

## Evaluation of the Antioxidant Properties of *Pediastrum duplex* and *Dactylococcopsis fascicularis* Microalgae

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Interest in plant-derived food additives has increased recently, with efforts to identify natural antioxidant sources to replace synthetic antioxidants. This study evaluated the antioxidant effects of organic solvent fractions of 80% methanol extracts from *Pediastrum duplex* and *Dactylococcopsis fascicularis* microalgae. Among the solvent fractions tested, the ethyl acetate and n-hexane fractions from *P. duplex* effectively scavenged 79.8% and 74.5% of DPPH free radicals, respectively. The chloroform fraction from *D. fascicularis* showed the strongest H<sub>2</sub>O<sub>2</sub> scavenging ability (49.7%). The greatest scavenging of hydroxyl radicals (73.1%) was exhibited by the 80% methanol extract from *P. duplex*. Aqueous residue and ethyl acetate fraction from *P. duplex* provided the strongest nitric oxide scavenging (57.7%) and metal chelating effect (82.1%), respectively. Chloroform and ethyl acetate fractions from *P. duplex* and n-hexane fraction from *D. fascicularis* exhibited significantly greater inhibition of lipid peroxidation than the commercial antioxidants. These results suggested that *P. duplex* and *D. fascicularis* microalgae having potential antioxidative compounds with various properties could be utilized in the food and pharmaceutical industries.

Key words: Microalgae, *Pediastrum duplex*, *Dactylococcopsis fascicularis*, Organic fractions, Antioxidant

### Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and superoxide anions are formed in living organisms by cellular processes such as mutagenesis, carcinogenesis, and premature aging that involve both exogenous (e.g., tobacco smoke, ionizing radiation, and certain pollutants) and endogenous (e.g., normal aerobic respiration) factors. ROS react with a large variety of easily oxidizable cellular components, including proteins, lipids, and lipoproteins (Fridovich, 1995). Oxidation is a primary cause of food deterioration, resulting in the destruction of fat-soluble vitamins and the development of off-colors and toxicants (Yang et al., 2000; Ukeda et al., 2002).

Antioxidants can prevent oxidative damage by free radicals and ROS. Commercial antioxidant supplements such as butylated hydroxyanisole (BHA), propyl gallate have been used to reduce oxidative

butylated hydroxytoluene (BHT),  $\alpha$ -tocopherol, and damage in the human body (Sherwin, 1990; Gülçin et al., 2002). However, these antioxidants are associated with side effects such as liver damage and carcinogenesis (Lindenschmidt et al., 1986). Thus, researchers and consumers seek to replace these synthetic products with natural antioxidants in foods and medicines.

Plants and algae are sources of natural antioxidants. Plants and algae absorb solar radiation and generate high levels of oxygen as a secondary metabolite during photosynthesis. This oxygen can be activated by ultraviolet radiation and solar heat to produce toxic ROS in plants and algae. The diverse antioxidative compounds present in plants and algae enhance their survival by neutralizing these ROS (Lu and Foo, 1995).

Many studies have investigated the antioxidant effects of macroalgae, but less attention has been paid to microalgae because of difficulties in the isolation and cultivation of these species. Recent research has

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focused on the use of microalgae as sources of nutraceuticals for the health food market. For example, *Chlorella* sp. and *Spirulina* sp. extracts have been included in pasta, bread, green tea, milk, beer, and candy (Liang et al., 2004). Microalgae such as *Chlorella* sp., *Spirulina* sp., and *Dunaliella* sp. are grown commercially for the production of algal products such as  $\beta$ -carotene, lutein, and phycocyanin. The antioxidative activity of phycocyanobilin from *Spirulina platensis* has been evaluated for prevention of methyl linoleate oxidation in hydrophobic systems and in phosphatidylcholine liposomes; phycocyanobilin effectively inhibited the peroxidation of methyl linoleate and produced a prolonged induction period (Hirata, 2000). *Aphanizomenon flos-aquae* (Cyanophyta) is rich in phycocyanin, a photosynthetic pigment with antioxidant and anti-inflammatory properties (Benedettia et al., 2004).

