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Characterization of Antioxidant Potential of a Methanolic Extract and Its Fractions of Highbush Blueberry (*Vaccinium corymbosum* L.)

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Abstract

The antioxidant potential of a 75% methanolic extract of highbush blueberry (*Vaccinium corymbosum* L.) and its different fractions was investigated using different reactive oxygen species (ROS), nitric oxide (NO·), metal chelating and lipid peroxidation assays. Methylene chloride and 75% methanol fractions showed equally high activities (IC_{50} 0.010 mg/mL) for hydroxyl radical (HO·) scavenging. Higher hydrogen peroxide (H_2O_2) scavenging values were reported for the ethyl acetate and methylene chloride fractions and their IC_{50} values were 0.20 and 0.15 mg/mL, respectively. Nitric oxide (NO·) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activities were higher in ethyl acetate and methylene chloride fractions. Chloroform and water fractions showed higher activities in superoxide (O_2·$^-$) scavenging. All fractions showed strong metal chelating capacities compared with the commercial antioxidants tested. The 0.1% ethyl acetate fraction showed notable capacity to suppress lipid peroxidation in both fish oil and linoleic acid. Phenolic content was measured in all the fractions and methanolic extract. Among the fractions, ethyl acetate fraction showed the highest phenolic content.

Key words: highbush blueberry (*Vaccinium corymbosum* L.), antioxidant potential, reactive oxygen species scavenging, methanolic extract, total phenolics

INTRODUCTION

Interest in potential antioxidant compounds from natural sources by the pharmaceutical and functional food industries has resulted in an enormous amount of research on natural sources of bioactive compounds and characterization of their modes of action.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously formed as a result of normal cellular functions, pathological process and toxic exposure during a cell's life. ROS and RNS include free radicals such as superoxide anion (O_2·$^-$), hydroxyl (HO·), nitric oxide (NO·), peroxynitrite ($ONOO^-$) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (1-3). ROS and RNS are able to damage the essential biomolecules in the body including nucleic acids, proteins, lipids and carbohydrates (1). The human body has various defense mechanisms to eliminate the ROS and RNS and reduce the oxidative damage. Antioxidants can interfere with the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as oxygen scavengers. Hence, the use of antioxidants may contribute to the prevention of oxidative damage caused by ROS and RNS.

Lipid oxidation is an important chemical change that lowers the sensory quality of food. The primary and secondary products of lipid oxidation are also detrimental to health. The unsaturated nature of fatty acids makes them subject to oxidation easily and produces a variety of lipid oxidation products. Further, fatty acids are recognized to play an essential role in human health and nutrition. Eicosapentaenoic and docosahexaenoic acids are essential polyunsaturated fatty acids in the omega-3 group that naturally occur in fish oil. Further, they are the precursors of effective anti-aggregatory substances with potential for reducing cardiovascular diseases, carcinogenesis and allergies (4,5). Linoleic acid is an essential omega-6 fatty acid, and is a common constituent of commercially available food oils, including fish oil and most vegetable oils.

As synthetic antioxidants have been suspected for car-

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cinogenic and a variety of unidentified health disorders (6,7), a number of studies have been conducted to investigate the potential for obtaining antioxidants from natural sources (8-10).

Highbush blueberry (*Vaccinium corymbosum* L.) is a shrub with many stems, which grows up to 10 feet in height and has clusters of bell shaped white flowers, which are abundantly grown in Canada and United State. Japan and Korea have also recently started to cultivate blueberries. A higher antioxidant capacity has been reported in blueberries than other fruits and vegetables (11). Lowbush blueberry has higher *in vitro* antioxidant capacity than the cultivated highbush blueberry (12). Further, blueberry contains chlorogenic acid, an important antioxidant compound, and it is also rich in phytochemicals such as anthocyanin (secondary plant metabolite). Numerous *in vitro* experiments have indicated that anthocyanins and other phenolics in berries have a wide range of potential anti-cancer and heart disease properties including antioxidant, anti-inflammatory, and cell regulatory effects.

The objective of the present work was to characterize the antioxidant activity of a 75% methanolic extract and its fractions from highbush blueberry using a variety of *in-vitro* methods to assess the stable DPPH, HO·, NO·, $O_2 \cdot^-$ radicals scavenging, H_2O_2 scavenging, reducing power and metal chelating ability. Additionally, the ability of the ethyl acetate fraction to inhibit lipid peroxidation in fish oil and linoleic acid was also evaluated.

MATERIALS AND METHODS

Materials

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-tocopherol, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium salt (NBT), xanthine, xanthine oxidase, fish oil, linoleic acid, thiobarbituric acid (TBA), trichloro acetic acid (TCA), Folin-Ciocalteu reagent, sodium nitroprusside, sulfanilic acid were purchased from Sigma Co. (St. Louis, USA). N-1-Naphthylethylene diamine dihydrochloride was purchased from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan). Ethylenediaminetetraacetic acid (EDTA), peroxidase, 2, 3-azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS), and deoxyribose were purchased from Fluka Co. (Buchs, Switzerland). All other chemicals used were of analytical grade supplied by Fluka or Sigma Co.

