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Sargassum sp. Attenuates Oxidative Stress and Suppresses Lipid Accumulation *in vitro*

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Oxidative stress causes tissue damage and facilitates the progression of metabolic diseases, including diabetes, cardiovascular heart diseases, and obesity. Lipid accumulation and obesity-related complications have been observed in the presence of extensive oxidative stress. As part of an ongoing study to develop therapeutic supplements, *Sargassum* sp. were tested for their ability to scavenge free radicals and intracellular reactive oxygen species (ROS), as well as to suppress lipid accumulation. Three species, *S. hemiphyllum*, *S. thunbergii*, and *Sargassum horneri*, were shown to scavenge free radicals in a di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay. In addition, *Sargassum* sp. was shown to scavenge intracellular ROS and to decrease nitric oxide (NO) production in H_2O_2 and lipopolysaccharide (LPS)-induced in RAW264.7 mouse macrophages, respectively. Taken together, the results suggest that *Sargassum* sp. possess huge potential to relieve oxidative stress and related complications, as well as lipid-induced oxidation. They indicate that *S. hemiphyllum*, *S. thunbergii*, and *S. horneri* are potent functional supplements that can produce beneficial health effects through antioxidant and antiobesity activities, with *S. hemiphyllum* being the most potent among the *Sargassum* sp. tested. A potential mechanism for the effect of *Sargassum* sp. on the suppression of lipid accumulation in differentiating 3T3-L1 mouse preadipocytes through deactivation of the peroxisome proliferator-activated receptor γ (PPAR γ) is presented.

Key words : Antioxidant, DPPH, lipid accumulation, ROS scavenging effect, *Sargassum* sp.

서 론

Failure to neutralize pro-oxidants results in oxidative stress in tissues and cells. Even though oxidation and its end products, free radicals are needed for survival, they are also incredibly damaging to tissues and leaving them untreated for a period of time can lead to more serious complications [14]. Numerous chronic states and diseases such as aging, diabetes and cancer have been reported to progress following elevated oxidative stress in the body [8, 30, 32]. In addition, it is also known that oxidative stress can lead to tissue damage if the oxidative defense mechanisms of cells fail to act against increased oxidative markers, especially reactive oxygen species (ROS). Cell damage mainly occurs by induction of ROS, namely hydrogen peroxide, the hydroxyl radical and the superoxide anion [9]. In general, ROS are produced as part of the normal protection mechanism. For instance, during an immune response, protective inflammation is carried out through the production of ROS by highly active leukocytes [24]. Nonetheless, excessive ROS lead to tissue damage if not scavenged. In order to fight against oxidative stress, cells express antioxidative enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. These enzymes and other natural antioxidative defense systems are regulated by the formation of ROS in the cells. However, an imbalance between ROS formation and neutralization can easily and rapidly damage tissues by breaking down antioxidant defenses. The imbalances may occur due to either overproduction of ROS and reactive nitrogen species (RNS), or deficiency in antioxidant systems [18]. In the case of oxidative stress, NO as

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well as free radicals are closely related in the development of inflammation [18]. Consequently, natural dietary antioxidants are necessary to aid antioxidant defense in the body.

Natural antioxidants are widely used and extensively studied for their ability to protect against oxidative stress-induced tissue damage and prevent crucial diseases such as coronary heart diseases, diabetes, cancer and even obesity [4, 34]. Recent studies demonstrated that most metabolic complications are closely linked to oxidative stress [12]. As a concerning problem of the modern world, obesity is also closely related oxidative stress-induced tissue damage as increased adipose tissue results in elevated oxidation of triglycerides [3]. In addition, recent research on the relationship between obesity and adipocyte differentiation was conducted by evaluating the cellular regulatory mechanisms of adipocyte differentiation [19]. The programmed differentiation of preadipocytes appears to be related to progressive stages of obesity [19]. Hence, obesity-related research efforts have been conducted in 3T3-L1 cells to identify new foods/agents with health benefits for obesity or weight control.

The past decades have witnessed the discovery of numerous amounts of secondary metabolites produced by natural sources as bioactive substances that act as antioxidants [28, 29]. As a result, marine organisms, especially plants, have been of much interest in recent studies due to the unique chemical composition of marine environments. Seaweed in particular, have been intensely studied for possible utilization as functional foodp and bioactive substance sources [2]. The Korean brown algae, *Sargassum species*, have been studied and promoted as possessing significant biological activities including antioxidant, anti-cancer, antiviral and antibacterial [26, 37]. In folk medicine, *Sargassum* sp. has been used for treatment of helminthic conditions, urination problems and immunological complications [21, 25]. Additionally, it has been reported that brown marine algae, which *Sargassum* sp. is a part of, produces polyunstaured fatty acids that are essential for balanced nutrition and health [27]. The chemical constituents of *Sargassum* sp. are also very broad, ranging from polysaccharides to polyphenols and glycosides. Therefore, in this study, three *Sargassum* sp., namely *Sargassum hemiphyllum*, *Sargassum thunbergii* and *Sargassum horneri*, were tested for their antioxidant as well as adipogenesis inhibitory activities as a part of an ongoing research to develop therapeutic agents for oxidative stress and related obesity complications.

