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Antioxidative and Nitric Oxide Production Inhibitory Activity of selected Vegetables

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ABSTRACT

Selected vegetables (broccoli, cabbage, and carrot) cultivated in Jeju were extracted using 80% methanol and fractionated using hexane, ethylacetate, butanol, and water, and their antioxidative and nitric oxide (NO) production inhibitory activities were measured. Ethylacetate fractions of cabbage and broccoli showed the highest DPPH radical scavenging activities with IC_{50} values of 539 μg/mL and 631 μg/mL, respectively. Ethylacetate fraction of cabbage had the highest NO scavenging activity with IC_{50} value of 433 μg/mL. Butanol fraction of broccoli had the highest superoxide anion scavenging activity with IC_{50} value of 108 μg/mL, while ethyl acetate fraction had the highest xanthine oxidase inhibitory activity. Specially, ethylacetate fractions of broccoli showed inhibitory activities of NO production in LPS-induced RAW 264.7 cells with IC_{50} value of 119 μg/mL without cytotoxicity. Simultaneous treatment with LPS and broccoli ethylacetate fraction was significantly reduced NO production in RAW 264.7 cells. The iNOS protein expression was decreased in a concentration-dependent manner (25-200 μg/mL) and decreased by about 80% at 100 μg/mL of broccoli ethylacetate fraction. These results revealed that solvent extracts of broccoli had the highest antioxidative and NO production inhibitory activities.

Keywords:

vegetables (broccoli, cabbage, carrot)

antioxidative activity

nitric oxide production inhibitory activity

1. Introduction

Epidemiological studies showed that a diet high in vegetables and fruits was associated with a reduced risk for the development of cancer, cardiovascular diseases, diabetes, and other diseases (Brandt et al., 2004; Block et al., 1992).

Vegetables contain lipid soluble vitamins A and E, α-carotene, the water soluble vitamin C, and a wide range of amphipathic molecules, broadly termed phenolic compounds that can potentially contribute to antioxidative activity and protect against several chronic diseases. These compounds are divided into several subclasses including phenolic acids, flavonoids, glucosides, and esters (Plumb et al., 1996; Ninfali et al., 2003).

Brassica vegetables including different genus of cabbage (white, red, savoy, swamp, chinese), cauliflower, broccoli, brussels sprouts, and kale belong to cruciferous family having the protective action that has been attributed to the presence of antioxidant phytochemicals, antioxidant vitamins including ascorbic acid, α-tocopherol and β-carotene. According to Plumb et al. (1996), *brassica* vegetables provide a large group of glucosinolates. It is well established that their breakdown products induce endogenous antioxidant defences such as quinone reductase and glutathione S-transferase in cells and *in vivo* and the products of their hydrolysis can protect against cancer (Podsedek, 2007; Singh et al., 2006).

Broccoli (*Brassica oleracea* italica) is one of the most commonly consumed green vegetables and a rich source of health promoting phytochemicals. Its consumption encourages a variety of functions

including providing antioxidants, regulating enzymes and controlling apoptosis. The organosulfur chemicals such as glucosinolates and the S-methyl cysteine sulphoxide found in broccoli are presumably responsible for various health benefits by cooperating with other constituents such as vitamins E, C, K, minerals and the polyphenols namely kaempferol, quercetin glucosides and isorhamnetin (Koh et al., 2009; Vasanthi et al., 2009).

Cabbage (*Brassica oleracea* L. var. capitata) is also cruciferous family and one of the most important vegetables grown worldwide. These shallow-rooted, cool-season crops are cultivated for its large leafy head and are different types of size, shape, color, and texture of leaves. Much research has been focused on beneficial phytochemicals in cabbage, particularly its indole-3-carbinole (I3C), sulforaphane and indoles, and the majority of the antioxidant activity of such vegetables may be originated from phenolic compounds such as flavonoids, isoflavone, flavones, anthocyanin, catechin, and isocatechin (Singh et al., 2006; Wang et al., 1996).

Carrot (*Daucus carota*) is an important cool season root crop in apiaceae family and a good source of natural antioxidants, especially carotenoids and phenolic compounds. Aliphatic C17-polyacetylenes of the falcarinol type in carrot have anti-inflammatory, cytotoxic activities, immune-stimulating effects, and anti-platelet-aggregatory effects (Chantaro et al., 2008; Young et al., 2007)

Reactive oxygen species (ROS) are created in the metabolism of aerobic cells by many redox processes that ordinary occur. Under normal conditions, ROS and free radicals are effectively eliminated in organisms

by the antioxidant defense systems, antioxidant enzymes and non-enzymatic factors. If not extinguished, ROS can attack important biological molecules, such as lipids, proteins, enzymes, DNA and RNA (Vrchovska et al., 2006; Phanturat et al., 2010). The occurrence of such oxidative damage may be a significant causative factor in the development of many chronic diseases, cancer and cardiovascular diseases, and various neurodegenerative diseases. In addition, that is intimately linked to other components of the degenerative process, mitochondrial dysfunction, excitotoxicity, nitric oxide (NO) toxicity and inflammation (Ismail et al., 2004; Behl et al., 2002; Jenner, 2003).

Consumption of vegetables has been associated with reduced risk of chronic diseases related to the oxidative stress induced by the production of ROS in the human body. Specially vitamin C, vitamin E, carotenoids and dietary flavonoids can play important roles in human nutrition (Triantis et al., 2004; Vrchovska et al., 2006).

Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing many pro-inflammatory molecules, and phagocytic cells that produce and release ROS in response to phagocytosis or stimulation with various agents and can also produce large amounts of NO. And they produce cytokines, growth factors, and proteolytic enzymes that are critical for tissue damage and repair. In the immune response, cytokines function as signal transducers and play critical roles in the execution and prohibition of inflammation (Formana et al., 2001; Mitani et al., 2005; Lin et al., 2007).

Inflammation is part of the non-specific immune response that occurs in reaction to any type of bodily injury. In some disorders, the inflammatory

process which under normal conditions is self-limiting becomes continuous, and chronic inflammatory diseases might develop subsequently (Miliani et al., 2007).

Nitric oxides (NOs) and prostaglandins (PGs) are well-known proinflammatory mediators in the pathogenesis of inflammation. NO is a short-lived free radical that mediates many biological function such a major role in the regulation of vascular tone, neurotransmission, platelet aggregation, and other homeostatic mechanisms (Kim et al., 2005; Lin et al., 2006). NO can be synthesized from L-arginine by a chemical reaction catalyzed by NO synthase (NOS) in living systems. Inducible nitric oxide synthase (iNOS) is only induced by various inflammatory stimulation, such as bacterial endotoxic lipopolysaccharide (LPS), interferon-Ɣ, and a variety of proinflammatory cytokines in macrophages, hepatocytes and endothelial cells (Palmer et al., 1988; Nathan et al., 1994; Uto et al., 2005). On the other hand, once iNOS is expressed, it produces large amounts of NO that profoundly influence cell and tissue function and damage. Large quantities of NO produced by LPS stimulation might play a critical role in LPS-induced tissue damage (Mu et al., 2001).

In this study, broccoli, cabbage, and carrot cultivated in Jeju were extracted using 80% methanol and fractionated using hexane, ethyl acetate, butanol, and water, and their antioxidative and NO production inhibitory activities in LPS-stimulated RAW 264.7 cells were measured.

2. Materials and methods

2-1. Chemicals and reagents

Methanol (MeOH), hexane (Hex), ethylacetate (EtOAc), and butanol (BuOH) were purchased from SK chemical Co. (Gyeonggi, Korea). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium nitroprusside dihydrate (SNP), phosphate buffered saline (PBS), sulphanilamide, beta-nicotinamide adenine dinucleotide (β-NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), xanthine, xanthine oxidase (XO) grade I from bovine milk, ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, butylated hydroxy anisole (BHA), allopurinol, curcumin, trolox, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), lipopolysaccharide (LPS) and β-actin antibody clone AC-74 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium Phosphate-monobasic, sodium Phosphate-dibasic were purchased from Bio basic Inc. (Markham, Ontario, Canada). N-1-napthylethylenediamine dihydrochloride were purchased from Yakuri pure Chemicals Co. (Osaka, Japan). Phosphoric acid were obtained from Junsei Chemical Co. (Tokyo, Japan). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from GIBCO Inc. (Carlsbad, CA, USA). Inducible nitric oxide synthase (iNOS) antibody and the peroxidase-conjugated secondary antibody were purchased from Calbiochem (La Jolla, CA, USA) and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), respectively.

2-2. Preparation of extracts and fractions

Fresh vegetables (broccoli, cabbage, and carrot) were purchased from a local market in Jeju, Korea. External and internal edible parts were separated and cut into small pieces, and then freeze-dried (Programmable freeze dryer, Ilshin Lab Co., Ltd, Korea) and stored in a freezer at -20℃ until needed.

100 g of freeze-dried sample was extracted 5 times using 80% methanol at room temperature with ultrasonification (Crest #1875, Crest Ultrasonics, Trenton, NJ, USA), and then filtered through a filter paper (100-mm; Whatman, Maidstone, UK). The methanol extract was concentrated by a rotary vacuum evaporator (BUCHI rotavapor R-200, BUCHI, Swiss) and freeze-dried. 10 g of freeze-dried methanol extract was dissolved in a 1 L of water and fractionated using 1 L of hexane, ethylacetate, butanol, and distilled water, respectively. Fractionated samples were concentrated by a rotary vacuum evaporator, freeze-dried, and stored at -20℃ until needed. A schematic diagram of extraction and fractionation process is shown in Fig. 1.

