Food Sci. Biotechnol. Vol. 16, No. 1, pp. 90 ~ 98 (2007)

Food Science and Biotechnology

Antioxidant Activities of the Ethanol Extract of *Hamcho* (*Salicornia herbacea* L.) Cake Prepared by Enzymatic Treatment

Ji-Hae Oh, Eun-Ok Kim, Sung-Kwon Lee, Mee-Hee Woo[1], and Sang-Won Choi*

Department of Food Science and Nutrition, Catholic University of Daegu, Gyeongsan, Gyeongbuk 712-702, Korea
[1]College Pharmacy, Catholic University of Daegu, Gyeongsan, Gyeongbuk 712-702, Korea

Abstract The antioxidant activities of water (H_2O) and ethanol (EtOH) extracts from *hamcho* (*Salicornia herbacea* L.) juice and cake prepared by enzymatic treatments were evaluated by *in vitro* assays against DPPH, superoxide, and hydroxyl radicals. Among the H_2O and EtOH extracts from five different carbohydrases treated, the EtOH extract from viscozyme-treated *hamcho* cake had higher yield and phenolic content, and exhibited the strongest radical scavenging activity against DPPH (IC_{50}=186.91 μg/mL), superoxide (IC_{50}=87.54 μg/mL), and hydroxyl radicals (IC_{50}=367.07 μg/mL). Antioxidant assay-guided fractionation and purification of the EtOH extract led to isolation and identification of five phenolic compounds, procatechuic, ferulic and caffeic acids, quercetin, and isorhamnetin. Most of these phenolic compounds exhibited considerable DPPH, superoxide, and hydroxyl radical scavenging activities, and in particular, caffeic and ferulic acids had stronger superoxide and hydroxyl radical scavenging activities than the well-known antioxidant radical scavenger, (+)-catechin (p<0.05). Quercetin and isorhamnetin were the primary compounds responsible for the strong antioxidant activity in the EtOH extract of the viscozyme-treated *hamcho* cake. Meanwhile, these five phenolic compounds were detected in the EtOH extract of the viscozyme-treated *hamcho* cake at the following levels (dry base of *hamcho*); procatechuic acid (1.54 mg%), caffeic acid (6.87 mg%), ferulic acid (8.45 mg%), quercetin (12.63 mg%), and isorhamnetin (6.65 mg%). However, three of these phenolic compounds (procatechuic, caffeic acid, and ferulic acids) were detectable in the H_2O extract of viscozyme-treated *hamcho* juice. These results suggest that the EtOH extract of viscozyme-treated *hamcho* cake may be a potential source of natural antioxidants.

Keywords: *Salicornia herbacea*, enzymatic treatment, *hamcho* juice and cake, antioxidant activity, phenolic compound

Introduction

Much attention has recently been focused on natural dietary antioxidants capable of inhibiting reactive oxygen radical-mediated lipid peroxidation, which is involved in several pathologic conditions, such as cancer, atherosclerosis, inflammation, and aging (1, 2). In particular, naturally occurring antioxidant phenolic compounds, including flavonoids, anthocyanins, cinnamic acids, phenolic acids, and tannins are receiving increasing interest as potential therapeutic agents against various oxidative stress-induced degenerated diseases (3, 4). Since several synthetic phenolic antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected to induce some toxicity (5, 6), an extensive search for safer and more effective natural phenolic antioxidants has been undertaken.

Seaweeds are rich in several minerals, amino acids, dietary fiber, carotenoids, and chlorophylls, which are important sources of functional foods (7). The crude drug 'glasswort' (Korean name: *hamcho*), a seaweed foodstuff (*Salicornia herbacea* L., Chenopodiaceae), has been used in traditional Korean folk medicine to treat hypertension, diabetes, cancer, hepatitis, and obesity, etc (8). *Hamcho* reportedly contains high contents of natural minerals, such as Mg, Ca, K, and Fe, and dietary fibers (9). Recently, *hamcho* has also been reported to possess many biological and pharmacological effects, including antidiabetic (10, 11), antioxidant (10-13), hypocholesterolemic (14), hypolipidemic (11), and anti-aging (15) activities. Several phytochemical constituents, such as flavonoids (16-18), phytosterols (17), betaine (19), tungtungmadic acid (20), and polysaccharides (21), have been isolated and identified as major active components for the biological activity of *hamcho*.