Materials and Methods

Materials

The dried *Sargassum* sp., including *S. hemiphyllum*, *S. thunbergii* and *S. horneri*, were ground to a powder. The samples (100 g) were extracted with ethanol (99.9%) for 24 hr at a room temperature and the procedure repeated 3 times using the same powdered samples. The crude extracts were concentrated under reduced pressure via rotary evaporator (RV 10 Series, IKA, NC, USA).

Determination of DPPH radical scavenging activity

The free radical scavenging activity was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) method [1, 10]. The DPPH (Sigma Chemical Co., St. Louis, MO, USA) solution (150 μM) was prepared in 99.9% ethanol. One hundred microliters of samples was added to 100 μl DPPH solution and hold for 30 min in the dark at a room temperature. Finally, the discoloration of the mixture was measured at 520 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). The control was prepared, which contained the same volume of 99.9% ethanol and DPPH solution without any sample. The 99.9% ethanol was used as the blank. Percent scavenging of the DPPH free radical was quantified compared to the control.

DPPH radical scavenging activity (%)

$$= 1-\{\frac{(\text{Sample} - \text{Sample Blank})}{(\text{Control} - \text{Control Blank})}\}\times 100$$

Cytotoxicity determination using MTT assay

Murine Raw 264.7 cells were grown as monolayers at 5% CO_2 and 37°C humidified atmosphere using Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 μg/ml penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD, USA). The medium was changed twice or three times each week. Cytotoxic levels of the *Sargassum* sp. on cultured cells were measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 hr, the cells were washed with fresh medium and were treated with control medium or the medium supplemented

with *Sargassum* sp. After incubation for 24 hr, cells were re-washed and 100 µl of MTT solution (1 mg/ml) was added and incubated for 4 hr. Finally, 100 µl of DMSO was added to solubilize the formed formazan crystals and the amount of formazan crystal was determined by measuring the absorbance at 540 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan crystal. Viability of cells was quantified as a percentage compared to the control cells supplemented without sample and dose response curves were developed.

Determination of intracellular formation of ROS using DCF-DA labeling

Intracellular formation of ROS was assessed using oxidation sensitive dye 2′, 7′-dichlorofluorescin diacetate (DCF-DA) as the substrate. Raw 264.7 cells growing in fluorescence microtiter 96-well plates were loaded with 20 µM DCF-DA in HBSS and incubated for 20 min in the dark. Nonfluorescent DCF-DA dye, that is freely penetrated into cells, is hydrolyzed by intracellular esterases to 2′, 7′-dichlorodihydrofluororescein (DCFH), and is tarpped inside the cells. Cells were then treated with different concentrations of test samples and incubated for 1 hr. After washing the cells with PBS three times, 500 µM H_2O_2 dissolved in HBSS was added to the cells. The formation of 2′, 7′-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of various ROS was read after every 30 min at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 528 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). Dose-dependent and time-dependant effects were plotted and compared with fluorescence intensity of control and blank groups.

Measurement of nitric oxide production

Raw 264.7 cells were seeded onto 96-well plates with 2×10^5 cells/well using DMEM without phenol red and allowed to adhere overnight with pre-treated samples at a concentration of 1 mg/ml for 1 hr. Cellular NO production was stimulated by adding 1 µg/ml final concentration of LPS and further incubated for 24 hr. After incubation, the production of NO was determined based on the Griess reaction. Briefly, 40 ml of 5 mM sulfanilamide, 10 ml of 2 M HCl and 20 ml of 40 mM naphthylethylenediamine were added to 50 ml of culture medium. After 15 min incubation at room temperature, absorbance was measured at 550 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). The concentrations of nitrite were calculated from regression analysis, using serial dilutions of sodium nitrite as a standard.

Adipocyte differentiations

Murine 3T3-L1 pre-adipocytes cells were seeded in 6-well plates at a density of 2×10^5 cells/well and grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. At 1 day postconfluence (designated "day 0"), cell differentiation was induced with a mixture of 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (0.25 mM) and insulin (5 mg/ml) in DMEM containing 10% FBS. After 48 hr (day 2), the induction medium was removed and replaced with DMEM containing 10% FBS supplemented with insulin (5 µg/ml). This medium was changed every 2 days. Extracts of *Sargassum* sp. were administered to the culture medium from day 6 to day 8 at the concentration of 1 mg/ml.