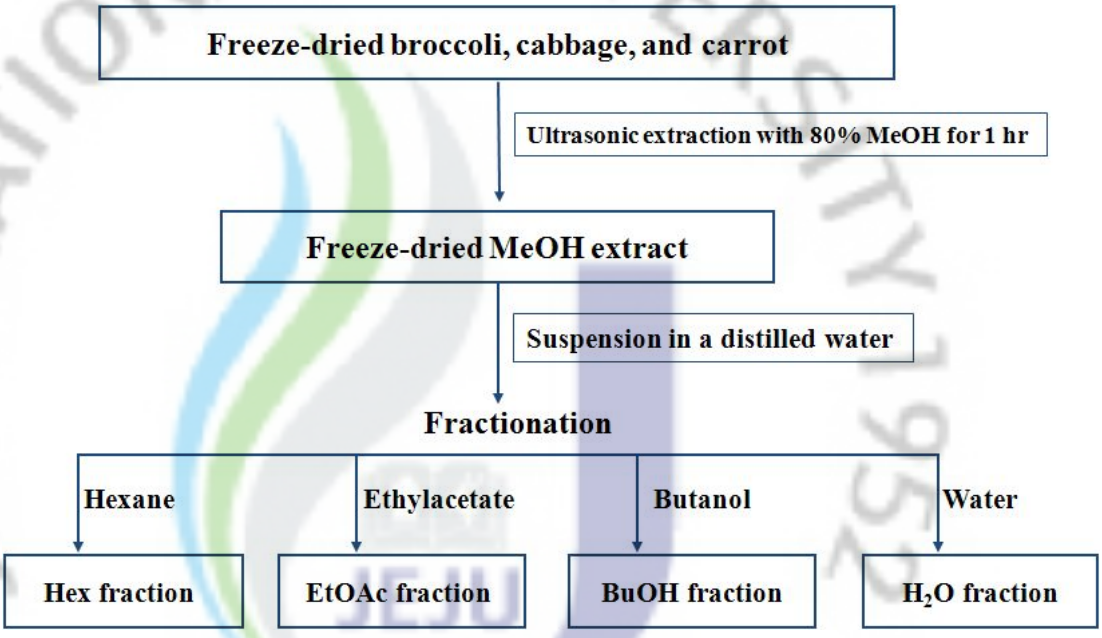

Fig. 1. Flow diagram of extraction and fractionation procedure from selected vegetables.

2-3. Determination of total phenolics content

The amount of total phenolics in the extracts and fractions was determined according to the Folin-Ciocalteu procedure (Singleton et al., 1965). Samples (final con., 5 mg/mL, 0.1 mL, two replicates) were introduced into test tubes; 0.9 mL of distilled water and 0.1 mL of Folin-Ciocalteu's reagent were added. The tubes were mixed and allowed to stand for 5 min. 0.3 mL 20% Na_2CO_3 was added, and then the volume was adjusted to 2 mL with distilled water. After being allowed to stand for 1 hr, absorption at 760 nm was measured. The total phenolics content was expressed as tannin acid equivalents in milligrams per gram dry material by comparison with a calibration curve built with standard tannin acid.

2-4. DPPH radical scavenging activity assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the extracts and its fractions was estimated according to the modified method of Blois (1958). 100 μL of methanol extracts (final con., 5000 μg/mL) and its fractions at various concentrations (0, 16, 31, 63, 125, 250, 500, 1000 μg/mL) were added to 0.4 mM DPPH (100 μL) in 96 well plate and mixed. The mixture solution was stood in the dark for 30 min at room temperature. The absorbance was read at 517 nm by microplate reader (MQX200, Bio-tek Instruments Inc., VT, USA). Ascorbic acid and BHA were used as positive controls. The results were expressed as the concentration required to decrease 50% of signal peak height (IC_{50}). Triplicate experiments were performed. The antioxidative activity of samples was calculated as follows:

DPPH radical scavenging activity (%) = $[(A_{control}-A_{sample})/A_{control}]\times100$

2-5. NO scavenging activity assay

NO was generated from sodium nitroprusside dihydrate (SNP) and measured by the Greiss reaction. SNP in an aqueous solution at physiological pH spontaneously generates NO (Green et al., 1982; Marcoci et al., 1994 a and b), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% N-1-napthylethylenediamine dihydrochloride). Scavengers of NO compete with oxygen leading to reduced production of NO (Marcocci et al., 1994 a and b).

10 mM SNP (50 μL) in a phosphate-buffered saline was mixed with different concentrations of the samples and incubated at 25°C for 30 min. The treated samples was reacted with Giess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme was read at 540 nm by microplate reader and referred to the absorbance of standard solutions of 2.5% phosphoric acid treated in the same way with Griess reagent. Curcumin was used as a positive control. Triplicate experiments were performed. The antioxidative activity of samples was calculated as follows:

Ntric oxide scavenging activity (%) = $[(A_{control}-A_{sample})/A_{control}]\times100$

2-6. Superoxide anion scavenging activity assay

Test solutions were made up with phosphate-buffered saline, 0.5 mM NADH, 0.25 mM NBT, and methanol extracts or its fractions at various

concentrations (16, 31, 63, 125, 250, 500, 1000 μg/mL). Control experiments were carried out simultaneously without test sample. The reaction was started by adding 8 μM PMS and continued at room temperature for 10 min, a period over which absorbance increased linearly from the third minute. The rate of NBT reduction was calculated from the difference in absorbance at 560 nm with respect to a blank solution in which PMS was replaced by buffer solution, and was expressed as increment of absorbance per min. BHA and trolox were used as positive controls. These experiments were performed in triplicates. The antioxidative activity of samples was calculated as follows:

Superoxide anion scavenging activity (%) = $[(A_{control}-A_{sample})/A_{control}]\times100$

2-7. Xanthine oxidase inhibition activity assay

The sample solutions of 200 mM sodium phosphate buffer containing different concentrations (16, 31, 63, 125, 250, 500, 1000 μg/mL) of methanol extracts and its fractions, 2 mM EDTA, and 1 mM xanthine were incubated for 15 min at room temperature. The reaction was started by adding 0.05 U of xanthine oxidase in a sodium phosphate buffer, and the rate of uric acid production was estimated from the difference in absorbance at 290 nm (measured at room temperature for 10 min by microplate reader) between the test solution and a blank solution in which xanthine oxidase was replaced by buffer solution. Allopurinol was used as a positive control. These experiments were performed in triplicates. The antioxidative activity of samples was calculated as follows:

Xanthine oxide inhibition activity (%) = $[(A_{control}-A_{sample})/A_{control}]\times100$

2-8. Cell viability assay

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, supplemented with 100 unit/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. Cell was maintained at 37℃ in an incubator (HEPA class 100, Themo electron Co., OH, USA) with humidified atmosphere of 5% CO_2.

Cell viability was determined by MTT assay (Mosmann, 1983). MTT solution (final con., 0.5 mg/mL) was added to each well and further incubated for 1 hr at 37℃. Media were discarded, and then dimethylsulfoxide was added to each well to dissolve the generated formazan. The absorbance was measured at 570 nm, and the percentage survival was determined by comparison with the control group.

2-9. NO assay

To evaluate the NO production suppression of methanol extracts and its fractions in LPS-stimulated RAW 264.7 cells, the cells were plated in 96-well (2×10^5 cells/well), incubated for 24 hr and then treated with either LPS (100 ng/mL) in the presence of the samples. After the cells were incubated for 24 hr, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. The percent inhibition was expressed as [1-(NO level of test samples/NO level of vehicle-treated control)] ×100. The IC_{50} value, equivalent to the sample concentration that inhibits NO production by

50%, was determined using non-linear regression analysis (% inhibition versus concentration).

2-10. Total protein extraction and western blot analysis

LPS (100 ng/mL)-stimulated RAW 264.7 cells were incubated in the presence of different concentrations of methanol extracts and its fractions for 6 hr. After the cells were lysed with RIPA buffer (Upstate Biotechnology, Waltham, MA, USA) containing 0.1 M PMSF, 0.1 M NaO_4, 0.5 M NaF, 5 mg/mL aprotinin and leupeptin, they were centrifuged at 12,000 rpm for 20 min at 4℃. The insoluble debris was removed by centrifugation, and the protein concentrations were determined using the Bio-Rad protein assay reagent. Whole cell lysates, equal amounts (30 μg/mL) of protein per sample were separated by 10% sodium dodecyl sulpate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to PVDF membrane (Millipore Co., Milford, MA, USA) at 200 mV for 90 min. PVDF membrane of spread protein was reacted in blocking buffer containing 5% skim milk for 1 hr at room temperature, and then the membrane blocking was incubated with a specific primary anti-body (1/5,000 iNOS antibody, 1/10,000 β-actin antibody clone AC-74), at 4℃ overnight, the membrane was washed 4 times with Tris-tween buffered saline and incubated for 30 min with a peroxidase-conjugated secondary antibody (1:5,000) at room temperature. And then, the membrane was detected using the WEST-ZOL Western Blot Detection System (iNtRON Biotechnology, Gyeonggi, Korea). The immuno reactive bands were visualized by image analyzer (UVP Labworks, Upland, CA, USA).

2-11. Statistical analysis

Experimental results are expressed as the mean ± SD. Significant difference from the respective controls for each experiment were tested using Student's t-test (SPSS, Inc., Chicago, IL, USA). A p-value <0.001 was considered statistically significant.

3. Results and discussion

3-1. Extraction yield of solvent extract and fractions

Conventional extraction and fractionation was carried out using different types of solvent (80% methanol, hexane, ethylacetate, butanol, and water) to extract phenolic compounds from broccoli, cabbage, and carrot (Table 1). Extraction yields of 80% methanol extracts were 51.5-66.2%. Among the fractions, distilled water fraction was the highest extraction yield as 61.7-81.8%, followed by butanol fraction (9.7-11.8%), hexane fraction (0.9-1.2%), and ethylacetate fraction (0.8-0.9%). Because of difference in polarity of the organic solvents used, the extraction yield could be different.

Table 1. Extraction yield of solvent extracts and fractions.

