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Potential of Benthic Diatoms *Achnanthes longipes*, *Amphora coffeaeformis* and *Navicula* sp. (Bacillariophyceae) as Antioxidant Sources

Seung-Hong Lee[1], Rohan Karawita[1], Abu Affan[2], Joon-Baek Lee[2], Ki-Wan Lee[1], Bae-Jin Lee[3], Dong-Woo Kim[4] and You-Jin Jeon[1]*

[1]*Faculty of Applied Marine Science, Cheju National University, Jeju 690-756, Korea*
[2]*Department of Oceanography, Cheju National University, Jeju 690-756, Korea*
[3]*Marine Bioprocess Co. Ltd., Pukyong National University, Busan 608-737, Korea*
[4]*Central Research Center, Natural F&P Co., Ltd., O-Chang, Chungbuk 363-883, Korea*

Recently, interest in plant-derived food additives has developed natural antioxidants, in order to alternate synthetic antioxidants with several disadvantages. In the present study, different organic fractions from solvent partitions of 80% methanol extract from Jeju benthic diatoms, *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis* was assessed for their potential antioxidant effects. Among the solvent fractions tested, *n*-hexane (80.4%) and 80% methanol extract (76.6%) from *A. longipes*, chloroform (63.2%) from *Navicula* sp. and *n*-hexane (67.4%) from *A. coffeaeformis* were effective in DPPH free radical scavenging. Fractions of chloroform (53.4%) and *n*-hexane (53.1%) from *A. longipes* exhibited higher activities on H_2O_2 scavenging. Fraction of *n*-Hexane from *A. longipes* exhibited the highest hydroxyl radical scavenging activity and NO· scavenging activity (56.5% and 75.6%, respectively). Aqueous residue from *A. coffeaeformis* (75.6%) showed the highest metal chelating effect. Chloroform and ethyl acetate fraction of all the diatoms exhibited significant antioxidant activities in lipid peroxidation inhibitory activity. In particular, both chloroform and the ethyl acetate fraction from *A. longipes* and *A. coffeaeformis* exhibited lipid peroxidation inhibitory activity significantly higher than that of α- tocopherol. These data suggest that the Jeju benthic diatoms tested are rich in hydrophilic and hydrophobic antioxidative compounds with different antioxidative properties that can be applied in food industry.

Key Words: *Achnanthes longipes*, *Amphora coffeaeformis*, Antioxidant, benthic diatoms, *Navicula* sp.

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and superoxide anions, which are produced because of exogenous factors (tobacco smoke, ionizing radiation and certain pollutants) and endogenous factors (normal aerobic respiration) have been implicated in cellular processes such as mutagenesis, carcinogenesis and premature aging. ROS have the ability to react with a large variety of easily oxidisable cellular components, such as proteins, lipids and lipoproteins (Fridovich 1995). Oxidation is one of the major reasons of food deterioration, resulting to the destruction of fat-soluble vitamins and development of off colors and toxicants (Yang *et al.* 2000; Ukeda *et al.* 2002). Besides, in the food industry lipid peroxidation is an important deteriorative reaction during processing and storage.

Commercial antioxidant supplements such as butylated hydroxyl anisole (BHA), butylated hydroxy tolouene (BHT), α-tocopherol and propyl gallate (PG) have been used in order to reduce oxidative damages in human body (Sherwin 1990; Gülcin *et al.* 2002). However, it is suspected that those antioxidants are responsible for some side effects such as liver damage and carcinogenesis. Antioxidants are involved with the oxidation mechanism by scavenging free radicals, chelating catalytic metals and by acting as oxygen scavengers (Shahidi and Wanasundara 1992; Büyükokuroğlu *et al.* 2001).

As a natural antioxidant source, plants have an ability to absorb the sun's radiation for generating high levels of oxygen as secondary metabolites of photosynthesis. Oxygen is easily activated by ultra violet (UV) radiation and heat from the sunlight to produce toxic ROS. Therefore, plants produce various antioxidative com-