Proximate chemical composition of blueberry

Proximate chemical composition of blueberry was determined according to AOAC guidelines (13). Crude protein was determined by the Kjeldahl method and crude carbohydrate was determined by phenol-sulphuric acid reaction (absorbance at 480 nm) using glucose as the calibration standard. Crude lipid was determined by Soxhlet extraction and crude ash was calculated by ashing in a dry-type furnace at 550°C.

Methanolic extraction and solvent fractionation

Blueberry was collected from a farm belonging to Jeju Nong San Co. Ltd. in Jeju-Do of Korea during a full month of May, 2004. The harvested berries were rinsed with deionized water, freeze-dried, and then pulverized into a fine powder using a grinder (MF 10 basic mill, GMBH & CO., Staufen, Germany) and sieved through a 300 mm standard testing sieve. A forty-gram sample of the ground blueberry powder was mixed in 75% methanol (1000 mL) and kept in a shaking incubator at 25°C for one day and vacuum filtered through Whatman No.1 (Whatman Ltd., England) filter paper. Later, solvent fractionation of 75% methanol extract was done with *n*-hexane, chloroform, ethyl acetate and methylene chloride, respectively (Fig. 1). After solvent fractionation, the methanol extract and its organic fractions were tested for ROS and NO scavenging activities, and reducing power and metal chelating activities together with final aqueous fraction. The ethyl acetate fraction was subjected to the lipid peroxidation analysis. In each assay, all activities were compared with the value of commercial antioxidants (BHT and α-tocopherol) dissolved in methanol.

DPPH radical scavenging assay

DPPH scavenging potential of the blueberry samples were measured based on the scavenging ability of stable 1, 1-diphenyl-2-picrylhdrazyl (DPPH) radicals. The method modified by Brand-Williams et al. (14) was employed to investigate the free radical scavenging activity. Freshly prepared 2 mL DPPH (3×10^{-5} M in DMSO) solution was thoroughly mixed with 2 mL of blueberry samples. The reaction mixture was incubated for 1 hr at room temperature. The absorbance of resultant mixture was recorded at 517 nm using a UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

Superoxide anion ($O_2 \cdot^-$) scavenging assay

The superoxide scavenging ability of the blueberry samples was assessed by the method of Nagai et al. (15). The reaction mixture contained 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 ethylenediaminetetraacetic acid (EDTA), 0.02 mL of 0.15% bovine serum albumin, 0.02 mL of 0.75 mM NBT and 0.02 mL of blueberry samples. After incubation at 25°C for 20 min, 6 mU XOD was added to the mixture to initiate the reaction,

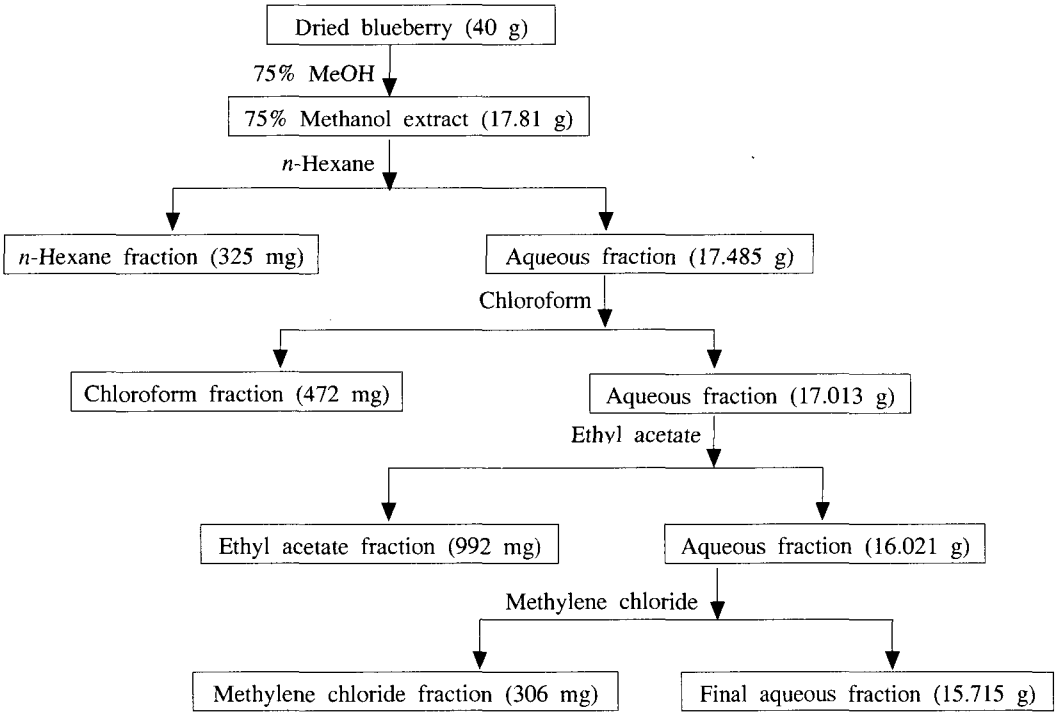

Fig. 1. Scheme of solvent fractionation of blueberry (*Vaccinium corymbosum* L.).

which was carried out at 25°C for 20 min. Reaction was terminated by adding 0.02 mL of 6 mM CuCl. The absorbance of the mixture was recorded at 560 nm.

