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Characterization of Antioxidant Potential of Methanolic and Enzymatic Extracts from Blueberry (*Vaccinium corymbosum* L.)

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요 약

블루베리는 철쭉과(科) 월귤나무속(屬)의 관목으로 줄기가 많으며, 백색 또는 핑크색을 띤 작은 종지 모양의 꽃이 피고 송이 모양의 열매를 맺으며, 높이가 2-3m 까지 자랄 수 있다. 서식지는 주로 캐나다와 미국이며 우리나라에서도 최근 들어 블루베리를 재배하기 시작하였다. 블루베리는 항산화, 항암 및 항염증 활성에 관하여 이미 연구가 이루어졌으나 앞으로 다양한 종의 블루베리에 대한 연구가 더욱 더 이루어져야 할 것으로 판단된다. 본 연구에서는 제주지역에서 재배되고 있는 블루베리 (*Vaccinium corymbosum* L.)를 채취하여 동결건조한 후 75% 메탄올 추출물과 메탄올 추출물 유래의 여러 가지 유기용매 분획물을 제조하여 활성산소종 (reactive oxygen species: ROS) 및 일산화질소 (NO)의 소거능력, 금속과의 킬레이트 활성 (metal chelating activity), 그리고 지질과산화 (lipid peroxidation) 억제능력 등에 대한 항산화 활성을 검토하였다. 75% 메탄올 추출물과 그것의 methylene chloride 분획물은 hydroxyl radical 소거활성이 높게 나타났다. 그리고 ethyl acetate 와 methylene chloride 분획물에서는 hydrogen peroxide, DPPH 자유라디칼 및 NO 소거활성이 우수하였으며, chloroform 과 수용성 분획물에서는 superoxide 소거활성이 높게 나타났다. Metal chelating 활성은 모든 분획물들이 합성 항산화제보다 높게 나타났다. 그리고 ethyl acetate 획분은 0.1% 농도에서도 지질과산화 억제 활성이 매우 높게 나타났다.

한편, 블루베리로부터 수용성 항산화 추출물을 얻기 위하여 5 개의 당분해 효소 (AMG, Celluclast, Termamyl, Ultraflo, Viscozyme) 및 5 개의 단백질 분해 효소 (Alcalase, Flavourzyme, Kojizyme, Nutrase, Protamex)를 이용하여 효소적 가수분해를 통하여 수행하였다. 블루베리의 Viscozyme 추출물은 DPPH 자유라디칼 소거활성이 가장 높았던 반면에, AMG 추출물에서는 NO 소거활성이 가장 높았다. 모든 효소적 가수분해물에서 metal chelating 활성이 높게 나타났으며, AMG 추출물에서는 헤모글로빈으로 유도된 linoleic acid 계에서 지질과산화 억제활성이 가장 높았다.

활성산소종과 자유라디칼은 세포내 DNA 분자에 산화적 손상에 크게 영향을 미치며 이로 인하여 암, 돌연변이, 노화 등과 같은 질병이 발생한다. 따라서 DNA 의 산화적 손상을 최소화하거나 예방하지 않으면 이러한 질병에 쉽게 노출될 수 있다. Comet assay 는 간단하고, 빠르며, 민감한 방법으로 DNA 손상 정도를 검출 할 수 있는 방법으로서, 이 방법을 이용하여 블루베리의 AMG 및 Alcalase 추출물이 DNA 의 산화적 손상의 억제에 미치는 영향을 조사한 결과 매우 긍정적인 효과를 나타낸 것으로 나타났다.

본 연구를 통하여 블루베리가 잠재적인 항산화 효과를 가지고 있는 천연자원이라는 사실을 알 수 있었으며 이 후에는 블루베리 유래 항산화 활성 물질의 순수 분리 정제를 통한 구조분석이 요구되고 있다. 이를 통하여 블루베리가 유용한 천연 항산화 소재로서의 가치를 인정받기를 기대한다.

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INTRODUCTION

Interest in potential antioxidant compounds from natural sources in pharmaceutical and functional food industries have resulted in enormous amount of research with natural sources to find bioactive compounds to characterize them and to find the mode of actions.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously formed as a result of normal cellular functions under pathological process and toxic exposure during cell life. ROS and RNS include free radicals such as superoxide anion ($O_2^{\cdot-}$), hydroxyl ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^{-}$) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Halliwell and Gutteridge, 1990; Brand, 2000; Sanchez-Moreno, 2002). ROS and RNS are able to damage the essential biomolecules in the body including nucleic acids, proteins, lipids and carbohydrates (Halliwell and Gutteridge, 1990). 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 prevent oxidative damages 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 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 omega-3 group, which possess in fish oil. Further, they are the precursors of effective anti-aggregatory substances potential in reducing cardiovascular diseases, carcinogenesis and allergies (Kinsella, 1987; Innis, 1991). Linoleic acid is in omega-6 series, which is also an essential fatty acid, and it is a common constituent of commercially available food oils including fish oil and most of the vegetable oils.

As synthetic antioxidants have been suspected for carcinogenic and a variety of unidentified health disorders (Safer and Al-Nughamish, 1999; Madsen and Bertelsen, 1995), a number of studies have been conducted to investigate the potential antioxidants from natural sources (Abdalla and Roozen, 1999; Siriwardhana et al., 2003; Athukorala et al., 2003).

The enzymatic hydrolysis has been reported excellent yields of desired compounds from the raw material tissues and cells and it present an easily accessible extraction and purification process in the industry (Chiang, Shih and Chu, 1999; Fleurennce, 1999; Heo et al., 2003; Jeon et al., 2000; Siriwardhana et al., 2004). Furthermore, enzymatic hydrolysis process possesses the number of advantages and characteristic features compared to conventional extraction procedures. Water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution and relatively less expensiveness are some of those advantages in the enzymatic hydrolysis process. The enzymes can work primarily by macerating the tissues and breaking down the cell walls and complex interior storage materials to release the bioactive compounds. Furthermore, high molecular weight polysaccharides and protein will be resulted in the

hydrolysis process, which can contribute to enhance the antioxidant potential (Ahn et al., 2004; Heo et al., 2003; Ramos and Xiong, 2002).

Alkaline comet assay (single-cell gel electrophoresis) is a simple, rapid and very sensitive technique for detecting DNA strands breaks at the individual cell level (Singh et al., 1998). It requires a small number of cells and it takes short time to complete the assay. The comet assay is now frequently used in genotoxicity testing, DNA repair studies and population biomonitoring.

Highbush blueberry (*Vaccinium corymbosum* L.) is a shrub with many stems, which grows up to 10 feet and has clusters of bell shaped white flowers, which are abundantly grown in Canada and United State. Japan and Korea have also started to cultivate blueberry recently. A higher antioxidant capacity has been reported in blueberries compared to other fruits and vegetables (Prior et al., 1998). Lowbush blueberry has higher *in vitro* antioxidant capacity than the cultivated highbush blueberry (Kalt et al., 1999). Further, blueberry contains chlorogenic acid as the important antioxidant compound and they are also rich in phytochemicals such as anthocyanin (secondary plant metabolite). Numerous *in vitro* experiments have been indicated that anthocyanins and other phenolics in berries have 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 75% methanolic extract and its fractions and enzymatic extract from highbush blueberry using variety of *in-vitro* methods to assess the stable DPPH, $HO\cdot$, $NO\cdot$, $O_2\cdot^-$ radicals scavenging, H_2O_2 scavenging, reducing power and metal chelating ability. Further, ability

of the ethyl acetate fraction of methanolic extract to inhibit the lipid peroxidation in fish oil and linoleic acid as well as the lipid peroxidation activity of enzymatic extracts in hemoglobin induced linoleic acid system were also evaluated in this work. A susceptibility of rat lymphocyte DNA to oxidative stress was also evaluated in this study exposing to hydrogen peroxide.

Part I

Characterization of antioxidant potential of methanolic extract and its fractions from highbush blueberry (*Vaccinium corymbosum* L.)

1. ABSTRACT

The antioxidant potential of 75% methanolic extract and its different fractions from highbush blueberry (*Vaccinium corymbosum* L.) were investigated using different reactive oxygen species (ROS), nitric oxide ($NO\cdot$), metal chelating and lipid peroxidation assays. Methylene chloride and 75% methanol fractions showed equal higher activities (IC_{50} 0.010 mg/mL) in hydroxyl radical ($HO\cdot$) 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 ($NO\cdot$) 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 ($O_2\cdot^-$) scavenging. All fractions showed strong activities in metal chelating compared with commercial antioxidants tested. 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. Of the fractions, ethyl acetate showed high phenolic content.

2. MATERIALS AND METHODS

2.1. 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 and 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-ethyl-benzthiazolin)-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.

2.2. Proximate chemical composition of blueberry

Proximate chemical composition of blueberry was determined according to AOAC guidelines (AOAC, 1990). Crude protein was determined by Kjeldahl method, 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 method and crude ash was calculated by keeping samples in the dry-type furnace at 550°C until white or light grey ash results.

2.3. Methanolic extraction and solvent fractionation

Blueberry were collected from the farm belongs to Jeju Nong San Co. Ltd. in Jeju-Do of Korea during a full month of May, 2004 and they were rinsed with deionised water before freeze-drying. Then, freeze dried blueberry was pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & CO., Staufen, Germany) and sieved through a 300 μm standard testing sieve. A forty-gram of ground blueberry powder was mixed in 75% methanol (1000 mL) and kept in the shaking incubator at 25°C for one day and filtered in a vacuum using 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, methanol extract and its organic fractions were tested for ROS, NO, reducing power and metal chelating activities together with final aqueous fraction. 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.

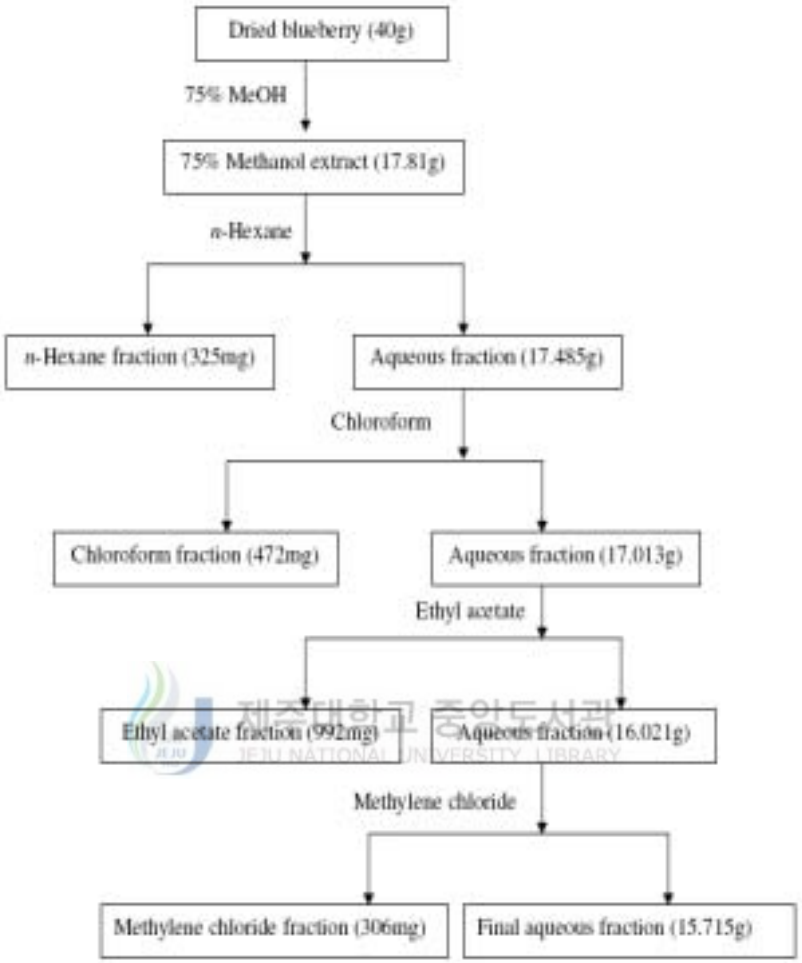

Fig. 1. Scheme of solvent fractionation of blueberry (*Vaccinium corymbosum* L.)