Samples	Extraction yield (g/100g)		
	Broccoli	Cabbage	Carrot
80% Methanol extract	51.7	56.7	66.2
Hexane fraction	1.1	0.9	1.2
Ethylacetate fraction	0.8	0.9	0.9
Butanol fraction	11.8	11.4	9.7
Distilled water fraction	61.4	68.5	81.8

3-2. Total phenolics content

Table 2 shows total phenolics content (TPC) of methanol extracts and its fractions from broccoli, cabbage, and carrot. Application of ethylacetate and butanol exhibited the highest TPC, followed by hexane and water in all vegetables tested. Ethylacetate is usually used for extraction of flavonoid aglycones, while ethanol, methanol, and water are used for medium polar and polar compounds such as flavonoid glycoside, phenolic acids, polysaccharides and sugars depending upon their polarity (Prasad et al., 2009).

Broccoli had higher TPC than cabbage and carrot in all the fractions. In broccoli, ethylacetate fraction showed the highest TPC (39.1 mg/g), followed by butanol (33.7 mg/g), distilled water (15.2 mg/g), and hexane (11.3 mg/g) fractions. In cabbage, ethylacetate fraction also had the highest TPC (36.3 mg/g), followed by butanol (9.3 mg/g), hexane (4.2 mg/g), and distilled water (2.5 mg/g) fractions. However, in carrot, butanol fraction had the highest TPC (9.2 mg/g), followed by ethylacetate (8.8 mg/g), hexane (2.6 mg/g), and distilled water (0.9 mg/g) fractions.

Vinson et al. (1998) measured TPC of broccoli, cabbage, and carrot based on dry and wet weight. It showed that TPC was 40.6, 19.2, and 15.3 μmol/g based on dry weight, and 3.6, 1.8, and 1.6 μmol/g based on wet weight in broccoli, cabbage, and carrot, respectively. These results were similar to our study.

Ninfali et al. (2003) reported that TPC was higher in fresh vegetables than in frozen vegetables. Fresh broccoli (69.3 mg/100 g) showed three times higher TPC than fresh carrot (20.3 mg/100g).

Similar results have been reported by Chu et al. (2002) saying that broccoli had the highest amount (80.8 mg/100 g), followed by cabbage and carrot (36.7 and 35.2 mg/100g of sample), respectively.

Table 2. Total phenolics content of methanol extracts and its fractions.

Samples	Total phenolics content (mg/g)		
	Broccoli	Cabbage	Carrot
80% Methanol extract	15.9±0.1	4.0±0.1	1.9±0.0
Hexane fraction	11.3±0.1	4.2±0.1	2.6±0.1
Ethylacetate fraction	39.1±0.2	36.3±0.1	8.8±0.1
Butanol fraction	33.7±0.3	9.3±0.1	9.2±0.0
Distilled water fraction	15.2±0.1	2.5±0.1	0.9±0.0

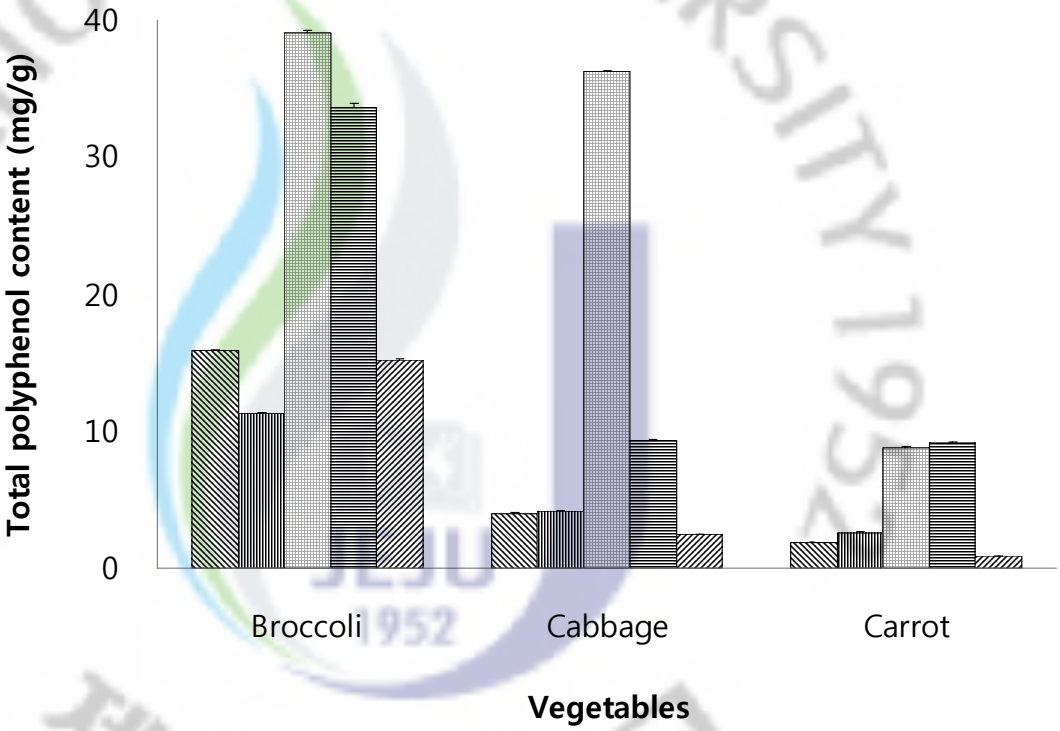

Fig. 2. Total phenolics content of methanol extracts and its fractions (▨ 80% Methanol extract, ▥ Hexane fraction, ▦ Ethylacetate fraction, ▤ Butanol fraction, ▨ Distilled water fraction; Mean±SD (n = 3)).

3-3. DPPH radical scavenging activity

DPPH is relatively stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The DPPH test is a non-enzymatic method currently used to give basic information on the ability of samples to scavenge free radicals and react with suitable reducing agent as a result of which electron become paired off forming the corresponding hydrazine. The solution therefore loses color stoichiometrically depending on the number of electrons consumed which is measured spectrometrically at 517 nm (Perumal et al., 2010; Vrchovska et al., 2006).

Table 3 and Fig. 3 show DPPH radical scavenging activities of 80% methanol extracts from broccoli, cabbage, and carrot. Broccoli was the one with the highest antioxidant potential (42.0%), followed by carrot (26.9%), and cabbage (19.4%) at the concentration of 2500 μg/mL.

Table 4 and Fig. 4 show DPPH radical scavenging activities of solvent fractions at the concentration of 500 μg/mL. In broccoli, butanol fraction had the highest DPPH radical scavenging activity (40.3%), followed by ethylacetate (39.4%), distilled water (20.7%), and hexane (8.4%) fractions. However, in cabbage, only ethylacetate fraction showed high DPPH radical scavenging activity (47.2%). Furthermore, in carrot, only butanol fraction had high activity (18.8%). In summary, ethylacetate fraction of cabbage showed the highest DPPH radical scavenging activity with IC_{50} value of 539 μg/mL, followed by ethylacetate and butanol fractions of broccoli with IC_{50} value of 630 μg/mL and 729 μg/mL, respectively. DPPH radical scavenging activities were concentration-dependent at 16-1000 μg/mL as shown in Fig. 4.

Triantis et al. (2005) reported that DPPH radical scavenging activity of aqueous extract of broccoli (6 μmol Trolox/g) was three times higher than that of carrot (2 μmol Trolox/g). Such compounds are polyphenols, which are known to have strong antioxidant activities and are widely distributed in fruits and vegetables.

Cho et al. (2006) measured the DPPH radical scavenging activities of methanol, dichloromethane, butanol, and distilled water fractions from the broccoli flowers. Among them, the butanol fraction exerted the strongest inhibition of DPPH formation by 50% at a concentration of 74.9 μg/mL, whereas the other fractions showed relatively weak DPPH radical scavenging activities. Antioxidative potential of broccoli butanol fraction assumed to be flavonol glycosides and hydroxycinnamic acid.

Table 3. DPPH radical scavenging activity of 80% methanol extracts.

Vegetables	DPPH radical scavenging activity	
	Scavenging activity (%) (at 2500 μg/mL)	IC_{50} (μg/mL)
Broccoli	42.0±1.8[a]	3063.3±94.3
Cabbage	19.4±1.7	>5000
Carrot	26.9±1.3	4055.7±72.0
Ascorbic acid (at 25 μg/mL)	96.2±0.0	12.5±0.1

[a]Mean±SD (n = 3).

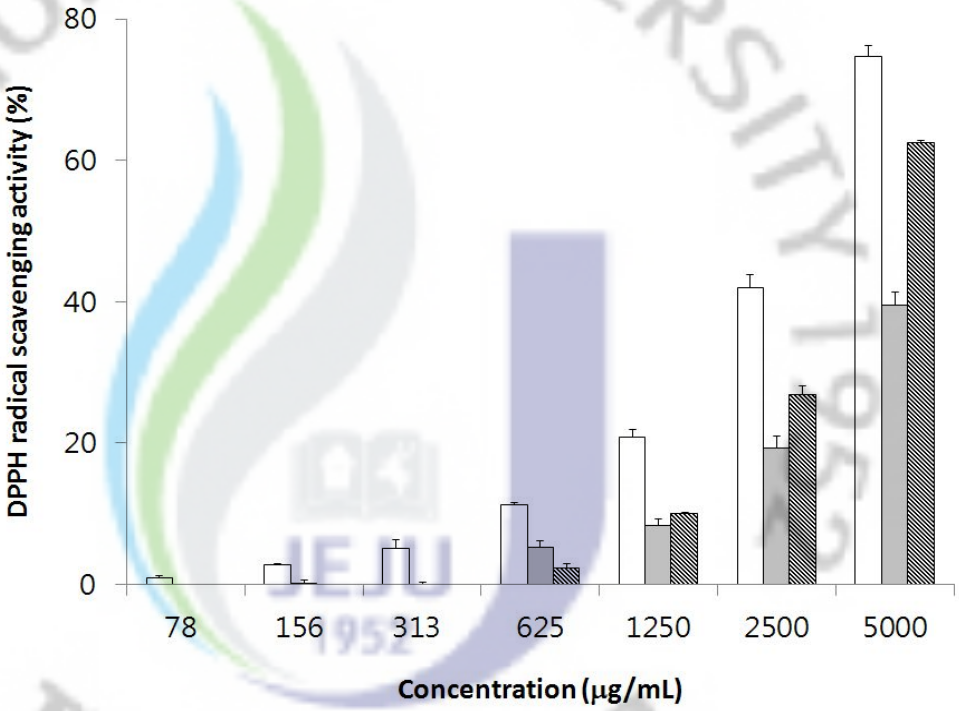

Fig. 3. DPPH radical scavenging activity of 80% methanol extracts (□ Broccoli, ▒ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

Table 4. DPPH radical scavenging activity of solvent fractions.