Hydrogen peroxide (H_2O_2) scavenging assay

The hydrogen peroxide scavenging abilities of the blueberry samples were investigated using a method based on the scavenging of hydrogen peroxide in the ABTS-peroxidase system described by Muller (16). Eighty microliter of each blueberry sample and 20 μL of 10 mM hydrogen peroxide were mixed with 100 μL of phosphate buffer (pH 5.0, 0.1 M) in a 96-microwell plate and the samples were incubated at 37°C for 5 min. Subsequently, 30 μL of freshly prepared ABTS (1.25 mM) and 30 μL of peroxidase were added and incubated at 37°C for another 10 min. The absorbance of the resulting mixture was recorded using an ELISA reader (Sunrise Tecan Co. Ltd., Austria) at 405 nm.

Hydroxyl radical (HO·) scavenging assay

Ability of the blueberry samples to scavenge the HO· generated by the Fenton reaction was measured according to the modified method of Chung et al. (17). The Fenton reaction mixture containing of 200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 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) containing 200 μL of blueberry samples. Thereafter, 200 μL of 10 mM H_2O_2 was added to the mixture before incubation at 37°C for 4 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and placed in the boiling water bath for 10 min. The resultant mixture was then allowed to cool to room temperature and centrifuged at 395× g for 5 min. The absorbance was recorded at 532 nm.

Nitric oxide radical (NO·) scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH (7.4) spontaneously produces nitric oxide, which reacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction (18). Griess Illosvoy reagent was slightly modified using naphthylethylenediamine dihydrochloride (0.1 % w/v) instead of 1-naphthylamine (5%). Scavengers of nitric oxide compete with oxygen and reduce the production nitric oxide (19). The reaction mixture (3 mL) containing 2 mL of 10 mM sodium nitroprusside, 0.5 mL of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 mL of extract was incubated at 25°C for 150 min. Thereafter, 0.5 mL of the reaction mixture containing nitrite was pipetted and mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotisation. Then, 1 mL of naphthylethylenediamine dihydrochloride (0.1%) was added, and allowed to stand for 30 min in diffused light. The absorbance of the pink colored chromophore was measured at 540 nm.

Ferrous ion chelating ability

A method by Decker and Welch (20) was used to investigate the ferrous ion chelating ability of blueberry

samples. Five milliliters of each blueberry sample was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. The absorbance at 562 nm was determined after 10 min. A complex of Fe^{2+}/ferrozine showed strong absorbance at 562 nm. The higher the ferrous ion chelating activity exhibit, the lower the absorbance.

Measurement of reducing power

Reducing power was investigated using the method developed by Oyaizu (21). A 2.5 mL blueberry sample was mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was placed in a water bath at 50°C for 20 min. The resulting solution was cooled rapidly, mixed with 2.5 mL of 10% trichloroacetic acid and centrifuged at 3000×g for 10 min. A 5.0 mL fraction from the supernatant was mixed with 5 mL of distilled water and 1 mL of 1% ferric chloride. The absorbance of the resultant mixture was measured at 700 nm after 10 min. The higher the absorbance value, the stronger the reducing power.

Oxidation of fish oil or linoleic acid

Fish oil or linoleic acid was exposed to accelerate oxidation conditions similar to the method used by Abdalla and Roozen (8). Fish oil or linoleic acid samples (50 g) containing 0.1%, 0.05% and 0.01% (w/w) of the blueberry extract were incubated at 60°C in darkness for 12 days. Initial 6 hr incubation was done without closing the cap of the bottles in order to remove the methanol, which was added to dissolve the organic solvent extract. Further, commercial antioxidants (BHT, BHA and α-tocopherol), which used for oil experiments were added at the concentration of 0.01% (w/w).

Thiobarbituric acid-reactive substances (TBARS) assay

This assay was based on the method described by Madsen et al. (22) and was conducted every 2 days for 12 days. One gram of the fish oil or linoleic acid was dissolved in 3.5 mL of cyclohexane and 4.5 mL of TCA-TBA mixture (7.5% TCA and 0.34% TBA) subsequently. The resultant mixture was vortexed for 5 min and centrifuged at 2780×g for 15 min. The TCA-TBA phase was removed and heated in a boiling water bath for 10 min. The absorbance was recorded at 532 nm and the antioxidant capacity was expressed as equivalent mol of malonaldehyde per kg oil. TBARS concentrations were obtained from a standard curve based on tetraethoxypropane.

Conjugated diene hydroperoxides (CDH) assay

Conjugated diene hydroperoxide content was detected at two-day intervals as described by Roozen et al. (23). Fifty milligrams of either the fish oil or linoleic acid sample (stored under accelerated oxidation conditions) were mixed with 5 mL of cyclohexane and vortexed. CDH absorbance was recorded at 234 nm.