*Corresponding author (youjinj@cheju.ac.kr)

pounds to protect them from harmful effects of ROS (Lu and Foo 1995). Although many studies regarding antioxidant effects from macroalgae are available, less attention has been paid for microalgae because of difficulties in the isolation and cultivation. Currently, microalgae are being paid more attention as nutraceutical and health food in the market. Extracts of *Chlorella* sp. and *Spirulina* sp. are being used with noodles, bread, green tea, milk, beer, and candy (Liang *et al.* 2004). Several microalgae, such as *Chlorella* sp., *Spirulina* sp. and *Dunaliella* sp. are grown commercially for the production of algal products such as β-carotene, lutein and phycocyanin. In addition, the antioxidative activity of phycocyanobilin from *Spirulina platensis* was evaluated against oxidation of methyl linoleate in a hydrophobic system or with phosphatidylcholine liposomes (Hirata *et al.* 2000). Phycocyanobilin effectively inhibited the peroxidation of methyl linoleate and produced a prolonged induction period. Another microalgae, *Aphanizomenon flos-aquae* (Cyanophyta) was reported to contain high amount of phycocyanin, a photosynthetic pigment with antioxidant and anti-inflammatory properties (Benedettia *et al.* 2004).

Jeju is a volcanic island of Korea, which belongs to the subtropical region where the benthic diatoms are used in commercial hatchery as a live feed for shellfish. The coastal water temperature and salinity of this island fluctuated widely and benthic diatoms are often found in high abundance in this coastal water throughout the year (Affan and Lee 2004). In the previous study, it was found that Jeju benthic diatom, *Grammatophora marina* that is commonly used as a live feed for shellfish in commercial hatcheries possessed antioxidant activity (Affan *et al.* 2006).

The objective of this study was to investigate the potential antioxidant effects of 80% methanol extract and its different organic solvent fractions from benthic diatoms, *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis* from Jeju Island, Korea in order to understand the usefulness of these algae in food industry.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium nitroprusside, sulphanilic acid, naphthylethylenediamine dihydrochloride, xanthine, xanthine oxidase from butter milk, nitro blue tetrazolium salt (NBT), butylated hydroxytoluene (BHT), α-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 were purchased from Sigma Co. (St Louis, USA). 2, 2-Azino-bis (3-ethylbenz-thiazolin)-6-sulfonic acid (ABTS), peroxidase and 2-deoxyribose were purchased from Fluka Chemie (Buchs, Switzerland). All the other chemicals used were of analytical grades.

Sample collection

Natural sample of benthic diatoms was collected from National Fisheries Research and Development Institute (NFRDI), Jeju Island, Korea. Benthic diatoms attached to wavy plastic plate (called Papan) which was used for abalone larval live food. Environmental conditions, especially temperature, pH and salinity were measured on the sampling spot and were 25°C, 8.02 and 30 psu, respectively. The attached benthic diatoms were recovered from the papan and diluted with the same seawater. The sample was again diluted and 1 mL was transferred to the S-R chamber. Single cell of benthic diatoms was picked up from the S-R chamber by using micropipette under inverted microscope (Olympus, IX71). The single cell was transferred into multi-well for subculture. Subculture of the isolated species was done with autoclaved seawater which was filtered through 0.4 μm filter membrane (Millipore Co., Bedford, MA) and enriched with F/2 nutrients media (Aquacenter Ltd. USA), trace metals and metasilicate anhydrous crystals (Na_2SiO_3). The isolation process was carried out until mono-strain of the benthic diatoms was obtained. The cultured benthic diatoms were observed under the phase-contrast microscope (Zeiss Axioplan) and the identification was done as described by Shim (1994).

Isolation of axenic strain

Bacteria were found to grow with the diatom mono-strain during culture which decomposes the microalgae. Further, it was suspected that the bacteria's own cell compounds might play a vital role in the determination of antioxidant properties of the phytoplankton. Thus, axenic strains of those benthic diatoms were prepared according to the method described by Affan *et al.* (2006). In brief, the mono-strain of each species was streaked on agar plate that was prepared with 2% agar (w/v) and 0.04% F/2 (v/v) nutrients media and autoclaved seawater. Mono-strain colony was transferred from the agar plate into the 250 mL flask which contained 100 mL of F/2 enriched culture media and antibiotics cocktail (Penicillin, Streptomycin and Neomycin) (SIGMA P

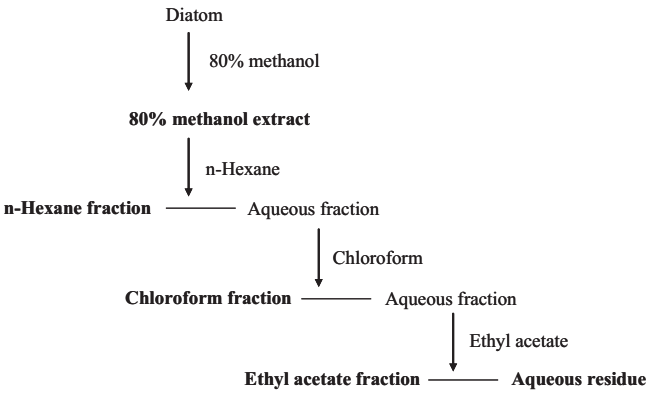

Fig. 1. Scheme of solvent fractionation of *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis*.

