging activity (\%)} = [1 - (C_1 / C_0)] \times 100$$

Where C₁ is the fluorescence intensity of cells treated with H₂O₂ and the test samples, and C₀ is the fluorescence intensity of cells treated with H₂O₂ and distilled water instead of the test samples.

Assessment of cell viability

Cell viability was estimated via a MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosman 1983). The cells were seeded in 96-well plate at a concentration of 1 × 10⁵ cells mL⁻¹ (180 mL). After 16 h incubation at 37°C under a humidified atmosphere, the cells were treated with various concentrations of the compound (10 µL), and further incubated for 30 min. Then, 10 µL of H₂O₂ (1 mM) was added to the cell culture medium, and incubated for 24 h at 37°C. The

50 µL of MTT stock solution (2 mg mL⁻¹) was then applied to the wells, to a total reaction volume of 250 mL. After 4 h of incubation, the plates were centrifuged for 5 min at 800 ×g, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 mL of DMSO, and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cell with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and / or fragmentation was indicative of apoptosis (Gschwind and Huber 1995, Lizard et al. 1995). The cells were placed in 24-well plates at a concentration of 1 × 10⁵ cells mL⁻¹ (950 µL). Sixteen h after plating, the cells were treated with various concentration of the compounds (50 µL), and further incubated for 1 h prior to expose to H₂O₂ (1 mM). After 24 h, 3 µL of Hoechst 33342 (stock 10 mg mL⁻¹), a DNA-specific fluorescent dye, were added to each well, followed by 10 min of incubation at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera (Maryland, USA), in order to examine the degree of nuclear condensation.

Cell cycle analysis

Cell cycle analysis was conducted to determine the proportion of apoptotic sub-G1 hypodiploid cells (Nicolletti et al. 1991). The Monkey kidney fibroblast cells (Vero cell) were plated in 6-well plates at a concentration of 4.0 × 10⁵ cells mL⁻¹ and incubated for 24 h at 37°C under a humidified atmosphere. The cells were then treated with different concentration of loliolide. After incubation for 1 h, 150 µL of 1 mM H₂O₂ was added, and the cells were incubated for an additional 6 h at 37°C. Finally the cells were harvested and fixed in 1 mL of 70% ethanol for 30 min at 4°C. The cells were washed twice with phosphate buffered saline (PBS) and incubated in darkness in 500 µL of PBS containing 50 µg PI and 50 µg RNase A for 30 min at 37°C. Flow cytometric analysis was conducted

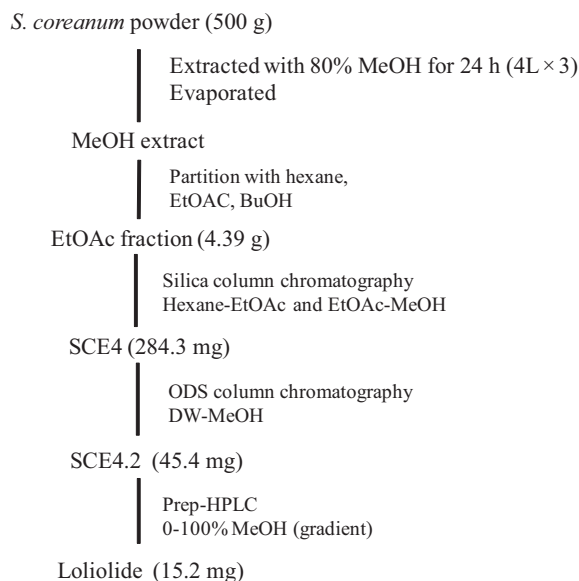


Fig. 1. Isolation procedure of loliolide from *Sargassum ringoldianum* subsp. *coreanum*. HPLC, high-performance liquid chromatography.

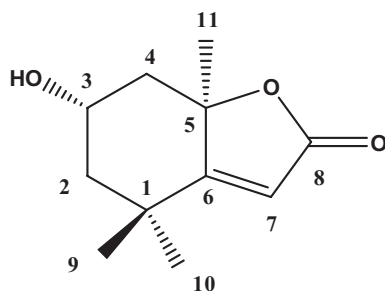


Fig. 2. Chemical structure of loliolide.