In recent years, as more has been learned about the nutritional benefits and functional properties of *hamcho*, development of functional foods using *hamcho* has increased (14, 22). However, *hamcho* powder and juice are not suitable for use as functional foods and cosmetic materials because they contain large amounts of nondigestible and insouble dietary fibers, and because of their fishy smell (9). So, a high quality, highly digestible, palatable, and functional *hamcho* extract must be developed through enzymatic treatments (23). We previously reported that enzymatic hydrolysates from *hamcho* exhibited considerable antioxidant, antithrombic, and antihyperlipidemic activities (24, 25). This prompted us to investigate the major principles for the biological activity of enzymatic hydrolysates from *hamcho*.

The objective of this study was to determine antioxidant activities of the water (H_2O) and ethanol (EtOH) extracts of enzyme-treated *hamcho* juice and cake using *in vitro* assays against DPPH radical, superoxide, and hydroxyl radicals. We also isolated and identified the antioxidant phenolic compounds in the EtOH extract of *hamcho* cake, and further quantified the phenolic compounds in *hamcho* juice and cake.

*Corresponding author: Tel: 82-53-850-3525; Fax: 82-53-850-3504
E-mail: swchoi@cu.ac.kr
Received September 21, 2006; accepted December 12, 2006

Materials and Methods

Materials The seaweed, *hamcho* (*Salicornia herbacea* L., glasswort) was directly harvested in early July from an abandoned salt farm in Haenam, Jeonnam, Korea. *Hamcho* was blanched for 5 min and desalted by washing continuously with running water; samples were then freeze-dried. The dried sample was milled to 100 mesh size and stored at -18°C until use.

Chemicals 1,1-Diphenyl-2-picrylhydrazyl (DPPH), xanthine oxidase (EC 1.2.3.2), xanthine, nitrobluetetrazolium chloride (NBT), thiobarbituric acid (TBA), H_2O_2, 2-deoxyribose, bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). (+)-Catechin and $FeCl_3 \cdot 6H_2O$ were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Sodium phosphate dibasic 12-hydrate and potassium phosphate monobasic were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). α-Amylase (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), amyloglucosidase (AMG) 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g), viscozyme L (EC 3.2.1.6, from *Aspergillus aculeatus*, 80 BGU/g), pectinex 100 L (pectinase, EC 3.2.1.15, from *A. niger*, 5,000 FDU/g) and celluclast 1.5 L (cellulase, EC 3.2.1.4, from *Trichoderma reesei*, 700 EGU/g) were purchased from Novo Nordisk (Bagsvaerd, Denmark). All other reagents were of analytical grade.

Preparation of water and ethanol extracts from *hamcho* juice and cake prepared by enzymatic treatment The schematic procedure for preparing water (H_2O) and ethanol (EtOH) extracts of *hamcho* juice and cake by enzymatic treatment is shown in Fig. 1. Desalted, ground *hamcho* powder (500 g) was suspended in ionic water (2.5 L) and each of 5 different carbohydrases (50 mL), α-amylase, amyloglucosidase, viscozyme L, pectinase, or cellulase, was added. The reaction mixtures were hydrolysed in a shaking water bath at 40°C for 12 hr. After hydrolysis, an equal volume of water (2.5 L) was added and refluxed with a boiling for 3 hr. The water extract was filtered through cheese-cloth and centrifuged at 700×g for 20 min; thus *hamcho* juice (supernatant) and cake (precipitate) were obtained. *Hamcho* juice was evaporated and redissolved in ionic water (100 mL), and then allowed to stand overnight. The upper layer was again evaporated to prepare the H_2O extract of *hamcho* juice (WEHJ). Meanwhile, the *hamcho* cake was extracted twice with ethanol in an ultrasonicator for 3 hr, filtered and evaporated *in vacuo*, thereby obtaining EtOH extract of *hamcho* cake (EEHC).

Antioxidant activity assay Antioxidant activity was assayed as previously described (26) for radical scavenging activity against DPPH, superoxide, and hydroxyl radicals induced by enzymatic and nonenzymatic reactions.