Determination of Oil-red O staining

For Oil-Red O staining, cells were fixed with 10% fresh formaldehyde in PBS for 1 hr at room temperature and stained with filtered Oil-red O solution (60% isopropanol and 40% water) for at least 1 hr. After staining, the Oil-red O staining solution was removed and the plates were washed with the distilled water or PBS and dried. Images of lipid droplets in 3T3-L1 adipocytes were collected by an Olympus microscope (Tokyo, Japan). Finally, the dye retained in the cells was eluted with isopropyl alcohol and quantified by measuring optical absorbance at 500 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria).

RNA extraction and reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from 3T3-L1 adipocytes treated with/without *Sargassum* sp. extracts using Trizol reagent (Invitrogen Co., CA, USA). For synthesis of cDNA, RNA (2 µg) was added to RNase-free water and oligo (dT), denaturated at 70°C for 5 min and cooled immediately. RNA was reverse transcribed in a master mix containing 1X RT buffer, 1mM dNTPs, 500 ng oligo (dT), 140 U M-MLV reserve transcriptase and 40 U RNase inhibitor at 42°C for 60 min and at 72°C for 5 min using an automatic T100 Thermo Cycler (Bio-Rad, UK). The target cDNA was amplified using the

following sense and antisense primers: forward 5′-TTT-TCA-AGG-GTG-CCA-GTT-TC-3′ and reverse 5′-AAT-CCT-TGG-CCC-TCT-GAG-AT-3′ for PPARɣ; forward 5′-CCA-CAG-CTG-AGA-GGG-AAA-TC-3′ and reverse 5′-AAG-GAA-GGC-TGG-AAA-AGA-GC-3′ for β-actin. The amplification cycles were carried out at 95°C for 45 sec, 60°C for 1 min and 72°C for 45 sec. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/ml ethidium bromide visualized by UV light using Davinch-Chemi imager™ (CAS-400SM, Wako Co., Japan).

Statistical analysis

The data were presented as mean ± SD. Differences between the means of the individual groups were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.1 (SAS Institute, Cary, NC, USA) with Duncan's multiple range tests. The significance of differences was defined at the $p<0.05$ level.

Results

The ability of three *Sargassum* species to scavenge free radicals and to protect cells from oxidative stress was evaluated. Cellular nitric oxide (NO) production in RAW264.7 mouse macrophages was also evaluated as an indicator of antioxidant potential in inflammatory responses. Additionally, the effects of *Sargassum* sp. on adipogenic differentiation of 3T3-L1 mouse pre-adipocytes was experimented in order to assess their capability to inhibit adipose tissue generation and lipid accumulation for efficient obesity-related oxidative stress reduction.

Antioxidant effect of *Sargassum* sp. in cell-free and RAW264.7 mouse macrophage models

In order to evaluate the antioxidant potential of *Sargassum* sp., their ability to scavenge free radicals was assessed using cell-free DPPH assay. All three samples were able to scavenge DPPH radicals to a certain extent, with *S. hemiphyllum* being the most active among all three (Fig. 1). At a concentration of 0.1 mg/ml, scavenging of DPPH was not statistically different between *S. hemiphyllum*, *S. thunbergii* and *S. horneri*. However, at the highest concentration tested (1 mg/ml), *S. hemiphyllum* exhibited approximately 90% scavenging activity on DPPH. Under the same conditions (1 mg/ml), the scavenging abilities of *S. thunbergii* and *S. horneri* were approx. 16% and 18%, respectively. At a concentration of 0.5 mg/ml, *S. hemiphyllum* also exhibited significantly higher scavenging activity at a rate of 65% in comparison to 15% and 13% for *S. thunbergii* and *S. horneri*, respectively. The results indicated a very notable free radical scavenging effect for *S. hemiphyllum* in a dose-dependent manner. Though *S. thunbergii* and *S. horneri* had some scavenging activity, it was at an insignificant level compared to *S. hemiphyllum* despite increasing the treatment dose.