4083). Seven different dosages of antibiotics cocktail (penicillin 100-250 unit mL^{-1}, streptomycin 100-250 μg mL^{-1} and neomycin 200-500 μg mL^{-1}) with interval of 25 unit-penicillin mL^{-1}, 25 μg-streptomycin mL^{-1} and 50 μg-neomycin mL^{-1} were used. The cultured sample was again streaked on the bacto-agar media for the observation of the presence of bacteria. Finally axenic strain of those benthic diatoms was obtained for succeeding studies.

Mass culture

The mass culture of benthic diatoms was done for 2 weeks in 10 liter high-density polypropylene bottles (Nalgene, USA) with autoclaved artificial seawater media, which was enriched with F/2 nutrients media, sodium silicate (Na_2SiO_3), and trace metals solution and with aeration of 0.03 kgf/cm^2 which provided 10 liter of air per minute (Btech, Korea). Mass culture condition was maintained with salinity, temperature, pH, L: D cycle and fluorescent light intensity of 30 psu, 25°C, 8, 12:12 and 180 μE m^{-1} s^{-1}, respectively. The standing crop was separated from the culture media by filtering and finally freeze-dried at -70°C.

Preparation of 80% methanol extract and solvent fractions

Freeze-dried benthic diatom sample was ground into a fine powder. The powdered sample (5 g) was extracted with 80% methanol (500 mL) for 24 h at 25°C. The mixture was filtered and the 80% methanol extract was collected and concentrated. The extracts were obtained in sequence fractionation in a separatory funnel with different organic solvents, such as *n*-hexane, chloroform, and ethyl acetate, respectively (Fig. 1). Each fraction was concentrated and redissolved in methanol to a concentration of 2 mg mL^{-1}. All activities of fractions were compared with commercial antioxidants (BHT and α- tocopherol) dissolved in methanol (2 mg mL^{-1}).

Proximate composition

Proximate chemical composition of freeze-dried benthic diatom samples were determined according to the AOAC methods (1995). Crude lipid content was determined by Soxhlet method and crude protein content was determined by Kjeldhal method. Ash content was determined by calcinations in furnace at 550°C and the moisture content was determined by keeping in a dry oven at 105°C for 24 h. In fractions and extracts, the crude protein content was determined by the Lowry spectrophotometric method and the polysaccharide content was determined by phenol-sulfuric method.

DPPH free radical scavenging assay

The DPPH free radical scavenging activity of extracts was measured by DPPH using the modified method of Brand-Williams *et al.* (1995). The sample (2 mL) was mixed thoroughly with 2 mL of freshly prepared DPPH solution (3×10^{-5} M). The reaction mixture was incubated for 1 h and the absorbance was measured at 517 nm using UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

Hydrogen peroxide scavenging assay

The ability of the diatom to scavenge H_2O_2 was determined according to the method of Muller (1995). Sample (80 μL) and 20 μL of 10 mM hydrogen peroxide were mixed with 100 μL of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37°C for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM ABTS and 30 μL of peroxidase (1 U mL^{-1}) were mixed and incubated at 37°C for 10 min and the absorbance was measured at 405 nm.

Superoxide anion scavenging assay

Measurement of superoxide anion scavenging activity of extracts was based on the method described by Nagai *et al.* (2003). A mixture of 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 (ethylenediaminetetraacetic acid), 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. Thereafter the reaction was started by adding 6 mU XOD and kept at 25°C for 20 min. The reaction was stopped by adding 0.02 mL of 6 mM CuCl. The

absorbance was measured in microplate reader (Sunrise; Tecan Co. Ltd., Austria) at 560 nm.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined according to Chung *et al.* (1977). The Fenton reaction mixture (200 μL of 10 mM $FeSO_4.7H_2O$, 200 μL of 10 mM EDTA and 200 μL of 10 mM 2-deoxyribose) was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) and with 200 μL of sample. Thereafter, 200 μL of 10 mM H_2O_2 was added and incubated (37°C for 4 h). After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were mixed and placed in a boiling water bath (10 min). After cooling, the mixture was centrifuged (5 min, 395 x g) and absorbance was measured at 532 nm.

