J Sci Food Agric 85:2341–2348 (2005)
DOI: 10.1002/jsfa.2246

Reduction of lipid peroxidation and H_2O_2-mediated DNA damage by a red alga (*Grateloupia filicina*) methanolic extract

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Abstract: The antioxidant activity of an extract of *Grateloupia filicina* was evaluated using linoleic acid and fish oil as substrates in an induct period at 65 °C. Furthermore, the algal extract was subjected to comet assay to evaluate its protecting ability for H_2O_2-induced DNA damage in rat lymphocytes. Progression of oxidation was examined using weight gain, peroxide value (PV), 2-thiobarbituric acid reactive substances and conjugated diene data. Effectiveness of the extract at 0.03 and 0.05% levels was superior to that of α-tocopherol at 0.01% and the activity of 0.05% extract was higher than that of butylated hydroxyanisole and butylated hydroxytoluene at 0.01%. Also, the algal extract significantly inhibited H_2O_2 induced DNA damage in comet assay. The maximum DNA damage inhibition (68.9%) was recorded from the 50 μg ml^{-1} alga extract when the rat lymphocyte cells were treated with 50 μM H_2O_2.

Keywords: *Grateloupia filicina*; lipid oxidation; linoleic acid; fish oil; DNA damage; comet assay

INTRODUCTION

Marine algae, including all photosynthetic plants, are exposed to a combination of light and high oxygen concentrations. This stress-induced proline accumulation in plants can arrest photoinhibitary damage by reducing photochemical activity losses. The absence of such damage in seaweeds is in spite of the proximity of the photosynthetically produced oxygen and suitable targets within the photosynthetic apparatus.

Dimethylsulphoniopropionate (DMSP) has recently been investigated as an effective antioxidant in marine algal species. Most algal species contain high concentrations of DMSP (100–400 mmol l^{-1}) and its concentration inside cells increases rapidly with the intensity of light. It is interesting that *in vitro* experiments suggest that DMSP can more effectively scavenge hydroxyl radical than glutathione and ascorbate.[1] The lysis of DMSP substantially increases the antioxidant protection in both aqueous and lipid membrane phases within the cells of alga. Furthermore, UV light (UV-A and UV-B) can stimulate antioxidant capability in many algae species as an adaptive response to the irradiation.[2,3]

In addition, seaweeds contain vitamins and vitamin precursors, including α-tocopherol, β-carotene, niacin, thiamin and ascorbic acid.[4,5] It has also been reported that *Laurencia obtuse* (Rhodophyta) contains sesquiterpenes.[6] These compounds directly or indirectly contribute to the scavenging, inhibition or suppression of radical production.

Among algal species, red seaweeds are a very heterogeneous group with respect to the chemical composition of the cell wall and intercellular matrix.[7] Bromophenol plays a major key role as an antioxygenic compound in marine red alga.[8] Phospolipids, phenols and chlorophyll related compounds are also lipid-soluble antioxidative compounds having antioxygenic activity in red algal extracts.[9] *Grateloupia elliptica,* one of the red algae, is rich in lipoxygenase inhibitors compared with *Porphyra* spp (red alga) and *Hizikia fusiformis* (brown alga).[7] Therefore, extraction and identification of natural antioxidative compounds from marine red algae are desirable but few efforts have been made in this area to study red algal species systematically.

Natural antioxidant compounds, such as rosemary extracts and ascorbic acid, play an effective role against oil peroxidation. However, the strong and characteristic flavor of rosemary might limit the use of this spice despite its high antioxidative capacity.[10] In

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Contract/grant sponsor: Brain Korea 21 Project
(*Received 10 July 2004; revised version received 7 January 2005; accepted 3 March 2005*)
Published online 28 June 2005

aqueous systems containing metals, ascorbic acid can act as a pro-oxidant by reducing metals, which become more active catalysts of oxidation in their lower valence state.[11] In non-aqueous media, ascorbic acid and esters are not good antioxidants.[12] Therefore, novel natural antioxidants are in high demand and higher plants and their constituents have been continuously investigated.

Grateloupia filicina is a traditional food in the Kangwando area of Korea, and it has been previously reported that this alga is a potential source of natural antioxidants.[13] Therefore, in this study, *G filicina* extract was introduced at different concentrations into linoleic acid and fish oil and stored under forced convection oven conditions (65 °C) to evaluate oxidative stability. Furthermore, in this study, we introduced *G filicina* extract to control hydrogen peroxide-induced DNA damage in rat lymphocytes. The damage was evaluated by means of single-cell gel electrophoresis (comet assay).