2.4. 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. (1995) was employed to investigate the DPPH radical scavenging activity. Freshly prepared 2 mL DPPH ($3x10^{-5}$ 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 UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

2.5. Superoxide anion ($O_2^{\cdot -}$) scavenging assay

The superoxide scavenging ability of the blueberry samples were assessed by the method of Nagai et al. (2001). 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), and 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, 6mU XOD was added to the mixture to initiate the reaction, 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.

2.6. Hydrogen peroxide (H_2O_2) scavenging assay

The hydrogen peroxide scavenging ability of the blueberry samples were investigated based on the scavenging of the hydrogen peroxide in ABTS-peroxidase system described by Muller (1995). Eighty microliter of each blueberry sample and 20 μL of 10 mM hydrogen peroxide was 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 ELISA reader (Sunrise; Tecan Co. Ltd., Austria) at 405 nm.

2.7. Hydroxyl radical ($HO\cdot$) scavenging assay

Ability of the blueberry samples to scavenge the $HO\cdot$ generated by Fenton reaction was measured according to the modified method of Chung et al. (1997). 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. Then, the resultant mixture was allowed to cool to room temperature and centrifuged at 395 x g for 5 min. The absorbance was recorded at 532

nm.

2.8. Nitric Oxide radical ($NO^{\cdot}$) scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH (7.4) spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions, which can be determined by the use of Griess Illosvoy reaction (Garrat, 1964). 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 of nitric oxide (Marcocci et al., 1994). 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, 0.5mL 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 coloured chromophore was measured at 540 nm.

2.9. Ferrous ion chelating ability

A method by Decker and Welch (1990) 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.

2.10. Measurement of reducing power

Reducing power was investigated using the method developed by Oyaizu (1986). 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 x 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. Higher the absorbance value exhibit, stronger the reducing power.

2.11. Oxidation of fish oil or linoleic acid

Fish oil or linoleic acid was exposed to accelerated oxidation condition similar to the method used by Abdalla and Roozen (1999). 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 the darkness for 12 days. Initial 6 h 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).

2.12. Thiobarbituric acid-reactive substances assay (TBARS)

This assay was based on the method described by Madsen et al. (1998) 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 vortex for 5 min and centrifuged at 2780 x 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 μmol of malonaldehyde per kg oil. TBARS concentration was obtained from a standard curve based on tetraethoxypropane.

2.13. Conjugated diene hydroperoxides (CDH) assay

Conjugated diene hydroperoxide content was detected every two-day interval as described by Roozen et al. (1994). Fifty milligrams of fish oil or linoleic acid sample (stored under accelerated oxidation conditions) was mixed with 5 mL of cyclohexane and vortex. CDH absorbance was recorded at 234 nm.

2.14. Weight gaining assay

This experiment was conducted according to the procedure forwarded by Wanasundara and Shahidi (1996) with slight modifications. Two grams from each sample, prepared for lipid peroxidation were separated into aluminium petri dishes and traces of water in the samples were removed keeping them in the vacuum oven

at 35°C for 12 hrs. Oxidation condition of the samples was accelerated in the forced air oven at 65°C, and percentage weight gaining was recorded for 12 days as in the procedure described by Yan et al. (25).

2.15. Total phenolic content assay

Total phenolic content was determined according to the protocol described by Chandler and Dodds (1993). One millilitre 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 dark for 1 h and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic content.

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

The concentration of the extract (mg/mL) that required scavenging 50% of radicals was calculated by using the percent scavenging activities of four different extract concentrations. Percent scavenging activity was calculated as $[1-(A_i- A_j)/Ac] X 100\%$.

Where; A_i is the absorbance measured with different blueberry fractions in the particular assay with ROS source; A_j is the absorbance measured with different blueberry fractions in the particular assay but without ROS source; A_c is the absorbance of control with particular solvent (without blueberry fractions).

2.17. 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 test ($P<0.05$).

3. RESULTS

Table 1. Proximate chemical compositions of blueberry(*Vaccinium corymbosum* L.)

Composition	Wet (%)	Dry (%)
Moisture	86.4±2.30	5.89±1.3
Ash	0.1±0.06	1.48±0.4
Protein	0.6±0.03	1.88±0.6
Fat	0.1±0.07	2.39±0.3
Carbohydrate	12.8±1.10	88.36±1.7

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

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

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

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

Significant differences at $P < 0.05$ indicated with different letters.

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

	DPPH	$O_2^{\cdot-}$	H_2O_2	$HO^{\cdot}$	$NO^{\cdot}$	Metal chelating
Total phenolics	0.458	0.089	0.135	0.002	0.208	0.607

All are (+) value.

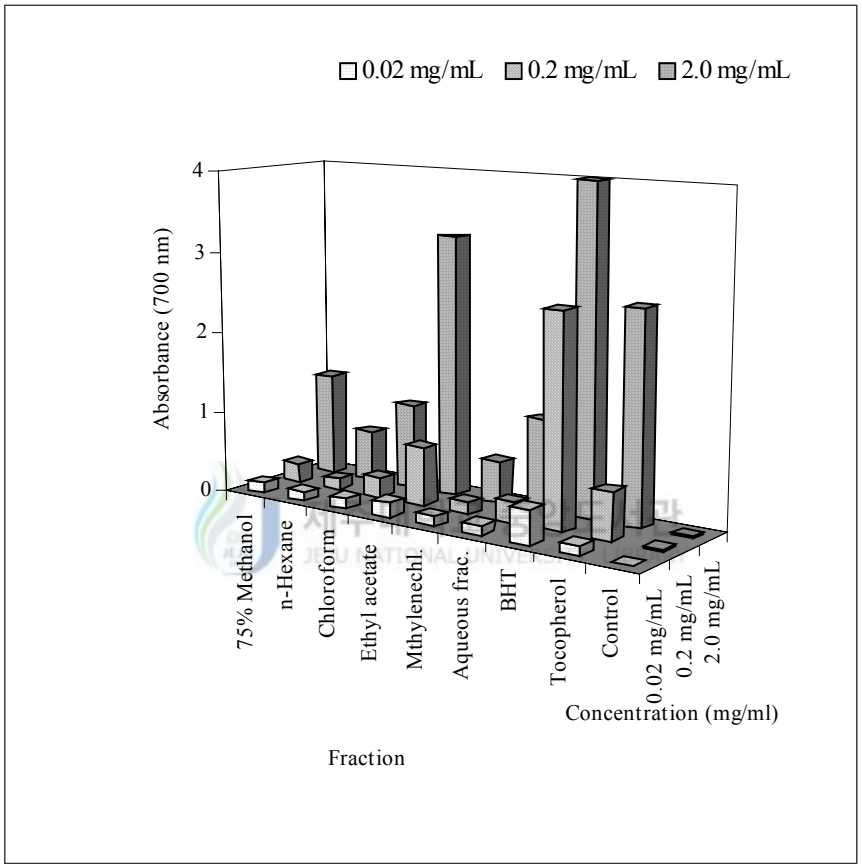

Fig. 2. Reducing power of 75% methanolic extract and its different fractions from blueberry.

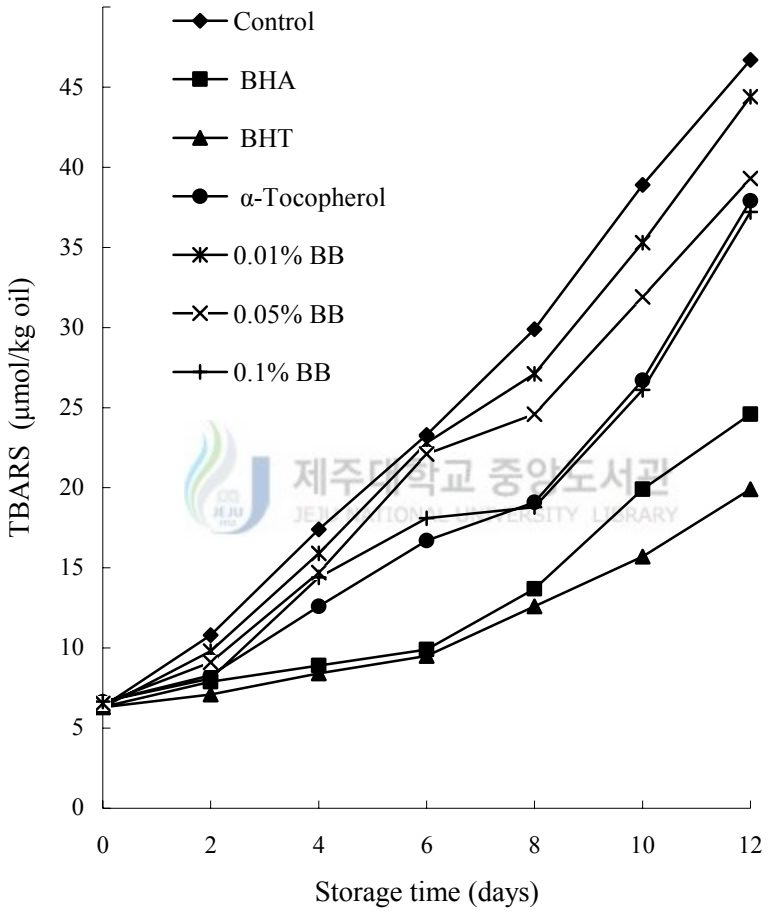

Fig. 3. Effect of ethyl acetate fraction of 75% methanolic extract from blueberry on the TBARS value of fish oil oxidation stored at 60ºC for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

