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Antioxidant Efficacy of Extracts from a Variety of Seaweeds in a Cellular System

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Abstract – As a part of an ongoing search for antioxidants from marine sources, antioxidant activities of 24 kinds of seaweeds (4 green algae, 8 brown algae, and 12 red algae) were investigated. The seaweeds were extracted by acetone/ dichloromethane and methanol, respectively. The antioxidant properties of both extracts were evaluated using four different activity tests, including degree of occurrence of intracellular reactive oxygen species (ROS), NO, lipid peroxidation, and GSH (glutathione) in mouse macrophage Raw 264.7 cells. The levels of intracellular reactive oxygen species (ROS) and GSH were measured using 2',7'-dichlorofluorescin diacetate (DCF-DA) and monobromobimane as fluorescence probe, respectively. Moreover, the generation of NO and lipid peroxidation products were determined by each method based on the Griess reaction and TBARS assay. Solvent extracts from seaweeds such as *Scytosiphon lomentaria*, *Prionitis cornea*, *Laruencia okamurae*, *Callophyllis japonica*, *Sargassum horneri*, *Dictyopteris divaricata*, *Lomentaria catenata*, *Corallina confuse*, *Ishige okamurae*, and *Ahnfeltiopsis flabelliformi* exhibited high antioxidant activities in cellular oxidizing systems.

Key words – seaweeds, reactive oxygen species, nitric oxide, lipid peroxidation, glutathione

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause cellular injury by oxidation process such as lipid and protein peroxidation. Nevertheless, the levels of intracellular reactive oxygen and nitrogen species such as hydrogen peroxide, hydroxyl and superoxide anion radicals, nitric oxide, and peroxynitrite anion, etc., uniformly were maintained because those have several functions to perform in the cell. Usually, balance between the formation of reactive species and antioxidant defenses is kept in the body, but oxidative stress may result when these systems fail to cope with the production of ROS/RNS. These imbalanced oxidative stresses are associated with several chronic degenerative diseases and pathological processes (Je and Kim 2006; Komaki-Yasuda *et al*. 2003).

The significance of seaweed as a source for biologically active natural products is well known (Ali *et al*. 2000). Recently we have reported antioxidant activity of crude extracts of some kinds of seaweeds (Lee *et al*. 2004a,b; Seo *et al*. 2004; Lee and Seo 2006; Jung *et al*. 2004; Lee *et al*. 2006). Nevertheless, we felt that there is a need to measure antioxidant activity of seaweeds in a cellular system which is closer to a living system.

In this study, antioxidant activities of 24 kinds of seaweeds (4 green algae, 8 brown algae, and 12 red algae) harvested in Cheju, Korea were evaluated in macrophage cellular oxidizing systems, including degree in occurrence of intracellular reactive species from ROS, NO, lipid peroxidation, and GSH.

2. Materials and Method

Sample collection and extraction

The seaweeds were collected by hand in February 2007, along the shores of Cheju Island, Korea. Shade-dried seaweeds were extracted with a volumetrically equal mixture of acetone and dichloromethane for 24 h at room

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temperature and then with methanol. Each step was repeated twice and then the extracts were evaporated under vacuum, yielding a dark and sticky material. Each of the crude extracts was used as experimental material. The prepared samples were then stored in a refrigerator at -20 °C, for further study.

Cell culture

Raw 264.7 murine macrophage cells were grown at 5% CO_2 and 37 °C in a humidified atmosphere using Dulbecco's modified eagle medium (DMEM, Gibco Co.) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 μg/mL penicillin-streptomycin (Gibco Co.). The medium was changed two or three times each week.

Cytotoxicity determination using MTT assay

Cytotoxic levels of the extracts 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 (Hansen *et al*. 1989). The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium and were treated with control medium or the medium supplemented with each solvent extract. After incubation for 48 h, cells were rewashed and 100 μL of MTT solution (1 mg/mL) was added and incubated for 4 h. Finally, 150 μL of DMSO was added to solubilize the formed formazan crystals and the amount of formazan crystal was determined by measuring the OD at 540 nm using a multidetection microplate fluorescene spectrophotometer synergy HT (Bio-Tek instruments, USA). 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 and dose response curves were developed.