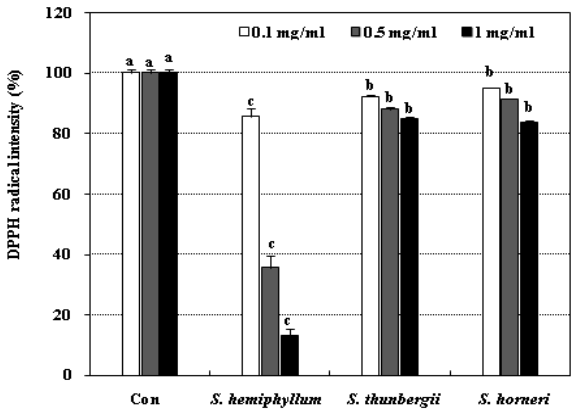

Fig. 1. DPPH radical scavenging effect of *Sargassum* sp. One hundred microliters of samples was added to 100 µl DPPH solution (150 µM in 99.9% ethanol) and hold for 30 min in the dark at a room temperature. Finally, the discoloration of the mixture was measured at 520 nm. The control was prepared, which contained the same volume of 99.9% ethanol and DPPH solution without any sample. Percent scavenging of the DPPH free radical was quantified compared to the control. Values are means ± SD ($n=3$). $^{a-c}$Means with the different letters in the same concentration are significantly different ($p<0.05$) in same concentration by Duncan's multiple range test.

Prior to conducting RAW264.7-based *in vitro* oxidative stress assays, the cytotoxicity of *Sargassum* sp. was evaluated using an MTT assay. None of the samples were cytotoxic to RAW264.7 mouse macrophages in any of the concentrations tested (0.1, 0.5 and 1 mg/ml) (Fig. 2). Consequently, further *in vitro* assays were performed accordingly. Cell viability was not affected by concentration-dependent exposure of *Sargassum* sp. and never went below 98% of untreated control cells which was determined to be significantly indifferent.

The effect of *Sargassum* sp. on ROS scavenging on H_2O_2-induced oxidative stress *in vitro* was observed in RAW264.7 cells. The ability to scavenge ROS was calculated using fluorescent intensity of DCF as an indicator of ROS activity. It

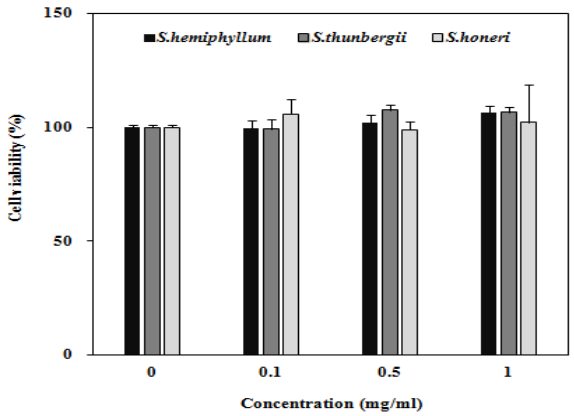

Fig. 2. Cytotoxicity of *Sargassum* sp. in Raw 264.7 cells. The cells were treated with the extracts of *Sargassum* sp. at the indicated concentrations. After incubation for 24 hr, cell viability was assessed by MTT assay. Percent scavenging of cell viability was quantified compared to the control cells supplemented without sample. Values are means ± SD (*n*=3).

was determined that *Sargassum* sp. acted on cells to protect them from oxidative stress caused by ROS, due to a reduction in the fluorescence intensity of intracellular DCF. Similarly, with the exception of 0.1 mg/ml *S. thunbergii*, all of the *Sargassum* sp. tested were able to lower intracellular oxidative stress in RAW264.7 cells in comparison to untreated H_2O_2-induced control cells, as indicated by DCF fluorescence intensity (Fig. 3). Among the three samples tested, *S. hemiphyllum* was once again the most active at scavenging intracellular ROS, followed by *S. horneri* and *S. thunbergii*, respectively. At a concentration of 0.1 mg/ml, *S. thunbergii* had no effect on ROS-related oxidative stress compared to untreated H_2O_2-induced control cells. However, *S. thunbergii* was able to lower oxidative stress in dose dependent manner starting from a concentration of 0.5 mg/ml which had more than 50% antioxidant potential in comparison to control and blank cells. On the other hand, *S. horneri* was the least effective of all three at 0.5 mg/ml. Overall, it can be concluded that *Sargassum* sp. is able to scavenge ROS and protect cells from H_2O_2-induced oxidative stress based on analysis of intracellular DCF levels.