The objective of this study was to investigate the antioxidant properties of 80% methanol extracts and their organic solvent fractions from *Pediastrum duplex* and *Dactylococcopsis fascicularis* microalgae from Jeju Island, Korea. Another objective was to extend the possibility to utilize these algae species in the food and pharmaceutical industries.

## Materials and Methods

### Chemicals

The following were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA): 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium nitroprusside, sulphaniic acid, naphthyl ethylenediamine dihydrochloride, xanthine, xanthine oxidase from buttermilk, nitroblue tetrazolium salt (NBT), BHT,  $\alpha$ -tocopherol, 3-(2-pyridyl)-5,6-di(p-sulfophenyl)-1,2,4-triazine disodium salt (ferrozine), potassium ferricyanide [ $K_3Fe(CN)_6$ ], Folin-Ciocalteu reagent, and linoleic acid. Peroxidase, 2-deoxyribose, and 2,2'-azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS) were purchased from Fluka Chemie (Buchs, Switzerland). All other chemicals were of analytical grade.

### Sample collection and isolation

Microalgal samples were directly collected in 1-L plastic bottles from several locations along the Sanji stream, Jeju Island, Korea. Environmental factors such as water temperature and pH were measured at each sampling spot. The recommended concentration of F/2 culture medium (Aquacenter Inc., Leland, MS, USA) was added to the samples, and the bottles were

immediately transferred to a plant growth chamber (Model VS-3D; Vision Scientific Co., Korea) for 3 days of incubation at temperatures of 20°C, 25°C, and 30°C. The samples were then removed from the chamber, the pH was again measured, and 1 mL of each sample was transferred to a Sedgwick-Rafter chamber for observation of phytoplankton abundance and community changes, under an inverted microscope. Single cells of *P. duplex* and *D. fascicularis* were collected with micropipettes and transferred into 12-microwell cell culture plates that contained F/2 culture medium, soil extract, and distilled water. Subculture was performed until phytoplankton monostrains were obtained.

### Mass culture

Mass culture of each algal strain was grown in F/2 nutrient culture medium, 10% soil extract, and distilled water, at a light intensity of 180  $\mu$ E/m/s and a 12-h light-dark cycle. The *P. duplex* culture was kept at a temperature of 25°C and pH 8.0, and the *D. fascicularis* culture was kept at 30°C and pH 8.5. After incubation for 2 weeks in 10-L transparent polycarbonate containers (Nalgene, Rochester, NY, USA), the microalgal biomass was separated by vacuum filtration, freeze-dried, and weighed.

### Proximate composition

Proximate chemical compositions of the freeze-dried microalgae samples were determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1980). The Soxhlet method was used to determine crude lipid content, and the Kjeldhal method was used to determine crude protein content. Ash content was determined by calcination in a furnace at 550°C, and moisture content was determined by placing the samples in a dry oven at 105°C for 24 h. The crude protein content of the solvent fractions and the 80% methanol extracts were determined by the method of Lowry (1951). The polysaccharide contents were determined by the phenol-sulfuric method.

### Preparation of 80% methanol extracts and solvent fractions

Powdered, freeze-dried algal samples (5 g) were extracted with 80% methanol (500 mL) at 25°C for 24 h under shaking conditions. The mixture was filtered, and the 80% methanol extract was collected and concentrated. The obtained extracts were fractionated in a separatory funnel with a sequence of organic solvents: n-hexane, chloroform, and ethyl acetate (Fig. 1). Each fraction was concentrated and