In the DPPH radical scavenging, each phenolic compound

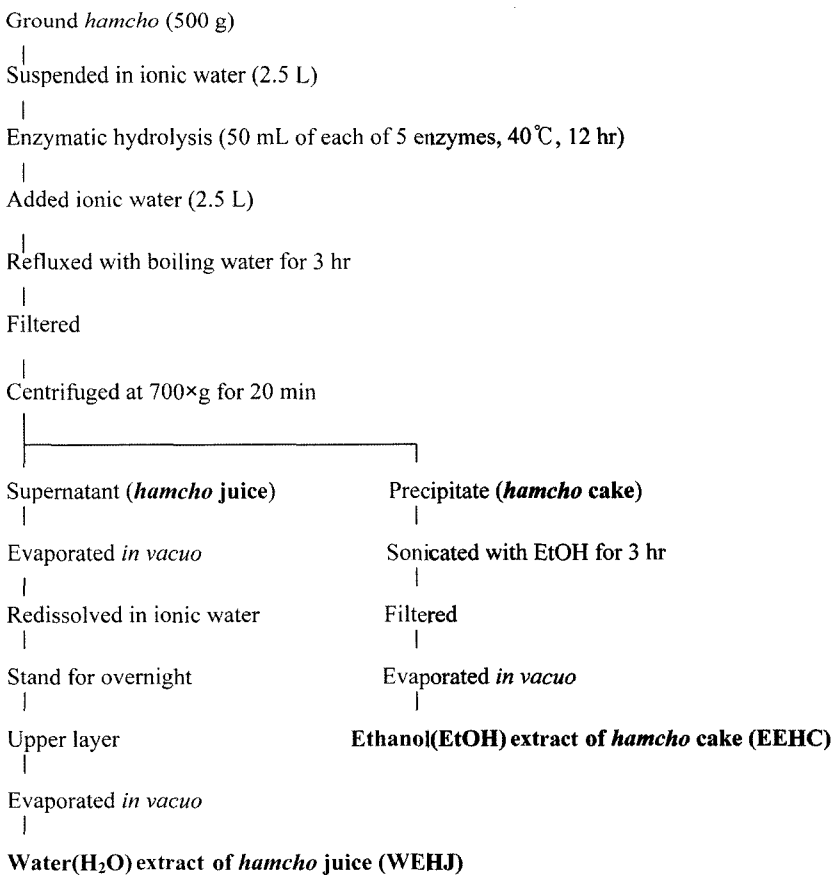

Fig. 1. Schematic procedure for preparation of water and ethanol extracts from *hamcho* juice and cake by enzymatic treatments.

was added to 100 μM DPPH in methanol, followed by incubation at 25°C for 10 min. Their reactivity with against DPPH was determined spectrophotometrically at 516 nm.

In the superoxide radical scavenging assay, the reaction mixture (3.0 mL), comprised of 0.05 M Na_2CO_3 buffer (pH 10.2) with 3 mM xanthine, 3 mM EDTA, BSA (1.5 mg protein/mL), 0.75 mM NBT, the test sample (in DMSO) and 5.0 U/mL xanthine oxidase, was incubated at 25°C for 20 min. The reaction was then terminated by the addition of 6 mM $CuCl_2$. Formazan production was determined at 560 nm.

In the hydroxyl radical scavenging assay, the reaction mixture (1.0 mL), comprised of 30 mM KH_2PO_4-KOH buffer (pH 7.4), 2 mM 2-deoxyribose, 0.1 mM $FeCl_3 \cdot 6H_2O$, 104 μM EDTA, test sample (in the same buffer), 1.0 mM H_2O_2, and 0.1 mM L-ascorbic acid, was incubated at 37°C for 1 hr. The thiobarbituric acid reactive substance (TBARS) level was determined after terminating the reaction by the addition of TBA reagent. IC_{50} values against three radical scavenging activities were determined by regression analysis of results obtained at three different sample concentrations.

Total phenolic content The total phenolic contents of WEHJ and EEHC were determined with Folin-Ciocalteu reagent (27). Each extract (0.5 mL, WEHJ and EEHC/100 mL) was diluted in a test tube with distilled water to 5.0 mL. Folin-Ciocalteu reagent (5.0 mL) was added, and mixed thoroughly. After 3.0 min, 10% Na_2CO_3 solution (5.0 mL) was added, and mixture was allowed to stand for 1 hr with intermittent mixing. The optical density (O.D.) was measured on an UV-vis Jasco spectrophotometer (Tokyo, Japan). The concentration of the total phenolic content of the *hamcho* extracts was determined by comparison with the O.D. values of various concentrations of gallic acid, a standard phenolic compound. The experiment was performed in triplicate, and the total phenolic contents were expressed as gallic acid equivalent per 100 g of dried *hamcho*.