Fractions	DPPH radical scavenging activity	
	Scavenging activity (%) (at 500 μg/mL)	IC_{50} (μg/mL)
Broccoli		
Hexane	8.4±0.7[a]	>1000
Ethylacetate	39.4±1.9	630.5±11.2
Butanol	40.3±2.5	729.3±42.6
Distilled water	20.7±0.4	>1000
Cabbage		
Hexane	<5	>1000
Ethylacetate	47.2±0.3	539.2±2.0
Butanol	7.0±0.8	>1000
Distilled water	<5	>1000
Carrot		
Hexane	<5	>1000
Ethylacetate	7.8±0.1	>1000
Butanol	18.8±1.5	945.3±12.1
Distilled water	<5	>1000
Ascorbic acid (at 25 μg/mL)	96.2±0.0	12.5±0.1
BHA (at 25 μg/mL)	92.0±1.0	12.3±0.4

[a]Mean±SD (n = 3).

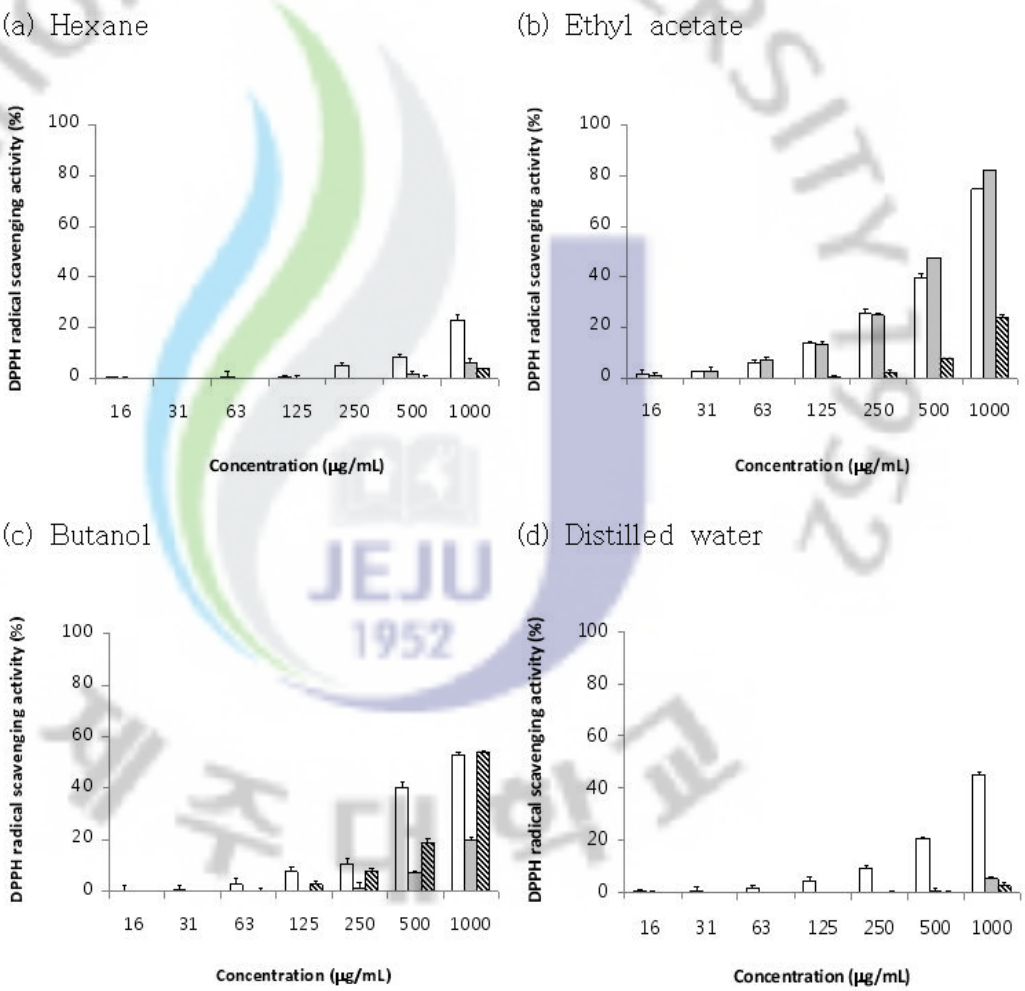

Fig. 4. DPPH radical scavenging activity of solvent fractions (□ Broccoli, ▒ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

3-4. NO scavenging activity

If a large amount of NO is generated, that gives cause for harmful effects by oxidation reactions and indirect effects such as nitrosation and nitration. Suppression of released NO may be partially attributed to direct NO scavenging, as the samples decreased the amount of nitrite generated from the decomposition of SNP which reacts with oxygen to form nitrite *in vitro* (Nakagawa et al., 2002).

Table 5 and Fig. 5 show NO scavenging activity of 80% methanol extracts from broccoli, cabbage, and carrot. Broccoli had the highest antioxidant potential (26.5%), followed by cabbage (12.5%), and carrot (3.4%) at the concentration of 2500 μg/mL, respectively.

Table 6 and Fig. 6 show NO scavenging activities of solvent fractions at the concentration of 500 μg/mL. In cabbage, ethylacetate fraction had the highest nitric oxide scavenging activity (53.8%) compared with other fractions by hexane (15.4%), butanol (11.1%), and distilled water (2.6%). In broccoli, ethylacetate fraction showed high NO scavenging activity (38.4%), followed by hexane (29.5%), butanol (17.0%), and distilled water (10.5%) fractions. In carrot, ethylacetate fraction showed high NO scavenging activity (11.4%), followed by hexane (7.4%), butanol (6.6%), and distilled water (2.5%) fractions. In summary, ethylacetate fraction of cabbage showed the highest NO scavenging activity with IC_{50} value of 433 μg/mL. NO scavenging activities were concentration-dependent at 16-1000 μg/mL as shown in Fig. 6.

Cho et al. (2006) measured NO scavenging activities of methanol, dichloromethane, butanol, and distilled water fractions from the broccoli

flowers. The butanol fraction showed high scavenging activities of 13.2, 31.9, 47.3% at concentrations of 12.5, 25, and 50 μg/mL, respectively, being significantly higher than those of the other fraction. The major components reported to be the flavonol glycosides and hydroxycinnamic acid.

Kang et al. (1996) reported that the high total phenolics content was related with superior nitrite scavenging activity by effectively decomposing nitrite.

Table 5. Nitric oxide scavenging activity of 80% methanol extracts.

Vegetables	Nitric oxide scavenging activity	
	Scavenging activity (%) (at 2500 μg/mL)	IC_{50} (μg/mL)
Broccoli	26.5±2.7[a]	>5000
Cabbage	12.5±0.4	>5000
Carrot	3.4±1.1	>5000
Curcumin (at 50 μg/mL)	59.2±0.7	27.8±0.7

[a]Mean±SD (n = 3).

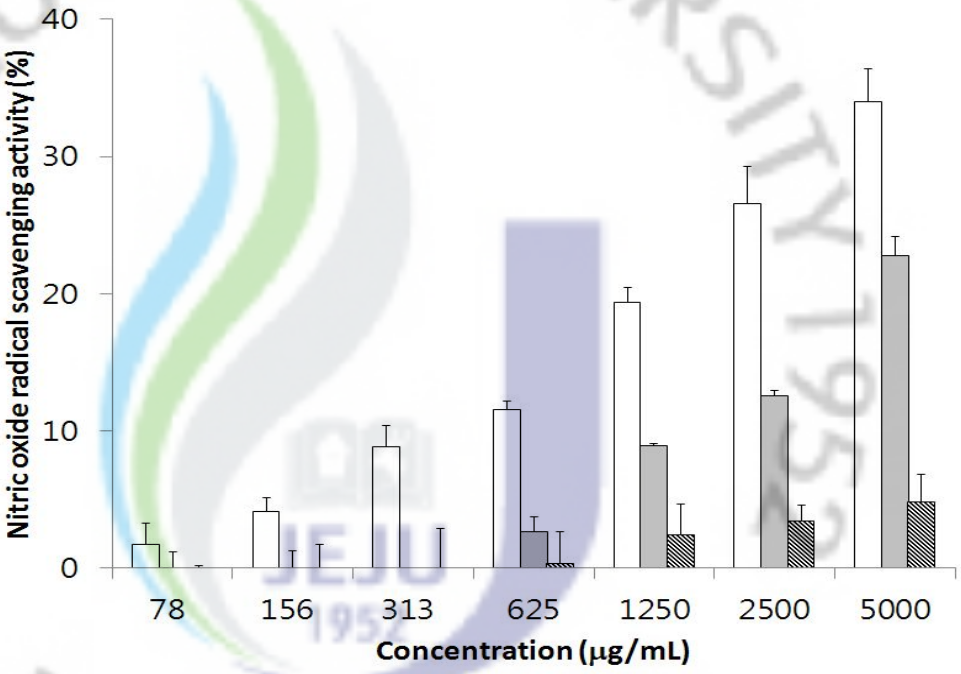

Fig. 5. Nitric oxide scavenging activity of 80% methanol extracts (□ Broccoli, ▒ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

Table 6. Nitric oxide scavenging activity of solvent fractions.