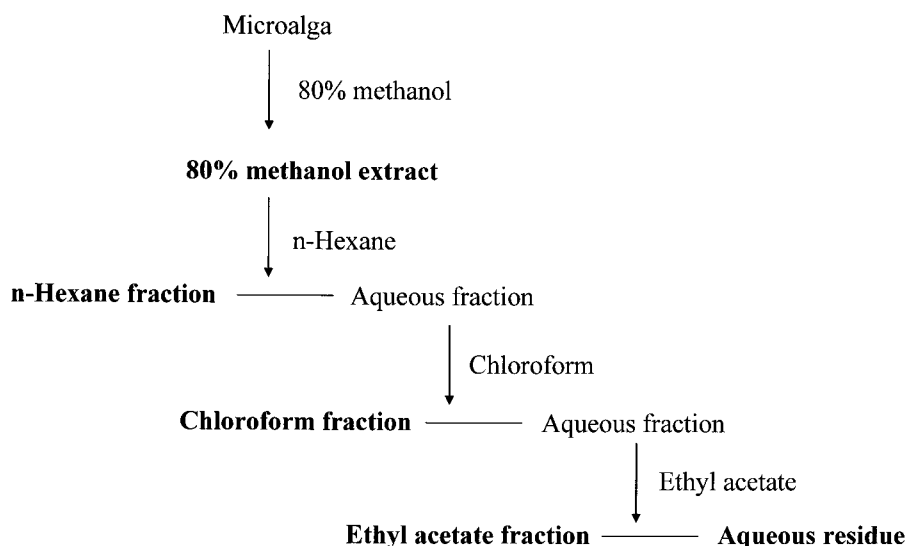


Fig. 1. Solvent fractionation procedure of *P. duplex* and *D. fascicularis*.

redissolved in methanol to a concentration of 2 mg/mL. All activities of the fractions were compared with those of commercial antioxidants (BHT and  $\alpha$ -tocopherol) dissolved in methanol (2 mg/mL).

#### DPPH free radical scavenging assay

DPPH free radical scavenging activity of the fractions was measured using a modified method of Brand-Williams et al. (1995). Each 2-mL sample was mixed with 2 mL of DPPH solution ( $3 \times 10^{-5}$  M) and incubated for 30 min. Then the absorbance at 517 nm was recorded using a UV-VIS spectrophotometer (Opron 3000; Hanson Tech. Co. Ltd., Seoul, Korea).

#### Hydrogen peroxide scavenging assay

Ability of the microalgae fractions to scavenge hydrogen peroxide ( $H_2O_2$ ) was determined according to the method of Muller (1995). To each 80  $\mu$ L sample, 20  $\mu$ L of 10 mM  $H_2O_2$  were added, and this was then mixed with 100  $\mu$ L of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37°C for 5 min. Thereafter, 30  $\mu$ L of 1.25 mM ABTS and 30  $\mu$ L of peroxidase (1 U/mL) were added, and the mixture was incubated at 37°C for 10 min. The absorbance at 405-nm was measured using a microplate reader (Sunrise; Tecan Co., Ltd., Grödig, Austria).

#### Superoxide anion scavenging assay

Superoxide anion scavenging activity of the microalgae fractions was measured using the method of Nagai et al. (2003). A mixture containing 0.48 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 mL of 3 mM xanthine, 0.02 mL of 3 mM EDTA, 0.02 mL

of 0.15% bovine serum albumin, 0.02 mL of 0.75 mM NBT, and 0.02 mL of sample was incubated at 25°C for 10 min. The reaction was started by adding 6-mU of xanthine oxidase, and the sample was maintained at 25°C for 20 min. Finally, 0.02 mL of 6 mM copper chloride was added, and the absorbance at 560 nm was measured.

#### Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined as described by Chung et al. (1977). The Fenton reaction mixture [200  $\mu$ L of 10 mM ferrous sulfate heptahydrate ( $FeSO_4 \cdot 7H_2O$ ), 200  $\mu$ L of 10 mM EDTA, and 200  $\mu$ L of 10 mM 2-deoxyribose] was mixed with 1.2 mL of phosphate buffer (0.1 M, pH 7.4) and 200  $\mu$ L of sample. Thereafter, 200  $\mu$ L of 10 mM  $H_2O_2$  were added, followed by incubation at 37°C for 4 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added, and the mixture was placed in a boiling water bath for 10 min. After cooling, the mixture was clarified by centrifugation, and the absorbance at 532 nm was measured.