Weight gaining

This experiment was conducted according to the procedure of Wanasundara and Shahidi (24) with slight modifications. Two grams from each sample were prepared for the lipid peroxidation assay and separated into aluminium petridishes and traces of water in the samples were removed keeping them in a vacuum oven at 35°C for 12 hr. Oxidation condition of the samples was accelerated in the forced air oven at 65°C, and percentage weight gain was recorded for 12 days as in the procedure described by Yan et al. (25).

Total phenolic content assay

Total phenolic content was determined according to the protocol described by Chandler and Dodds (26). One milliliter of blueberry sample was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Foiln-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. It was mixed thoroughly and placed in the dark for 1 hr and then the absorbance was recorded at 725 nm in the UV-VIS spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic content.

Calculation of 50% inhibition concentration (IC_{50})

The concentration of the extract (mg/mL) required for scavenging 50% of radicals was calculated by using the percent scavenging activities of four different extract concentrations. Percent scavenging activity was calculated as [1−(Ai−Aj)/Ac]×100%. Where; Ai is the absorbance measured with different blueberry fractions in the particular assay with ROS source; Aj is the absorbance measured with different blueberry fractions in the particular assay but without ROS source; Ac is the absorbance of the control with solvent (without blueberry fractions).

Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using Duncan's test ($p<0.05$).

RESULTS AND DISCUSSION

Proximate chemical composition

The proximate chemical composition of blueberry (*Vac*

cinium corymbosum L.) is shown in Table 1. It contained 13.13% carbohydrate as a major chemical constituent of its dry weight. Fat and protein contents were 2.39 and 1.88%, respectively on a dry basis. Ash content was 1.48% of the dry weight.

DPPH radical scavenging activity

The free radical scavenging activities of blueberry extracts were evaluated using the DPPH assay. DPPH possesses a proton free radical having characteristic absorption, which decreases on exposure to radical scavengers (27). Due to hydrogen/electron donating ability of antioxidants, they can scavenge the DPPH radicals. The ethyl acetate fraction showed significantly higher (IC_{50} 0.025±0.00 mg/mL) DPPH radical scavenging activity than that of BHT (IC_{50} 0.374±0.03 mg/mL). The second highest scavenging activity was detected in the methylene chloride fraction (IC_{50} 0.079±0.01 mg/mL). All the other fractions also showed significantly higher ($p<0.05$) values than that of BHT (Table 2). The differences in antioxidant activity in a particular assay are largely a function of the ratio of the hydrophilic and hydrophobic natures of phenolic compounds. Our data also proved that different organic solvent fractions show different DPPH scavenging activities. This demonstrates the dis tribution of polar and non-polar antioxidant compounds of blueberry. Further, the antioxidant potential of phenolic compounds depends on the redox properties and chemical structures of those compounds which may take part in neutralizing free radicals, chelating metal ions and quenching singlet oxygen, by delocalization or decomposing peroxides (28,29). Rossi et al. (30) have also reported the same results as DPPH scavenging by highbush blueberry juice. The results presented in this study suggest that most of the free radical scavenging components tended to be concentrated in hydrophilic solvent fractions.

Table 1. Approximate chemical composition of blueberry (*Vaccinium corymbosum* L.)

Composition	%
Moisture	81.12±2.30
Ash	1.48±0.06
Protein	1.88±0.03
Fat	2.39±0.07
Carbohydrate	13.13±1.10

All data are means of three determinations (mean±SD, n=3).

Superoxide anion ($O_2\cdot^-$) scavenging activity

Superoxide anion radicals are produced in cellular oxidation processes and act as a precursor for some of the other reactive oxygen species (H_2O_2 and HO·) in living cells (31). Superoxide scavenging activities of blueberry extracts were estimated using xanthine-xanthine oxidase system (NBT method) and the results are shown in Table 2. The chloroform and the final aqueous fraction showed the equally highest (IC_{50} 2.3±0.11 mg/mL) activities at superoxide anion scavenging, but significantly lower than those of BHT and α-tocopherol (IC_{50} 0.18±0.01 and 1.60±0.08 mg/mL, respectively). Superoxide anions indirectly initiate lipid peroxidation by producing singlet oxygen and hydroxyl radicals. Hence, the $O_2\cdot^-$ scavenging ability of blueberry shown in this study suggests that blueberry has beneficial effects for decreasing toxicity of the superoxide anions.

Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide is a non-radical reactive oxygen species, which is derived from normal metabolism. Hydrogen peroxide can cross membranes and may gradually oxidize a number of compounds. All fractions of the methanolic extract showed significantly higher ($p<0.05$) activities than those of commercial antioxidants (Table 2) for H_2O_2 scavenging. The methylene chloride and the ethyl acetate fractions showed strong activities (IC_{50} 0.15±0.01 and 0.20±0.01 mg/mL, respectively) and these were significantly higher ($p<0.05$) than those of BHT and α-tocopherol (IC_{50} 2.2±0.04 and 3.2±0.04 mg/mL, respectively). The methanolic extract and the final

Table 2. Antioxidative effect of different fractions of blueberry (*Vaccinium corymbosum* L.)