Fractions	Nitric oxide scavenging activity	
	Scavenging activity (%) (at 500 μg/mL)	IC_{50} (μg/mL)
Broccoli		
Hexane	29.5±1.6[a]	>1000
Ethylacetate	38.4±2.2	>1000
Butanol	17.0±0.9	>1000
Distilled water	10.5±2.6	>1000
Cabbage		
Hexane	15.4±0.5	>1000
Ethylacetate	53.8±1.7	432.5±25.9
Butanol	11.1±1.0	>1000
Distilled water	2.6±1.5	>1000
Carrot		
Hexane	7.4±0.5	>1000
Ethylacetate	11.4±2.4	>1000
Butanol	6.6±0.8	>1000
Distilled water	2.5±1.4	>1000
Curcumin (at 50 μg/mL)	59.2±0.7	27.8±0.7

[a]Mean±SD (n = 3).

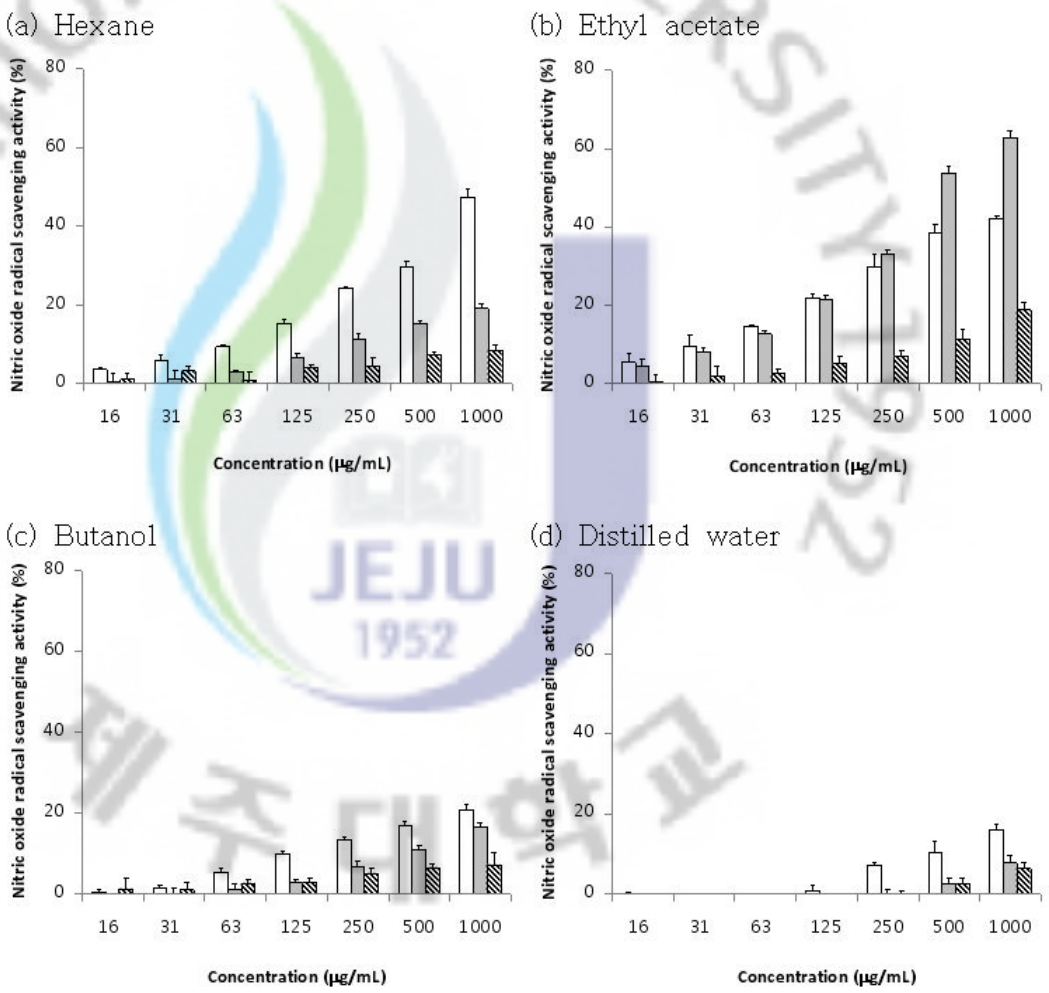

Fig. 6. Nitric oxide scavenging activity of solvent fractions (□ Broccoli, ▒ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

3-5. Superoxide anion scavenging activity

Superoxide anions were generated *in vitro* in a non-enzymatic phenazine methosulfate (PMS)-NADH system through the reaction of PMS, NADH, and oxygen (Valentao et al., 2001).

Table 7 and Fig. 7 show superoxide anion scavenging activity of 80% methanol extracts from broccoli, cabbage, and carrot. Broccoli exhibited the highest antioxidant potential (21.9%) at the concentration of 2500 μg/mL. However, in cabbage and carrot, no activities were found even at 2500 μg/mL.

Table 8 and Fig. 8 show superoxide anion scavenging activities of solvent fractions at the concentration of 500 μg/mL. In broccoli, butanol fraction had the highest superoxide anion scavenging activity (74.9%), followed by distilled water (70.9%), ethylacetate (50.5%) fractions. In cabbage, butanol fraction showed high superoxide anion scavenging activity (23.9%), followed by distilled water (14.0%) fractions. In carrot, butanol fraction showed high superoxide anion scavenging activity (42.6%), followed by distilled water (7.7%) fractions. In summary, butanol fraction of broccoli showed the highest superoxide anion scavenging activity with IC_{50} value of 108 μg/mL, followed by distilled water and ethylacetate fractions of broccoli with IC_{50} value of 309 μg/mL and 488 μg/mL, respectively. Superoxide anion scavenging activities were concentration-dependent at 16-1000 μg/mL as shown in Fig. 8.

Yuan et al. (2010) reported that methanol isothiocyanate (ITC) extracts from fresh broccoli was found to slow down the pyrogallol autoxidation reaction in superoxide anion radical assay. The rate of pyrogallol

autoxidation reaction was inhibited by 17.1% and 64.1% in the solution containing 0.5 mL and 1.0 mL, respectively. Sulforaphane in methanol ITCs extracts from broccoli was obtained using the clean-up silica SPE column. Sulforaphane showed outstanding superoxide anion scavenging activity in a dose-dependent manner. Sulforaphane was abundant in broccoli and exhibits anticancer, antidiabetic, and antimicrobial properties.

Table 7. Superoxide anion scavenging activity of 80% methanol extracts.

Vegetables	Superoxide anion scavenging activity	
	Scavenging activity (%) (at 2500 μg/mL)	IC_{50} (μg/mL)
Broccoli	21.9±2.9[a]	3578.9±45.6
Cabbage	<5	>5000
Carrot	<5	>5000
BHA (at 50 μg/mL)	76.8±1.1	11.7±0.5

[a]Mean±SD (n = 3).

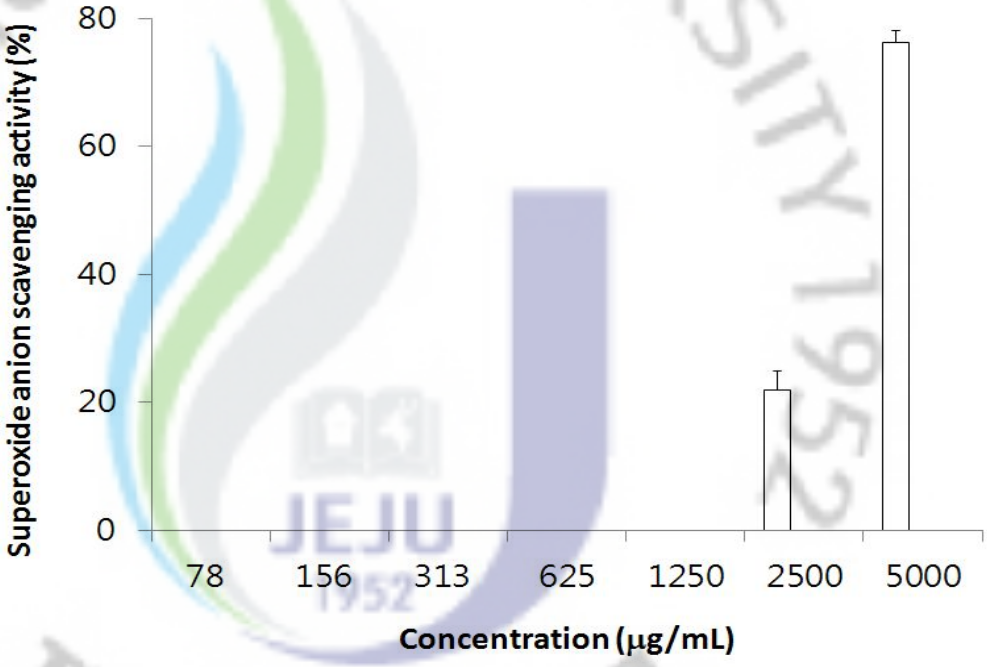

Fig. 7. Superoxide anion scavenging activity of 80% methanol extracts (□ Broccoli, ▨ Cabbage, ▩ Carrot; Mean±SD (n = 3)).

Table 8. Superoxide anion scavenging activity of solvent fractions.

Fractions	Superoxide anion scavenging activity	
	Scavenging activity (%) (at 500 μg/mL)	IC_{50} (μg/mL)
Broccoli		
Hexane	<5	>1000
Ethylacetate	50.5±1.5[a]	487.9±50.3
Butanol	74.9±2.0	108.2±3.4
Distilled water	70.9±1.5	308.5±13.0
Cabbage		
Hexane	<5	>1000
Ethylacetate	<5	>1000
Butanol	23.9±1.5	>1000
Distilled water	14.0±1.2	>1000
Carrot		
Hexane	<5	>1000
Ethylacetate	<5	>1000
Butanol	42.6±0.9	>1000
Distilled water	7.7±1.9	>1000
Trolox (at 100 μg/mL)	72.4±2.7	46.5±4.7

[a]Mean±SD (n = 3).