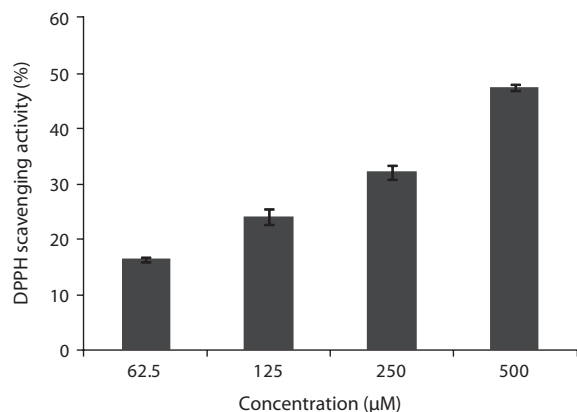


Fig. 3. DPPH radical scavenging activity of loliolide isolated from *Sargassum coreanum*.

with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect on the cell cycle was determined by changes in the percentage of cell distribution at each cell cycle phase, and assessed by histograms generated by the Quest and Mod-Fit computer programs (Wang et al. 1999).

RESULTS

Isolation and structure determination of loliolide from *Sargassum ringoldianum* subsp. *coreanum*

The EtOAc fraction was purified by silica gel and ODS column chromatography (Fig. 1). A monoterpene compound, loliolide, was successively isolated using HPLC from the sub-fraction. The chemical structure of the compound was elucidated on the basis of spectral analysis of MS and NMR (^1H and ^{13}C) data, and also comparing with the previous published data (Zhang et al. 2010). The compound, loliolide, was obtained from this species for the first time. The structure was shown in Fig. 2.

Antioxidant activities of loliolide

To evaluate antioxidant activities of loliolide isolated from *S. coreanum*, DPPH free radical, H_2O_2 and intracellular ROS scavenging assays were adopted.

DPPH is a free-radical generating compound and has been widely used to evaluate the free-radical scavenging ability of various samples. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Li et al. 2009). Hydrogen peroxide is a product derived from normal metabolism, but is not an inherently reactive non-radical compound. However, hydrogen peroxide can be converted into highly reactive and deleterious products such as oxygen, and hydroxyl radical (Heo et al. 2005a). However, the compound showed moderate scavenging activities on both DPPH free radical and hydrogen peroxide. Up to 500 μM , the scavenging activity on DPPH (Fig. 3) was 47.5%, and up to 250 μM , hydrogen peroxide scavenging activity was 55.2% (Fig. 4). Furthermore, the intracellular ROS scavenging activities of loliolide was also determined. The fluorescence intensity of the negative control cells (non-treated cells) was recorded as 3,299.33; whereas it was 13,003 in the positive control (H_2O_2 treated cells). After addition of loliolide to the cells mixed with H_2O_2 , the intensity was reduced to 6,236.33, 5,816.67 and 5,312.33 at concentration of 125, 250, and 500 μM . The intracellular

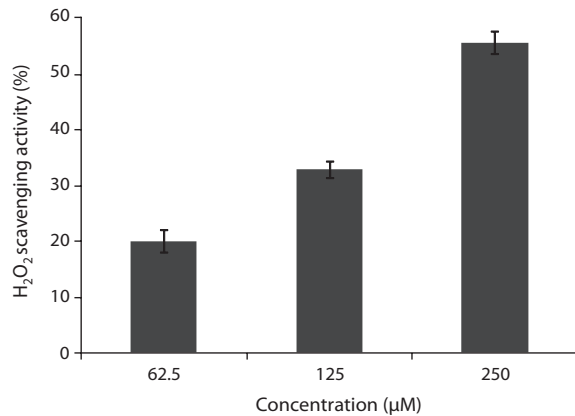


Fig. 4. H₂O₂ scavenging activity of loliolide isolated from *Sargassum coreanum*.