MATERIALS AND METHODS

Materials

The marine red alga, *G filicina*, used in this study was collected close to the shores of Jeju Island in Korea during October 2003. Salt, sand and epiphytes were removed from samples by washing with tap water. Seaweed samples were finally rinsed carefully with fresh water and freeze-dried at −20 C for further experimentation. Fish oil, linoleic acid, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol, 1,1,3,3-tetraethoxypropane, Na_2EDTA, agarose and H_2O_2 were purchased from Sigma Chemical Co (St Louis, MO). All other chemicals used were of 99% or greater purity and were used as such.

Extract preparation

The dried alga sample was ground to a fine powder in a MFC SI mill (Janke and Kunkel Ika-Wreck, Staufen, Germany). The material was then sieved through a #50 standard testing sieve. The ground alga (25 g) was extracted with methanol (200 ml) overnight in a shaking water bath at room temperature. The extracts were filtered (Whatman No1) and re-extracted under the same conditions. The combined filtrates were concentrated under vacuum at 40 °C in a rotary evaporator and directly introduced into the oils.

Introduction of extract into the oils

The crude extracts were mixed with the oils (50 g) at 0.01, 0.03 and 0.05%, with a magnetic stirrer during 10 min at room temperature. Commercial antioxidants, BHT, BHA and α-tocopherol (0.01 [w/v]), were dissolved in methanol and used as reference substances for comparative purposes. A control treatment with no additive was also used as a blank sample. Finally, methanol was removed from the oil in a vacuum oven over 12 h at 35 C. Each sample (50 g) was stored separately in the oven (OF-22 GW, Jeio Tech, Korea) at 65 C for 168 h in 100-ml beakers (4.5 cm diameter and 6 cm height) and treated samples were removed after 0, 24, 48, 72, 96, 120, 144 and 168 h for further analysis. The position of the sample inside the oven was placed in a zone where the heat and airflow would be constant throughout the entire experiment.

Weight gain

Oil (2 g) was mixed with antioxidants and placed in aluminum Petri dishes (7 cm diameter and 1.5 cm height). Any trace of water in the sample was removed by keeping the samples in vacuum oven for 12 h at 35 C. The time required to attain a 0.5% weight increase in oil was taken as an index of oil stability.[14]

Peroxide value (PV)

An acetic acid–chloroform method was used for determination of PV of oil samples. The results presented are mean values of triplicate determination.[15]

Thiobarbituric acid-reactive substances (TBARS)

The oil sample (1 g) was dissolved in 3.5 ml of cyclohexane, 4.5 ml of 7.5% trichloroacetic acid (TCA)/0.34% thiobarbituric acid (TBA) were subsequently added and the resultant sample was shaken for 5 min. After centrifuging for 15 min at $1000 \times g$ samples were heated in a water bath at 100 C for 10min. TBARS values were calculated using 1,1,3,3-tetramethoxypropane as a standard precursor of malondialdehyde. Inhibition of TBARS formation was determined using the equation: percentage inhibition = 100 [1-(TBARS value for the treated sample/TBARS value for the control sample)].

Conjugated diene (CD)

Oil (50 mg) was dissolved in 5 ml of cyclohexane and the conjugated diene absorbance measured at 234 nm using spectrophotometer (Opron 3000, Hanson Tech Co Ltd, Seoul, Korea).[16]

Isolation of rat lymphocytes

Blood samples were obtained from the inferior vena cava of five male Sprague–Dawley rats aged 15 weeks (Samtako, Korea). For each treatment 100 µl of fresh whole blood was added to 900 µl of phosphate-buffered saline (PBS) and layered onto 100 µl of Histopaque 1077 (System Histopaques, Sigma Chemical, St Louis, MO, USA). After centrifugation for 5 min at 2000 rev min^{-1} at room temperature, the lymphocytes were collected from just above the boundary with Histopaque 1077, and washed with 1 ml of PBS, centrifuging under the same condition.

Incubation of lymphocytes

Lyophilized algal extract was dissolved in PBS and diluted into concentrations 0, 1, 10, 25 and 50 µg ml^{-1}. Lymphocyte samples containing 2×10^5

cells ml^{-1} were incubated with each diluted extract for 60 min at 37 °C in a dark incubator together with untreated control. After pre-incubation, samples were centrifuged at 2000 rev min^{-1} for 5 min at 4 °C. The incubated cells were re-suspended in PBS with 50 μM hydrogen peroxide (H_2O_2) for 5 min on ice. The untreated control sample was re-suspended only in PBS without H_2O_2. Cells were centrifuged as described above and then washed with 1 ml of PBS. Lymphocytes were checked for viability by trypan blue exclusion. All the experiments were repeated three times on other days.