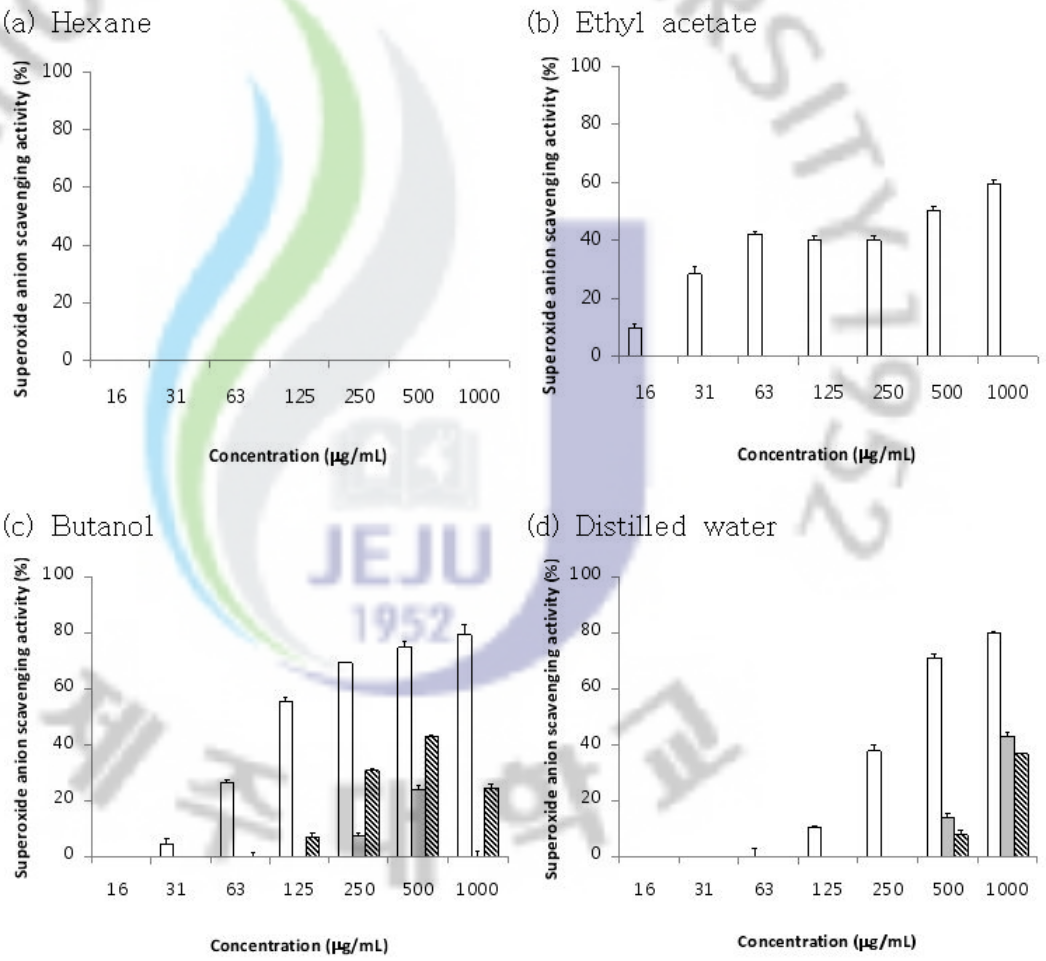

Fig. 8. Superoxide anion scavenging activity of solvent fractions (□ Broccoli, ▒ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

3-6. Xanthine oxide inhibition activity

Xanthine oxide (XO) plays an important role in various forms of ischemic, tissue and vascular injuries, inflammatory diseases, and chronic heart failure. There is significant evidence for the pathogenetic role of XO in some but not all colitis and inflammatory bowel disease and duodenal ulceration in murine experimental models. Electron leakage from the mitochondrial electron transport chain and in the conversion of xanthine to uric acid produce superoxide radical *in vivo* by activated phagocytes. Much of the molecular damage that can be done by superoxide radical is due to its conversion into much more reactive species, namely hydroxyl radical and peroxynitrite (Pacher et al., 2006; Vrchovska et al., 2006).

Xanthine was generated by xanthine oxide of a xanthine/xanthine oxide system and was measured by uric acid formation. Table 8 and Fig. 8 show XO inhibitory activity of 80% methanol extracts from broccoli, cabbage, and carrot. Broccoli was the highest XO inhibitory potential (33.0%), followed by cabbage (12.6%) fractions at the concentration of 2500 μg/mL. However, in carrot no activities were found even at 2500 μg/mL.

Table 9 and Fig. 9 show XO inhibitory activities of solvent fractions at the concentration of 500 μg/mL. In broccoli, ethylacetate fraction had the highest XO inhibitory activity (26.1%), followed by hexane (14.2%) fractions. In carrot, hexane fraction showed high XO inhibitory activity (22.2%), followed by ethylacetate (15.3%) fractions. In cabbage, the activities were 10% or less. In summary, ethylacetate fraction of broccoli showed the highest XO inhibitory activity, followed by hexane and ethylacetate fractions of carrot. XO inhibitory activities were concentration-dependent at 16-1000 μg/mL as shown in Fig. 9.

Table 9. Xanthine oxidase inhibitory activity of 80% methanol extracts.

Vegetables	Xanthine oxidase inhibitory activity (%)	
	Inhibitory activity (%) (at 2500 μg/mL)	IC_{50} (μg/mL)
Broccoli	33.0±0.5[a]	4426.9±415.2
Cabbage	12.6±0.2	>5000
Carrot	<5	>5000
Allopurinol (at 12.5 μg/mL)	81.8±2.5	3.1±0.2

[a]Mean±SD (n = 3).

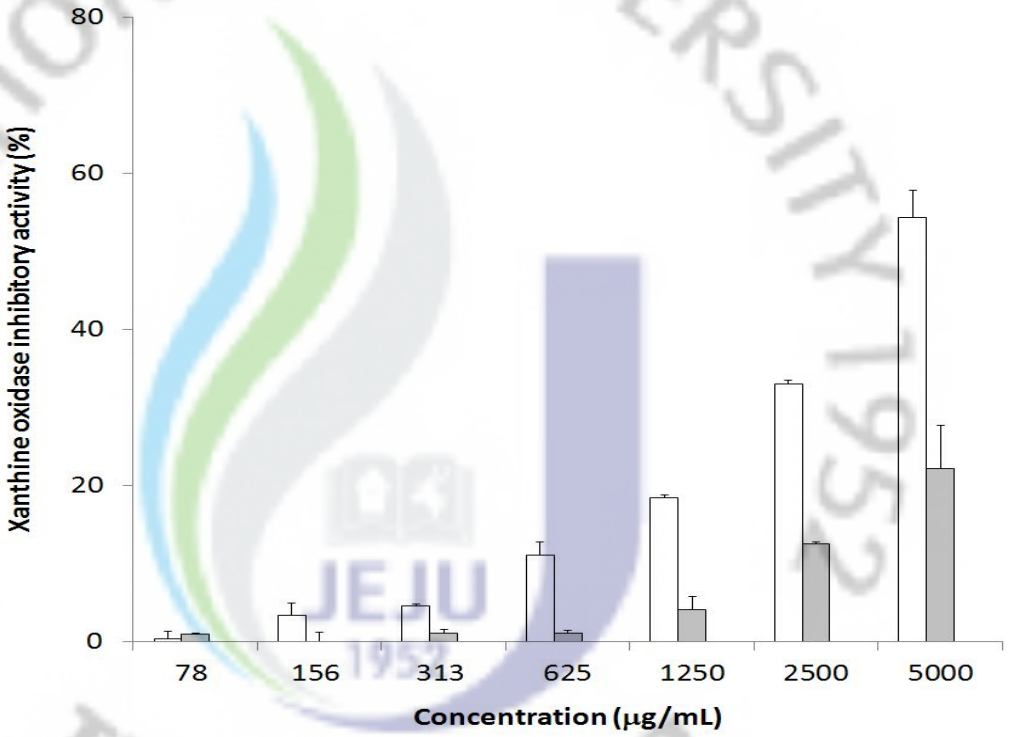

Fig. 9. Xanthine oxidase inhibitory activity of 80% methanol extracts (□ Broccoli, ▨ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

Table 10. Xanthine oxidase inhibitory activity of solvent fractions.

Fractions	Xanthine oxidase inhibitory activity (%)	
	Inhibitory activity (%) (at 500 μg/mL)	IC_{50} (μg/mL)
Broccoli		
Hexane	14.2±0.9[a]	>1000
Ethylacetate	26.1±1.0	>1000
Butanol	6.7±1.7	>1000
Distilled water	<5	>1000
Cabbage		
Hexane	7.4±1.5	>1000
Ethylacetate	9.1±0.4	>1000
Butanol	<5	>1000
Distilled water	<5	>1000
Carrot		
Hexane	22.2±2.1	>1000
Ethylacetate	15.3±2.9	>1000
Butanol	<5	>1000
Distilled water	<5	>1000
Allopurinol (at 12.5 μg/mL)	81.8±2.5	3.1±0.2

[a]Mean±SD (n = 3).