#### Nitric oxide radical scavenging assay

Nitric oxide radical scavenging was determined according to the method of Garrat (1964). Two milliliters of 10 mM sodium nitroprusside in 0.5 mL of phosphate-buffered saline (pH 7.4) were mixed with 0.5 mL of sample and incubated at 25°C for 150 min. After incubation, 0.5 mL of the mixture was added to 1.0 mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 mL of naphthyl ethylenediamine dihydrochloride (0.1% w/v) was

added, and the mixture was incubated at room temperature for 30 min. The absorbance at 540 nm was measured.

#### Metal chelating ability

Metal chelating ability was determined according to the method of Decker and Welch (1990). The sample (5 mL) was added to 0.1 mL of 2 mM ferric chloride (FeCl<sub>2</sub>), then mixed with 0.2 mL of 5 mM ferrozine, and incubated for 10 min at room temperature under shaking conditions. After incubation, the absorbance at 562 nm was measured.

#### Determination of lipid peroxidation inhibitory effect

Lipid peroxidation inhibitory effect of the microalgae samples was determined according to the ferric thiocyanate (FTC) method (Kikuzaki and Nakatani, 1993). Each sample (2 mL) was mixed with 2 mL of 2.51% linoleic acid in ethanol, 4 mL of 0.05 M phosphate buffer (pH 7), and 2 mL of distilled water. The reaction mixture was kept at 40°C in a darkroom. Then, 0.1 mL of the reaction mixture was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. After 5 min, 0.1 mL of 0.02 M FeCl<sub>2</sub> in 3.5% HCl was added, and the absorbance at 500 nm was measured every 24 h for 7 days.

#### Total phenolic content

Folin-Ciocalteu reagent was used to determine the total phenolic compounds in the samples, according to the method of Chandler and Dodds (1993). Gallic acid was used as a standard phenolic compound. Each microalgae sample (1 mL) was mixed with 1 mL of 95% ethanol, 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. After 5 min, 1 mL of 5% sodium bicarbonate was added, and the mixture was placed in a darkroom for 1 h. The absorbance at 725 nm was measured.

#### Statistical analysis

Statistical analyses were conducted with SPSS version 11.5 software, using triplicate (n=3) test data. The mean values of the fractions were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. A P-value of less than 0.05 was considered to be significant.

## Results

Water temperature at the collection sites varied from 25°C to 30°C, and the pH values ranged from 7.6 to 8.2. The cultures of *P. duplex* showed better

growth at 25°C and pH 8.1; and *D. fascicularis* grew best at 30°C and pH of 8.5. The dry-weight biomass productions of *P. duplex* and *D. fascicularis* were 0.8 g/L and 0.6 g/L, respectively.

Proximate compositions of freeze-dried *P. duplex* and *D. fascicularis* are shown in Table 1. The moisture contents of *P. duplex* and *D. fascicularis* were 6.1% and 5.5%, respectively. The ash, carbohydrate, and lipid contents of *P. duplex* were 15.2, 30.0, and 2.4%, respectively; those of *D. fascicularis* were 8.9, 33.7, and 4.5%, respectively. Protein was the primary component of both microalgae: 46.3% for *P. duplex* and 47.4% for *D. fascicularis*. The total phenolic, polysaccharide, and protein contents in the 80% methanol extracts and solvent fractions are listed in Table 2. Significant differences in the total phenolic, polysaccharide, and protein contents were observed between species and among fractions.

Table 1. Proximate composition of *P. duplex* and *D. fascicularis*

Nutrient	<i>P. duplex</i>	<i>D. fascicularis</i>
Moisture (%)	6.1 ± 0.3	5.5 ± 0.3
Carbohydrate (%)	30.4 ± 0.2	33.7 ± 0.1
Protein (%)	46.3 ± 0.1	47.4 ± 0.2
Lipid (%)	2.4 ± 0.2	4.5 ± 0.3
Ash (%)	15.2 ± 0.4	8.9 ± 0.4

Values are means of three replicates ± SD.