Fraction	Total phenolic content (mg/100 g dried sample)	IC_{50} (mg/mL)					
		DPPH	$O_2\cdot^-$	H_2O_2	HO·	NO·	Metal chelating
75% Methanol extract	1659±9.4	0.099±0.03^{c}	7.60±0.31^{f}	0.68±0.01^{c}	0.010±0.00^{a}	4.70±0.16^{e}	0.011±0.003^{a}
n-Hexane	790±4.5	0.097±0.03^{c}	9.50±0.42^{g}	1.10±0.03^{e}	0.012±0.00^{a}	9.60±0.21^{g}	0.010±0.001^{a}
Chloroform	1176±6.7	0.120±0.04^{c}	2.30±0.11^{c}	1.30±0.02^{f}	0.017±0.00^{a}	3.90±0.11^{d}	0.010±0.001^{a}
Ethyl acetate	2430±8.2	0.025±0.00^{ab}	2.70±0.12^{d}	0.20±0.01^{b}	0.012±0.00^{a}	3.00±0.13^{c}	0.012±0.004^{a}
Methylene chloride	764±4.3	0.079±0.01^{bc}	3.50±0.14^{e}	0.15±0.01^{a}	0.010±0.00^{a}	3.10±0.09^{c}	0.011±0.003^{a}
Aqueous	1379±8.7	0.100±0.06^{c}	2.30±0.11^{c}	0.97±0.03^{d}	0.011±0.00^{a}	5.50±0.21^{f}	0.011±0.003^{a}
BHT		0.374±0.03^{d}	0.18±0.01^{a}	2.20±0.04^{g}	0.027±0.00^{a}	1.63±0.06^{a}	3.300±0.024^{b}
α-Tocopherol		0.018±0.00^{a}	1.60±0.08^{b}	3.20±0.04^{h}	0.051±0.00^{a}	2.34±0.06^{b}	4.300±0.027^{c}

All data are means of three determinations (mean±SD, n=3).
Significant differences at $p<0.05$ indicated with different letters.

aqueous fraction also showed higher activities (IC_{50} 0.68 ±0.01 and 0.97±0.03 mg/mL, respectively) in H_2O_2 scavenging. From our results, it is clear that there are different scavenging activities between polar and non-polar extracts. This may be due to the presence of different H_2O_2 scavenging phenolic antioxidants in those fractions. Further, most H_2O_2 scavenging compounds present in these fractions are concentrated into the ethyl acetate and the methylene chloride fractions, which are hydrophilic in nature.

Hydroxyl radical (HO·) scavenging activity

The hydroxyl radical is the most reactive oxygen species among ROS. It can be formed from H_2O_2 under a variety of stress conditions and is involved in various cellular disorders (32,33). The methanol extract and the methylene chloride fraction showed the highest activity, which was IC_{50} 0.010±0.00 mg/mL, while the final aqueous fraction showed the second highest activity (IC_{50} 0.011±0.00 mg/mL) for hydroxyl radical scavenging (Table 2). The ethyl acetate and the n-hexane fraction also showed slightly higher activities (IC_{50} 0.012±0.00 mg/mL) while the chloroform fraction exhibited IC_{50} 0.017±0.00 mg/mL in the scavenging. The activities of all the fractions from blueberry were higher than those of commercial antioxidants tested; BHT and α-tocopherol (IC_{50} 0.027±0.00 and 0.0051±0.00 mg/mL respectively). However, there was no significant difference between all the fractions and the commercial antioxidants tested. Hydroxyl radicals are capable of abstracting hydrogen atoms from the membranes and bring about peroxidic reactions of lipids (34). Hence, it can be expected that blueberry extract would show antioxidant effects against lipid peroxidation of biomembranes and scavenge the hydroxyl radicals at the stage of initiation and termination of peroxy radicals. Further, our values were higher than the values obtained by Rossi et al. (30) for highbush blueberry juice. Both hydrophobic and hydrophilic fractions showed strong scavenging activity, indicating that both kinds of polyphenolic antioxidants are present in blueberry. The ability to quench hydroxyl radicals by the antioxidant compounds present can be caused by the direct prevention of propagation of lipid peroxidation processes.

Nitric oxide radical (NO·) scavenging activity

Nitric oxide radical is very reactive and has a short half-life. NO· is generated from the amino acid, L-arginine by nitric oxide synthase and its isoforms (35, 36). The ethyl acetate, the methylene chloride and the chloroform fractions showed the highest activities (IC_{50} 3.0±0.13, 3.1±0.09 and 3.9±0.11 mg/mL, respectively) for NO· radical scavenging among all the fractions. However, these activities were significantly ($p < 0.05$) lower than those of BHT and α-tocopherol (IC_{50} 1.63±0.06 and 2.34±0.06 mg/mL, respectively). Nitric oxide plays important roles as a neurotransmitter, vasodilator and in the immunological system as a defense against tumor cells, bacteria and parasites under physiological conditions, but excess production of NO· during ischemia-reperfusion is considered to act as a toxic radical and to cause renal dysfunction as well (37,38). Since ROS and RNS have been proved to produce mutations and cause DNA damage (39-41), it might be beneficial to human health, if consumed foods could scavenge ROS and RNS.