Sargassum sp. was introduced into LPS-treated RAW264.7

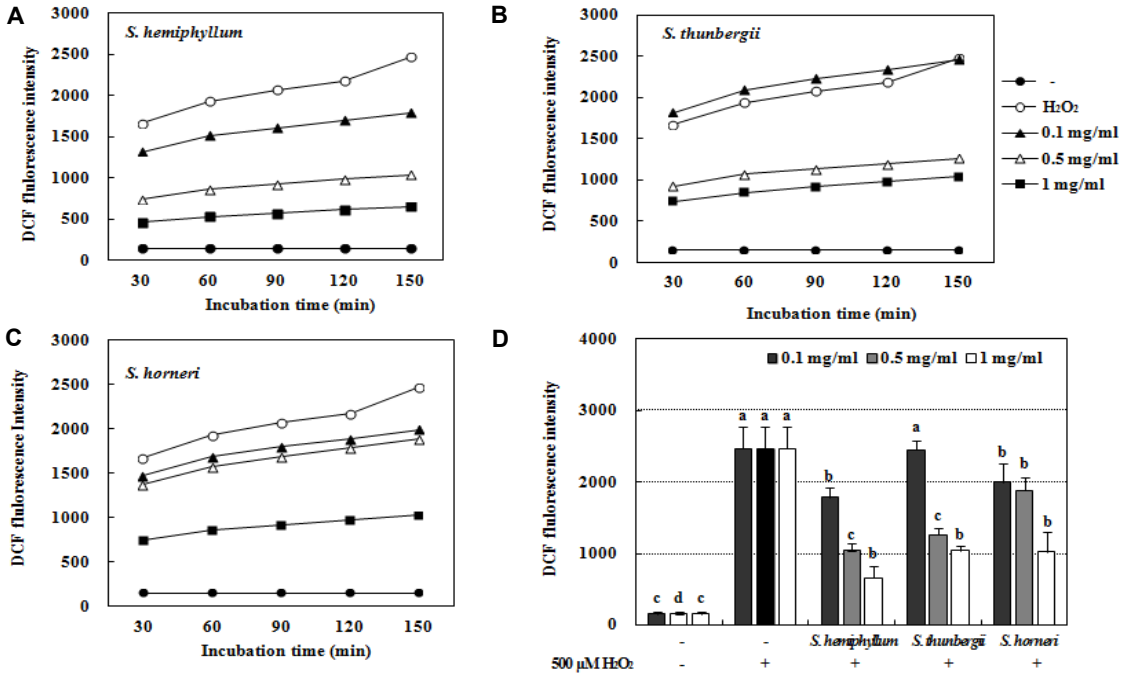

Fig. 3. ROS scavenging effect of *Sargassum* sp. in hydroperoxide-exposed Raw 264.7 cells. Raw 264.7 cells growing in fluorescence microtiter 96-well plates were preincubated in 20 μM DCF-DA for 20 min. The cells were then treated with different concentrations of test samples and incubated for 1 hr. After washing the cells with PBS three times, 500 μM H_2O_2 dissolved in HBSS was added to the cells. The formation of 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of various ROS was read after every 30 min at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 528 nm. Dose-dependent and time-dependant effects of *Sargassum hemiphyllum* (A), *Sargassum thunbergii* (B) and *Sargassum horneri* (C), separately, were plotted and compared with fluorescence intensity of control and blank groups. And the fluorescence intensity after 120 min was in comparison altogether (D). $^{a-d}$Means with the different letters in the same concentration are significantly different (p<0.05) by Duncan's multiple range test.

cells in order to evaluate their efficiency against NO production. The measured NO amount was elevated in control cells where only LPS-induction was performed without exposure to *Sargassum* sp.. Cells treated with *Sargassum* sp. also showed limited elevation in NO production. However, in comparison to control cells, all three samples had lower amounts of NO, indicating a potential antioxidant effect in response to inflammation (Fig. 4). The results indicate that *S. hemiphyllum* lowered NO production to less than half of the amount in the control cells, followed by *S. thunbergii* and *S. horneri* in efficiency. The effect of *S. thunbergii* was similar to that of *S. hemiphyllum* statistically, while *S. horneri* only lowered NO production by approximately 10% compared to control cells.

Effect of *Sargassum* sp. on adipogenesis and lipid accumulation in 3T3-L1 pre-adipocytes

Following assessment of the antioxidant potential of *Sargassum* sp., their anti-obesity effects were also evaluated in differentiating 3T3-L1 pre-adipocytes by means of intracellular triglyceride. First, *Sargassum* sp. was introduced to differentiating pre-adipocytes to observe any potential bioactivity that capable of the lowing lipid accumulation. The results strongly suggested that *Sargassum* sp. decreased the Oil-Red O stained triglycerides used as an indicator of accumulated lipid in comparison to control cells (Fig. 5). Not surprising, *S. hemiphyllum* and *S. thunbergii* were the most active samples reducing lipid accumulation and both samples decreased differentiation-related triglycerides by a statistically similar amount. *S. horneri* had a small effect on lipid accumulation, which was not statistically significant, in comparison to fully differentiated control adipocytes. In detail, expression of the key transcription factor for adipogenesis, PPARɣ, was monitored in the presence and absence of *Sargassum* sp. in order to evaluate whether *Sargassum* sp. had an effect on adipogenic differentiation as well as on lipid accumulation. As shown in Fig. 6, mRNA expression of PPARɣ was strongly elevated by differentiation in control cells. However, introduction of *Sargassum* sp. suppressed the expression levels indicating a possible adipogenesis prevention effect. The results revealed *S. thunbergii* as the most potent adipognesis inhibitor. *S. horneri* was also observed to possess some effect on lipid accumulation and adipogenesis, although to a lesser extent than the remaining *Sargassum* sp..