DPPH scavenging activities of the 80% methanol extracts and the solvent fractions are shown in Table 3. Significant differences in activity were observed between species and among fractions. The 80% methanol extract and all *P. duplex* fractions displayed remarkably high DPPH scavenging activity, with the highest activity observed in the ethyl acetate (79.8%) and n-hexane (74.5%) fractions. The fractions from *D. fascicularis* produced lesser effects; the chloroform and ethyl acetate fractions exhibited the highest activity among the *D. fascicularis* samples (49.4% and 39.8%, respectively).

Table 3 presents the H<sub>2</sub>O<sub>2</sub> scavenging activities of the 80% methanol extracts and solvent fractions. Among the *P. duplex* samples, the chloroform (47.2%) and ethyl acetate (48.7%) fractions demonstrated the greatest activity. For *D. fascicularis*, the chloroform fraction (49.7%), 80% methanol extract (47.7%), and n-hexane fraction (42.9%) demonstrated the greatest H<sub>2</sub>O<sub>2</sub> scavenging activity.

Chloroform (40.5%) and ethyl acetate (31.5%) fractions from *P. duplex* exhibited the greatest superoxide anion scavenging effect among all microalgae samples (Table 3).

The greatest hydroxyl radical scavenging effects

Table 2. Total polyphenolic, polysaccharide, and protein contents of 80% methanol extracts, and its different fractions from *P. duplex* and *D. fascicularis*

Microalgae	Fractions	Yield (g/100 g)	Total phenolic <sup>a</sup> (mg/100 g)	Polysaccharide <sup>b</sup> (mg/100 g)	Protein <sup>c</sup> (mg/100g)
<i>P. duplex</i>	Methanol	15.8	172.4 ± 12.2	1,398 ± 98.5	1,069 ± 79.4
	n-Hexane	5.5	41.7 ± 2.8	571.1 ± 27.1	240.3 ± 19.2
	Chloroform	0.5	15.5 ± 0.7	50.1 ± 2.4	25.1 ± 1.2
	Ethyl acetate	1.0	16.2 ± 0.6	209.1 ± 15.6	180.2 ± 13.1
	Aqu. resi.	8.8	88.3 ± 4.6	567.9 ± 23.4	563.6 ± 32.3
<i>D. fascicularis</i>	Methanol	14.8	245.3 ± 19.1	1352 ± 93.5	753.1 ± 44.2
	n-Hexane	2.3	10.8 ± 0.3	153.2 ± 11.2	48.8 ± 2.3
	Chloroform	10.0	185.7 ± 13.4	843.8 ± 46.7	540.2 ± 24.2
	Ethyl acet.	1.0	12.6 ± 0.7	80.7 ± 4.9	73.3 ± 3.4
	Aqu. resi.	1.5	16.8 ± 1.2	306.1 ± 17.6	89.7 ± 5.3

Values are means of three replicates ± SD.

<sup>a</sup>As equivalent to gallic acid, <sup>b</sup>As equivalent to glucose, <sup>c</sup>As equivalent to bovine serum albumin.

Table 3. Antioxidants activity of 80% methanol extracts and solvent fractions from *P. duplex* and *D. fascicularis*