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging was determined according to the method reported by Garrat (1964). Two milliliter of 10 mM sodium nitroprusside in 0.5 mL of phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of sample and incubated at 25°C for 150 min. From the incubated mixture 0.5 mL was taken out and added into1.0 mL sulphanilic acid reagent (0.33% in 20% glacial acetic acid) then incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1 % w/v) was mixed and incubated at room temperature for 30 min and absorbance was measured at 540 nm.

Ferrous ion chelating effect

The chelating of ferrous ions by the extracts was estimated by the method of Decker and Welch (1990). Sample (5 mL) was added to a solution of 0.1 mL of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 mL of 5 mM ferrozine solution and reaction mixture was incubated for 10 min at a room temperature in a shaking incubator. After incubation, the absorbance of reaction mixture was measured at 562 nm.

Determination of lipid peroxidation inhibitory activity with the ferric thiocyanate (FTC) method

The lipid peroxidation inhibitory activity of the benthic diatom was determined according to the FTC method (Kikuzaki and Nakatani 1993). Two milliliter of Sample (100 mg L^{-1}) was mixed with 2 mL of 2.51% linoleic acid in ethanol, 4 mL of 0.05 M of phosphate buffer (pH 7) and 2 mL of distilled water and kept at 40°C in the dark. A total of 0.1 mL of the above mixture was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate and after 5 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl was mixed. The absorbance was measured every 24 h for 7 days.

Table 1. Proximate composition of *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis*

Nutrient	*A. longipes*	*Navicula* sp.	*A. coffeaeformis*
Moisture (%)	8.1 ± 0.3	3.6 ± 0.3	5.9 ± 0.3
Carbohydrate (%)	16.4 ± 0.2	13.5 ± 0.1	15.8 ± 0.4
Protein (%)	6.5 ± 0.1	16.9 ± 0.2	15.6 ± 0.3
Lipid (%)	1.1 ± 0.2	2.1 ± 0.3	6.9 ± 0.3
Ash (%)	67.9 ± 0.4	63.9 ± 0.4	55.8 ± 0.3

Values are means of three replicates ± SD

Total phenolic content

Total phenolic compounds in the extracts were determined with Folin-Ciocalteu reagent according to the method of Chandler and Dodds (1993) using gallic acid as a standard phenolic compound. 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. The mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. After mixing thoroughly, the mixture was placed in the dark for 1 h then absorbance was measured at 725 nm.

Statistical analysis

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

RESULTS

The benthic diatoms were found as an axenic strain with the dosages of 200 units-penicillin mL^{-1}, 200 μg-streptomycin mL^{-1} and 400 μg-neomycin mL^{-1} among different dosages of antibiotics cocktail. The mass culture of the isolated benthic diatoms was done according to the natural environmental conditions. The yield of *Achnanthes longipes*, *Navicula* sp., *Amphora coffeaeformis* was 1.25, 0.94 and 1.08 g L^{-1} on dry weight basis respectively.

Proximate composition of freeze dried *A. longipes*, *Navicula* sp and *A. coffeaeformis* are shown in Table 1.

Table 2. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract, and its different fractions from *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis*

Algae	Fractions	Yield (g/100 g)	Total phenol[1)] (mg/100 g)	Polysaccharide[2)] (mg/100 g)	Protein[3)] (mg/100 g)
A. longipes	Methanol	13.9	123.6 ± 11	1531 ± 89	541.7 ± 35
	n-Hexane	1.6	19.3 ± 0.8	196.9 ± 15	88.5 ± 03
	Chloroform	2.3	23.1 ± 1.3	512.1 ± 32	242.5 ± 13
	Ethyl ace.	0.8	10.1 ± 0.4	127.9 ± 11	91.3 ± 04
	Aqu. res.	5.2	78.1 ± 3.6	637.8 ± 39	139.7 ± 11
Navicula sp	Methanol	17.7	58.9 ± 2.3	517.8 ± 34	376.1 ± 19
	n-Hexane	1.6	17.4 ± 0.7	265.1 ± 12	72.7 ± 03
	Chloroform	0.5	8.3 ± 0.4	124.5 ± 11	63.5 ± 03
	Ethyl ace.	1.7	6.8 ± 0.3	33.9 ± 02	21.9 ± 01
	Aqu. res.	14.0	24.5 ± 1.2	97.8 ± 06	207.8 ± 11
A. coffeaeformis	Methanol	14.7	104.5 ± 6.7	466.2 ± 27	335.1 ± 16
	n-Hexane	1.6	16.6 ± 0.5	121.7 ± 11	131.7 ± 08
	Chloroform	1.4	77.2 ± 4.1	281.7 ± 13	167.1 ± 12
	Ethyl ace.	0.03	0.06 ± 0.0	0.279 ± 0.0	0.077 ± 0.0
	Aqu. res.	11.8	14.5 ± 0.7	59.2 ± 03	39.7 ± 0.2