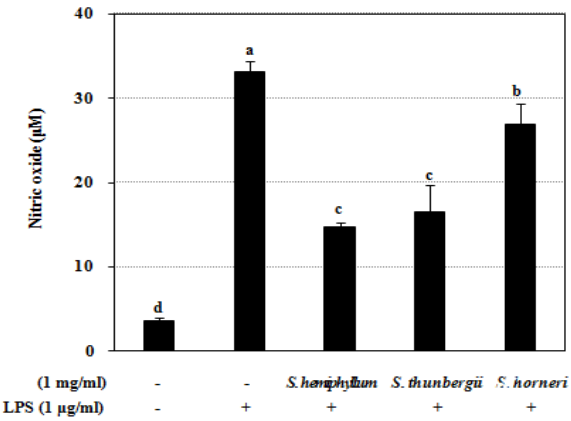

Fig. 4. Effect of *Sargassum* sp. on NO production in LPS-stimulated Raw 264.7 cells. The cells were grown in 96-well plates using DMEM without phenol red at a density of 2×10^5 cells/well and allowed to adhere overnight. After incubation, the cells were pretreated with *Sargassum* sp. for 1 hr, stimulated with LPS (1 μg/ml) and further incubated for 24 hr. The nitrite content of culture media was analyzed based on the Griess reaction. [a-d]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test. Values are means ± SD ($n=3$).

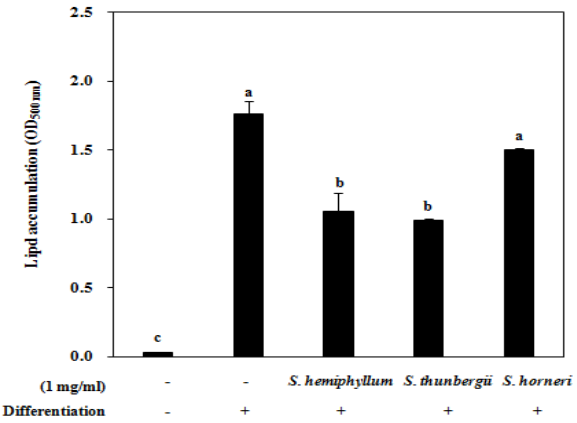

Fig. 5. Effect of *Sargassum* sp. on intracellular lipid accumulation in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes with 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 mM dexamethasone and 5 mg/ml insulin_for 6 days in 6-well plates and the cells were treated with crude extracts of *Sargassum* sp. at the end of differentiation period for 2 days (from day 6 to day 8). Absorbance values indicate lipid accumulation by dilution of Oil-Red O from wells. Values are means ± SD ($n=3$). [a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Discussion

Oxygen, an element that is indispensable for survival, can have some deleterious effects in the human body. The harm-

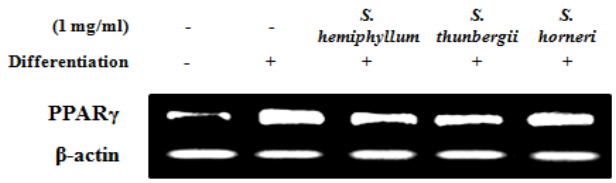

Fig. 6. Effect of *Sargassum* sp. on PPARγ expression in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes with 0.5 mM 3-isobutyl-1- methylxanthine, 0.25 mM dexamethasone and 5 mg/ml insulin for 6 days in 6-well plates and the cells were treated with crude extracts of *Sargassum* sp. at the end of differentiation period for 2 days (from day 6 to day 8). The expression levels of adipogenic transcription factor PPARγ were determined by RT-PCR analysis. Fully differentiated adipocytes and preadipocytes untreated with sample were used as control and blank, respectively.