Determination of intracellular formation of reactive oxygen species using DCF-DA labeling

Intracellular formation of reactive oxygen species (ROS) was assessed using oxidation sensitive dye 2',7'-dichlorofluorescin diacetate (DCF-DA) as the substrate (Okimoto *et al*. 2000). The 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, which freely penetrates into cells, gets hydrolyzed by intracellular esterases to 2',7'-dichlorodihydrofluororescein (DCFH), and traps inside the cells. Cells were then treated with test extracts and incubated for 1 h. After washing the cells with PBS three times, 500 μM H_2O_2 dissolved in HBSS was added to cells. The formation of 2',7'-dichlorofluorescein (DCF), a fluorescent compound ($\lambda_{excitation}$=485 nm; $\lambda_{emission}$=528 nm), due to oxidation of DCFH in the presence of various ROS, was read after every 30 min using a multidetection microplate fluorescene spectrophotometer synergy HT (Bio-Tek instruments, USA). Time-dependent effects of the compound groups were plotted and compared with fluorescence intensity of control and blank groups.

Measurement of intracellular GSH level

GSH level on cultured cells in fluorescence microtiter 96-well plates was tested with monobromobimane as a thiol-staining reagent (Poot *et al*. 1986). The Raw 264.7 cells were seeded at a density of 1×10^7 cells/mL and allowed to attach completely. Cells were treated with prepared extracts and incubated for 30 min. Monobromobimane in 1% DMSO was added to cells at a final concentration of 40 μM and staining was carried out for 30 min at 37 °C in the dark. Afterwards, staining fluorescence intensity was measured ($\lambda_{excitation}$=360 nm; $\lambda_{emission}$=465 nm) using a multidetection microplate fluorescene spectrophotometer synergy HT (Bio-Tek instruments, USA). The averaged fluorescence values of cell populations were plotted and compared with a control group in which cells were grown without treatment of the extracts.

Assessment of cellular nitric oxide (NO) 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 test samples for 1 h. Cellular NO production was stimulated by adding 1 μg/mL final concentration of LPS and further incubated for 48 h. After incubation, the production of NO was determined based on the Griess reaction (Green *et al*. 1982). Briefly, 40 mL of 5 mM sulfanilamide, 10 mL of 2 M HCl and 20 mL of 40 mM naphthylethylenediamine were added to 150 mL of culture medium. After a 15 min incubation period at room temperature, absorbance was measured with a multidetection microplate fluorescene spectrophotometer synergy HT (Bio-Tek instruments, USA) at 550 nm.

Membrane lipid peroxidation assessment by TBARS method

Generation of lipid peroxidation products was carried out by a modification of the thiobarbituric acid-reactive substances (TBARS) method on the Raw 264.7 cells (Heath and Packer, 1968). Briefly, 200 μL of PBS-suspended Raw 264.7 cells was incubated for 10 min with test samples or distilled water as a control. To induce cellular oxidation, 2 mM H_2O_2 and 0.1 M $FeSO_4$ were added to the cells, and then incubated at 37 °C for 10 min. Two-fold volume of cold trichloroacetic acid (TCA, 10% w/v) was added to stop oxidation. After adding an equal volume of TBA (1% w/v), the mixture was heated at 90°C for 30 min and then cooled to room temperature. Finally, reaction mixture was centrifuged and absorbance of the supernatant was measured at 528 nm.

Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS Institute 1999-2001). Significant differences between treatment means were determined by using Duncan's multiple range tests. Significance of differences was defined at the $p<0.05$ level.

3. RESULTS AND DISCUSSION

Raw 264.7 cells, mouse macrophage cell line, were used to study antioxidant effects of 24 kinds of seaweed extracts. There is a close connection between these cells and ROS/

Table 2. Effect of seaweed extracts on the viability of Raw 264.7 cells. Cells were treated with various seaweed extracts at 50 μg/mL concentrations. After 24 h, cell viability was determined by the MTT assay