Determination of DNA damage (comet assay)

The alkaline comet assay was conducted with little modification to evaluate extract for DNA protection.[17] The cell suspension was mixed with 75 μl of 0.5% low-melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, slides were covered with another 75 μl of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4 C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4 C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4 C, and then treated with ethanol for another 5 min before staining with 50 μl of ethidium bromide (20 μg ml^{-1}). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, Liverpool, UK) and fluorescence microscope (LEICA DMLB, Germany) in order to determine the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

Statistical analysis

Data were analyzed using the SPSS package for Windows (version 10). Values were expressed as mean ± standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A p-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The addition of oxygen to unsaturated fatty acids to form hydroperoxides is reasonably quantitative during the initial stages of peroxidation.[18] The weight gain data for both oil treated with methanol extract and commercial antioxidants are exhibited in Figs 1 and 2. All samples treated in this experiment showed delayed induction periods in comparison with their control counterparts. The time required to attain a 0.5% weight increase in oil system was taken as an index of oil stability.[14] It has been suggested that each storage date in Schaal oven condition at 65 °C is equivalent to one month of storage at ambient temperatures.[19] The rate of weight gain was slightly higher for fish oil than linoleic acid. This observation clearly explains the fact that the rate of addition of oxygen to lipid molecules to form hydroperoxides is higher for fish oil. The primary purpose of adding antioxidants to lipids is to delay the onset of oxidation and accumulation of oxidative products. Thus, *G filicina* extracts delayed the accumulation

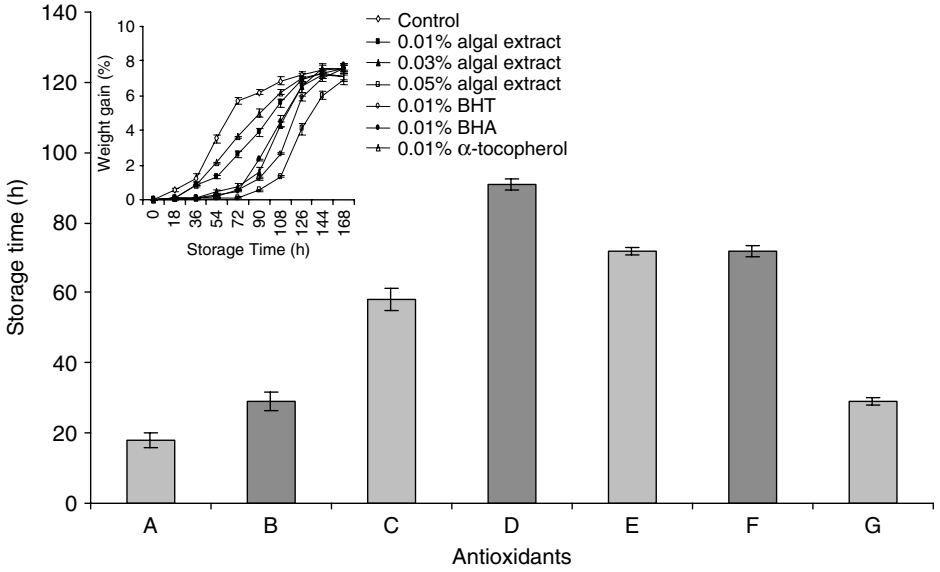

Figure 1. Effect of *G filicina* extract and conventional antioxidants on the weight gain (%) in linoleic acid. Vertical bars represents the time (h) required to achieve a 0.5% weight increase for each sample. The inset graph indicates weight gaining (%) over the entire storage period. (A) Control; (B) 0.01% algal extract; (C) 0.03% algal extract; (D) 0.05% lagal extract; (E) 0.01% BHT; (F) 0.01% BHA; (G) 0.01% α-tocopherol. ($n = 3$).