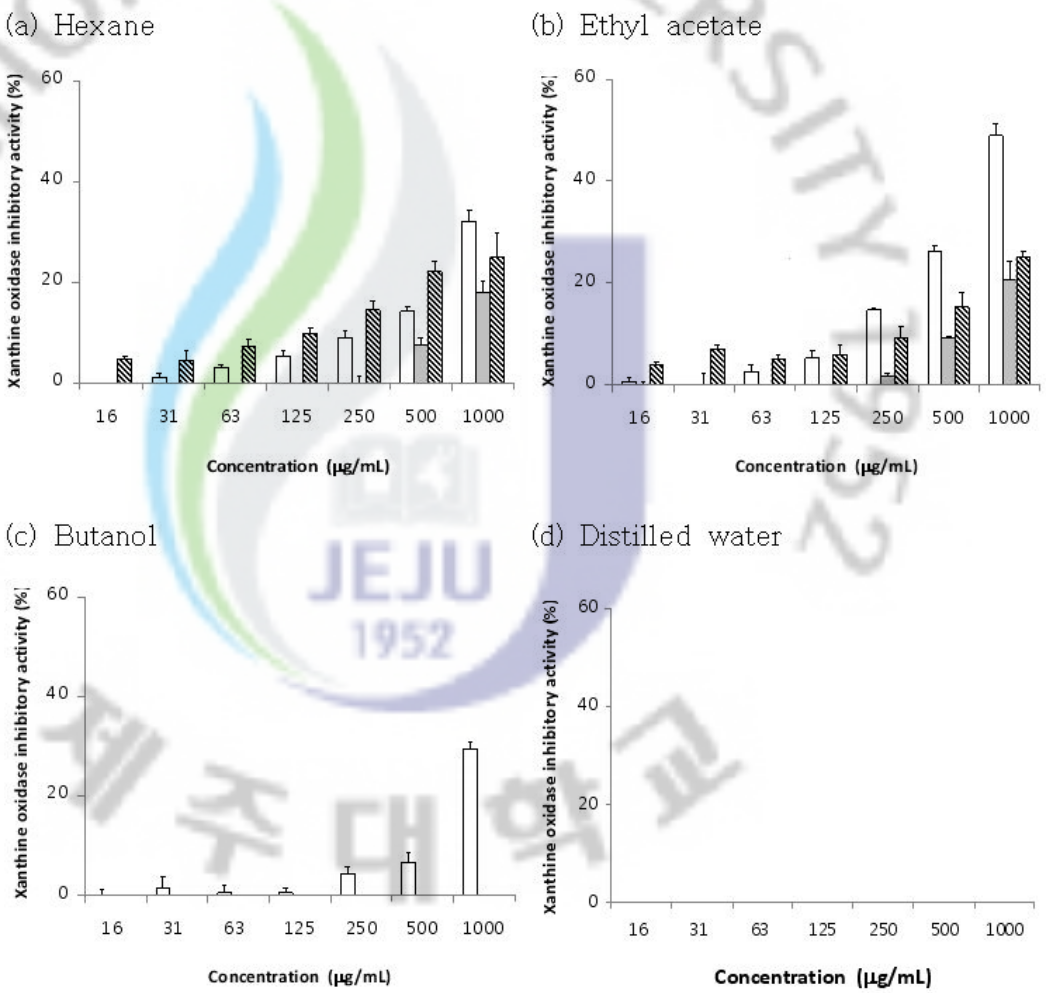

Fig. 10. Xanthine oxidase inhibitory activity of solvent fractions (□ Broccoli, ▒ Cabbage, ▨ Carrot; Mean±SD (n = 3)).

3-7. Cytotoxicity and nitric oxide production inhibitory activity in LPS-stimulated RAW 264.7 cells

NO is important messenger molecule involved in many pathological and physiological processes within the mammalian body. Exogenous NO sources constitute a powerful way to supplement NO when the body can not generate enough for normal biological functions (Hou et al., 1999).

Table 11 shows cytotoxicity and nitric oxide (NO) production inhibitory activity of 80% methanol extracts in LPS-stimulated RAW 264.7 cells. In broccoli methanol extract had the highest NO production inhibitory activity with IC_{50} value 690.4 μg/mL, followed by carrot 869.9 μg/mL. Cytotoxicity was not detected in all samples. NO production inhibitory activities were concentration-dependent at 16-1000 μg/mL.

Table 12 shows cytotoxicity and NO production inhibitory activity of solvent fractions in LPS-stimulated RAW 264.7 cells. Carrot hexane fraction showed the highest NO production inhibitory activity (IC_{50} = 34.0 μg/mL) with the cytotoxicity (TC_{50} = 192.2 μg/mL). However, broccoli ethylacetate fraction was nontoxic with the highest selectivity index (TC_{50}/IC_{50} = 8.42). Therefore, subsequent experiments were performed using the broccoli ethyl acetate fraction.

Table 11. Cytotoxicity and nitric oxide (NO) production inhibitory activity of 80% methanol extracts in LPS-stimulated RAW 264.7 cells.

Vegetables	TC_{50}[1)] (μg/mL)	IC_{50}[2)] (μg/mL)	Selectivity Index[3)]
Broccoli	>1000	690.4±24.8[a]	>1.45
Cabbage	>1000	>1000	>1
Carrot	>1000	869.9±29.9	>1.15
Curcumin (reference)	5.3±0.2	1.3±0.1	4.08

[1)]TC_{50} is the concentration producing 50% toxicity in RAW 264.7 cells.

[2)]IC_{50} is the concentration producing 50% inhibition of NO production in RAW264.7 cells.

[3)]Selectivity Index = TC_{50}/IC_{50}

[a]Mean±SD (n = 3).

Table 12. Cytotoxicity and nitric oxide (NO) production inhibitory activity of solvent fractions in LPS-stimulated RAW 264.7 cells.

Fractrions	TC_{50}[1)] (μg/mL)	IC_{50}[2)] (μg/mL)	Selectivity Index[3)]
Broccoli			
Hexane	737.4±5.3[a]	98.8±2.8	>7.46
Ethylacetate	>1000	118.8±3.0	>8.42
Butanol	>1000	>1000	>1
Distilled water	>1000	>1000	>1
Cabbage			
Hexane	>1000	182.5±13.7	>5.48
Ethylacetate	>1000	165.8±0.1	>6.03
Butanol	>1000	945.2±64.3	>1.06
Distilled water	>1000	>1000	>1
Carrot			
Hexane	192.2±0.5	34.0±1.4	>5.65
Ethylacetate	>1000	158.6±1.7	>6.30
Butanol	>1000	>1000	>1
Distilled water	>1000	>1000	>1
Curcumin (reference)	5.3±0.2	1.3±0.1	4.08

[1)]TC_{50} is the concentration producing 50% toxicity in RAW 264.7 cells.

[2)]IC_{50} is the concentration producing 50% inhibition of NO production in RAW264.7 cells.

[3)]Selectivity Index = TC_{50}/IC_{50}

[a]Mean±SD (n = 3).

3-8. iNOS protein expression of broccoli ethylacetate fraction in LPS-stimulated RAW 264.7 cells

1) NO production inhibitory activity

NO production inhibitory activity of broccoli ethylacetate fraction were investigated in RAW 264.7 cells (Fig. 11). When cells were treated with LPS, NO production was significantly increased. However, the simultaneous treatment with LPS and broccoli ethylacetate fraction was significantly reduced NO production.

2) iNOS protein expression

Inducible nitric oxide synthase (iNOS) is one of the major inflammatory mediator that contribute to the pathogenesis of cancer and inflammation. In response to LPS, the iNOS of macrophages is induced and sequentially leads to NO overproduction, which may play an important role in the pathogenesis of various inflammatory diseases (Mitani et al., 2005).

To check whether NO production inhibition of broccoli ethylacetate fraction is due to reduction of iNOS protein rather than NO scavenging activity of the sample, iNOS protein expression was analyzed by Western blotting. When treated with LPS alone, iNOS protein expression was increased. However, treatment with broccoli ethylacetate fraction decreased iNOS protein expression in a concentration-dependent manner as shown in Fig. 12. The approximately 80% of protein inhibition was observed in broccoli ethylacetate fraction (100 μg/mL) treated sample compared with untreated sample, which may partly explain its anti-inflammatory effect.

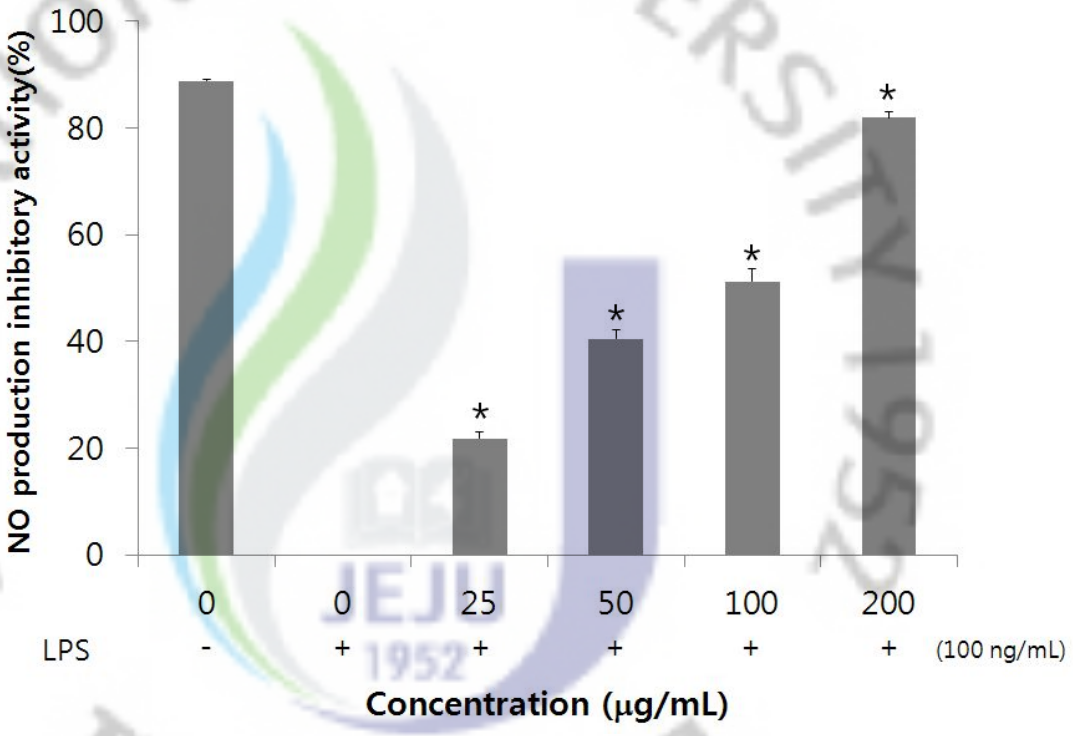

Fig. 11. The Nitric oxide production inhibitory activity of broccoli ethylacetate fraction in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/mL) alone or LPS plus the indicated concentrations of broccoli ethylacetate fraction for 24 hr. * $p < 0.001$ vs LPS alone-treated cells. Mean±SD ($n = 3$).