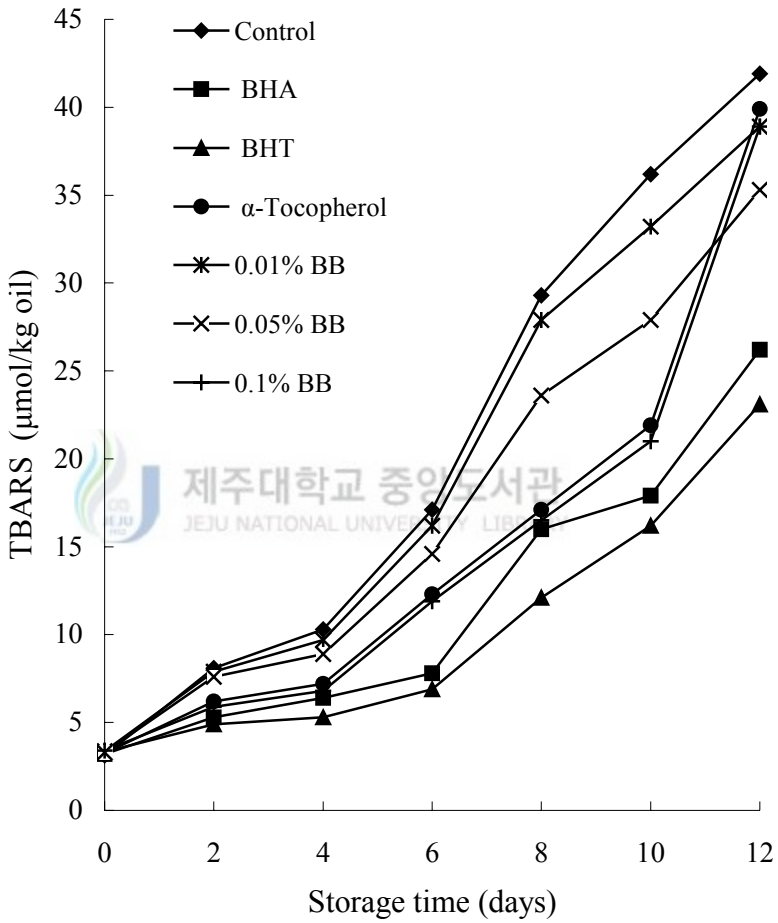

Fig. 4. Effect of ethyl acetate fraction of 75% methanolic extract from blueberry on the TBARS value of linoleic acid oxidation stored at 60ºC for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

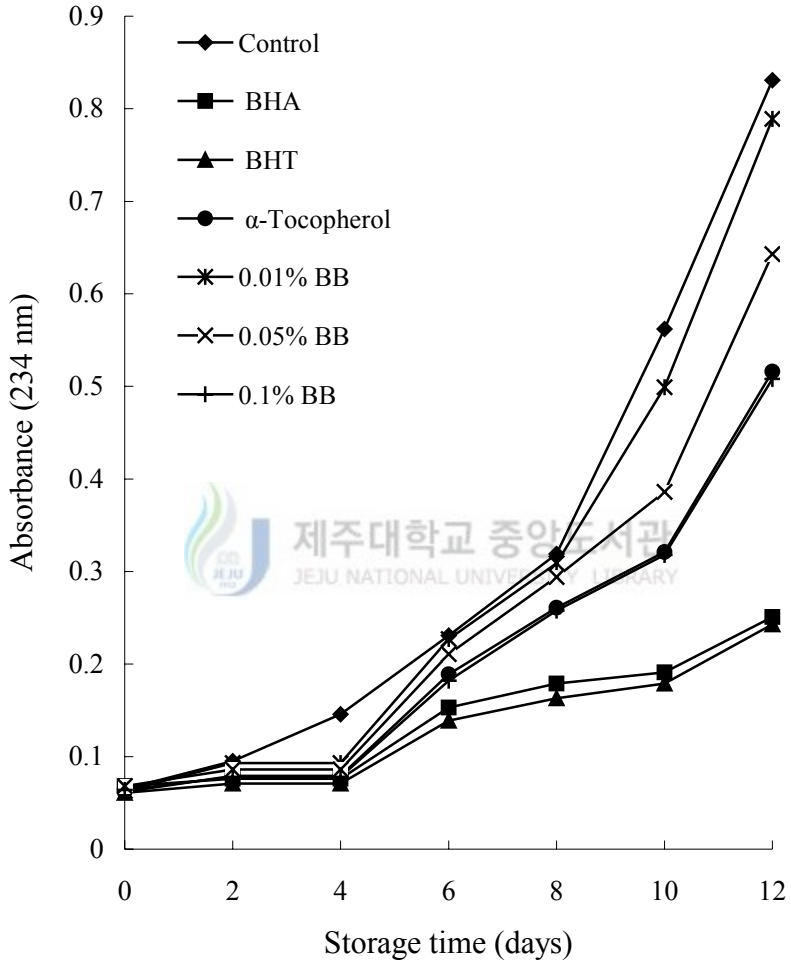

Fig. 5. Effect of ethyl acetate fraction of 75% methanolic extract from blueberry on the formation of conjugated diene hydroperoxides (absorbance at 234 nm) in fish oil stored at 60ºC for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

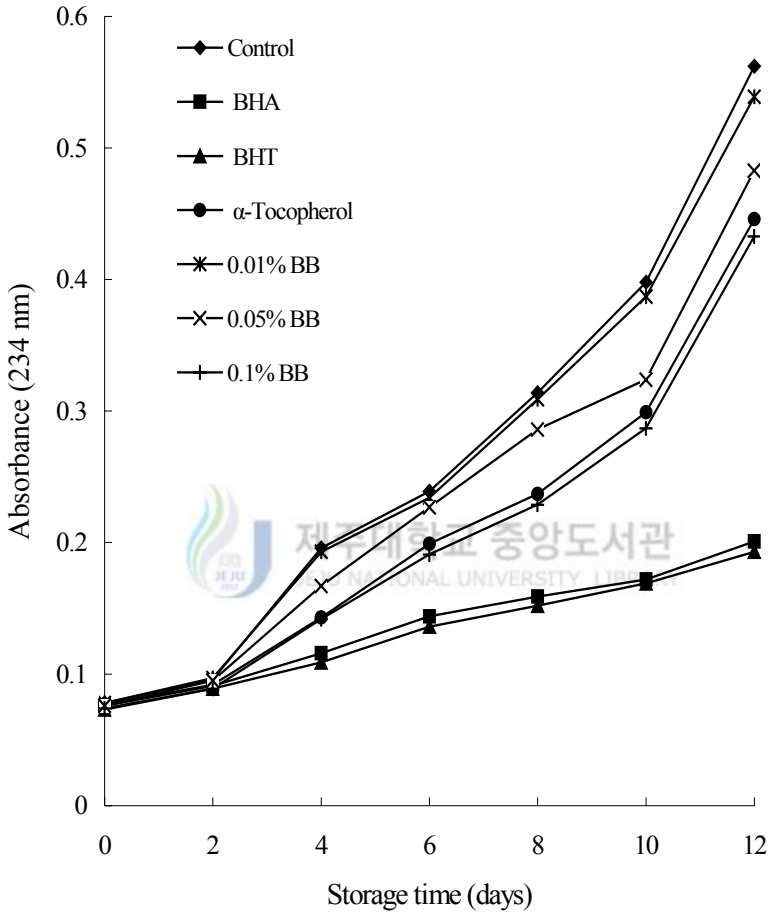

Fig. 6. Effect of ethyl acetate fraction of 75% methanolic extract from blueberry on the formation of conjugated diene hydroperoxides (absorbance at 234 nm) in linoleic acid stored at 60ºC for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

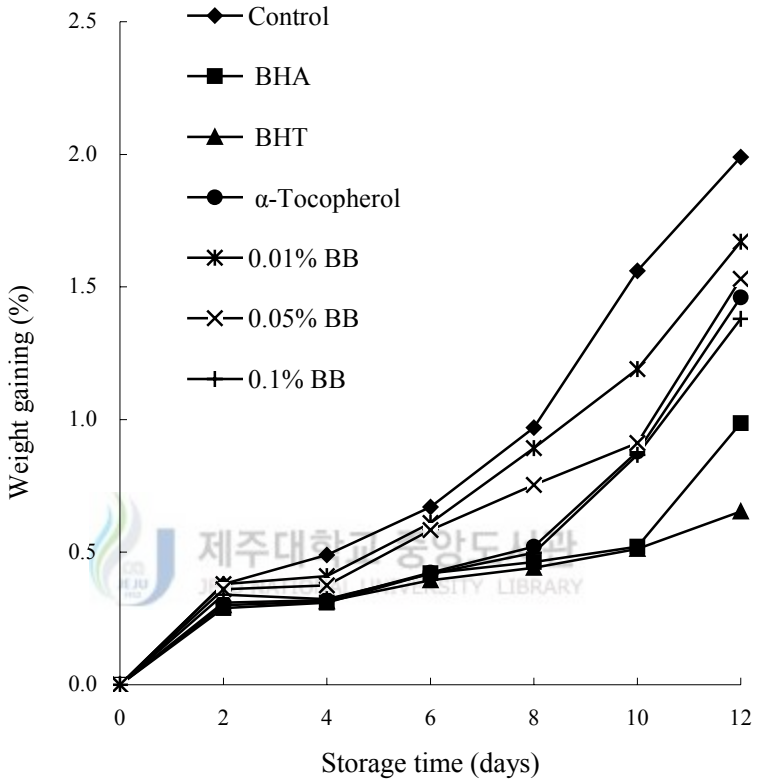

Fig. 7. Effect of ethyl acetate fraction of 75% methanolic extract from blueberry on weight gain in fish oil stored at 60ºC for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

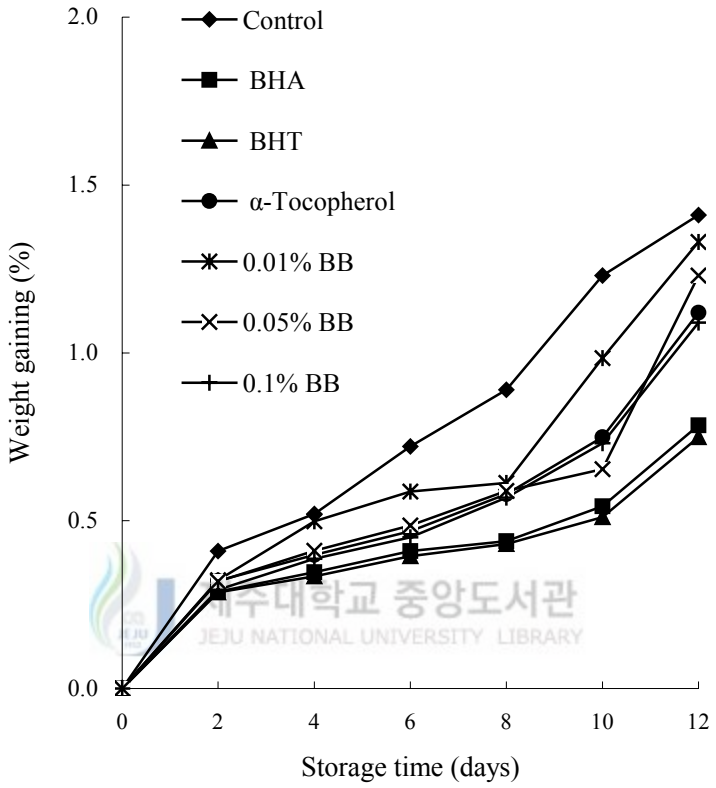

Fig. 8. Effect of ethyl acetate fraction of 75% methanolic extract from blueberry on weight gain in linoleic acid stored at 60ºC for 12 days. BHA, BHT and α-tocopherol were investigated at 0.01% (w/w) concentration. BB=blueberry.