Extraction, isolation, and purification of phenolic compounds Phenolic compounds in the EtOH extract of viscozyme-treated *hamcho* cake, which possesses a high antioxidant activity (Table 2), were isolated and purified by the following method: The EtOH extract (250 g) was solubilized in 80% aq. MeOH and washed with *n*-hexane to remove lipids and pigments. The defatted aqueous MeOH extract was evaporated *in vacuo* and successively partitioned further with ether (Et_2O), ethylacetate (EtOAc), and *n*-butanol (*n*-BuOH). The Et_2O-soluble fraction (4.14 g) with strong antioxidant activity (Table 3) was chromatographed on a silica gel column (6.5×50 cm) with hexane-ethylacetate-TFA (100:100:0.2, v/v) as an eluent to yield ten fractions; Fr. 1 (0.13 g), Fr. 2 (0.13 g), Fr. 3 (0.11 g), Fr. 4 (0.19 g), Fr. 5 (0.22 g), Fr. 6 (0.18 g), Fr. 7 (0.12 g), Fr. 8 (0.24 g), Fr. 9 (0.20 g), and Fr. 10 (0.03 g). Fr. 2 (0.13 g) and Fr. 3 (0.11 g) were combined and chromatographed on a Sephadex LH-20 (Pharmacia Biotech., Sweden) column (2.5×30 cm) with MeOH to produce five subfractions: fr. 1 (18.3 mg), fr. 2 (12.7 mg), fr. 3 (9.1 mg), fr. 4 (13.3 mg), and fr. 5 (12.5 mg). Subfraction 3 (9.1 mg) was solubilized in MeOH and evaporated to yield a white powder (Comp. **1**: 7.7 mg). Fr. 5 (0.22 g) and Fr. 6 (0.18 g) were combined and chromatographed on a Sephadex LH-20 column with MeOH to obtained five subfractions: fr. 1 (10.2 mg), fr. 2 (35.3 mg), fr. 3 (33.1 mg), fr. 4 (10.7 mg), and fr. 5 (9.2 mg). Subfraction 2 (35.3 mg) and 3 (33.1 mg) were separately chromatographed on YMC gel ODS-A column (2.5×30 cm) with 60% MeOH, and two pure white powders (Comp. **2**: 12.5 mg, Comp. **3**: 10.2 mg), respectively. Finally, Fr. 8 (0.24 g) and Fr. 9 (0.20 g) were combined and subjected to the same purification procedure on Sephadex LH-20 with 100% MeOH on the ODS-A column with 80% MeOH, which afforded two light yellow needles (Comp. **4**: 15.8 mg, Comp. **5**: 10.3 mg).

Identification of phenolic compounds UV spectra were obtained on a Jasco 3334 UV-vis spectrophotometer (Osaka, Japan). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of phenolic compounds were measured in CD_3OD (phenolic acids) and DMSO (flavonoids) on a Varian Unity Plus 500 spectrometer (Palo Alto, CA, USA) and chemical shifts are given as δ values with tetramethylsilane (TMS) as an internal standard. Fast-atom bombardment mass spectrometry (FAB-MS) was recorded on a Jeol JMS-700 mass spectrometer (ion source, Xe atom beam; accelerating voltage, 10 kV; Tokyo, Japan), using glycerol and *m*-butylalcohol as a mounting matrix. Column chromatography was performed with silica gel (70-230 mesh; Merck, Damstadt, Germany), ODS-A (12 nm, 150 μm; YMC Inc., Milford, MA, USA), and Sephadex LH-20 (Pharmacia Biotech) columns. All solvents used for this study were of analytical and HPLC grades.

HPLC analysis The EtOH extract of viscozyme-treated *hamcho* cake was solubilized in 80% aq. MeOH and washed with *n*-hexane to remove lipids and pigments. The lower layer was evaporated *in vacuo* and redissolved in 80% aq. MeOH. The extract was left to stand overnight, and then the upper layer was brought up to 100 mL with the same solvent. Meanwhile, the H_2O extract of viscozyme-treated *hamcho* juice was fully solubilized in 40% aq. MeOH and brought up to 100 mL with the same solvent. Each MeOH-souble extract was diluted ten-fold passed through a 0.45 μm PVDF syringe filter (Whatman, Maidstone, England) and then injected onto YMC-Pack Pro C_{18} colum (46 mm i.d. × 250 mm, YMC Inc.) with a Guard-Pak C_{18} precolumn insert on a Gilson 506B HPLC System (Middleton, WI, USA) coupled with Gilson 170 UV-vis detector and Gilson 231 XL autosampler with a 10 μL loop. Separation was achieved with a linear gradient from 0.05% v/v H_3PO_4 in H_2O (solvent A) to 40% CH_3CN (solvent B) for 50 min at a flow rate of 0.8 mL/min with UV detection at 270 nm. The elution profile was as follows: 0-5 min, 60% A, 40% B; 10-15 min, 40% A, 60 % B; 20-40 min, 0% A, 100% B; 45-50 min, 60% A, 40 % B. The column was returned to initial conditions for 10 min before the next injection. Individual phenolics were identified by a comparing their retention with those of the five phenolics isolated previously in Materials and Methods. Peaks were identified by co-chromatography with previously isolated authentic samples. Duplicate