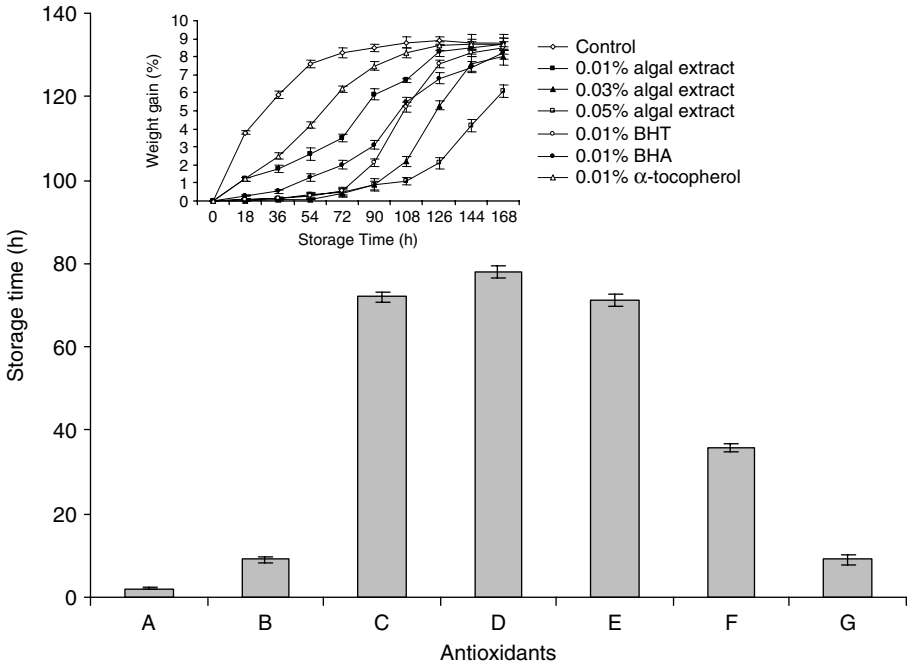

Figure 2. Effect of *G filicina* extract and conventional antioxidants on the weight gain (%) of fish oil. Vertical bar represents the time (h) required to achieve a 0.5% weight increase for each sample. The inset graph indicates weight gaining (%) over the entire storage period. (A) Control; (B) 0.01% algal extract; (C) 0.03% algal extract; (D) 0.05% algal extract; (E) 0.01% BHT; (F) 0.01% BHA; (G) 0.01% α-tocopherol. ($n = 3$).

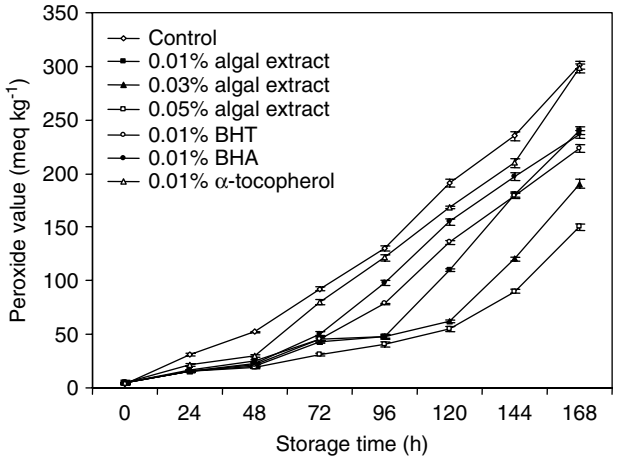

Figure 3. Effect of *G filicina* extract and conventional antioxidants on peroxide values (meq kg^{-1}) of refined linoleic acid stored under forced air convection oven at 65 °C. All data points are means of three determinations.

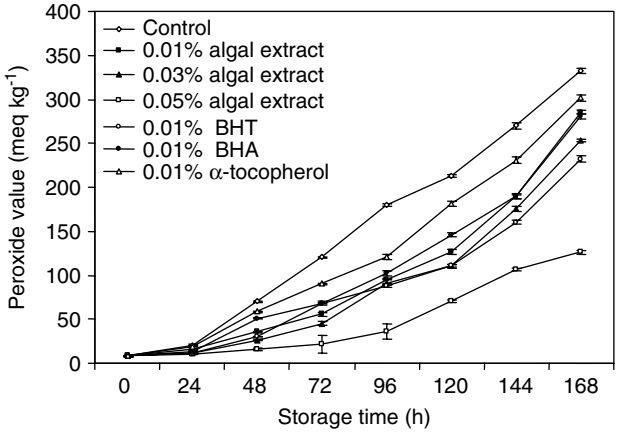

Figure 4. Effect of *G filicina* methanol extract and conventional antioxidants on peroxide values (meq kg^{-1}) of refined Fish oil stored under forced air convection oven at 65 °C. All data points are means of three determinations.

of oxidative products in both fish oil and linoleic acid.

The primary products of lipid peroxidation are hydroperoxides. Therefore, the result from determining the concentration of peroxides for oxidized oil is a clear index of lipid peroxidation. Peroxide values (PV) of linoleic acid and fish oil samples containing *G filicina* methanol extracts, α-tocopherol, BHA and BHT are presented in Figs 3 and 4. Under accelerated oxidation conditions, linoleic acid and fish oil without the addition of algal extracts were accompanied by a rapid increase in peroxide values. However, samples treated with α-tocopherol, BHA and BHT showed comparatively higher PVs than *G filicina* methanol extract at 0.05%. Moreover it was clear that effect of the extract on the reduction of peroxide value was dependent upon concentrations in both oils. Methanol extract at 0.05% showed much lower PV than α-tocopherol, BHA and BHT at 0.01%, respectively. Furthermore, the inhibitory effects of lipid peroxidation, in both oils containing methanol extract of *G filicina* were remarkably dependant upon concentration. However, measurement of peroxide values reveals information regarding the initial oxidation potential of the oil. To elucidate the antioxidants effect of the extract on other stages of lipid peroxidation, it is necessary to find whether the extract has an inhibitory effect at a later stages; hence it is important that more than one method be used to monitor the oxidation process.[20]