4. DISCUSSION

4.1. Proximate chemical composition

The proximate chemical composition of blueberry (*Vaccinium corymbosum* L.) is shown in Table 1.

4.2. DPPH radical scavenging activity

The free radical scavenging activities of blueberry extracts were evaluated using DPPH assay. DPPH possesses a proton free radical having characteristic absorption, which decreases on the exposure to radical scavengers (Yamaguchi et al., 1998). Due to hydrogen/electron donating ability of antioxidants, they can scavenge the DPPH radicals.

Ethyl acetate fraction showed significantly higher ($P<0.05$) activity (IC_{50} 0.025±0.00 mg/mL) in DPPH radical scavenging than that of BHT (IC_{50} 0.374±0.03 mg/mL). The second highest scavenging activity was detected in 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. The differences in antioxidant activity in a particular assay are largely a function of the ratio of hydrophilic and hydrophobic nature of phenolic compounds. Our data also proved that different organic solvent fractions show different activity in DPPH scavenging. This demonstrates the distribution 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 (Zheng and Wang, 2001; Pizzale et al., 2002). Rossi et al. (2003) also have reported the same results in DPPH radical scavenging by highbush blueberry juice. The results present in this study suggest that most of the free radical scavenging components tended to be concentrated in hydrophilic solvent fractions.

4.3. Superoxide anion ($O_2^{\cdot -}$) scavenging activity

Superoxide anion radicals are produced in cellular oxidation process and act as a precursor for some of other reactive oxygen species (H_2O_2 and $HO^{\cdot}$) in living cells (Liu and Ng, 2000).

Superoxide scavenging activities of blueberry extracts were estimated using xanthine-xanthine oxidase system (NBT method) and the results are shown in Table 2. Chloroform and final aqueous fractions showed the equal highest (IC_{50} 2.3±0.11 mg/mL) activities in superoxide anion scavenging but significantly lower ($P<0.05$) 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 the 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.

4.4. Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide is a non-radical reactive oxygen species, which

derived from normal metabolism. Hydrogen peroxide can cross membranes and may gradually oxidize a number of compounds. All fractions of methanolic extract showed significantly higher ($P<0.05$) activities than those of commercial antioxidants (Table 2) in H_2O_2 scavenging. Methylene chloride and 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). Methanolic extract and the final 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 it shows different scavenging activities in polar and non-polar extracts. This may be due to 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 ethyl acetate and methylene chloride fractions, which are more hydrophilic in nature.

4.5. Hydroxyl radical ($HO^{\cdot}$) scavenging activity

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 are involved in various cellular disorders (Halliwell and Gutteridge, 1984; Tiedemann, 1997).

Methanol extract and mthylene chloride fractions 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±00 mg/mL) in hydroxyl radical scavenging (Table 2). Ethyl acetate and *n*-

hexane fractions also showed slightly higher activities (IC_{50} 0.012±0.00 mg/mL) while 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; BHT and α-tocopherol (IC_{50} 0.027±0.00 and 0.051±0.00 mg/mL respectively). However, there is no significant difference ($P<0.05$) 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 (Kidata et al., 1997). Hence, it can be expected that blueberry extract would show antioxidant effects against lipid peroxidation on 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. (2003) for highbush blueberry juice. Both hydrophobic and hydrophilic fractions showed strong activity in the scavenging, indicating that both kinds of polyphenolic antioxidants present in blueberry. The ability to quench hydroxyl radicals by the antioxidant compounds present can be caused the direct prevention of propagation of lipid peroxidation process.

4.6. Nitric Oxide radical ($NO^{\cdot}$) scavenging activity

Nitric oxide radical is very reactive and has a short half-life. $NO^{\cdot}$ is generated from the amino acid, L-arginine by nitric oxide syntheses with isoforms (Forstermann et al., 1994; Fortermann and Kleinert, 1995).

Ethyl acetate, methylene chloride and chloroform fractions showed

the highest activities (IC_{50} 3.0±0.13, 3.1±0.09 and 3.9±0.11 mg/mL respectively) in 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 (Paller, Hoidal and Ferris, 1984; Gengaro et al., 1994). Since ROS and RNS have been proved to produce mutations and cause DNA damage (Salgo et al., 1995a and 1995, Yu and Anderson, 1997) it might be beneficial to human health if consumed foods could scavenge ROS and RNS.

4.7. Ferrous ion chelating ability

Transition metal ions such as iron, copper and manganese are present abundantly in living organisms and foods of both plant and animal origin and can initiate the lipid oxidation directly or indirectly (Schaich, 1980). 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.

Methanol extract and its fractions showed significantly higher activities ($P<0.05$) than those of BHT and α-tocopherol in metal chelating. Chloroform and *n*-hexane fractions were involved in the highest activity group, which IC_{50} 0.010±0.001 mg/mL in metal chelating (Table 2). Methanol extract, methylene chloride and aqueous

fraction showed the second highest activity group in metal chelating (IC_{50} 0.011±0.003 mg/mL). Further, ethyl acetate fraction also showed strong activity (IC_{50} 0.012±0.004 mg/mL). Phenolic compounds present in plants are known to participate in metal chelating (Van Acker et al., 1996). 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 that of five-membered ring complexes (Thompson and Williams, 1976). Therefore higher ion chelating capacities of blueberries may be attributed by the properly arranged structure in the antioxidant compounds present.

4.8. Reducing power

The reducing capability of $Fe^{3+} \rightarrow Fe^{2+}$ transformation was investigated in the presence of different blueberry extracts using the method deployed by Oyaizu (1986).

The reductive capabilities of blueberry extracts compared to BHT and α-tocopherol is shown in Fig 2. Ethyl acetate fraction showed higher activities than that of α-tocopherol at same concentration but lower than that of BHT at the concentration of 2mg/mL. Methanolic extract and the final aqueous fraction also showed higher activities than other fractions but lower than that of BHT and α-tocopherol at the same concentration. All the fractions showed the higher activities than that of control and those were dose-dependent. In case of reducing power, these higher reducing activities can be attributed by the higher amount of polyphenolics and the reducing capacity of a compound may

reflect its antioxidant potential.

4.9. Thiobarbituric acid reactive substances (TBARS)

Malonaldehyde is the breakdown product of oxidized fatty acids and results in some 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 that of linoleic acid. Addition of 0.1% (w/w) ethyl acetate fraction, BHT, BHA and α-tocopherol showed relatively higher inhibitory activities compared with 0.05 and 0.01% (w/w) ethyl acetate fraction. The inhibitory effects suddenly decreased after 8th day in fish oil and 10th day in linoleic acid. 0.1% (w/w) ethyl acetate fraction was compatible with the values of α-tocopherol but lower than that of BHT and BHA. The TBARS formation inhibitory effect of blueberry constituents indicates the total antioxidant potential of the blueberry.

4.10. Conjugated diene hydroperoxide (CDH)

CDH are formed by re-arrangement of lipid radicals and then undergo further radical formation. Lipid peroxidation inhibition in early stages is important as it can prevent the further generation of reactive lipid radicals.

The rate of CDH formation was decreased considerably in the fish oil and linoleic acid treated with ethyl acetate fraction at a

concentration of 0.1% (w/w) and the commercial antioxidants. Those activities were dose-dependent. However during the storage period of 12 days, the formation of CDH increased rapidly in the control samples as compared to the others (Fig.5 and 6). However, the 0.1% (w/w) level was almost compatible with the inhibition of α-tocopherol in both fish oil and lenoleic acid. Furthermore, the activity of 0.1% (w/w) level was lesser that that of BHT and BHA. The rate of oxidation of fish oil was higher than that of linoleic acid at the same concentration. This is principally due to high amount of unsaturated 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 antioxidant in the oil systems during the initial and final steps of lipid peroxidation

4.11. Weight gaining

The addition of oxygen to the lipid radicals can produce the lipid peroxy radicals and the result is weight gaining. The results we obtained for weight gaining depicted the ability of antioxidative compounds in ethyl acetate fraction to retard the lipid peroxidation through competitive binding of oxygen and minimize the further reactions.

Fig.7 and 8 show the effect of ethyl acetate fraction on weight gaining in fish oil and linoleic acid stored at 60℃ for 12 day. Ethyl acetate fraction and synthetic antioxidant inhibit the weight gaining compared to the control counterpart in both fish oil and linoleic acid. Inhibitory effect of 0.1% (w/w) ethyl acetate fraction was almost

compatible with α-tocopherol. It showed a sudden increase in weight gaining after 8th day both in fish oil and linoleic acid.

4.12. Total phenolic content

It is well known that plant phenolic extracts act as free radical scavengers and act 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 assay and the correlation between total phenolic content and antioxidant assays are given in Table 3. Total phenolic content is varies in different fractions due to their polarity. Although methylene chloride shows relatively less amount of phenolic content, it shows higher activities for $HO\cdot$ and H_2O_2 scavenging and metal chelating assays. This indicates that the other factors or compounds except 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 chelating 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 activities we speculate that it may be related to the phenolic compounds in the crude extract.

Part II

Antioxidant potential of enzymatic extracts from highbush blueberry (*Vaccinium corymbosum* L.)

1. ABSTRACT

Enzymatic extracts were prepared from the blueberry (*Vaccinium corymbosum* L.) collected in Jeju, Korea. Five carbohydrases namely AMG, Celluclast, Termamyl Ultraflo and Viscozyme, and five proteases namely Alcalase, Flavourzyme, Kojizyme, Nutrase and Protamex were used to prepare the enzymatic extracts. Antioxidant properties of each extract was studied using stable 1, 1-diphenyl-2-picrylhdrazyl (DPPH), reactive oxygen species (ROS), nitric oxide ($NO^{\cdot}$) scavenging, metal chelating assays and lipid peroxidation activity in hemoglobin-induced linoleic acid system.The phenolic content of all enzymatic extracts was in the range of 517.85 – 597.96 mg/100 g dried sample. DPPH, $NO^{\cdot}$ and metal chelating assays exhibited prominent activities. Viscozyme extract showed the highest DPPH activity (0.046± 0.002 mg/mL) while AMG extract showed the highest activity in $NO^{\cdot}$ scavenging (0.339± 0.011 mg/mL). All the extracts exhibited strong metal chelating activities. Blueberry enzymatic extracts also showed relatively good activity in hydrogen peroxide scavenging. AMG extract showed the highest lipid peroxidation activity (0.28± 0.01 mg/mL) in hemoglobin-induced linoleic acid system. In this results, the blueberry, which has potential antioxidant components may be a good candidate as a natural antioxidant source.