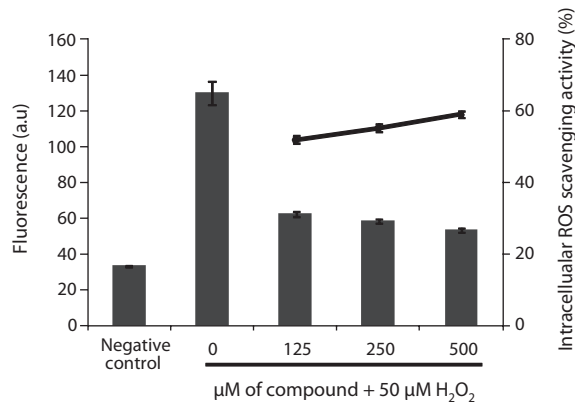


Fig. 5. Intracellular reactive oxygen species (ROS) scavenging activities of loliolide isolated from *Sargassum coreanum*. Cells were treated with loliolide at concentrations of 125, 250, and 500 μM. After an additional 30 min, the intracellular ROS generated was detected by spectrofluorometry after the DCFH-DA treatment. (■) Fluorescence intensity; (●) Intracellular ROS scavenging activity.

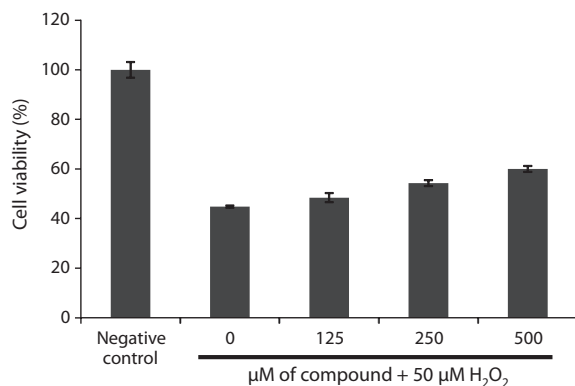


Fig. 6. Protective effect of loliolide isolated from *Sargassum coreanum* on H₂O₂-induced oxidative cell damage. The cells were treated with loliolide at concentrations of 125, 250, and 500 μM. After 1 h, 1 mM H₂O₂ was added to the plate. After an incubation of 24 h, cell viability was determined by MTT assay.

ROS scavenging activities of loliolide was 52.0, 55.3 and 59.1% at concentration of 125, 250, and 500 μM, respectively (Fig. 5).

Determination of cell protective effects of loliolide

Because loliolide showed DPPH free radical, H₂O₂ and intracellular ROS scavenging activity, the cell protective effects of loliolide on H₂O₂-induced cell damage were also determined via different assays, including MTT, nuclear staining and cell cycle analysis. The protective effect of loliolide on cell viability against H₂O₂-induced Vero cell damage was assessed using MTT assay. As shown in Fig. 6, cell viability in non-treated cell was assigned as 100%, whereas in the H₂O₂-treated cells, the cell viability was reduced to 44.8%. However, loliolide could prevent the H₂O₂-induced cell damage. The cell viability was increased to 48.4, 54.3 and 60.0%, as increasing the concentrations from 125 to 500 μM.

The protective effects of loliolide on H₂O₂-induced apoptosis were investigated via nuclear staining with Hoechst 33342. Hoechst 33342 is used for specifically staining the nuclei of living or fixed cells and has been used extensively for the detection of nuclear shrinkage, including chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, all of which are indicative of apoptosis. As shown in Fig. 7, uninjured nuclei were clearly observed in the non-treated group (Fig. 7A). When the cell was treated with H₂O₂, cell apoptosis which was expressed as a significant level of nuclear fragmentation was appeared (Fig. 7B). However, the cells co-incubated with different concentration of loliolide and H₂O₂ could reduce the apoptosis of cells (Fig. 7C & D). Particularly, at the concentration of 500 μM, a significant reduction of fragmentation was observed. This result suggested that loliolide show an ability to protect cells against H₂O₂-induced nuclear fragmentation.