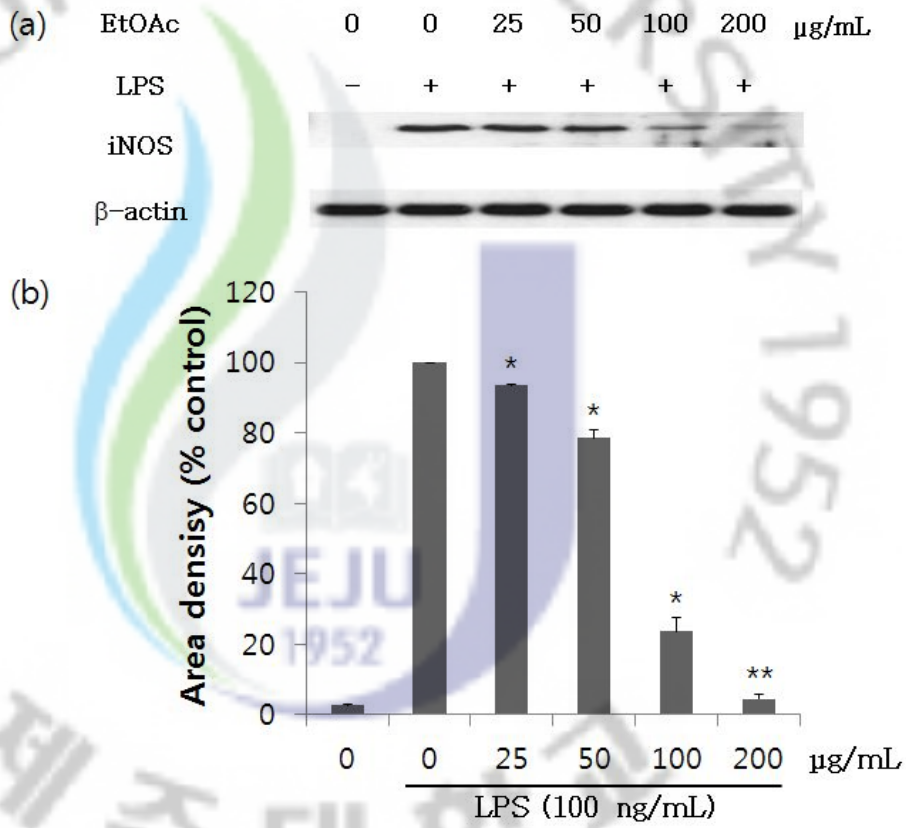

Fig. 12. Effect of ethylacetate fraction on the inducible nitric oxide synthase (iNOS) protein expression level in LPS stimulated RAW 264.7 cells. (a) Cells were treated with LPS (100 ng/mL) alone or LPS plus the indicated concentrations of ethylacetate fraction for 24 hr. (b) Quantification of iNOS protein expression was performed by densitometric analysis. The relative level was calculated as the ratio of iNOS protein expression to β-actin protein expression. * $p < 0.05$, ** $p < 0.001$ vs LPS alone-treated cells. Mean±SD ($n = 3$).

국문 요약

제주산 채소류, 특히 브로콜리, 양배추, 당근을 대상으로 80% 메탄올로 추출한 후 핵산, 에틸아세테이트, 부탄올, 증류수로 분획하여 추출물과 분획물의 총페놀 함량, DPPH radical 소거활성, nitric oxide radical 소거활성, superoxide radical 소거활성, xathine oxidase 저해능 및 nitric oxide 생성 저해능을 측정하였다.

브로콜리, 양배추, 당근의 80% 메탄올 추출물의 수율은 각각 51.7%, 56.7%, 66.2% 이었고, 핵산, 에틸아세테이트, 부탄올, 증류수 분획물의 수율은 브로콜리의 경우 각각 1.1%, 0.8%, 11.85, 61.4% 이며, 양배추의 경우 각각 0.9%, 0.9%, 11.4%, 68.5% 이고, 당근의 경우 각각 1.2%, 0.9%, 9.7%, 81.1% 이였다.

브로콜리에서 총페놀 함량은 에틸아세테이트 분획물이 39.1 mg/g으로 가장 높았고, 부탄올 분획물은 33.7 mg/g으로 많은 함량을 나타내었다. 양배추도 에틸아세테이트 분획물에서 36.3 mg/g으로 가장 높은 함량을 나타냈으며, 당근의 경우는 부탄올 분획물에서 9.2 mg/g, 에틸아세테이트 분획물에서 8.8 mg/g의 함량을 나타내었다.

DPPH free radical 소거활성 (IC_{50})은 양배추 에틸아세테이트 분획물에서 539.2 μg/mL, 브로콜리 에틸아세테이트 분획물에서 630.5 μg/mL로 다른 분획물 보다 에틸아세테이트 분획물에서 높은 활성을 보였다. Nitric oxide radical 소거활성 (IC_{50})은 양배추 에틸아세테이트 분획물에서 432.5 μg/mL로 가장 높은 활성을 나타내었으며, Superoxide anion 소거활성 (IC_{50})은 브로콜리 부탄올 분획물에서 108.2 μg/mL 가장 높았고, 브로콜리 증류수와 에틸아세테이트 분획물에서도 높은 활성을 나타내었다. Xanthine oxidase 저해능은 모든 시료에서 실험시 최고 농도보다 높은 IC_{50} 값을 나타내었고, 브로콜리 에틸아세테이트 분획물과 당근 헥산 분획물에서 다른 분획물 보다 높은 활성을 나타내었다.

Nitric oxide 생성저해능은 브로콜리 에틸아세테이트 분획물에서 농도가 증가할수록 높았으며, iNOS 단백질 발현을 확인한 결과 최고농도인 200 μg/mL에서 90% 이상 발현이 저해됨으로써 항염증 활성에 효과가 있을 것으로 추정되었다.

현재 국내외적으로 채소의 항산화 활성에 대한 연구가 활발히 진행되고 있어 청정지역인 제주에서 재배되고 있는 채소류의 항산화 효과에 대한 연구는 그 효용가치가 높다고 여겨진다. 따라서 본 연구결과를 토대로 항산화 효과 및 nitric oxide 생성저해능이 우수한 채소류에 대하여 단일 물질에 대한 분리 작업과 함께 항염증이나 항노화 등의 연구가 더 깊이 있게 진행된다면 새로운 천연물 유래 생리 활성 물질로서 활용 가능성이 높을 것으로 기대된다.

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감사의 글

뒤늦게 대학원에 입학하면서 실험실 생활을 잘 극복하고 부족한 논문이나마 완성될 수 있도록 도움을 주신 모든 분들께 감사의 뜻을 전하고자 합니다. 먼저 학부과정부터 지금까지 많은 가르침을 주시고 어려운 시기를 잘 극복할 수 있도록 이끌어 주신 임상빈 교수님께 고개숙여 감사드립니다. 부족한 논문을 정성껏 심사해 주시고 격려해주신 고영환 교수님, 박은진 교수님께 감사드리며 늘 관심을 두고 지켜봐 주신 강영주 교수님, 하진환 교수님께도 감사드립니다. 그리고 아낌없는 조언과 자상하신 가르침으로 부족한 제자를 일깨워 주셨던 학과 명예교수님이신 송대진 교수님, 김수현 교수님께도 깊이 감사드립니다.

논문실험을 위해 많이 부족한 저를 하나하나씩 정성껏 가르쳐 주신 제주대학교 생명과학기술혁신센터(RIC) 연구개발부 오유성 팀장님과 황준호 선생님께 깊은 감사드리며 바쁜 스케줄을 뒤로하고 연구일정에 지장이 없게 실험에 도움을 주신 RIC 직원분들께도 감사드립니다.

오랜 실험실 생활동안 즐겁게 지낼 수 있었던 것은 식품분리공정실험실의 많은 선배, 동기, 후배들의 도움이 있었기 때문입니다. 항상 신경써 주시는 좌미경 선생님, 따뜻한 말로 할 수 있다며 응원해준 성근이형, 선희, 재성이 그리고 실험실에 충우형에게도 고마운 마음을 전합니다. 부족한 저를 항상 믿어주시고 격려해 주셨기 때문에 지금의 제가 있을 수 있었습니다.

가까이에서 힘들 때마다 술 한잔 사주시며 용기를 주셨던 김지훈 박사님, 동생처럼 챙겨주신 RIC 김용환 팀장님과 조교생활을 함께했던 강호정 선생님께도 감사드리며, 가장 큰 응원을 보내주었던 좋은 친구들 경국, 경백, 민수, 보람, 종철, 예준아빠 형철이, 힘들 때 옆에서 더 못 해주는 것에 미안해하는 우리 혼수상태 팀 성우형과 창윤이, 동네친구들 명섭, 정모, 재민, 현철, 효철, 사랑하는 후배 영준, 현석, 같이 살면서 항상 챙겨주는 공대 고시반 후배들을 비롯한 많은 분들께 일일이 말로는 다 못할 고마움을 전합니다.

논문 수정 말기 바쁘신 가운데도 논문 마무리가 잘 될 수 있도록 도와주신 한국에너지기술연구원 에너지소재센터 김동국 박사님과, 이곳에서 함께 일하며 불편함이 없게 해준 연구실 홍란, 성일, 영민에게도 감사드립니다.

마지막으로 오늘의 제가 있기까지 한결같이 희생과 사랑으로 지켜봐 주시고 항상 저를 믿고 큰 힘이 되어주시는 부모님과 든든한 지원자인 누나에게 더없는 감사와 사랑을 바칩니다.

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다시 한번 이 글을 쓸 수 있도록 도와주시고 소중한 추억을 함께 만들어주신 모든 분들게 감사의 마음을 전합니다. 사랑합니다.