Microalgae	Fractions	DPPH <sup>a</sup>	H <sub>2</sub> O <sub>2</sub> <sup>b</sup>	O <sub>2</sub> <sup>-c</sup>	OH <sup>d</sup>	NO <sup>e</sup>	FC <sup>f</sup>
<i>P. duplex</i>	Methanol	65.9d ± 3.2	38.9d ± 1.6	19.6g ± 0.3	73.1c ± 5.8	13.3e ± 0.6	26.6b ± 1.7
	n-Hexane	74.5c ± 5.2	32.1e ± 1.3	22.4d ± 1.6	15.5fg ± 2.3	28.3d ± 0.7	13.9h ± 0.1
	Chloroform	65.9d ± 4.3	47.2b ± 2.4	40.5b ± 2.7	113.5f ± 0.4	12.7f ± 0.1	11.5g ± 0.2
	Ethyl ace.	79.8b ± 5.7	48.7b ± 2.6	31.5c ± 2.1	17.4g ± 0.3	27.6d ± 1.1	82.1a ± 6.4
	Aqu. res.	59.9e ± 1.3	32.5e ± 1.1	15.5f ± 0.6	18.7e ± 0.4	57.7a ± 2.7	11.8g ± 0.4
<i>D. fascicularis</i>	Methanol	23.1h ± 1.3	47.7b ± 2.3	13.5h ± 0.1	23.3e ± 0.9	12.7e ± 0.1	14.1e ± 0.7
	n-Hexane	39.8g ± 1.7	42.9c ± 2.1	15.5f ± 0.3	34.1d ± 1.4	45.7b ± 1.8	14.5e ± 0.8
	Chloroform	49.4f ± 2.1	49.7b ± 1.7	19.7e ± 0.7	36.4d ± 1.3	12.3f ± 0.1	18.8e ± 0.8
	Ethyl ace.	39.8g ± 2.4	25.2f ± 0.8	18.9e ± 0.3	11.4f ± 0.6	42.7c ± 1.8	37.6c ± 1.6
	Aqu. res.	38.9g ± 2.7	15.2g ± 0.7	15.3h ± 0.4	11.2f ± 0.3	16.5f ± 0.4	49.2b ± 1.9
	BHT	94.6a ± 6.4	60.1a ± 4.2	63.2a ± 4.3	76.6b ± 4.6	26.1d ± 0.9	11.5g ± 0.1
	Tocopherol	94.3a ± 7.1	62.5a ± 4.9	61.5a ± 4.7	79.5a ± 4.7	25.2d ± 0.6	10.3g ± 0.1

Sample concentration is 2 mg/mL; Values are Means ± SE (n=3); values in each column followed by different letters denote significant difference at  $P < 0.05$ .

<sup>a</sup>DPPH free radical scavenging activity, <sup>b</sup>hydrogen peroxide scavenging activity, <sup>c</sup>superoxide anion scavenging activity, <sup>d</sup>hydroxyl radical scavenging activity, <sup>e</sup>nitric oxide scavenging activity, <sup>f</sup>ferrous ion chelating effect.

were observed in the 80% methanol extract from *P. duplex* (73.1%; Table 3). The n-hexane (34.1%) and chloroform (36.4%) fractions showed the greatest hydroxyl radical scavenging activity among the *D. fascicularis* samples. No significant effects were observed in the other fractions.

Aqueous residue (57.7%) and the n-hexane (28.3%) and ethyl acetate (27.6%) fractions from *P. duplex* demonstrated significant nitrous oxide scavenging activity, which was greater than that of the commercial antioxidants ( $P < 0.05$ ; Table 3). The n-hexane (45.7%) and ethyl acetate (42.7%) fractions from *D. fascicularis* also exhibited significant activity ( $P < 0.05$ ).

Significantly strong metal chelating effects were observed in the ethyl acetate fraction (82.1%) and 80% methanol extract (26.6%) from *P. duplex*, as

well as in the aqueous residue (49.2%) and ethyl acetate fraction (37.6%) from *D. fascicularis* (Table 3). These effects were stronger than those of the commercial antioxidants ( $P < 0.05$ ).

As shown in Fig. 2, an increased absorbance of the linoleic acid emulsion was observed without the addition of extracts (control sample). The chloroform and ethyl acetate fractions from *P. duplex* and the n-hexane fraction from *D. fascicularis* exhibited significantly greater inhibition of lipid peroxidation than the commercial antioxidants. No significant antioxidant activity was present in the other fractions.