ful effects of oxygen are due to the formation of several chemical compounds, known as ROS, via essential bodily functions [5]. The presence of these free radicals and ROS, results in oxidative stress in cells which can lead to cell death unless they are removed by the body's defense mechanisms, specially, through free radical scavenging and the activity of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase [22]. However, intracellular oxidative stress results from our daily activities and occasionally cause harm until it is relieved. Otherwise, tissue damage occurs due to the presence of ROS in amounts too great for the cellular defense mechanism to handle. Oxidative stress has also been linked with numerous diseases by recent developments in research and evaluation of disease progression mechanisms. Lack of scavenging or oxidative stress relief is credited as the cause or result of several metabolic complications as well as diseases including heart diseases, diabetes and obesity [3, 36]. Dietary and supplementary antioxidants are therefore needed for improved quality of life. As a result, recent studies have focused on developing natural antioxidant compounds that can scavenge free radicals and ROS in cells and/or interrupt the radical chain reaction of oxidation. Medicinal plants have been an important source of natural antioxidant compounds with increasing utilization of marine-based sources, paving the way for further development of natural antioxidant compounds with exceptional bioactivities [16]. Marine plants are being studied in detail in order to treat or prevent oxidative stress and related complications [2]. The importance of algal functional ingredients, especially marine brown algae, has recently been confirmed due to their effectiveness in relieving harmful complications, reducing disease progression and preventing further onset of illnesses [23]. A broad range of ingredients have been isolated and characterized from the Korean brown algae, *Sargassum* sp., possessing recognized bioactivities such as antitumor, antiviral, antibacterial, antiobesity and antioxidant properties [7, 13, 17]. Hence, this study focused on the evaluation of antioxidant potential and the ability to lower lipid accumulation of three *Sargassum* sp.; *S. hemiphyllum*, *S. thunbergii* and *S. horneri*.

The ability of *Sargassum* sp. to scavenge free radicals and ROS were tested in both cell-free and mouse macrophage models. All three species showed some level of antioxidant potential according to DPPH assay results, with *S. hemiphyllum* being the most notable and active by exhibiting scavenging activity above 80%. Although the remaining two *Sargassum* sp. were not as potent as *S. hemiphyllum*, they were active enough for further assessment of antioxidant potential in cells. Cytotoxicity experiments revealed no toxic effects on RAW264.7 mouse macrophages which enables utilization of *Sargassum* sp. without causing any cell death. As the intent of the study was to develop dietary health beneficial supplements, observing steady cell viability in the presence of *S. hemiphyllum*, *S. thunbergii* and *S. horneri* indicates that *Sargassum* sp. can be safely used in the human diet.

The intracellular free radical scavenging efficiency of *Sargassum* sp. was assessed by monitoring the oxidation of DCFH-DA, a chemical that easily passes into the cytoplasm and remains trapped there following the removal of –DA. Next, excessive intracellular ROS leads to oxidation of DCFH into DCF, with a fluorescence intensity that can be measured [35]. Measured fluorescence intensity clearly indicates an intracellular ROS scavenging effect for *Sargassum* sp. Several studies have reported protective effects of numerous *Sargassum* sp. from excessive generation of ROS. Heo *et al.* indicated that *Sargassum siliquastrum* can protect human fibroblast from UV-B induced ROS generation which results in cell damage [11]. In addition, it was also shown that *Sargassum wightii* can protect against cyclosporine A-induced oxidative liver damage due to its sulfated polysaccharide content [15]. Furthermore, polyphenol constituents of *Sargassum* sp. are credited for several of their reported bioactivities including ROS scavenging. Other *Sargassum* sp. with anti-inflammatory effects includes *Sargassum micracanthum* and *Sargassum fulvellum*, which reduce NO production resulting from oxidative stress damage [20]. The results of the current study are in complete agreement with reported

Sargassum sp. bioactivities as our samples were able to scavenge intracellular ROS in a dose-dependent manner and also significantly lower LPS-induced NO production. In light of current results, *Sargassum* sp., specifically *S. hemiphyllum*, *S. thunbergii* and *S. horneri*, can be promoted as potentially significant sources for antioxidant defense, with *S. hemiphyllum* being the most potent. Dietary inclusion of *Sargassum* sp. can prove protection against oxidative stress, as they can scavenge free radicals and intracellular ROS at the same time in addition to lowering NO production and lipid peroxidation. Hence, total antioxidant efficiency can be obtained by utilizing *Sargassum* sp. for health beneficial purposes.