No.	Cell viability (% of control)	
	Acetone/dichloromethane Ext.	Methanol Ext.
Con	100.0 ± 0.9[1]	100.0 ± 0.6
1	86.6 ± 0.3*	97.9 ± 3.2
2	101.8 ± 8.6	68.9 ± 3.7*
3	104.0 ± 7.3	109.0 ± 4.3
4	99.9 ± 5.1	83.9 ± 5.0*
5	65.3 ± 4.5*	96.5 ± 6.0
6	43.4 ± 1.9*	48.0 ± 1.1*
7	107.7 ± 12.9	101.1 ± 0.3
8	49.3 ± 1.0*	84.0 ± 2.9*
9	51.7 ± 1.7*	92.7 ± 2.1
10	100.8 ± 3.5	81.2 ± 1.8*
11	58.0 ± 3.2*	98.3 ± 3.1
12	103.1 ± 12.1	102.8 ± 1.0
13	104.9 ± 15.9	110.6 ± 6.5
14	108.8 ± 19.0	95.0 ± 6.8
15	100.0 ± 8.6	73.2 ± 1.6*
16	43.0 ± 2.8*	99.4 ± 2.8
17	91.3 ± 1.0	60.7 ± 2.1*
18	104.7 ± 9.1	103.1 ± 5.7
19	97.7 ± 3.6	77.6 ± 4.8*
20	91.5 ± 3.2	98.1 ± 7.2
21	81.3 ± 7.7*	107.2 ± 4.2
22	90.2 ± 1.0	79.3 ± 2.6*
23	86.7 ± 2.2*	81.8 ± 6.3*
24	88.3 ± 3.2*	94.5 ± 8.5

[1]Means ± S.D., *$p<0.05$

Table 1. List of collected seaweeds in Jeju, Korea.

No.	Species	No.	Species
	Green algae		Red algae
1	*Enteromorpha compressa*	13	*Lomentaria catenata*
2	*Cladophora sakaii*	14	*Prionitis cornea*
3	*Ulva pertusa*	15	*Corallina confusa*
4	*Codium cylindricum*	16	*Gloiopeltis complanata*
	Brown algae	17	*Gracilaria verrucosa*
5	*Sargassum nigrifolium*	18	*Laruencia okamurae*
6	*Sargassum coreanum*	19	*Gelidium amansii*
7	*Sargassum horneri*	20	*Callophyllis japonica*
8	*Hizikia fusiformis*	21	*Pachymeniopsis lanceolata*
9	*Colpomenia sinuosa*	22	*Chondrus ocellatus*
10	*Dictyopteris divaricata*	23	*Corallina officinalis*
11	*Ishige okamurae*	24	*Ahnfeltiopsis flabelliformis*
12	*Scytosiphon lomentaria*		

RNS-mediated cellular events because they can produce high amounts of reactive species after stimulation (Arató *et al*. 2006). The cytotoxic effects of seaweed extracts were examined using MTT assay in order to determine non-toxic concentrations for antioxidant activity test. At the concentration of 50 μg/mL, 14 acetone/dichloromethane and 14 methanol extracts of seaweeds didn't show any significant toxic effect ($P < 0.05$) on the growth of cells (Table 2). On the basis of these results, non-toxic extracts of each solvent were used for the following experiments.

Intracellular radical scavenging effect of seaweed extracts was assessed using Raw 264.7 cells labeled fluorescence probe 2',7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA has been used for detecting several ROS and RNS in biological media (Okimoto *et al*. 2000). Cellular esterases first hydrolyse DCF-DA to non-fluorescent 2',7'-dichlorodihydrofluororescein (DCFH), which is then oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by reactive species (Gomes *et al*. 2005). DCF fluorescence of each extract was described only as the result after incubation for 2 h. As a result, DCF fluorescence intensity increased by H_2O_2 significantly decreased time-dependently in the presence of each extract as shown in Table 3B. Most of the acetone/dichloromethane extracts were significantly effective at 50 μg/mL concentration. In particular, 7 extracts among them showed high ROS-scavenging effect at more than 50%, which decreased in the following order: *Prionitis cornea, Lomentaria catenata, Laruencia okamurae, Scytosiphon lomentaria, Callophyllis japonica, Corallina confuse,* and *Gelidium amansii*. Extracts of *Sargassum horneri* and *Dictyopteris divaricata* also showed significant ROS-scavenging effect. Similarly, several methanol extracts of seaweeds also exhibited strong ROS-scavenging effect in the order of *Ahnfeltiopsis flabelliformis* > *Laruencia okamurae* > *Scytosiphon lomentaria* > *Callophyllis japonica* > *Prionitis cornea* > *Sargassum nigrifolium* > *Ishige okamurae* (Table 3).