## Discussion

Recent research has sought safe and effective natural antioxidants that can be substituted for the

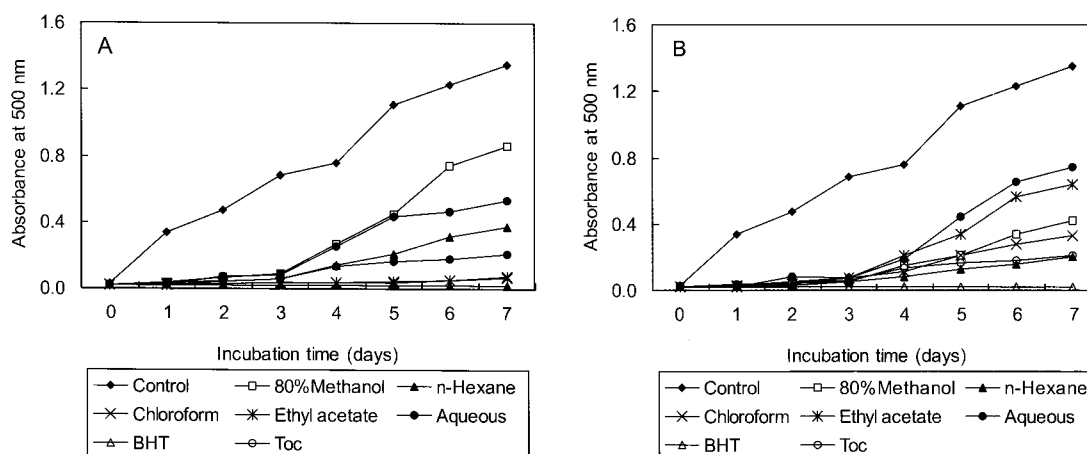


Fig. 2. Lipid peroxidation inhibitory activity of 80% methanol extracts and solvent fractions from (A) *P. duplex* and (B) *D. fascicularis* compared to BHT and  $\alpha$ -Tocopherol at 1 mg/mL of ethanol concentration as assessed by linoleic acid.

currently used commercial synthetic antioxidants BHA and BHT. Microalgae have become good candidates for sources of natural antioxidants, as revealed by a number of recent studies (Hirata et al., 2000; Benedettia et al., 2004; Karawita et al., 2007; Lee et al., 2008). The present study investigated the antioxidant effects of *P. duplex* and *D. fascicularis* by testing organic fractions from these microalgae.

DPPH is a free radical-generating compound that has been widely used to evaluate the free radical scavenging ability of various antioxidative compounds. All *P. duplex* solvent fractions in this study efficiently scavenged free radicals, indicating that DPPH-scavenging biochemical compounds with both hydrophilic and hydrophobic properties were dispersed in the aqueous residue and organic fractions. Oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of many chronic conditions (Gülçin et al., 2003). Radical scavengers may protect tissues from free radical damage, thereby preventing diseases such as cancer (Nakamura et al., 1996).

Produced during normal metabolism,  $H_2O_2$  is converted in the body to water and singlet oxygen, a powerful oxidizing agent (Halliwell, 1991). All solvent fractions in this study efficiently scavenged  $H_2O_2$ , indicating that the potential biochemical compounds exhibited both hydrophilic and hydrophobic properties. Although  $H_2O_2$  is not very reactive, it can convert to more reactive species such as singlet oxygen and hydroxyl radicals (Halliwell, 1991). Thus, the removal of  $H_2O_2$  is important for the protection of living systems.

Superoxide anion and hydroxyl radicals are the two

most effective representative free radicals. All solvent fractions in this study exhibited superoxide anion scavenging activity, indicating the presence of both hydrophilic and hydrophobic compounds. In cellular oxidation reactions, superoxide anion radicals are normally formed first. The effects of this radical are magnified when it produces free radicals and oxidizing agents that induce cell damage (Liu and Ng, 2000). Superoxide anion radical is a precursor to active free radicals that may react with biological macromolecules and thereby induce tissue damage (Halliwell and Gutteridge, 1989).

Hydroxyl radical scavenging activity of the microalgae solvent fractions was determined as the percentage of hydroxyl radical inhibition generated in the Fenton reaction mixture. Solvent fractions from both species exhibited considerable activity. The 80% methanol extract from *P. duplex* showed the strongest scavenging effect, implying that the bioactive compound may be hydrophilic. Both hydrophilic and hydrophobic properties were demonstrated by *D. fascicularis* fractions. Hydroxyl radical is the most reactive of the ROS because of its strong ability to react with a wide range of biomolecules. This ROS can oxidatively react with biological materials through hydrogen withdrawal, double bond addition, electron transfer, and radical formation; it also initiates auto-oxidation, polymerization, and fragmentation (Liu and Ng, 2000).