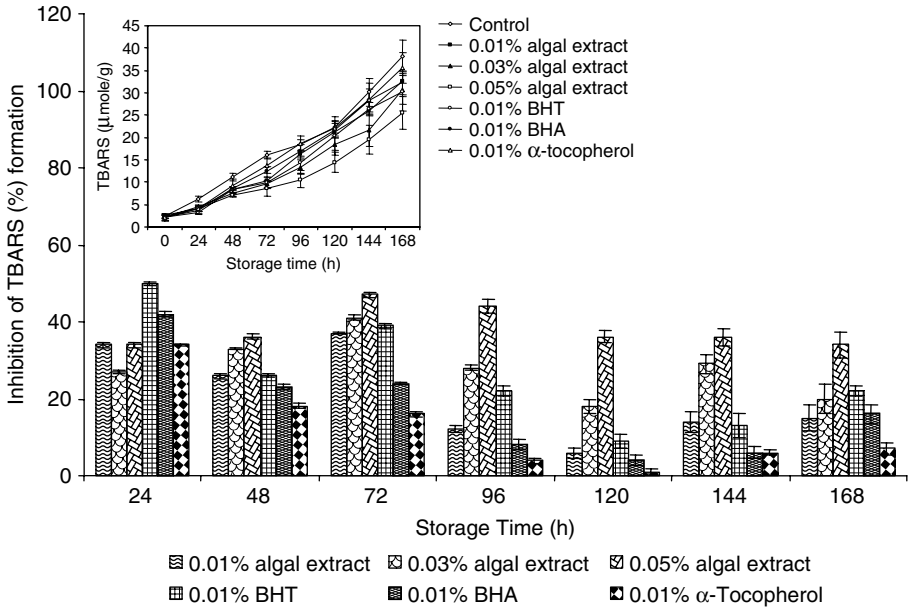

Figure 5. Effect of *G filicina* methanol extract and conventional antioxidants on 2-thiobarbituric acid-reactive substances (TBARS) values (μmol g^{-1}) of refined linoleic acid stored under forced air convection oven at 65 °C. All data points are means of three determinations.

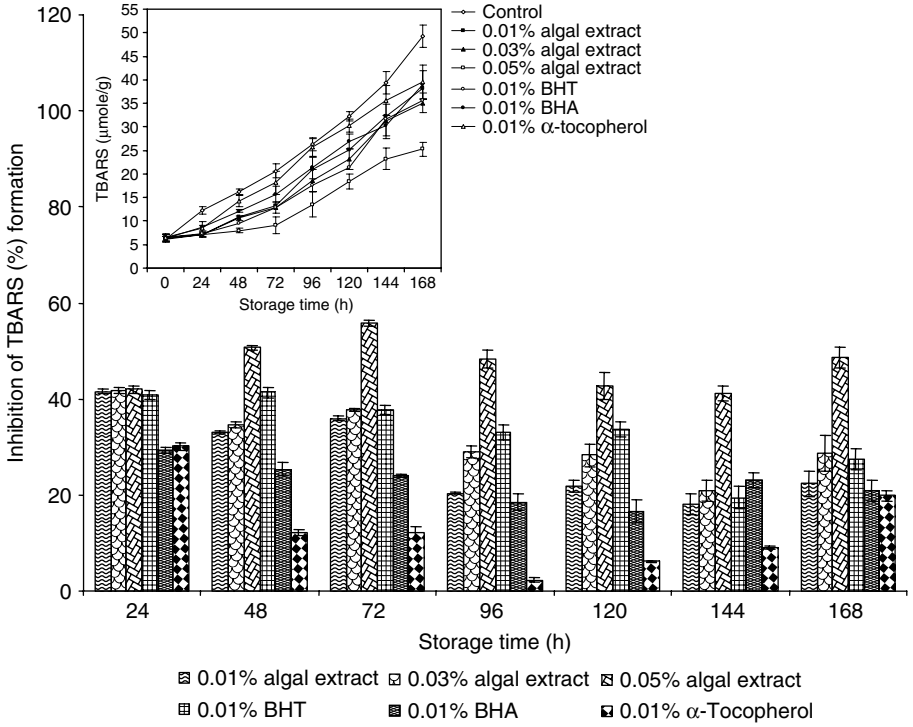

Figure 6. Effect of *G filicina* methanol extract and conventional antioxidants on 2-thiobarbituric acid-reactive substances (TBARS) values (μmol g^{-1}) of refined fish oil stored under forced air convection oven at 65 °C. All data points are means of three determinations.