2. MATERIALS AND METHODS

2.1 Materials

Blueberry samples were collected from the farm belong Jeju Nong San Co., Ltd, Jeju, in Korea. Carbohydrases such as Viscozyme L, Celluclast 1.5 L FG, AMG 300 L, Termamyl 120 L and Ultraflo L, and Protease such as Protomex, Kojizyme 500 MG, Neutrase 0.8 L, Flavourzyme 500 MG and Alcalase 2.4 L were purchased from Navo Co.(Novozyme Nordisk, Bagsvaed, Denmark). Butylated hydroxytoluene (BHT), α-tocopherol, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium salt (NBT), xanthine, xanthine oxidase, thiobarbituric acid (TBA), trichloro acetic acid (TCA), Folin Ciocalteu reagent, sodium nitroprusside, sulfanilic acid were purchased from Sigma Co. (St. Louis, USA) and N-1-naphthylethylene diamine dihydrochloride was purchased from Hayashi pure chemical Industries Ltd. (Osaka, Japan). Ethylinediamine tetra-acetic acid (EDTA), peroxidase, 2, 3-Azino-bis (3-ethyl-benzthiazolin)-6-sulfonic acid (ABTS), and deoxyribose were purchased from Fluka Co. (Buchs, Switzerland). All other chemicals used were analytical grade supplied by Fluka or Sigma Co.

2.2 Proximate chemical composition of blueberry

Proximate chemical composition of blueberry was determined according to AOAC methods (AOAC, 1990). Crude protein content was determined by Kjeldahl method, crude carbohydrate content was

determined by phenol-sulfuric acid reaction (absorbance at 480 nm,) using glucose as the calibration standard, crude lipid content was determined by Soxhlet method and ash content was determinated by calcinations in a furnace at 550°C until white or light grey ash results while moisture content was determined at 105°C for 24 hrs.

2.3 Preparation of enzymatic extracts

Method described by Heo et al. (2003) was used with slight modifications to perform the enzymatic extracts from blueberry. One gram of ground blueberry was mixed with 100 mL of buffer solution and then, 100 μL (or 100 mg) of enzyme was mixed. Then the relevant pH and temperature was adjusted to optimize the digestion process in each sample (Table 4). Later, enzymatic hydrolysis was performed for 12 hrs to reach an optimum degree of hydrolysis. Afterwards, the samples were kept in a boiling water bath (100°C) for 10 min to inactivate the enzyme. Enzymatic extracts were obtained after filtering with Whatman filter paper and pH was adjusted to 7. Each enzymatic extract was tested for ROS, NO, metal chelating and lipid peroxidation assays together with measurement of phenolic content. In each assay, all activities were compared with the values of commercial antioxidants (BHT and α-tocopherol) dissolved in methanol.

Table 4. Characteristics of different carbohydrases and proteases in hydrolysis process

Enzymes	Sources	Characteristics	Optimal pH	Optimal T (℃)
AMG	*Aspergillus niger*	Hydrolyzes 1,4- and 1,6-α-linkages in liquefied starch	4.5	60
Celluclast	*Trichoderma reesei*	Catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose ploymers	4.5	50
Termamyl	*Bacillus licheniformis*	Hydrolyses 1,4-α-glucosidic linkages in amylose and amylopectin.	6.0	60
Ultraflo	*Humicola insolens*	Breakdown of β-glucans, pentosans and other gums	7.0	60
Viscozyme	*Aspergillus aculeatus*	Ability to liberate bound materials and to degrade non-sratch polysaccharides.	4.5	50
Alcalase	*Bacillus licheniformis*	Endopeptidase activity.	8.0	50
Flavourzyme	*Aspergillus oryzae*	Endoprotease and exopeptidase activities	7.0	50
Kojizyme	*Aspergillus oryzae*	Amino-and carboxy peptidase activities	6.0	40
Neutrase	*Bacillus amyloliquefaciens*	Endopeptidase activities.	6.0	50
Protamax	*Bacillus protease*	Production of non-bitter protein hydrolysis	6.0	40

2.4 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. (1995) was employed to investigate the DPPH radical scavenging activity. Freshly prepared 2 mL DPPH ($3x10^{-5}$ 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 UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

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

The superoxide scavenging ability of the blueberry samples were assessed by the method described by Nagai et al. (2001). 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 EDTA, and 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, 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.

2.6 Hydrogen peroxide (H_2O_2) scavenging assay

The hydrogen peroxide scavenging ability of the blueberry samples were investigated based on the scavenging of the hydrogen peroxide in ABTS-peroxidase system described by Muller (1995). Eighty microliter of each blueberry sample and 20 μL of 10 mM hydrogen peroxide was 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 ELISA reader (Sunrise; Tecan Co. Ltd., Austria) at 405 nm.

2.7 Hydroxyl radical ($HO\cdot$) scavenging assay

Ability of the blueberry samples to scavenge the $HO\cdot$ generated by Fenton reaction was measured according to the modified method of Chung et al. (1997). 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 0.1M 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 hrs. 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. Then, the resultant mixture was allowed to cool to room temperature and centrifuged at 395 x g for 5 min. The absorbance was recorded at 532 nm.

2.8 Nitric Oxide radical ($NO^{\cdot}$) scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH (7.4) spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions and it can be determined by the use of Griess Illosvoy reaction (Garrat, 1964). 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 of nitric oxide (Marcocci et al., 1994). 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, 0.5mL of the reaction mixture containing nitrite was pipetted and mixed with 1.0 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotisation. Then, 1.0 mL of naphthylethylenediamine dihydrochloride (0.1%) was added, and allowed to stand for 30 min in diffused light. The absorbance of the pink coloured chromophore was measured at 540 nm.

2.9 Ferrous ion chelating ability

A method described by Decker and Welch (1990) 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.

2.10 Measurement of reducing power

Reducing power was investigated using the method developed by Oyaizu (1986). 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 x 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. Higher the absorbance value exhibit, stronger the reducing power.

2.11 Lipid peroxidation inhibitory activity in a hemoglobin-induced linoleic acid system

The lipid peroxidation inhibitory activity of blueberry extract was determined according to the method described by Kuo et al. (1999) with slight modifications. Each sample fraction (0.1 mL) was thoroughly mixed with 0.025 mL of 0.1 M linoleic acid/ethanol and 0.075 mL of 0.2 M phosphate buffer (pH 7.2). Afterwards, 0.08% hemoglobin (0.05 mL) was added to start the autoauxidation and mixture was incubated at 37°C for 1 hr. The lipid peroxidation was stopped by adding 5 mL of 0.6% HCl/ethanol. The peroxidation value of the mixture (0.2 mL) was measured colouring with 0.02 mL of 20 mM $FeCl_2$ and 0.01 mL of 30% ammonium thiocyanate. The absorbance was recorded at 490 nm with an ELISA reader (Sunrise;

Tecan Co., Austria)

2.12 Total phenolic content assay

Total phenolic content was determined according to the method described by Chandler and Dodds (1993). One millilitre 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 dark for 1 hr and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic content.

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

The concentration of the extract (mg/mL) that required scavenging 50% of radicals was calculated by using the percent scavenging activities of four different extract concentrations. Percent scavenging activity was calculated as $[1-(A_i- A_j)/Ac] X 100\%$.

Where; A_i is the absorbance measured with blueberry extracts in the particular assay with ROS source; A_j is the absorbance measured with blueberry extracts in the particular assay without ROS source; A_c is the absorbance of control with particular solvent (without blueberry extracts).

2.14 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 test ($P<0.05$).

3. RESULTS

The proximate chemical composition of blueberry (V*accinium corymbosum* L.) was: moisture 86.4±2.30% (5.89± 1.3%) carbohydrate 12.8±1.10 % (88.36±1.7%), fat and protein content were 2.39±0.3% (0.1±0.07%) and 0.6±0.03% (1.88±0.6%), respectively. Ash content was 0.1±0.06% (1.48±0.06).

Table 5. Antioxidative effect of enzymatic extracts (carbohydrases) from blueberry (*Vaccinium corymbosum* L.) on DPPH, ROS, NO· scavenging and metal chelating

Extracts	Total phenolic content (mg/100g dried sample)	IC_{50} (mg/mL)					
		DPPH	$O_2^{\cdot-}$	H_2O_2	HO·	NO·	Metal chelating
AMG	589.38±9.4	0.057^{c}±0.005	3.77^{f}±0.13	2.10^{a}±0.12	3.84^{c}±0.09	0.339^{a}±0.011	0.120^{a}±0.007
Celluclast	540.74±8.4	0.104^{e}±0.003	2.23^{d}±0.13	2.11^{a}±0.12	3.94^{c}±0.15	0.486^{bc}±0.013	0.108^{a}±0.002
Termamyl	546.63±7.6	0.057^{c}±0.004	1.83^{c}±0.14	2.50^{b}±0.14	3.25^{b}±0.14	0.442^{b}±0.014	0.089^{a}±0.003
Ultaraflo	563.63±8.7	0.064^{d}±0.001	2.56^{e}±0.17	2.83^{c}±0.09	3.86^{c}±0.17	0.405^{b}±0.015	0.117^{a}±0.009
Viscozyme	532.12±7.3	0.046^{b}±0.002	3.71^{f}±0.03	2.72^{bc}±0.19	3.11^{b}±0.15	0.675^{d}±0.012	0.103^{a}±0.005
BHT		0.374^{f}±0.003	0.18^{a}±0.01	2.20^{a}±0.11	0.03^{a}±0.00	1.630^{e}±0.060	3.300^{b}±0.140
α-Tocopherol		0.018^{a}±0.000	1.60^{b}±0.08	3.20^{d}±0.14	0.05^{a}±0.00	2.340^{f}±0.060	4.300^{c}±0.170

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

Significant differences at $P < 0.05$ indicated with different letters.

Table 6. Antioxidative effect of enzymatic extracts (proteases) from blueberry (*Vaccinium corymbosum* L.) on DPPH, ROS, NO· scavenging and metal chelating

Extracts	Total phenolic content (mg/100g dried sample)	IC_{50} (mg/mL)					
		DPPH	$O_2^{\cdot-}$	H_2O_2	HO·	NO·	Metal chelating
Alcalase	597.96±3.1	0.062^{c}±0.004	0.89^{b}±0.12	2.10^{a}±0.14	3.98^{c}±0.08	0.349^{a}±0.011	0.149^{b}±0.006
Flavourzyme	577.93±5.1	0.045^{b}±0.001	2.84^{e}±0.12	2.35^{bc}±0.13	3.72^{b}±0.10	0.697^{c}±0.013	0.117^{a}±0.001
Kojizyme	532.16±4.1	0.113^{d}±0.003	3.28^{f}±0.15	2.75^{d}±0.12	3.90^{c}±0.04	0.429^{b}±0.015	0.129^{b}±0.003
Neutrase	517.85±3.2	0.061^{c}±0.001	2.46^{d}±0.11	2.41^{c}±0.11	3.97^{c}±0.05	0.482^{b}±0.015	0.117^{a}±0.001
Protomax	549.32±6.2	0.152^{e}±0.002	2.83^{e}±0.13	2.16^{ab}±0.11	3.97^{c}±0.05	0.420^{b}±0.011	0.115^{a}±0.006
BHT		0.374^{f}±0.003	0.18^{a}±0.01	2.20^{ab}±0.11	0.03^{a}±0.00	1.630^{d}±0.060	3.300^{b}±0.140
α-Tocopherol		0.018^{a}±0.000	1.60^{c}±0.08	3.20^{e}±0.14	0.05^{a}±0.00	2.340^{e}±0.060	4.300^{c}±0.170

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

Significant differences at $P < 0.05$ indicated with different letters.