Nitric oxide is a gaseous free radical that serves important roles in many physiological and pathological conditions. Nitric oxide scavenging effect of *P. duplex* was produced primarily by a hydrophilic compound, as indicated by a significantly higher

activity level in the aqueous residue. On the other hand, n-hexane fraction from *D. fascicularis* exhibited significant nitric oxide scavenging activity, indicating the presence of bioactive compounds with both hydrophilic and hydrophobic properties. Marcocci et al. (1994) reported that scavengers of nitric oxide compete with oxygen, leading to reduced nitric oxide production.

As a result of its high reactivity, ferrous is known as the most important lipid oxidizing pro-oxidant among the transition metals. Ferrozine produces red complexes with ferrous ions. The formation of these complexes is interrupted in the presence of chelating agents, thereby decreasing the red coloration. In this study, the formation of the ferrozine-Fe<sup>2+</sup> complex was interrupted in the presence of solvent fractions from microalgae, demonstrating significant chelating ability. The bioactive compounds exhibiting this metal chelating effect were present in the ethyl acetate fraction from *P. duplex* and the aqueous fraction from *D. fascicularis*, indicating their hydrophilic status. Ferrous ions accelerate peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals (Halliwell, 1991; Fridovich, 1995). The organic solvent fractions of microalgae exhibited a noticeable capacity for iron binding, suggesting the ability to function as peroxidation protectors (Gülçin et al., 2004).

FTC method was used in this study to evaluate the inhibition of lipid peroxidation by microalgal fractions by comparing the effects of our samples with those of commercial antioxidants. The FTC method determines the amount of peroxide formed in emulsion during an incubation period by measuring optical absorbance. The chloroform and ethyl acetate fractions from *P. duplex*, as well as the n-hexane fraction from *D. fascicularis*, exhibited significant antioxidant effects and were able to reduce the formation of peroxides. These results suggest that different antioxidant components were released from the microalgae cells in different solvent fractions. These fractions contained high levels of polysaccharides, proteins, and polyphenols, which are components that may influence inhibitory effects.

Studies conducted by Lu and Foo (2000) and Siriwardhana et al. (2004) reported a high correlation between free radical scavenging activities and total polyphenolics. Some solvent fractions of microalgae in our study did not exhibit antioxidant activity, although they contained as many phenolic compounds as the other extracts from the sample. Other bioactive components such as proteins, polysaccharides, and pigments in the microalgae extracts

may therefore contribute to the antioxidant activity (Kardošová and Machová, 2006; Moure et al., 2006). For example, oligosaccharides, sulfates, and glycol-protein components of the red microalga *Porphyridium* sp. have been shown to have antioxidant activities (Spitz et al., 2005). We thus conclude that numerous factors influence antioxidant activity.

The cell walls of *D. fascicularis* consist of murein, a peptidoglycan with peptide side chains attached to linked, alternating residues of N-acetylglucosamine and N-acetylmuramic acid. The cell wall layers outside the murein layer consist primarily of lipopolysaccharides, and the mucilage sheaths are predominantly composed of complex polysaccharides. In *P. duplex*, the cell walls consist of a fibrillar fraction embedded in an amorphous matrix. Proteins in these structures have bonded with polysaccharides to form glycoprotein. Both 80% methanol and organic solvents were used in this study to extract hydrophilic and hydrophobic antioxidant compounds from the microalgae, thereby using solvent partitioning to disperse potential biological compounds according to their polarity.

This study has shown that microalgae have different antioxidative activities. Both polar and non-polar components exhibited appreciable antioxidative effects. Therefore, microalgae are good candidate sources of natural antioxidants that act against ROS. Their demonstrated antioxidant properties may be applicable in the food and pharmaceutical industries.

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