Table 3. Effect of seaweed extracts on intracellular ROS level induced by hydrogen peroxide at 50 μg/mL. After preincubation of the Raw 264.7 cells in 20 μM DCF-DA, cells were treated with seaweed extracts for 2 h. DCF fluorescence was measured following addition of 500 μM H_2O_2 at $\lambda_{excitation}$=485 nm and $\lambda_{emission}$ =528 nm

DCF fluorescence intensity			
No.	Acetone/dichloromethane Ext.	No.	Methanol Ext.
Con	259 ± 11[1]	Con	215 ± 7
2	186 ± 8* (28)[2]	1	192 ± 16 (11)
3	183 ± 26 (29)	3	183 ± 26 (15)
4	155 ± 1* (40)	5	124 ± 4* (42)
7	139 ± 13* (47)	7	208 ± 4 (3)
10	141 ± 26* (46)	9	148 ± 6* (31)
12	108 ± 4* (58)	11	130 ± 19* (40)
13	78 ± 14* (70)	12	108 ± 4* (50)
14	70 ± 6* (73)	13	166 ± 19* (23)
15	115 ± 28* (56)	14	116 ± 9* (46)
17	194 ± 17 (25)	16	153 ± 40 (29)
18	103 ± 11* (60)	18	106 ± 1* (51)
19	121 ± 23* (53)	20	113 ± 7* (47)
20	113 ± 7* (56)	21	176 ± 6 (18)
22	222 ± 18 (14)	24	105 ± 21* (51)
BK	64 ± 4 (75)	BK	88 ± 5 (59)

[1]Means ± S.D., [2]The values in parentheses are the inhibition rates (%)
*$p<0.05$

With reactive oxygen species, correlation between the protective effect of seaweeds and the LPS-induced production of nitric oxide (NO) was examined. Reactive NO has been known to generate peroxynitrite ($ONOO^-$) that is a latent oxidizing agent, by the chemical reaction between NO and superoxide. Various negative effects of $ONOO^-$ have been described, such as oxidation and nitration of proteins (Lee *et al*. 2004a,b; Seo *et al*. 2004; Patel *et al*. 1999; Virag *et al*. 2003; Reiter *et al.* 2002; Chen *et al*. 2005; Tsao *et al*. 2005). Therefore, NO production is an important step in the regulation of NO-mediated diseases. To measure cellular NO inhibition by seaweed extracts, Raw 264.7 cells were treated with extracts at the concentration of 50 μg/mL for 1 h and then stimulated with LPS for another 48 h. Among them, acetone/dichloromethane extracts from *Corallina confuse* (55.2±1.2%), *Lomentaria catenata* (52.9±0.3%), *Sargassum horneri* (52.5±0.1%), *Dictyopteris divaricata* (51.8±1.8%), and *Prionitis cornea* (49.1±0.3%) exhibited potent inhibitory activities compared to control (Table 4). And methanol extracts from *Scytosiphon lomentaria* (52.5±0.1%), *Ishige okamurae* (52.1±0.3%), *Ulva pertu* (50.0±0.0%), and *Ahnfeltiopsis flabelliformis* (44.3±3.2%) also exhibited strong activities (Table 4).

Glutathione (γ-glutamylcysteinylglycine or GSH) is a naturally occurring tripeptide made of three amino acids: cysteine, glutamic acid, and glycine. GSH is an important antioxidant that protects cells from toxins such as free radicals (Sies H. 1999; Watson and Jones 2003). Therefore, GSH levels in sample-treated cells were measured for comparison between reactive species and antioxidant. At the concentration of 50 μg/mL, GSH levels were increased

Table 4. Effect of seaweed extracts on intracellular NO level induced by LPS. Raw 264.7 macrophages were pretreated with 50 μg/mL concentration of seaweed extracts for 1 h before stimulation with LPS (1 μg/mL) for another 48 h. The nitrite content of culture media was analyzed

NO reduction (% of control)			
No.	Acetone/dichloromethane Ext.	No.	Methanol Ext.
Con	100.0 ± 0.1[1]	Con	100.0 ± 0.1
2	52.0 ± 0.1*	1	66.2 ± 0.1*
3	62.4 ± 0.8*	3	50.1 ± 0.0*
4	71.9 ± 1.6*	5	61.4 ± 0.3*
7	47.5 ± 0.1*	7	63.5 ± 2.5*
10	48.2 ± 1.8*	9	85.6 ± 0.5*
12	54.2 ± 2.7*	11	47.9 ± 0.3*
13	47.1 ± 0.3*	12	47.5 ± 0.1*
14	50.9 ± 0.3*	13	77.6 ± 2.8*
15	44.9 ± 1.2*	14	69.1 ± 0.2*
17	62.8 ± 0.2*	16	81.0 ± 0.5*
18	57.9 ± 0.7*	18	71.7 ± 1.1*
19	51.6 ± 0.5*	20	73.8 ± 0.3*
20	51.8 ± 1.6*	21	59.6 ± 0.1*
22	54.9 ± 1.8*	24	55.7 ± 3.2*