The values for TBARS represent the content of secondary lipid oxidation products, mainly aldehydes (or carbonyls) that contribute to off-flavor in oxidized oils. The results of this study are presented in Figs 5 and 6. Addition of *G filicina* extract, α-tocopherol and synthetic antioxidants to linoleic acid and fish oil had the effect in lowering the formation of TBARS compared with the control sample. Among these additives, *G filicina* extract was most effective in TBARS formation at 0.05% concentration. The ability of BHA and BHT to lower TBARS values of the oils stored at 65 °C was slightly lower than that of 0.05% extract. The addition of extract at the levels of 0.01–0.03% was able to lower TBARS values more effectively than α-tocopherol. TBARS formation followed a similar pattern in both oils; however it seems that algal extract was more effective in retarding TBARS formation in fish oil than in linoleic acid.

The peroxidation of lipids can be measured as an increase in the absorbance of UV light due

to conjugated diene formation. The amount of conjugated diene is directly proportional to the amount of hydroperoxides formed as the major initial product of the lipid peroxidation reaction. At the same experimental condition, all treated oil samples delayed the formation of conjugated diene compared with their control samples (Figs 7 and 8). Among the additives, methanol extract at 0.05% was most effective in retarding conjugated diene formation in both oils. The effect of methanol extract (0.03%) was equivalent or slightly lower than that of BHT in fish oil for conjugated diene formation. In this experiment BHA did not show clear activity in retarding lipid peroxidation in fish oil.[21]

α-Tocopherol is generally regarded as an acceptable, consumer friendly antioxidant, but α-tocopherol is not heat stable. In addition, at concentration above 250 ppm, it has been reported to function as a pro-oxidant.[22] Furthermore, the pro-oxidant activity of α-tocopherol is depend on the test system, the concentration, the oxidation time and the method used to evaluate the oxidation.[23] Even though α-tocopherol is a natural antioxidant, it does not provide effective protection against *in vitro* oxidation[24] and this was confirmed in this study. In early stages, the oxidation of linoleic acid without added algal extracts was accompanied by a rapid increase of conjugated diene value. According to the results (Fig 7) it is evident that addition of all the extracts delayed the formation of conjugated diene, as shown by the low rate of accumulation of oxidative product (diene) compared with commercial antioxidants.

Apparently the methanolic extract of *G filicina* used in this study plays an important role in prevention of oil oxidation in both fish oil and linoleic acid. The antioxidative effectiveness in natural sources has been reported to be dependent on the contents of phenolic compounds. The total phenolic content of the methanolic extract of *G filicina* is approximately 200 mg 100 g^{-1}.[25] In addition to phenolic compounds, algae contain vitamins and vitamin precursors, including α-tocopherol, β-carotene, niacin, thiamine, and ascorbic acid.[4,5] *G filicina* also may contains those compounds or related compounds, which could prevent fish oil and linoleic acid from on going rancidity. Apart from being an effective antioxidant, the methanolic extract of *G filicina* did not show any visible color or odor change to the treated linoleic acid and fish oil, thus this could benefit its use in different food formulations.

In this study, the presence of *G filicina* extract prevented hydrogen peroxide induced DNA damage dose dependently (Fig 9) in rat lymphocytes. The maximum inhibition of >69% was recorded at the concentration of 50 $\mu g\,ml^{-1}$. For algal extract

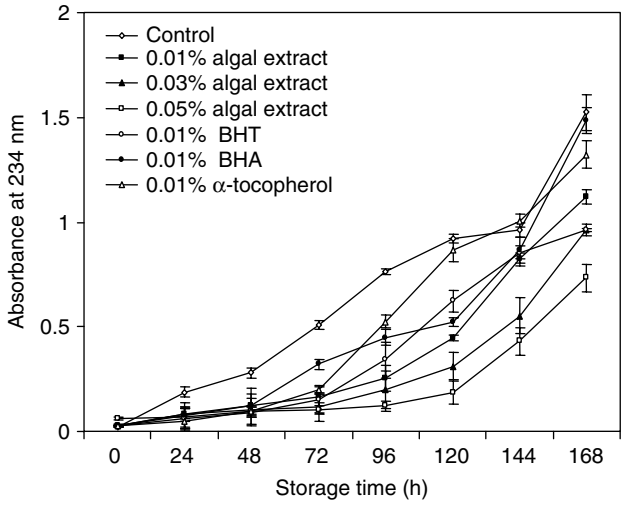

Figure 7. Effect of *G filicina* methanol extract and conventional antioxidants on the formation of conjugated diene in linoleic acid during storage under forced air convection oven at 65 °C. All data points are means of three determinations.