Values are means of three replicates ± SD
[1)] As equivalent gallic acid
[2)] As equivalent to glucose
[3)] As equivalent to bovine serum albumin

Moisture contents of *A. longipes*, *Navicula* sp. and *A. coffeaeformis* were 8.1%, 3.6% and 5.9%, respectively. Carbohydrate, protein and lipid content for *A. longipes* were 16.4%, 6.5% and 1.1%, respectively, while those for *Navicula* sp. were 13.5%, 16.9% and 2.1%, respectively, and *A. coffeaeformis* were 15.8%, 15.6% and 6.9%, respectively. In the case of ash, the three benthic diatom had 67.9%, 63.9% and 55.8%, respectively. Ash was the most major component in approximate compositions of the three benthic diatom. Total phenolic, polysaccharide and protein content in 80% methanol extract and its solvent fractions are depicted in Table 2. Significant differences in the total phenolic, polysaccharide and protein content among the different diatom species and among different fractions were observed.

The percentage scavenging activity of 80% methanol extract and each solvent fractions against DPPH was shown in Table 3. Significant differences in the activities among the different diatom species and among different fractions were observed. According to Table 3, *A. longipes* had higher scavenging effect than the rest of diatoms. Each fraction from *A. longipes* showed higher effect more than 45% and among the rest of chloroform fractions of *Navicula* sp. (63.2%) and *n*-hexane from *A. coffeaeformis* was prominent in giving higher activity.

As depicted in Table 3, solvent fractions of chloroform (53.4%), *n*-hexane (53.1%) and the ethyl acetate (52.9%) from *A. longipes*, *n*-hexane (47.5%) and chloroform (46.1%) from *A. coffeaeformis* demonstrated higher H_2O_2 scavenging effects. However, other fractions provided less effect.

As shown in Table 3, ethyl acetate fraction (47.6%) and 80% methanol extract (37.6%) from *Navicula* sp. exhibited higher superoxide anion scavenging effects in all solvent fractions of tested diatoms.

According to Table 3, *n*-hexane fraction of *A. longipes*, *A. coffeaeformis* and *Navicula* sp. exhibited higher hydroxyl radical scavenging effects (56.5%, 55.5%, and 42.2%, respectively). However, no significant effects were observed from the rest of fractions.

As shown in Table 3, *n*-hexane (75.6%) and ethyl acetate fraction (26.3%) from *A. longipes*, *n*-hexane (28.8%) and ethyl acetate (27.6%) fraction from *Navicula* sp., aqueous fraction (29.6%) from *A. coffeaeformis* exhibited significant results, even higher than that of the commercial antioxidants ($P < 0.05$). However, the rest of fractions showed less significant effects.

As shown in Table 3, the aqueous residue (75.6%) and the 80% methanol extract (51.4%) from *A. coffeaeformis*, as well as the ethyl acetate fraction and the 80% methanol

Table 3. Antioxidants activity of 80% methanol extract and solvent fractions from *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis*