Ferrous ion chelating ability

Transition metal ions such as iron, copper and manganese are abundantly present in living organisms and foods of both plant and animal origins, and can initiate lipid oxidation directly or indirectly (42). Ferrous ion is a higher valence state metal, which is known to participate in direct initiation of lipid oxidation through electron transfer and lipid alkyl radical formation. The methanol extract and its fractions showed significantly higher activities than those of BHT and α-tocopherol in metal chelating. The chloroform and the n-hexane fraction had the highest metal chelating activity, with an IC_{50} 0.010±0.01 mg/mL (Table 2). The methanol extract, the methylene chloride and the aqueous fraction showed the second highest activity groups for metal chelating (IC_{50} 0.011±0.03 mg/mL). Further, the ethyl acetate fraction also showed strong activity (IC_{50} 0.012± 0.04 mg/mL). Phenolic compounds present in plants are known to participate in metal chelating (43). Furthermore, phenolic compounds possess properly oriented functional groups that can chelate metal ions. Also, six-membered ring complexes show higher stability of metal-antioxidant complexes than do five-membered ring complexes (44). Therefore, higher ion chelating capacities of blueberries may be attributed to the properly arranged structure in the antioxidant compounds present.

Reducing power

The reducing capability of $Fe^{3+} \rightarrow Fe^{2+}$ transformation was investigated in the presence of different blueberry extracts using the method of Oyaizu (21). The reductive capabilities of blueberry extracts compared to BHT and α-tocopherol are shown in Fig. 2. The ethyl acetate fraction showed higher activity than that of α-tocopherol at the same concentration, but lower than that of BHT at a 2 mg/mL concentration. The methanolic extract and the final aqueous fraction also showed higher ac-

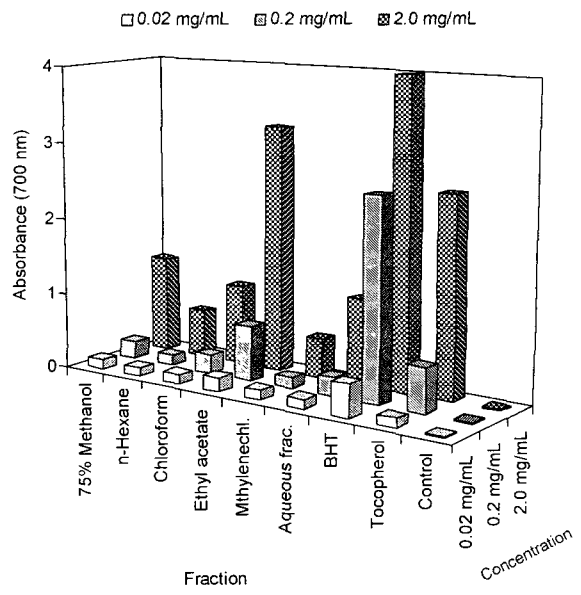

Fig. 2. Reducing power of different fractions of the 75% methanolic extract from blueberry.

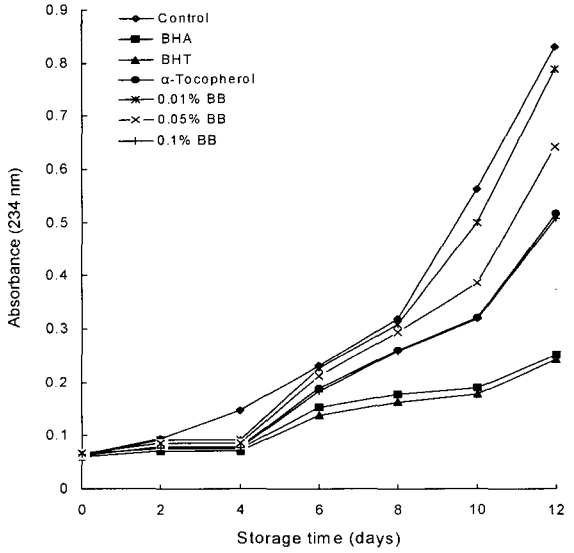

Fig. 3. Effect of the ethyl acetate fraction of the 75% methanolic extract from blueberry on the TBARS value of fish oil stored at 60°C for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