Table 7. Lipid peroxidation inhibitory activity of enzymatic extracts from blueberry in a hemoglobin-induced linoleic acid system

Sample	IC_{50} (mg/mL)
AMG	0.28±0.01
Celluclast	0.97±0.04
Termamyl	0.38±0.05
Ultaraflo	1.06±0.08
Viscozyme	0.66±0.04
Alcalase	0.65±0.05
Flavourzyme	0.95±0.06
Kojizyme	1.98±0.04
Neutrase	0.96±0.03
Protomax	2.14±0.09
BHT	0.13±0.02
α-Tocopherol	0.16±0.03

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

Table 8. Coefficient of correlation between total phenolics and other antioxidant assays in carbohydrase extracts

	DPPH	$O_2^{\cdot -}$	H_2O_2	HO·	NO·	Metal chelating
Total phenolics	0.028	0.113	0.117	0.316	0.719	0.465

All are (+) values

Table 9. Coefficient of correlation between total phenolics and other antioxidant assays in protease extracts

	DPPH	$O_2^{\cdot -}$	H_2O_2	HO·	NO·	Metal chelating
Total phenolics	0.121	0.430	0.392	0.066	0.002	0.349

All are (+) values

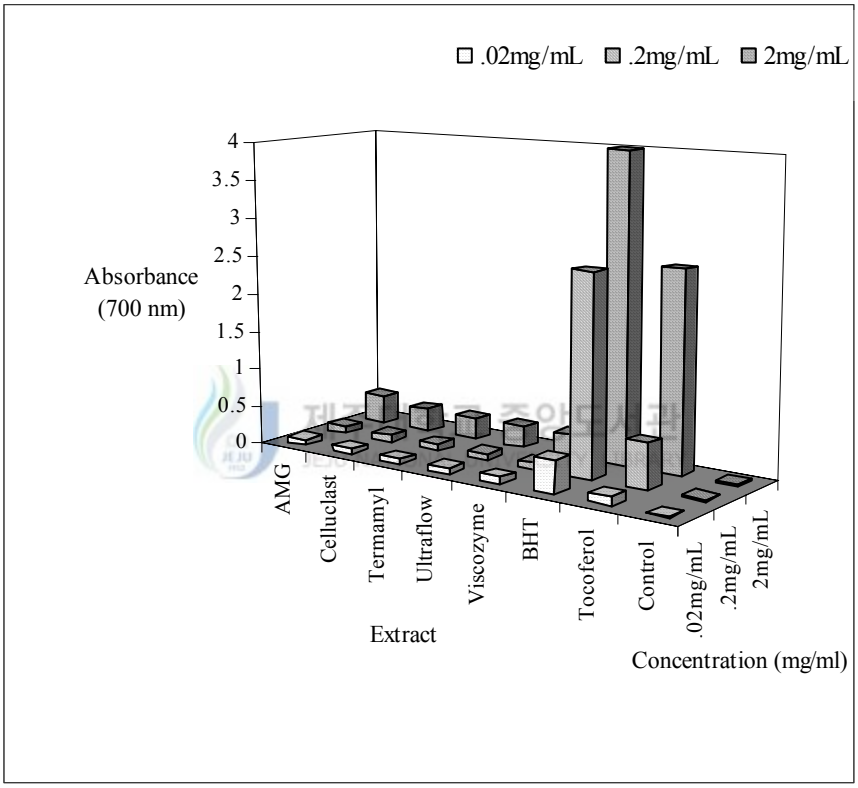

Fig. 9. Reducing power of carbohydrase extracts from blueberry

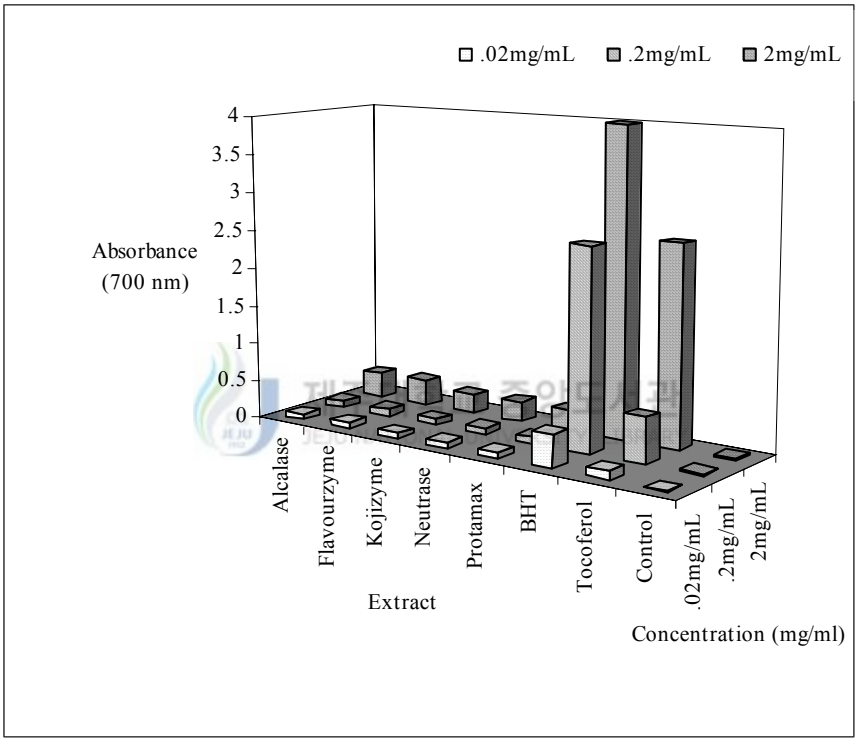

Fig. 10.Reducing power of protease extracts from blueberry

4. DISCUSSION

4.1. DPPH radical scavenging activity

Proton-radical scavenging activity is an important attribute of antioxidants, which can be measured using DPPH radical scavenging assay. DPPH, a protonated radical, has characteristic maximum absorbance peak at 517 nm, which decreases with the scavenging of the proton radical (Yamaguchi et al, 1998). Further, the 2,2-diphenyl-2-picrylhydrazyl radical has been widely used to evaluate the free radical scavenging capacity of antioxidants (Brand-Williams, Cuvelier and Berset, 1995; Espin, Soler-Rivas and Wichers, 2000). Hydrogen-donating ability of the antioxidant molecule contributes to its free radical scavenging nature (Chen and Ho, 1995).

Viscozyme and Flavourzyme extracts showed significantly higher ($P<0.05$) scavenging activities (0.046±0.002 and 0.045±0.001 mg/mL respectively) (Table 5 and 6) when compared with the values of BHT (0.374±0.003 mg/mL) but significantly lower scavenging activities ($P<0.05$) than that of α-tocopherol (0.018±0.0 mg/mL). All the enzyme extracts showed higher values than that of BHT but lower values than that of α-tocopherol. Furthermore, DPPH scavenging activity of carbohydrases treated extracts was relatively higher than the extracts treated with proteases. Oki et al. (2002) have reported that the radical scavenging ability may increase with the increase of the phenolic content. Clear correlation coefficient between total phenolic content and DPPH activity could not be observed (Tables 8 and 9). In this study,

however, some enzymatic extracts showed high activities, even though they contained low amount of phenolics. This may be due to presence of other materials such as low molecular weight polysaccharides, pigments, proteins and peptides. The results reveal that enzymatic extracts from blueberry might contain radical scavenging compounds.

4.2. Superoxide anion ($O_2^{\cdot -}$) scavenging activity

Superoxide radicals are generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Dahl and Richardson, 1978; Meyer and Isaken, 1995). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

Superoxide anion scavenging activity of blueberry extracts is illustrated in Tables 5 and 6. Alcalase extract showed significantly higher ($P<0.05$) activity (0.89±0.12 mg/mL) than that of α-tocopherol (1.60±0.08 mg/mL) but significantly lower ($P<0.05$) activity than that of BHT (0.18±0.01 mg/mL) while Termamyl extract showed higher activity (1.83±0.14 mg /mL) among carbohydrases extracts but significantly lower ($P<0.05$) than those of commercial antioxidants. Furthermore, all the other extracts showed relatively moderate values in supeoxide scavenging activity when compared with the commercial antioxidants. The superoxide scavenging activity of proteases treated extracts was comparatively higher than that of the extracts treated with carbohydrases. Correlation coefficient between polyphenolic content

and superoxide scavenging activity in extracts treated with protease was relatively higher compared with the extract treated with carbohydrases. Higher results may attribute the presence of low molecular weight protein or peptides after hydrolysis process in addition to phenolic content they pocess.

4.3. Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide converts into singlet oxygen ($^1O_2$) and hydroxyl radical, and thereby become a very powerful oxidizing agent. Further, it can cross membranes and may oxidize number of compounds. Thus, removing of H_2O_2 is very important for antioxidant defense in cell or food systems.

In the results of hydrogen peroxide scavenging activity, AMG extract exhibited the highest activity (2.10±0.12 mg/mL) followed by Celluclast extract (2.11±0.12 mg/mL) among carbohydrase extracts (Table 5). Further, Alcalase extract showed the highest activity (2.10±0.14 mg/mL) among protease extracts (Table 6). Both activities were significantly higher ($P<0.05$) than that of α-tocopherol (3.20±0.14 mg/mL). All extracts showed higher activities than that of α-tocopherol. There is no clear correlation between hydrogen peroxide activity and total phenolic content. Therefore, we may conclude that another kind of bioactive compounds may also contribute to the hydrogen peroxide activity other than phenolic compounds.

4.4. Hydroxyl radical (HO·) scavenging activity

An antioxidant ability to scavenge hydroxyl radicals is an important antioxidant action because of the high reactivity of hydroxyl radicals that enables the radical to react with a wide range of molecules found in living cells, such as amino acids, lipids, nucleotides and sugars. Hydroxyl radicals can be generated *in situ* by decomposition of hydrogen peroxide by high redox potential EDTA–Fe^{2+} complex (non-site specific) and, in the presence of 2-deoxy-D-ribose substrate, it forms thiobarbituric acid reactive substances which can be measured photometrically (Aruoma, 1994).

Viscozyme extract showed the highest activity (3.11±0.15 mg/mL) among the carbohydrase extracts (Table 5) while Flavourzyme extract showed the highest activity (3.72±0.10 mg/mL) among the protease extracts (Table 6). However, both activities were significantly lower ($P<0.05$) than those of commercial antioxidants, BHT and α-tocopherol (0.03±0.0 and 0.05±0.0 mg/mL respectively). Furthermore, all the enzymatic extracts showed significantly lower ($P<0.05$) activities in hydroxyl radical scavenging when compared with the commercial antioxidants.