analyses were conducted on duplicate samples. Linear correlation coefficients were superior to 0.998 for each phenolics. The calibration lines (y = 2.4695x - 6.6162 for procatechuic acid, y = 1.5635x - 2.6322 for caffeic acid, y = 1.2502x - 5.5221 for ferulic acid, y = 2.2168x - 2.9880 for quercetin, y = 2.0725x - 0.2439 for isorhamnetin) were determined by a least squares regression method. The concentrations of phenolics were determined by calibration curves of the five standard phenolics and expressed as mg% of dried weight. Recovery rates of the phenolic acid, hydroxycinnamic acids, and flavonoids were 93, 91, and 87%, respectively.

Statistical analysis All experiments were performed in triplicate unless otherwise noted. Statistical analysis was performed using Duncan's multiple range test (28).

Results and Discussion

Yields and phenolic contents of WEHJ and EEHC prepared by enzymatic treatment The yield (%) of WEHJ and EEHC prepared by five different carbohydrase treatments is shown in Table 1. The yields of the H_2O and EtOH extracts from untreated *hamcho* juice and cake (control) were 16.96 and 3.52%, respectively. However, the yields of the H_2O extracts from enzyme-treated *hamcho* juice increased in the following order; amyloglucosidase (18.92%) < pectinase (23.18%) < cellulase (35.08%) < viscozyme (42.20%) ≤ α-amylase (44.86%). The yields of the EtOH extracts from enzyme-treated *hamcho* cake increased in the following order: amyloglucosidase (3.08%) < α-amylase (3.14%) < pectinase (3.84%) < viscozyme (5.10%) < cellulase (5.85%). Among the enzyme-treated *hamcho* extracts, the yields of H_2O and EtOH extracts were highest in enzymes α-amylase- and viscozyme-treated *hamcho* juice and viscozyme- and cellulase-treated *hamcho* cake, respectively, and these yields were higher about 1.5-3 times higher than those of control. In contrast, the yields of H_2O and EtOH extracts were lowest in amyloglucosidase-treated *hamcho* juice and cake, respectively. Thus, of the five carbohydrases viscozyme was the most effective treatment to increase the yields of the H_2O and EtOH extract from *hamcho* juice and cake, while amyloglucosidase did not greatly affect the yields of the two extracts. *Hamcho* is known to contain large amounts of dietary fiber (9). However, no data on dietary fiber composition of *hamcho* is currently availabl. From above results on great increase in the yield of H_2O extract from *hamcho* juice treated by α-amylase, viscozyme (multi-enzyme complex containing a wide range of carbohydrase, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) and cellulase, the cell wall of *hamcho*, which comprise the majority of its dietary fiber, may be composed of cellulose or hemicellulose mixed with β-glucans (23).

Table 1. Yield and phenolic content of water and ethanol extracts from *hamcho* juice and cake prepared by enzymatic treatments[1)]

Enzyme treatment	Extract	Yield (%)[2)]	Phenolics (%, dry base of *hamcho*)[3)]
Control (Untreated)	Water	16.96±0.23	0.37±0.05 (2.18)[4)]
	Ethanol	3.52±0.15	0.12±0.03 (3.39)
α-Amylase	Water	44.86±1.32[a]	0.48±0.10 (1.07)
	Ethanol	3.14±0.10	0.10±0.02 (3.28)
Amyloglucosidase	Water	18.92±0.24	0.27±0.02 (1.45)
	Ethanol	3.08±0.21	0.13±0.04 (4.12)
Viscozyme	Water	42.20±1.21[a]	0.59±0.12 (1.40)
	Ethanol	5.10±0.23	0.20±0.04 (3.89)
Pectinase	Water	23.18±0.42	0.27±0.03 (1.18)
	Ethanol	3.84±0.18	0.09±0.01 (2.43)
Cellulase	Water	35.08±1.28	0.40±0.04 (0.91)
	Ethanol	5.85±0.26	0.21±0.02 (2.13)

[1)]Values are mean±SD of triplicate analyses.
[2)]Dry base of *hamcho*. Values with the same superscript in the column are not significantly different at $p<0.05$.
[3)]Gallic acid equivalent.
[4)]Dry base of *hamcho* extracts.