Diatom	Fractions	DPPH[1)]	H_2O_2[2)]	$O_2^{-\cdot}$[3)]	OH·[4)]	NO·[5)]	FC[6)]
A. longipes	Methanol	76.6c ± 4.7	32.3e ± 1.2	17.3g ± 1.1	9.3g ± 0.8	12.8f ± 0.7	11.6d ± 0.3
	n-Hexane	80.4b ± 6.3	53.1b ± 2.3	7.7h ± 0.4	56.5c ± 2.8	75.6a ± 4.7	6.4g ± 0.3
	Chloroform	65.1d ± 4.3	53.4b ± 2.4	3.5i ± 0.1	19.3e ± 0.9	6.3g ± 0.1	12.9c ± 0.7
	Ethyl ace.	59.4f ± 3.7	52.9b ± 2.5	2.8i ± 0.1	7.3g ± 0.4	26.3b ± 1.3	3.8g ± 2.9
	Aqu. res.	46.5f ± 2.1	8.1h ± 0.1	10.7h ± 0.7	2.9f ± 0.7	20.6e ± 1.1	7.5f ± 0.6
Navicula Sp.	Methanol	31.6j ± 1.3	43.2c ± 1.3	37.6c ± 1.3	4.9i ± 0.2	19.5e ± 0.7	50.1c ± 2.8
	n-Hexane	39.2h ± 1.4	37.9d ± 1.9	26.7e ± 0.8	42.2d ± 2.5	28.8b ± 1.7	4.9g ± 0.2
	Chloroform	63.2e ± 2.7	32.1e ± 1.2	22.1f ± 0.9	8.3g ± 0.7	12.3f ± 0.4	7.1f ± 0.3
	Ethyl ace.	57.3f ± 2.5	23.6f ± 0.7	47.6b ± 2.1	2.7i ± 0.2	27.6b ± 1.3	58.1b ± 2.7
	Aqu. res.	31.9j ± 1.3	14.7h ± 0.3	4.1i ± 0.1	4.2i ± 0.1	15.2f ± 0.7	14.2c ± 0.7
A. coffeaeformis	Methanol	22.7k ± 0.9	23.3f ± 0.8	28.1e ± 1.4	4.1i ± 0.1	14.8f ± 0.4	51.4b ± 2.4
	n-Hexane	67.4b ± 3.2	47.5c ± 2.3	27.4e ± 1.3	55.5c ± 2.1	3.4g ± 0.1	1.7g ± 0.1
	Chloroform	35.5i ± 2.3	46.1b ± 2.1	15.2g ± 0.7	13.3f ± 0.2	6.1g ± 0.2	2.1g ± 0.1
	Ethyl ace.	23.3k ± 1.2	21.1g ± 1.2	34.1d ± 1.7	6.4h ± 0.3	16.8f ± 0.7	43.2d ± 2.7
	Aqu. res.	18.9l ± 0.9	19.1h ± 0.7	31.3d ± 1.6	7.9g ± 0.4	29.6b ± 1.4	75.6a ± 3.8
	BHT	94.6a ± 6.4	60.1a ± 4.2	63.2a ± 4.3	76.6b ± 4.6	26.1b ± 0.9	11.5d ± 0.1
	Tocopherol	94.3a ± 7.1	62.5a ± 4.9	61.5a ± 4.7	79.5a ± 4.7	25.2c ± 0.6	10.3e ± 0.1

Sample concentration is 2 mg mL^{-1}; Data are Means ± SE (n = 3); values in each column followed by different letters denote significant difference at $P < 0.05$

[1)] DPPH free radical scavenging activity, [2)] hydrogen peroxide scavenging activity, [3)] superoxide anion scavenging activity, [4)] hydroxyl radical scavenging activity, [5)] nitric oxide scavenging activity, [6)] ferrous ion chelating effect

extract (50.1%) from *Navicula* sp. exhibited significantly strong metal chelating effects which were even higher than the activities of commercial antioxidants ($P < 0.05$).

As shown in Fig. 2, the absorbance of linoleic acid emulsion without the addition of any extract increased. Among the solvent fractions of the three diatoms, the chloroform and the ethyl acetate fraction exhibited significant antioxidant activities in lipid peroxidation inhibitory activity ($P < 0.05$) (Fig. 2). In particular, both chloroform and the ethyl acetate fraction from *A. longipes* and *A. coffeaeformis* exhibited lipid peroxidation inhibitory activity significantly higher than that of α- tocopherol.

DISCUSSION

Recently, many researchers are interested in finding any natural antioxidants safety and effectiveness, which can be substituted for current and commercial synthetic antioxidants. Benthic diatoms have become good candidates for sources of natural antioxidants, as revealed by a number of recent studies (Affan *et al.* 2006; Karawita *et al.* 2007; Lee *et al.* 2008). Thus, in the present study, different organic fractions from solvent partitions of 80% methanol extract from Jeju benthic diatoms, *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis* was assessed for their potential antioxidant effects.