Finally, the protective effects of loliolide were also investigated via flow cytometry. Compared with non-treated cells (Fig 7A), the sub-G1 DNA content was increased to 47.5% when the cells were treated with H₂O₂ (Fig. 7B). This result revealed that addition of H₂O₂ could induce the apoptosis. Whereas, the cells pretreated with 250 and 500 μM of loliolide, the sub-G1 contents were significantly reduced to 30.33 and 26.24%, respectively.

DISCUSSION

Recently, marine brown and red algae have been well-

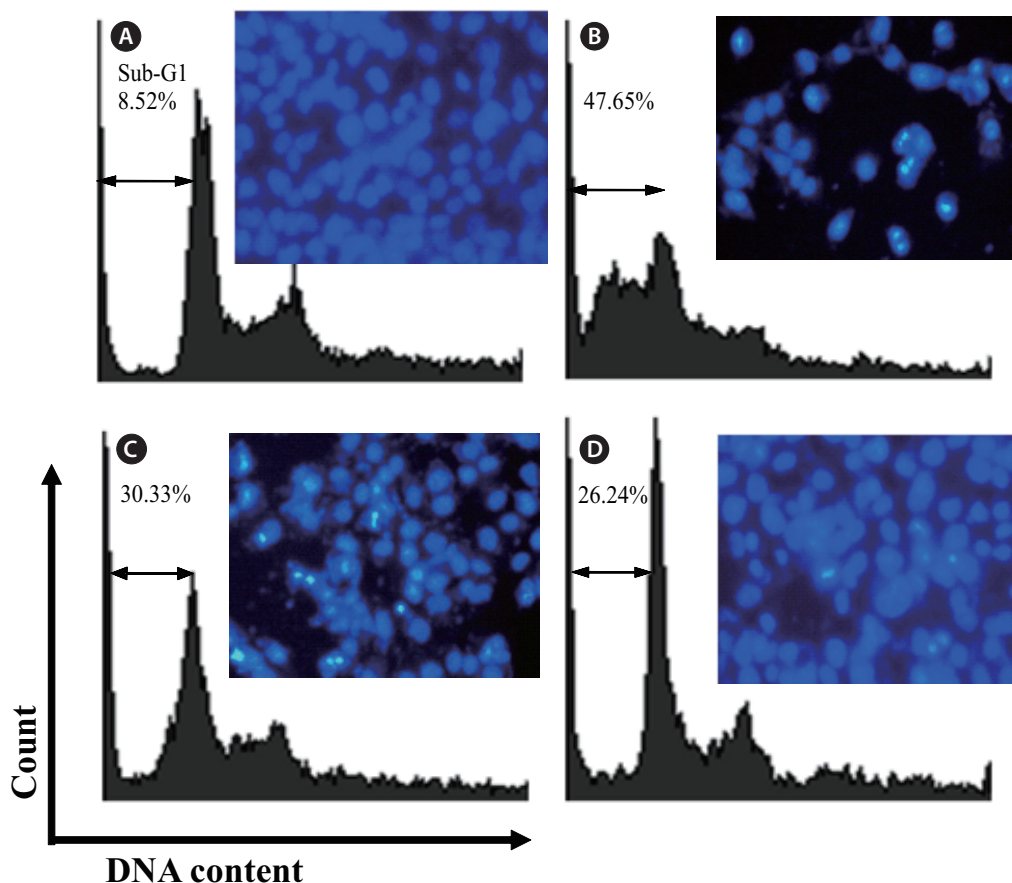


Fig. 7. Protective effect of loliolide isolated from from *Sargassum coreanum* on cell cycle pattern and H_2O_2 -induced apoptosis in Vero cells. (A) Control. (B) H_2O_2 -treated. (C) H_2O_2 + 250 μM loliolide-treated. (D) H_2O_2 + 500 μM loliolide-treated.

known as an important source of bioactive components. Many reports showed that these components exhibited various bioactivities. Marine brown alga, *S. coreanum* has been used as animal feed for long time in Jeju Island, Korean. Our research group has reported that either the enzymatic or the hot water extract of *S. coreanum* exhibited antioxidant activities on several free radicals. Hence, this made us take attempt to investigate the chemical composition of this species and evaluate the antioxidant activity. For the chemistry efforts in this study, the successively isolation and purification of ethyl acetate fraction led to a monoterpenoid compound, loliolide. This compound has been isolated from terrestrial plants (Valdes 1986) and marine algae (Hodges and Porte 1964, Kimura and Maki 2002). To our best knowledge, it was isolated for the first time from *S. coreanum*. This compound is known to have immunosuppressive (Okada et al. 1994), germination inhibitory (Kuniyoshi 1985) and ant-repellent activities (Okunade and Wiemer 1985).