[1]Means ± S.D., *p<0.05

Table 5. Effect of seaweed extracts on regulation of GSH level at 50 μg/mL. Cellular GSH level was determined using mBBr as a thiol-staining reagent according to the method described in the text measuring mBBr-GSH fluorescence intensity at $\lambda_{excitation}$=360 nm and $\lambda_{emission}$=465 nm

GSH-mBBr fluorescence intensity			
No.	Acetone/dichloromethane Ext.	No.	Methanol Ext.
Con	1334 ± 4[1]	Con	1297 ± 3
2	1658 ± 37* (24)[2]	1	1713 ± 29* (32)
3	1678 ± 18* (26)	3	1585 ± 0* (22)
4	1662 ± 5* (25)	5	1659 ± 39* (28)
7	1482 ± 24* (11)	7	1592 ± 20* (23)
10	1606 ± 9* (20)	9	1716 ± 6* (32)
12	1623 ± 12* (22)	11	1485 ± 18* (14)
13	1546 ± 44* (16)	12	1648 ± 37* (27)
14	1685 ± 7* (26)	13	1648 ± 6* (27)
15	1609 ± 26* (21)	14	1719 ± 1* (32)
17	1684 ± 51* (26)	16	1762 ± 24* (36)
18	1762 ± 40* (32)	18	1704 ± 16* (31)
19	1582 ± 13* (19)	20	1771 ± 22* (37)
20	1560 ± 3* (17)	21	1764 ± 26* (36)
22	1729 ± 10* (30)	24	1671 ± 23* (29)

[1]Means ± S.D., [2]The values in parentheses are the increasing rates compared with control (%)
*p<0.05

considerably in cells treated with each extract of all seaweeds. However, difference in the increase of GSH level was not so high between seaweed extracts (Table 5).

Table 6. Effect of seaweed extracts on membrane lipid peroxidation at 50 μg/mL. Membrane lipid peroxidation determined by TBARS method. Raw 264.7 cells were exposed to ·OH generated via Fenton's reaction and oxidation products of membrane lipids which can react with TBA were determined spectroscopically at 528 nm

Lipid peroxidation intensity (% of control)			
No.	Acetone/dichloromethane Ext.	No.	Methanol Ext.
Con	100.0	Con	100.0
2	79.1	1	81.5
3	105.4	3	87.1
4	104.9	5	84.2
7	93.2	7	83.2
10	114.8	9	88.3
12	104.9	11	58.4
13	93.1	12	56.0
14	92.0	13	112.5
15	83.8	14	86.5
17	91.5	16	86.8
18	101.6	18	81.2
19	100.8	20	55.1
20	96.3	21	99.9
22	110.8	24	66.5

Lipid peroxidation is a free radical-related process in biologic systems and is associated mostly with cellular damage as a result of oxidative stress. Therefore, determining the degree of lipid peroxidation and antioxidant activity is significantly important in order to screen the antioxidant from natural product. Experiment of lipid peroxidation was performed by formation of TBARS. TBARS assay is an indicator of free-radical generation in the tissues (Heath and Packer 1968). Our data demonstrated that lipid peroxidation intensity was decreased in most of the methanol extracts, except for *Lomentaria catenata*. Especially *Callophyllis japonica*, *Scytosiphon lomentaria*, *Ishige okamurae*, and *Ahnfeltiopsis flabelliformis* showed the most strong inhibitory effects against lipid peroxidation of all methanol extracts, whereas in the case of acetone/dichloromethane extracts, the significant inhibitory effects were observed only in *Cladophora sakaii* and *Corallina confuse* (Table 6).

In conclusion, on the basis of the above results, we could suggest that *Scytosiphon lomentaria*, *Ishige okamurae*, and *Ahnfeltiopsis flabelliformis*, which showed a potent antioxidizing effect in all bioactivity tests performed above, may be useful as antioxidant sources for protection against radical-mediated oxidation of cellular biomolecules and the treatment of oxidative damage by reactive species, particularly

in the macrophage cells. *Prionitis cornea*, *Lomentaria catenata*, *Corallina confuse*, and *Sargassum horneri*, which revealed significant effects in all bioactivities tests, may be also used as good antioxidant sources. However, at this stage the components responsible for antioxidant activity of seaweed species are currently unclear. Therefore, it is suggested that further works should be performed on the isolation and identification of the antioxidant components in their active extracts.

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