DPPH free radical scavenging assay is a commonly used method to evaluate antioxidant activity in a comparatively short period compared with other relative methods. The effects of antioxidants on DPPH free radical scavenging was considered to be due to their hydrogen donating ability. In this study, the organic solvent fractions of 80% methanol extract exhibited strong effects in DPPH free radical scavenging. Thus, it is revealed that the active compound has been dispersed according to their polarity. Specially, the *n*-hexane fraction from *A. longipes* and *A. coffeaeformis* demonstrated notable free radical scavenging effect. Therefore, it was clear that the biochemical compounds exhibiting the activity were concentrated in the *n*-hexane fraction suggesting that the bioactive compounds are fat-soluble and could be made up of pigments and steroids. Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer (Nakamura *et al.* 1996). Even though it is unclear whether active constituents in extracts are active against free radicals after being absorbed and metabolized by cells in the body, free radical-scavenging assays have gained acceptance for their

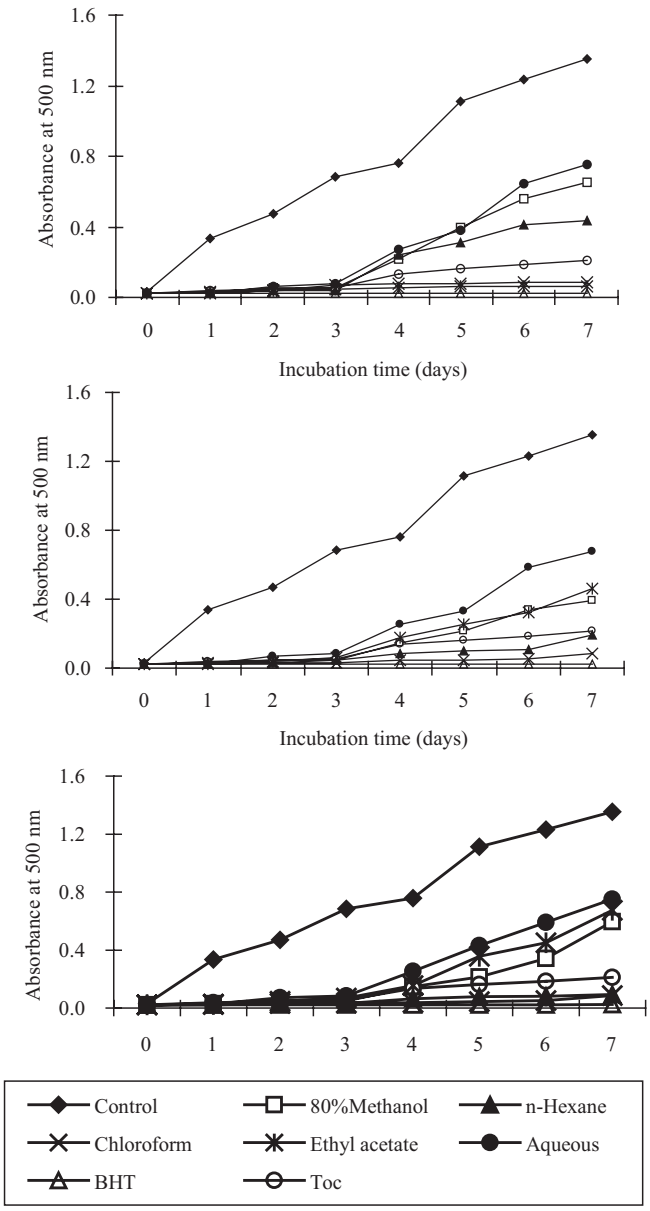

Fig. 2. Lipid peroxidation inhibitory activity of 80% methanol extract and solvent fractions from (A) *Achnanthes longipes* (B) *Navicula* sp. and (C) *Amphora coffeaeformis* compared to BHT and α- Tocopherol at 1 mg mL^{-1} of concentration of ethanol as assessed by linoleic acid.

capacity to rapidly screen materials of interest.

H_2O_2, a reactive non-radical compound, is very important as it can penetrate biological membranes. The higher H_2O_2 activity exhibited by organic solvent fractions (*n*-hexane and chloroform) of three diatoms and the decline in the activity of *Navicula* sp. extract with successive partition obviously implies that the H_2O_2 scavenging compounds present in these fractions have more hydrophobic properties. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals. Therefore, it is very important to remove H_2O_2 for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent OH· mediated oxidative DNA damage (Halliweil 1991).

Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Halliwell and Gutteridge 1989; Liu and Ng 2000). In this study, the superoxide scavenging activities of all the diatoms tested were distributed among the fractions. Therefore, it was obvious that the superoxide scavenging compounds present in these fractions have both hydrophilic and hydrophobic properties. Although superoxide anion is itself a weak oxidant, it can be converted into the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Dahl and Richardson 1978).

Hydroxyl radical scavenging activity of the solvent fractions from the benthic diatoms was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the solvent fractions, only *n*-hexane showed comparatively higher activities implying that the bioactive compounds in it might be lipophilic compounds. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules. Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from phospholipid membranes bringing about peroxidic reactions of lipids (Kitada *et al.* 1979). Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer, and radical formation, and initiates autoxidation, polymerization and fragmentation (Liu and Ng 2000).

Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions. In solvent fractions, NO· inhibition effect was dispersed according to polarity. The reactivities of the NO· and $O_2^{-\cdot}$ were found to be relatively low, but their metabolite ONOO- (peroxynitrite) is extremely reactive and directly induce toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Radi *et al.* 1991; Moncada *et al.* 1991). Therefore, the scavenging ability of NO· from the diatom may help to interrupt the chain reactions initiated by excessive production of NO· that are harmful to human health.

Ferrozine can make red color complexes with ferrous

ions. In presence of chelating agents, complex formation is interrupted and as a result, the red color of the complex is decreased. It was noted that bioactive compounds that exert ion chelating effect was not dissolved efficiently in non-polar solvents such as n-hexane and chloroform. However, as ethyl acetate is a moderately polar solvent, iron chelating activity was detected in *A. coffeaeformis* and *Navicula* sp.. Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliweill 1991; Fridovich 1995). In this study, different organic solvent fractions of the benthic diatoms demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protector which relates to its ferrous binding capacity (Gülin *et al.* 2004).

FTC method is used to determine the amount of peroxide generated at the initial stage of lipid peroxidation. During the linoleic acid oxidation, peroxides are formed and these compounds oxidize Fe^{2+} to Fe^{3+} and complexes with SCN^{-}. In this method, the concentration of peroxide decreases as the antioxidant activity increases. Thus, high absorbance is an indication of high concentration of formed peroxides. Fractions of ethyl acetate and chloroform of all the diatoms provided higher antioxidant effects and were able to reduce the formation of peroxides. In particular, both chloroform and the ethyl acetate fraction from *A. longipes* and *A. coffeaeformis* exhibited lipid peroxidation inhibitory activity significantly higher than that of α- tocopherol. It is suggested that according to solvent fractionation, different antioxidant components are released from the inside of diatom cells. These fractions contained high level of polysaccharides, proteins and polyphenols and it could be assumed that these components are responsible for the reduced lipid peroxidation.

The two studies conducted by Lu and Foo (2000) and Siriwardhana *et al.* (2003) reported a high correlation between free radical scavenging activities and total polyphenolics. In this study, some solvent fractions of benthic diatoms did not possess antioxidant activity, although they contained as much phenolic compounds as the other extracts of benthic diatoms. It is thought that other bioactive components in benthic diatoms extracts, such as proteins, polysaccharides and different kinds of pigments present in this microalga (Kardošová and Machová 2006; Moure *et al.* 2006). For example, it was found that oligosaccharides, sulfate and glycoprotein components in red microalga *Porphyridium* sp. have exhibited antioxidant activities (Spitz *et al.* 2005). Thus, it can be concluded that all those factors influenced the antioxidant activity.

The characteristic feature of the benthic diatoms is their ability to secrete an external wall composed of silica known as frustule. The cell wall consists predominantly of polymerized silica acid with no crystalline structure. It also contains protein, polysaccharide, and lipids. Diatoms produce insoluble mucilage that combines with glycoprotein and form a mucilaginous skin which aid in preventing erosion by waves and tidal scour. In this study, 80% methanol and its different organic solvent fractions were used for extraction of antioxidant compounds. As a solvent, 80% methanol has a potential to extract different kinds of bioactive compounds from microalgae which showed both hydrophilic and hydrophobic characteristics. By solvent partitions, potential biological compounds can be dispersed according to their polarity. Further studies are required for identification and purification of antioxidative compounds from the organic solvent fractions of benthic diatoms.

CONCLUSION

The study has shown that Jeju benthic diatoms have different antioxidative activities. Both polar and non-polar components exhibited appreciable antioxidative activities. In conclusion, Jeju benthic diatoms have exhibited potential antioxidant against ROS and it would be a potential candidate as a natural antioxidant source, which can be applied in food industry.

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