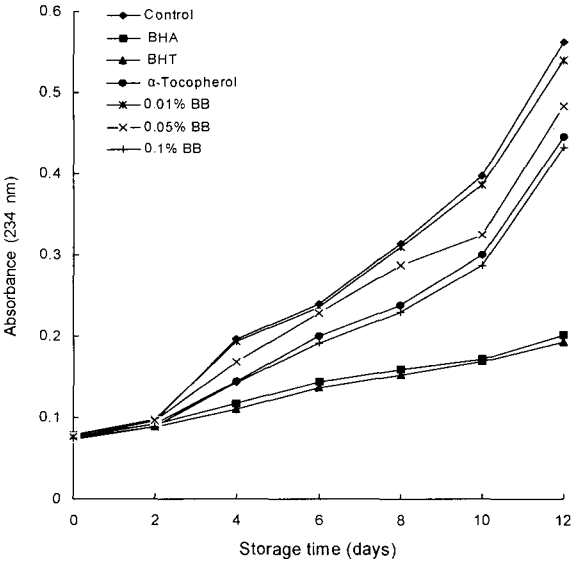

Fig. 4. Effect of the ethyl acetate fraction of the 75% methanolic extract from blueberry on the TBARS value of linoleic acid stored at 60°C for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

tivities than other fractions, but lower than those of BHT and α-tocopherol at the same concentration. All the fractions showed higher activities than that of control and were dose-dependent. In case of reducing power, these higher reducing activities can be attributed to the higher amount of polyphenolics and the reducing capacity of a compound may reflect its antioxidant potential.

Thiobarbituric acid reactive substances (TBARS)

Malonaldehyde is the breakdown product of oxidized fatty acids and results in a rancid flavor in oxidized oils that can be estimated through the reaction with TBA. The inhibitory effect of the ethyl acetate fraction on TBARS formation was higher than that of its control sample in both fish oil and linoleic acid (Fig. 3 and 4). Furthermore, fish oil showed a higher rate of oxidation than linoleic acid. Addition of 0.1% (w/w) ethyl acetate fraction, BHT, BHA and α-tocopherol showed relatively higher inhibitory activities for the 0.1% ethyl acetate fraction compared with 0.05 and 0.01% (w/w) ethyl acetate fractions. The inhibitory effects suddenly decreased after the 8th day in fish oil and 10th day in linoleic acid. The 0.1% (w/w) ethyl acetate fraction was compatible with the values of α-tocopherol, but lower than that of BHT and BHA. The inhibition of TBARS formation by blueberry constituents indicates the total antioxidant potential of blueberry.

Conjugated diene hydroperoxides (CDH)

CDH are formed by re-arrangement of lipid radicals, which then undergo further radical formation. Lipid peroxidation inhibition in the early stages is important as it can prevent the further generation of reactive lipid radicals. The rate of CDH formation was decreased considerably, and dose-dependently, in the fish oil and linoleic acid treated with either the ethyl acetate fraction at a concentration of 0.1% (w/w) or with the commercial antioxidants. However, during the 12-day storage period, the formation of CDH increased rapidly in the control samples as compared to the others (Fig. 5, 6). However, the 0.1% (w/w) level was almost compatible with the

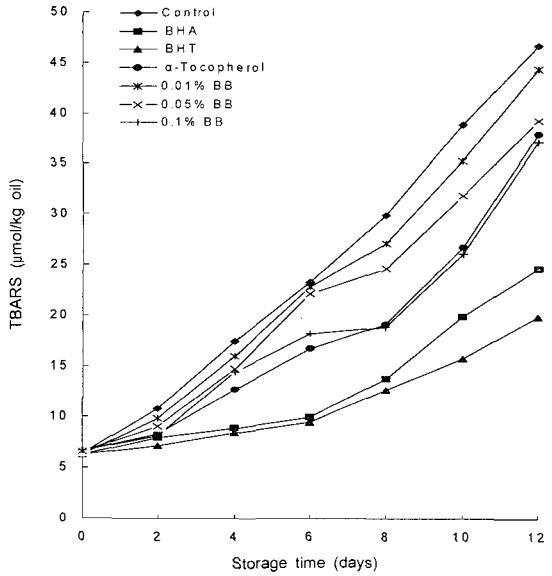

Fig. 5. Effect of the ethyl acetate fraction of the 75% methanolic extract from blueberry on the formtion of conjugated diene hydroperoxides (absorbance at 234 nm) in fish oil stored at 60°C for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

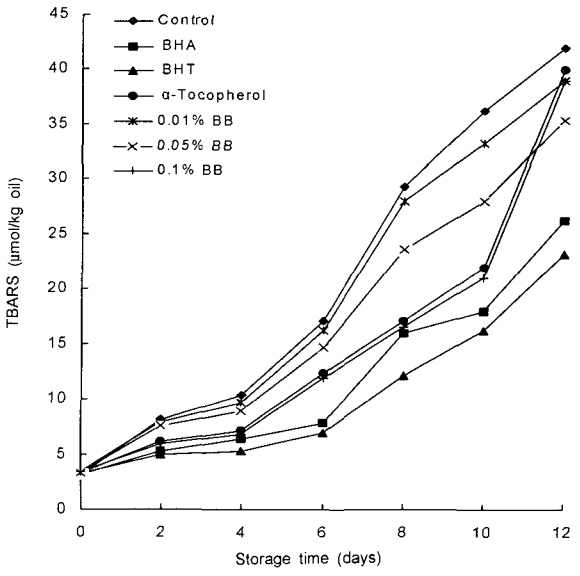

Fig. 6. Effect of the ethyl acetate fraction of the 75% methanolic extract from blueberry on the formation of conjugated diene hydroperoxides (absorbance at 234 nm) in linoleic acid stored at 60°C for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

inhibition of α-tocopherol in both fish oil and linoleic acid. Furthermore, the activity at the 0.1% (w/w) level was less than those of BHT and BHA. The rate of oxidation of fish oil was higher than that of linoleic acid with the same antioxidant concentrations. This is principally due to the higher degree of unsaturation of some fatty acids present in fish oil than that of linoleic acid, which has only two double bonds in its molecule. These results demonstrate that the blueberry extract contains active natural antioxidants in the oil systems during the initial and final steps of lipid peroxidation.