ROS including superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical

($^{\bullet}OH$), peroxy (ROO^{\bullet}), alkoxy (RO^{\bullet}), nitric oxide and hydrogen peroxide (H_2O_2) is thought to be one of the causes of chronic diseases such as heart disease, inflammation, aging, and cancer. ROS can be produced by endogenous sources such as normal aerobic respiration, simulated polymer phonuclear leukocytes and macrophages, peroxisomes, and exogenous sources such as smoking, ionizing radiation, certain pollutants, organic solvents, pesticides, and a high polyunsaturated fatty acid diet. Among the ROS, H_2O_2 is one of the most crucial as it can generate other species of ROS, such as hypochlorous acid ($HOCl$), via the enzymatic (myeloperoxidase) oxidation of chloride ions. $HOCl$ can also result in the production of highly reactive singlet oxygen or even hydroxyl peroxy-carbonate. Finally, these ROS could react with bimolecular substance *in vitro*.

According to our knowledge, there is no literature regarding to the antioxidant activity of loliolide. This is first report focusing on the antioxidant properties of loliolide isolated from *S. coreanum*. In this study, DPPH and H_2O_2

radicals were employed to evaluate the antioxidant activity of loliolide. The results showed that the monoterpene, loliolide has a moderate antioxidant activity against DPPH free radical, H_2O_2 . In order to detail elucidate the antioxidant activity exerted by loliolide, the intercellular ROS scavenging activity of loliolide was also carried out using an oxidant-sensitive fluorescent probe DCF-DA. DCF-DA was hydrolyzed by intracellular esterase and converted to non-fluorescent DCFH. Then the DCFH was oxidized to highly fluorescent DCF. The results of intercellular ROS scavenging activity of loliolide indicated that pre-treatment of loliolide could reduce the ROS production induced by H_2O_2 within cells.

As loliolide showed antioxidant effects against DPPH, H_2O_2 radicals and intercellular ROS, the cell protective effects of loliolide against the cellular damage induced by H_2O_2 were also evaluated. The cell protective of loliolide against H_2O_2 -induced cell death or damage can be observed by enhance the cell viability. Furthermore, the morphology changes regarding to the cell protective effect was also investigated via nuclear staining with Hoechst 33342. Hoechst 33342 is used for specifically staining DNA, and has been used extensively for the detection of nuclear shrinkage, including chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, all of which are indicative of apoptosis. High ROS level in the cell could lead to apoptosis and necrosis, both of which processes have been implicated in cancer, aging, heart diseases and neurodegenerative disorders. In this study, the obvious apoptosis can be observed when the cell was challenged with H_2O_2 . H_2O_2 are able to induce damage to various biological macromolecules, including DNA, RNA, proteins and lipids. Thus it is very important to study the substances that scavenge H_2O_2 . Our results showed that the treatment of different concentration of loliolide was found to significantly reduce the nuclear fragmentation in the H_2O_2 -treated cells. Finally, the cell protective effects of loliolide associated with influence of cell cycle arrest in G1 phase were also studied. Treatment with different concentration of loliolide in H_2O_2 -treated Vero cells could reduce the sub-G1 DNA contents.

In conclusion, a monoterpene, loliolide isolated from *S. coreanum*, showed antioxidant activity against several radicals and cell protective effect against H_2O_2 -induced cell damage or apoptosis. This is the preliminary study on chemical composition of marine brown alga, *S. coreanum*. Since the extract and fractions of *S. coreanum* exhibited excellent biological activities, this result encourages us to isolated and identified more compounds,

with interesting bioactivities. Thus, *S. coreanum* might be developed as a functional food or cosmetic material.

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