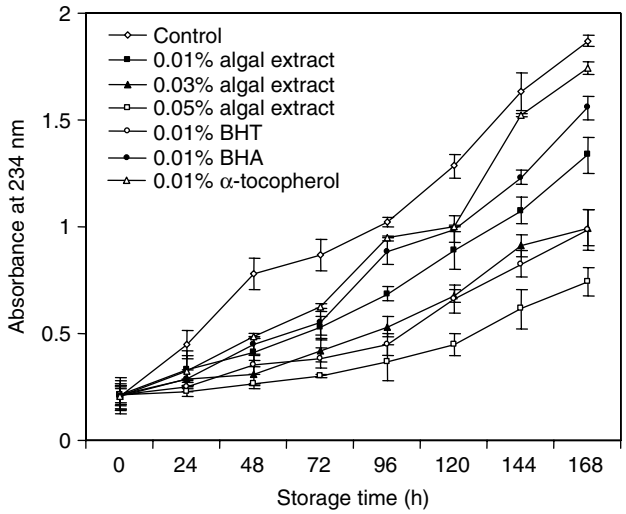

Figure 8. Effect of *G filicina* methanol extract and conventional antioxidants on the formation of conjugated diene in Fish oil during storage under forced air convection oven at 65 °C. All data points are means of three determinations.

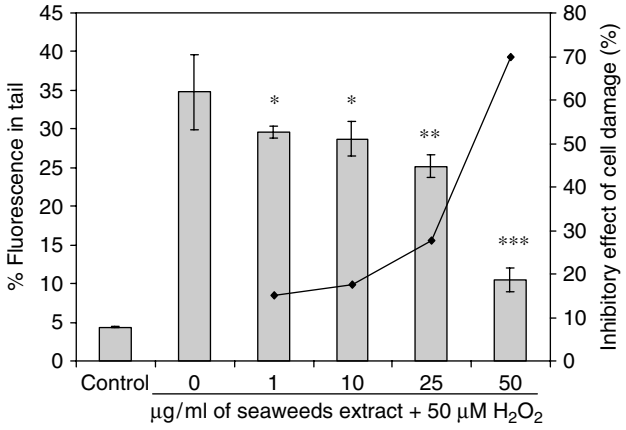

Figure 9. The effect of supplementation *in vitro* with different concentrations of *G filicina* methanol extract on H_2O_2 induced rat lymphocytes DNA damage. Values are means with standard error of duplicated experiments. Significant difference to values for samples treated with 50 μM H_2O_2 only using LSD: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. □ % fluorescence in tail; —●— inhibitory effect of cell damage.