4.5. Nitric Oxide radical (NO·) scavenging activity

In addition to reactive oxygen species, nitric oxide is also implicated in cancer, inflammation and other pathological conditions (Moncada, Palmer and Higgs, 1991). The plant or plant products may have the property to counteract the effect of NO· and in turn may be of

considerable interest in preventing the bad effects of excessive generation of NO· in the human body. Therefore, the scavenging ability of NO· may also help to disrupt the chain reactions initiated by excessive generation of NO· that are detrimental to human health. AMG extract exhibited significantly higher activity ($P<0.05$) than other carbohydrase extracts (0.339±0.011 mg/mL) (Table 5) and Alcalase extract showed significantly higher activity ($P<0.05$) than other protease extracts (Table 6) (0.349±0.011mg/mL). Further, all values were significantly higher ($P<0.05$) than those of commercial antioxidants; BHT and α-tocopherol (1.630±0.06 and 2.340±0.06 mg/mL respectively). Relatively moderate correlation coefficient was shown between nitric oxide scavenging activity and phenolic content in carbohyrase extracts.

4.6. Ferrous ion chelating ability

Iron is known as the most important lipid oxidizing pro-oxidant among the transition metals due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals via the Fenton type reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\cdot$). Ferrozine was used to indicate the presence of chelator and it forms a complex with free Fe^{2+}. In the presence of chelating agent, the complex formation of ferrous and ferrozine is disrupted, resulting in a decrease in red color of the complex. The measurement of red color reduction therefore allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi et al., 2000).

Tables 5 and 6 showed the metal chelating activity of enzymatic extracts from blueberry. Both, the protease and carbohydrase extracts from blueberry showed significantly higher ($P<0.05$) chelating activities when compared with the commercial antioxidant. Termamyl extract exhibited the highest activity among carbohydrases and Protomax extract showed the highest activity among proteases. However, all the enzymatic extracts showed almost equal activities and higher activities than those of the commercial antioxidants, towards metal chelating. Furthermore metal chelating activity showed relatively lower correlation coefficient with total phenolic content. It has been reported that chelating agents, which form σ-bonds with metal are effective as secondary antioxidants because these chelating agents reduce the redox potential and thereby stabilizing the oxidized form of the metal ion (Gordon, 1990). Therefore, values obtained from enzymatic extracts from blueberry demonstrate an effective capacity of iron binding, suggesting that function as peroxidation protector.

4.7. Reducing power

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of blueberry enzymatic extraction. Fig. 9 and 10 depicts the reductive effects of blueberry enzymatic extracts compared with BHT and α-tocopherol. Similar to antioxidant activity, the reducing power of blueberry enzymatic extracts increased with increasing dosage. All the extracts showed moderately higher activities than that of control but significantly lower activities

than that of commercial antioxidants tested.

4.8. Lipid peroxidation inhibitory activity in a hemoglobin-induced linoleic acid system

Lipid peroxidation is a critical problem, which affects the quality of food leading to rancidity, toxicity and destruction of bioactive components. Fats and oils are generally used as the substrates for evaluating lipid peroxidation activity from natural sources (Duh and Yen, 1997; Tian and White, 1994). As shown in Table 7, the highest inhibitory activity of lipid peroxidation in a hemoglobin-induced linoleic acid model system was found in AMG extract (0.28±0.01 mg/mL) among carbohydrases extracts and in Alcalase extract (0.65±0.05 mg/mL) among proteases extracts. Those activities were compared with the values of BHT and α-tocopherol. Although Alcalse and AMG extracts showed the highest activity in lipid peroxidation, they do not show the highest activity in DPPH radical scavenging. However, all the enzymatic extracts showed moderate activities in this assay. Coefficient correlation of DPPH radical scavenging and lipid peroxidation was relatively high (0.713). However, this method could assess the results with only 1 hr of oxidation time. Su and Silva (2005) also have reported good activity in lipid peroxidation of by-products from rabiteye blueberry (*Vaccinium ashei*).

4.9. Total phenolic content

Hydrogen donating ability of the polyphenolic compounds is responsible for the inhibition of free radical induced lipid peroxidation (Yen, Duh and Tsai, 1993). In the present study, seemed to be no clear correlation between the phenolic content and antioxidant activity of the extracts as some enzymatic extracts with lower phenolic content showed relatively higher antioxidant activity. The correlation between total phenolic content and antioxidant assays are given in Tables 8 and 9. However, it is known that non- phenolic antioxidants could also contribute to the antioxidant activity of an extract (Harish and shivanandappa, 2005; Mariko et al., 2005).

In conclusion, the results obtained in the present study clearly demonstrate that the enzymatic extract of blueberry may contain a number of antioxidant compounds, which can effectively scavenge various reactive oxygen species and chelating ferrous ions under *in vitro* conditions. Additionally, higher phenolic content also dispersed in the extracts giving different antioxidant activities. Thus, these results indicate that highbush bluberry containing potential antioxidant components may be a good candidate as a natural antioxidant source and as a food supplement or in pharmaceutical industry. Further investigations are needed to find-out the bioactive compounds present in the blueberry.

Part III

Inhibitory activity of Alcalase and AMG extracts from highbush blueberry (*Vaccinium corymbosum* L.) on H_2O_2 induced DNA damage

1. ABSTRACT

The DNA Comet assay has been described as a sensitive, rapid and inexpensive screening test for evaluating oxidative stress caused by reactive oxygen species (ROS) on individual cells. The DNA damage inhibitory effect of blueberry enzymatic extracts prepared by means of carbohydrase (AMG) and protease (Alcalase) was examined using comet assay. With the increased concentrations, the inhibition effect of cell damage decreased significantly ($P<0.05$). The highest concentration (50 μg/mL) of the extract indicated significantly higher effect ($P<0.05$) against DNA damage, as compared to the control. Inhibitory effects of cell damage were 72% for the AMG extract and 60% for the Alcalase extract at 50 μg/mL of concentration. Photomicrographs of different DNA migration profiles obtained from rat lymphocyte cells, when treated with different concentrations of extract, depicted that with the addition of extracts, the damage which caused by hydrogen peroxide was reduced. The DNA migration changed with the increment of the concentrations; specially, at highest concentrations of both enzymatic extracts indicated protecting effect against DNA damage in contrast to the control. Thus, these results indicate that blueberry may be a good candidate as a natural antioxidant source, which contain strong DNA inhibition potential.

2. MATERIALS AND METHODS

2.1. Materials

Blueberry samples were collected from the farms of Jeju Nong San Co., Ltd, Jeju, in Korea. Carbohydrase such as AMG 300 L and Protease such as Alcalase 2.4 L were purchased from Navo Co. (Novozyme Nordisk, Bagsvaed, Denmark). All the other chemicals used were analytical grade supplied by Fluka or Sigma Co. (St Louis, USA).

2.2. Preparation of enzymatic extracts

Method described by Heo et al. (2003) was used with slight modifications to perform the enzymatic extracts from blueberry. One gram of ground blueberry was mixed with 100 mL of buffer solution and then, 100 μL (or 100 mg) of enzyme was mixed. Then the relevant pH and temperature was adjusted to optimize the digestion process in each sample. Later, enzymatic hydrolysis was performed for 12 hrs to reach an optimum degree of hydrolysis. Afterwards, the samples were kept in a boiling water bath (100°C) for 10 min to inactivate the enzyme. Enzymatic extracts were obtained after filtering with Whatman filter paper and pH was adjusted to 7.

2.3. Isolation and cryoconservation of rat lymphocytes.

Blood samples were obtained from rats. A 5 mL of fresh whole blood was added to 5 mL of phosphate buffered saline (PBS) and

layered onto 5 mL of Histopaque 1077. After centrifugation for 30 min. at 400g at room temperature, the lymphocytes were collected from just above the boundary with the Histopaque 1077, washed with 5 mL PBS, Finally, they were freshly used for comet assay or resuspended in freezing medium (90% fetal calf serum, 10% demethyl sulfoxide) at 6×10^6 cells/mL. The cells were frozen to -80 °C using a Nalgene Cryo 1°C freezing container and stored in liquid nitrogen. The cells were thawed rapidly prior to each experiment in a water bath at 37°C.

2.4. Incubation of lymphocytes with enzymatic extracts from highbush blueberry

Each blueberry extract was dissolved in PBS and diluted into concentrations of 12.5, 25.0 and 50.0 μg /mL. The diluted samples (1.0 mL) were added into the lymphocyte suspension containing 2×10^4 cell/mL and incubated for 60 min at 37°C together with untreated control sample. After preincubation, samples were centrifuged at 2000 rpm for 5 min at 4°C. The incubated cells were resuspended in PBS with 50 μM H_2O_2 for 5 min on ice. The untreated control sample was resuspended only in PBS without H_2O_2. Cells were centrifuged as described above and then washed with 1.0 mL PBS. All the experiments were repeated twice with lympocytes

2.5. Determination of DNA damage reduction (Comet assay).

The alkaline comet assay was conducted according to Singh et al. (1988) with slight modifications. The cell suspension was mixed with

75 μL of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose. After solidifaction of the agarose, slides were covered with another 75 μL of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 hr at 4°C. The slides were next placed in the electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 minutes for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 minutes at 4°C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

3. RESULTS

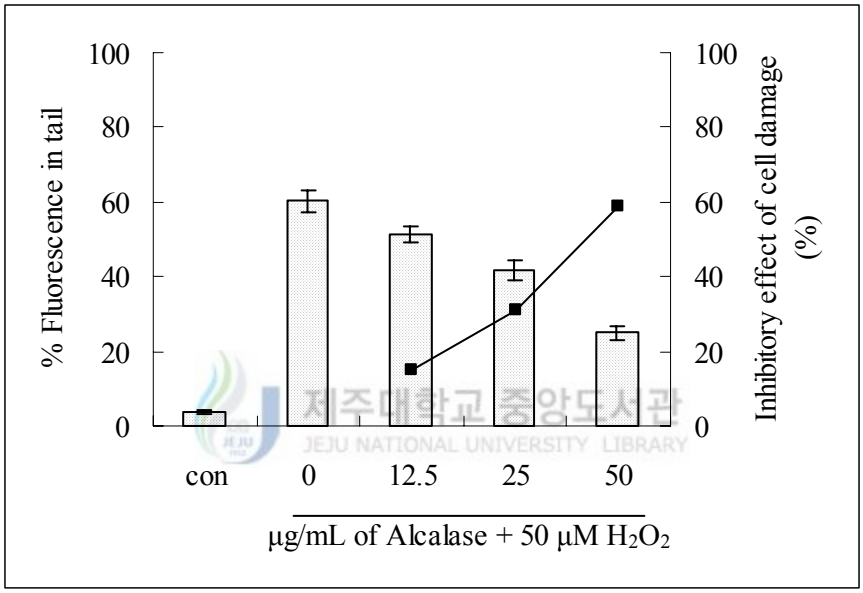

Fig. 11. The effect of different concentrations of Alcalse extract on H_2O_2 induced rat lympocytes DNA damage. Values are means of three replicates. (% fluorescence in tail, Inhibitory effect of cell damage)

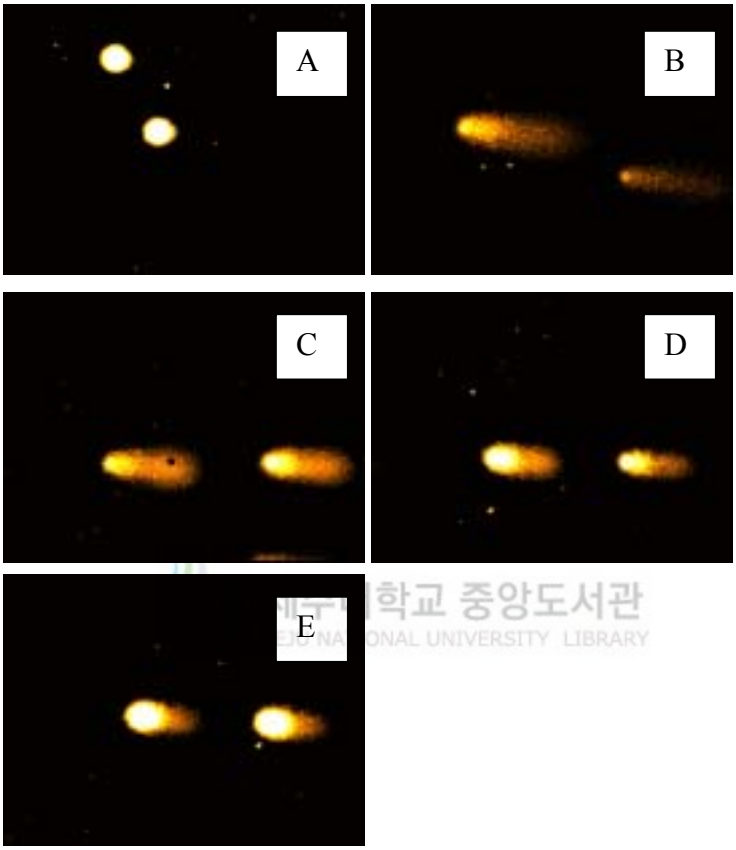

Fig. 12. Comet images of rat lymphocytes.