Oxidative stress is also known to lead to several bodily disorders, obesity being the most concerning in the developed world. The relationship between excessive lipid accumulation and oxidative stress has been the focus of much attention and the target of many studies aimed at relieving obesity-related complications [6]. Obesity is defined by excessive lipid accumulation due to elevated adipogenesis. Excessive amounts of triglycerides results in elevated free fatty acids in the blood, which triggers oxidative stress via peroxidation [31]. On the other hand, excessive oxidative stress is also known to cause increased lipid accumulation via activation of the sterol regulatory element-binding protein (SREBP) 1c which is a cascade step for the PPARɣ pathway [33]. Previous reports have revealed a close relation between obesity and oxidative stress and urge future research towards an efficient regulation of obesity relief through the suppression of oxidative stress and lipid accumulation. In this context, *Sargassum* sp. was evaluated for their potential ability to decrease lipid accumulation through a PPARɣ related mechanism since their antioxidant activity was already evident. Following their significant ability to scavenge free radicals and ROS, *S. hemiphyllum*, *S. thunbergii* and *S. horneri* were introduced to differentiating adipocytes. The results of Oil-Red O staining showed lower amounts of stored intracellular triglycerides in the presence of *Sargassum* sp. compared to mature control adipocytes. In addition to their antioxidant capabilities, their potential to suppress lipid accumulation promotes *Sargassum* sp. as a promising source of functional ingredients. We examined a possible action mechanism for their effect on suppressing lipid accumulation by monitoring the mRNA expression of PPARɣ. As expected, *Sargassum* sp. lowered the PPARɣ gene expression, indicating that their action mechanism involves deactivation of the SREBP1c and PPARɣ pathway. Sekiya *et al.* suggested a mechanism for the activation of SREBP1c by oxidative stress, which results in lipid accumulation [33]. Our results therefore suggest an anti-obesity effect for *Sargassum* sp., possibly due to their antioxidant properties, that also prevents activation of the PPARɣ pathway and hinders lipid accumulation.

In conclusion, *Sargassum* sp., specifically *S. hemiphyllum*, *S. thunbergii* and *S. horneri*, are presented as potential antioxidant supplements with the ability to lower lipid accumulation and relieve obesity-related oxidative stress complications. Future *in vivo* studies to elucidate detailed mechanisms of action and dose efficiency will reveal the true potential of *Sargassum* sp. as therapeutic functional supplements with antioxidant and antiobesity properties. In addition, *Sargassum* sp. can potentially relieve intracellular oxidative stress by scavenging free radicals and ROS, decreasing NO production and suppressing lipid accumulation. These bioactivities suggest possibly application of *Sargassum* sp. for promoting health benefits through dietary supplementation.

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초록 : 모자반추출물의 항산화활성 및 지방세포 생성억제 효과

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인간 생체 내 산화스트레스는 조직적 손상을 일으켜 당뇨병, 심장혈관계질환, 비만 등의 대사성 질환의 발병에 관여하는 것으로 알려져 있다. 본 연구에서는 질병예방용 천연 보조제 개발의 일환으로 모자반 3종류(*Sargassum hemiphyllum*, *Sargassum thunbergii*, *Sargassum horneri*)의 에탄올 추출물을 이용하여 항산화활성 및 지방생성억제효과를 비교 검토하였다. 항산화활성은 DPPH 라디칼 소거능, 세포 내 ROS 활성 및 NO 함량의 측정을 통해 검토하였다. 지방세포생성억제활성은 3T3L1세포를 이용하여 지방세포의 축적 정도와 PPARɣ 유전자의 발현 정도를 각각 Oil-Red O 염색법과 RT-PCR로 측정하였다. DPPH 라디칼 소거능 결과, 모자반의 처리에 의해 농도 의존적으로 DPPH 라디칼이 줄어드는 경향을 보였으며, 모자반 중에서 *S. hemiphyllum* 처리군에서 가장 높은 소거 효과를 볼 수 있었다. MTT assay을 통해 모자반의 에탄올 추출물들이 RAW 264.7 cell에 대한 독성을 보이지 않는 농도에서 세포내 실험을 진행하였다. 세포 내 ROS 소거능의 측정 결과, 농도 의존적으로 DCF 형광도 값이 낮게 나왔으며, 시간이 지남에 따라 형광도 값이 일정하여 모자반의 에탄올 추출물에는 세포 내 ROS 생성을 억제하는 효과가 있음을 알 수 있었다. LPS 처리에 의해 증가한 NO 값은 모자반의 추출물들의 처리에 의해 유의적으로 감소하였으며, 감소 정도는 *S. hemiphyllum*과 *S. thunbergii* 처리 군에서 높게 나타났다. 모자반의 추출물들을 3T3-L1지방세포에 유도물질과 함께 처리한 결과 모자반 추출물 중 *S. hemiphyllum*과 *S. thunbergii*이 세포 내 지방 축적 및 PPARɣ 유전자의 발현을 유의적으로 감소시키는 것으로 나타났다. 이상의 결과로부터 모자반 추출물들 중 *S. hemiphyllum*과 *S. thunbergii*이 높은 항산화 활성과 지방세포 생성억제 효능을 보유하고 있는 천연소재임을 확인할 수 있었다.