Weight gaining

The addition of oxygen to the lipid radicals can produce lipid peroxy radicals, with a resulting weight increase. The results we obtained for weight gain depicted the ability of antioxidative compounds in the ethyl acetate fraction to retard lipid peroxidation through competitive binding of oxygen, thereby minimizing further reactions. Fig. 7 and 8 show the effect of the ethyl acetate fraction on weight gaining in fish oil and linoleic acid stored at 60°C for 12 days. The ethyl acetate fraction and the synthetic antioxidants inhibited the weight gain compared to the control counterpart in both fish oil and linoleic acid. Inhibition by 0.1% (w/w) ethyl acetate fraction was almost comparable with α-tocopherol. However, there was a sudden increase in weight gain after the 8th day both in fish oil and linoleic acid.

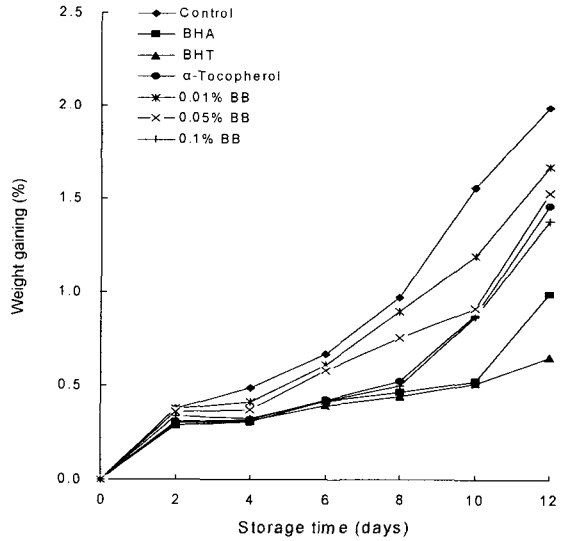

Fig. 7. Effect of the ethyl acetate fraction of the 75% methanolic extract from blueberry on weight gain in fish oil stored at 60°C for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

Total phenolic content

It is well known that plant phenolic extracts act both as free radical scavengers and as antioxidants. Hence, the amount of phenolic content was estimated in this study. Different fractions from the methanolic extract exhibited different amounts of total phenolic content with the organic solvents used. The phenolic compounds present in these fractions may contribute to the antioxidant potential in particular assays and the correlation between total phenolic content and antioxidant assays are given in Table 3. Total phenolic content varies in different fractions due to their polarity. Although the meth-

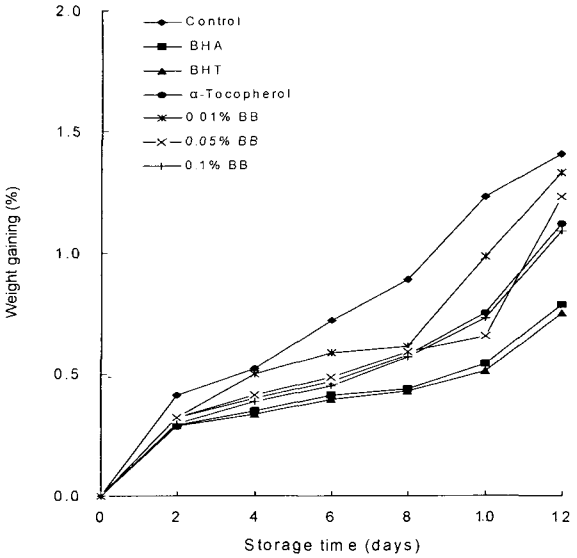

Fig. 8. Effect of the ethyl acetate fraction of the 75% methanolic extract from blueberry on weight gaining in linoleic acid stored at 60°C for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

Table 3. Coefficient of correlation among total phenolics and other antioxidant assays

	DPPH	$O_2\cdot$	H_2O_2	HO·	NO·	Metal chelating
Total phenolics	0.458	0.089	0.135	0.002	0.208	0.607

All are (+) value.

ylene chloride shows relatively lower phenolic content, it shows higher activities for HO· and H_2O_2 scavenging and metal chelating assays. This indicates that the other factors or compounds, other than polyphenols, may also contribute to the activities.

In conclusion, the results obtained in the present study clearly demonstrate that the methanolic extract of blueberry may contain a number of antioxidant compounds, which can effectively scavenge various reactive oxygen species and chelate ferrous ions under *in vitro* conditions. The broad range of activities of the extract suggests that multiple mechanisms are responsible for the antioxidant activity of blueberry. Although we have not isolated the compounds responsible for the antioxidant activites, we speculate that they may be related to the phenolic compounds in the crude extract.

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