(A) negative control (B) lymphocytes treated with 50 μM H_2O_2 (C) lymphocytes treated with 12.5 μg/mL blueberry Alcalase extract + 50 μM H_2O_2 (D) lymphocytes treated with 25 μg/mL blueberry Alcalase extract + 50 μM H_2O_2 (E) lymphocytes treated with 50 μg/mL blueberry Alcalase extract + 50 μM H_2O_2

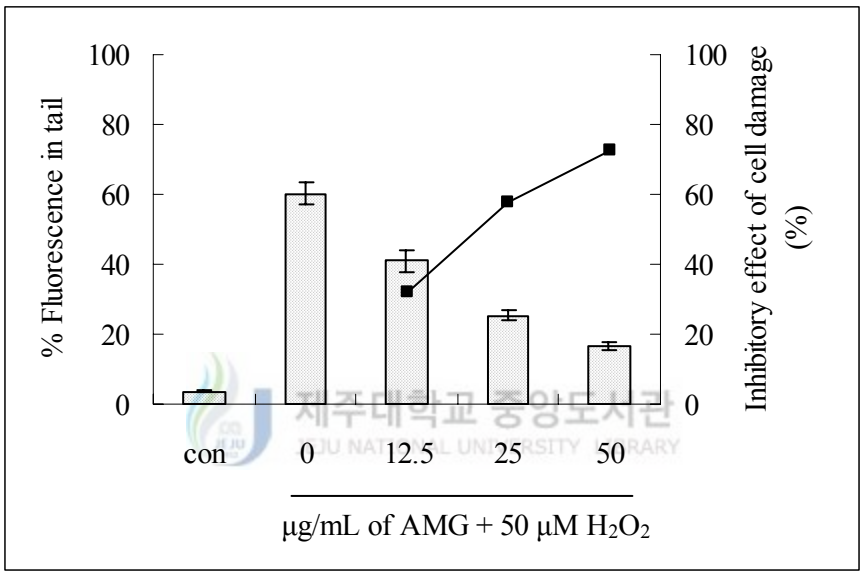

Fig. 13. The effect of different concentrations of AMG extract on H_2O_2 induced rat lymphocytes DNA damage. Values are means of three replicates. (% Fluorescence in tail, Inhibitory effect of cell damage)

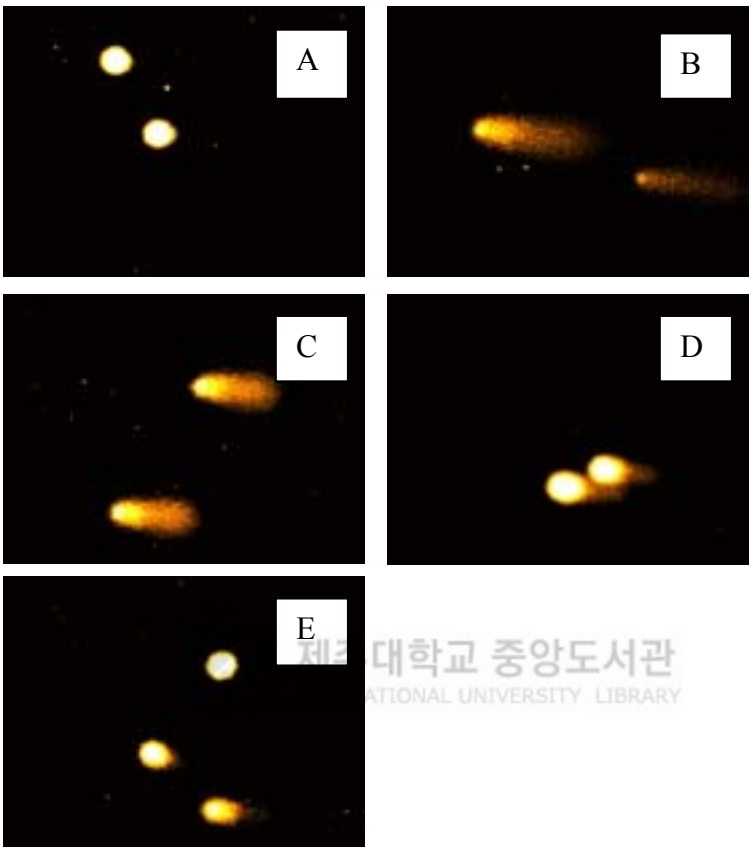

Fig. 14. Comet images of rat lymphocytes.

(A) negative control (B) lymphocytes treated with 50 μM H_2O_2

(C) lymphocytes treated with 12.5 μg/mL blueberry AMG extract + 50 μM H_2O_2 (D) lymphocytes treated with 25 μg/mL blueberry AMG extract + 50 μM H_2O_2 (E) lymphocytes treated with 50 μg/mL blueberry AMG extract + 50 μM H_2O_2

4. DISCUSSION

DNA which is the genetic material in our cells and controls cellular functions can be damaged as a result of several factors such as reactive oxygen species, smoke heat, toxic chemicals and ultraviolet light. The sequence of the DNA base pares can be changed and leads errors/ disorders in replicating DNA If the damage could not be repaired by the existing DNA repair mechanisms. The DNA damage of cultured rat lymphocytes was artificially induced by H_2O_2 and the ability of blueberry extract to inhibit the damage was extrapolated by the fluorescence intensity of the tail extent movement using comet assay.

The DNA damage inhibitory effect of enzymatic extracts of Alcalase and AMG were investigated using comet assay. DNA damage is well known to be one of the most sensitive biological markers for evaluating oxidative stress caused by ROS (Kassie et al., 2000). Comet assay (single cell gel electrophoresis), which is a rapid and sensitive fluorescence microscopic method for detection of primary DNA damage on the individual cell level, is extensively used to evaluate the genotoxicity of test substances (Olive et al., 1990;Fairbairn et al., 1995).

As shown in Fig. 11 and 13 the inhibition of DNA damage by blueberry extracts was investigated with three different concentrations. With increased concentration of extracts the percentage of inhibitory effect of cell damage increased significantly ($P<0.05$) indicating these activities were dose dependants. The highest concentration (50 μg/mL) of both extracts (Alcalase and AMG) indicated strong inhibitory effect against DNA damage, as compared to the control. Inhibitory effect of cell damage was 60.0% by Alcalase extract and 72.0% for AMG extract

at 50 μg/mL of concentration. Furthermore, the fluorescence intensity in tail was decreased with increased concentrations. The H_2O_2 induced DNA damage was successfully overcome/repaired by the blueberry extract at all the concentrations tested. Photomicrographs of different DNA migration profiles obtained from rat lymphocytes, when treated with different concentrations of enzymatic extracts are shown in (Fig.12 and 14). In the group treated with only hydrogen peroxide, the DNA was completely damaged but the addition of enzymatic extracts with hydrogen peroxide reduced the damage. The DNA migration changed with the increment of the concentrations; specially, at 50 μg/mL of the extract indicated protecting effect against DNA damage in contrast to control.

This finding suggests that the constituents in blueberry enzymatic extracts have a potential to act against DNA damage and is an effective chemotherapeutic agent for complications induced by DNA damage.

SUMMARY

Highbush blueberry (*Vaccinium corymbosum* L.) is a shrub with many stems, which grows up to 10 feet and has clusters of bell shaped white flowers, which are abundantly grown in Canada and United State. Korea has also started to cultivate blueberry recently. Numbers of blueberry varieties are available and several studies have reported their antioxidative, anticancer and antiinflammatory activities.

The antioxidant potential of 75% methanolic extract and its different fractions from highbush blueberry were investigated using different reactive oxygen species (ROS), nitric oxide ($NO\cdot$), metal chelating and lipid peroxidation assays. Methylene chloride and 75% methanol fractions showed equal higher activities in hydroxyl radical ($HO\cdot$) scavenging. Higher hydrogen peroxide (H_2O_2) scavenging values were reported for the ethyl acetate and methylene chloride fractions. Nitric oxide ($NO\cdot$) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activities were high in ethyl acetate and methylene chloride fractions. Chloroform and water fractions showed high activities in superoxide ($O_2\cdot^-$) scavenging. All fractions showed strong activities in metal chelating compared to commercial antioxidants. Ethyl acetate 0.1% fraction showed notable capacity to suppress lipid peroxidation in both fish oil and linoleic acid.

Enzymatic hydrolysates of blueberry were prepared using five carbohydrases namely AMG, Celluclast, Termamyl, Ultraflo and Viscozyme, and five proteases namely Alcalase, Flavourzyme, Kojizyme, Neutrase and Protamex. Antioxidant properties of each

extract were studied using different antioxidant assays. Viscozyme showed the highest DPPH activity while AMG showed the highest activity in $NO^{\cdot}$ scavenging. All the extracts exhibited strong metal chelating activities. AMG showed the highest lipid peroxidation activity in hemoglobin-induced linoleic acid system among all the enzymatic extracts.

Reactive oxygen species and free radicals can cause the oxidative damage in DNA of vital molecules in human body. Therefore, it is necessary to minimize or prevent this damage and protect the DNA as it may lead to diseases like cancer or various genetic disorders. Comet assay is a simple, rapid and sensitive method that can be used to detect the amount of damage. Thus, blueberry enzymatic extracts (AMG and Alcalase) were examined for its potential DNA damage inhibitory effect using this method and both Alcalase and AMG extracts showed strong inhibitory activities against DNA damage.

In conclusion, this work has shown that the highbush blueberry is a natural source of potential antioxidants. Further research should be carried out to purify and isolate the bioactive compounds available in blueberry, which later, can be used as a candidate in pharmacutical industry.

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