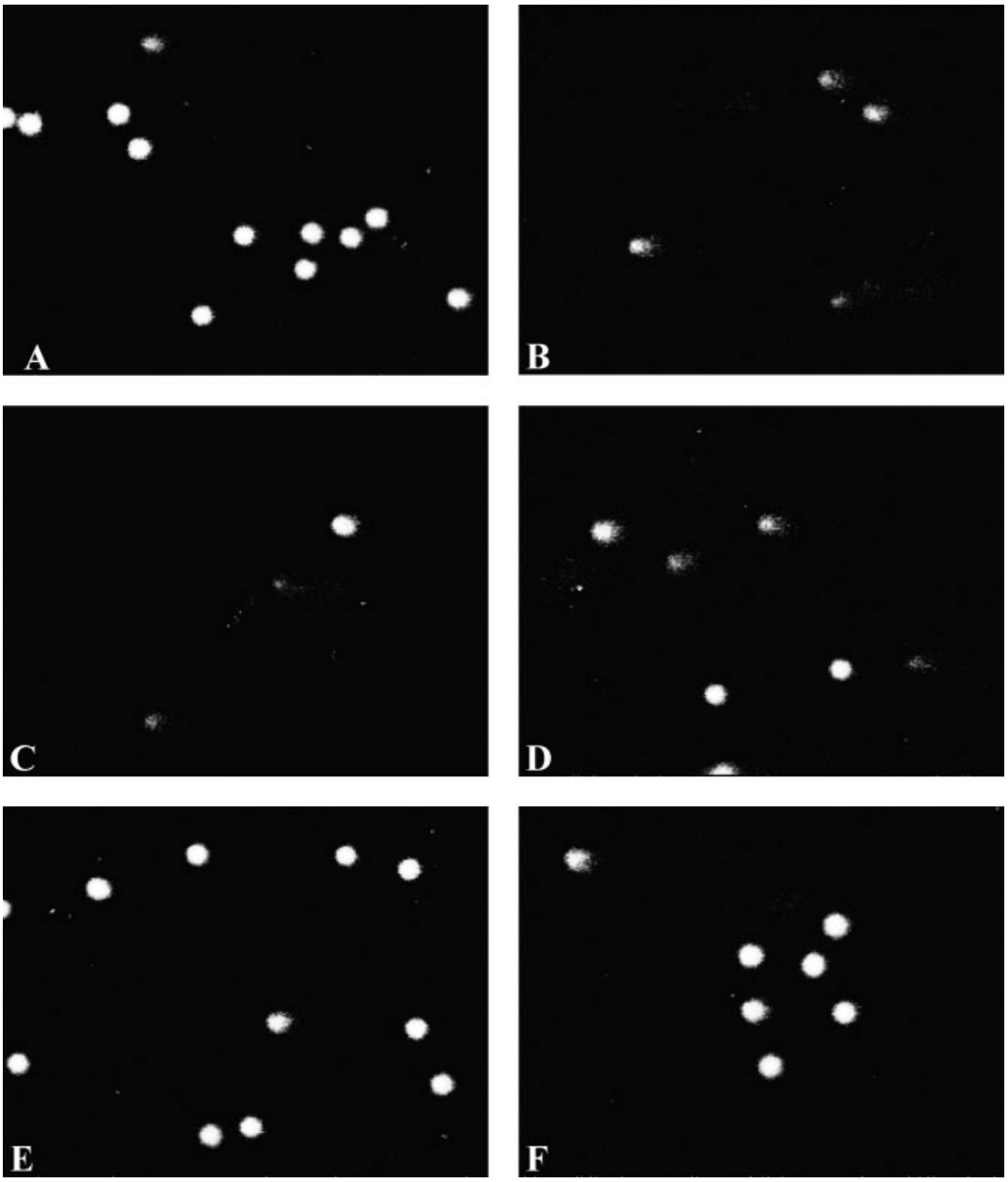

Figure 10. Comet images of rat lymphocytes. A: (A) negative control; (B) lymphocytes treated with 50 μM H_2O_2; (C) lymphocytes treated with 1 μg ml^{-1} algal extract +50 μM H_2O_2; (D) lymphocytes treated with 10 μg ml^{-1} algal extract +50 μM H_2O_2; (E) lymphocytes treated with 25 μg ml^{-1} algal extract +50 μM H_2O_2; (F) lymphocytes treated with 50 μg ml^{-1} algal extract +50 μM H_2O_2.

concentrations from 1 to 10 μg ml^{-1}, there was no significant DNA damage inhibition, but, from 10 to 25 μg ml^{-1} extract concentration, there was significant inhibition ($p < 0.01$). The inhibitory effect of cell damage increased more than twofold from 25 to 50 μg ml^{-1} algal extract concentration, which outweights the ability of the extract to inhibit H_2O_2 induced DNA damage.

In the present study, inhibition images of hydrogen peroxide induced DNA damage is shown in Fig 10. These images confirm the ability of *G filicina* crude extract to protect DNA against H_2O_2 radical attack. The DNA damage decreases with the increased concentration from 1 to 50 μg ml^{-1}, and consequently the relative tail intensity linearly decreased with the concentration. The photomicrographs of DNA migration show low strand breaks with high extract concentration, where untreated sample shows maximum strand breaks at the same experimental condition. The negative control, without algal extract, has a clear image suggesting that minor/no strand breaks can take place without hydrogen peroxides. In agreement with the present experiment, it has been shown that the methanol extract of *G filicina* has >65% hydrogen peroxide radical inhibition ability over the hydrogen peroxide scavenging assay.[25]

DNA damage is responsible for several degenerative diseases, including Alzheimer's disease, Parkinson disease, Hodgkin's disease and Bloom's syndrome. Therefore, inhibition of DNA damage may be one

of the strategies in the chemoprevention of clinical disorders.[26] The comet assay, which is known as single-cell gel electrophoresis, is an ideal technique to detect DNA breaks and damage in eukaryotic cells. DNA loops containing breaks extend under electrophoreses to form 'comet tails', and the relative intensity of the tail indicates the DNA break frequency.[27] This result implies that the *G filicina* extract contains compounds with high antioxidant activity. As a popular edible alga, its potential antioxidant activity can be used to make different food formulations. In addition, the effects were seen at concentrations readily achievable at a reasonable intake of *G filicina*, provided that bio-availability is not a problem.

CONCLUSION

The methanolic extract of *G filicina* showed good antioxidant activities in the tested assays including the reduction of lipid peroxidation in oils and DNA damage in rat lymphocytes.

ACKNOWLEDGEMENT

This work was supported by the Brain Korea 21 Project in 2004.

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