The total phenolic contents of the H_2O and EtOH extracts from *hamcho* juice and cake prepared by 5 different enzyme treatments are also shown in Table 1. The phenolic contents of the H_2O and EtOH extracts from untreated *hamcho* juice and cake (control) were 0.37 and 0.12% (dry base of *hamcho*), respectively. Among the enzyme-treated extracts, the total phenolic content of the H_2O extract was highest from *hamcho* juice treated with viscozyme (0.59%), followed by α-amylase (0.48%), cellulase (0.40%), amyloglucosidase, and pectinase (0.27 %), while that of the EtOH extracts was highest from *hamcho* cake treated with cellulase (0.21%), followed by viscozyme (0.20%), amyloglucosidase (0.13%), α-amylase (0.10%), pectinase (0.09%). Thus, the total phenolic contents of the H_2O extracts from enzyme-treated *hamcho* juice were higher than those of the EtOH extracts from enzyme-treated *hamcho* cake, by-product of enzymatic treatment of *hamcho*. In particular, phenolic contents of the H_2O and EtOH extracts were highest in viscozyme-treated *hamcho* juice and cakes, respectively, when considering the extract recovery rates. From these results, therefore, viscozyme is the most effective enzyme for preparing high quality extracts from *hamcho* juice and cake due to higher yield and phenolic content. We therefore prepared H_2O and EtOH extracts from *hamcho* juice and cake and compared their antioxidant activity against DPPH, superoxide, and hydroxyl radicals.

Antioxidant activity of the H_2O and EtOH extracts The antioxidant activity of H_2O and EtOH extracts from viscozyme-treated *hamcho* juice and cake was determined against DPPH radical, superoxide anion radical induced by xanthine-xanthine oxidase, and hydroxyl radical generated via the Fenton reaction; their activities were compared to those of untreated *hamcho* (control). These four *hamcho* extracts exhibited considerable dose-dependent radical scavenging activity (data not shown), and the 50% inhibitory concentration (IC_{50}) of each extract is given in Table 2. Among four extracts, the EtOH extract of the viscozyme-treated *hamcho* cake had the strongest DPPH (IC_{50}=186.91 μg/mL), superoxide (IC_{50}=87.54 μg/mL),

Table 2. DPPH, superoxide, and hydroxyl radical scavenging activities of water and ethanol extracts from viscozyme-treated *hamcho* juice and cake

Enzymatic treatment	Extracts of *hamcho* juice and cake	IC_{50} (μg/mL)[1)]		
		DPPH radical	Superoxide radical	Hydroxyl radical
Control (Non-treatment)	H_2O extract of *hamcho* juice	292.25±4.34	118.43±12.93	534.77±28.43
	EtOH extract of *hamcho* cake	250.61±5.82	289.25±13.42	397.20±15.75[a]
Viscozyme	H_2O extract of *hamcho* juice	621.26±12.01	174.56±8.64	670.86±25.76
	EtOH extract of *hamcho* cake	186.91±2.83	87.54±2.74	367.07±12.20[a]

[1)]IC_{50} represents the concentration of a sample required for 50% inhibition of the DPPH, superoxide, and hydroxyl radicals. Values are mean±SD of triplicate analyses. Values with the same superscript in the column are not significantly different at $p<0.05$.

Table 3. DPPH, superoxide, and hydroxyl radical scavenging activities (IC_{50}) of four solvent fractions from the ethanol extract of viscozyme-treated *hamcho* cake

Solvent fraction	IC_{50} (μg/mL)[1)]		
	DPPH radical	Superoxide radical	Hydroxyl radical
Et_2O fr.	154.92±2.03	47.74±2.53	424.24±12.45
EtOAc fr.	472.72±3.95	67.27±3.28	392.18±9.73
n-BuOH fr.	837.17±5.26	184.35±5.18	873.56±12.64
H_2O fr.	>3,000	224.84±4.76	>2,000

[1)]IC_{50} represents the concentration of a compound required for 50% inhibition of DPPH, superoxide, and hydroxyl radicals. Values are mean± SD of triplicate analyses. All values in column are significantly different at $p<0.05$.

and hydroxyl (IC_{50}=367.07 μg/mL) radical scavenging activities, which were than those of the EtOH extract from the untreated *hamcho* cake. However, the H_2O extract from the viscozyme-treated *hamcho* juice has less radical scavenging activity than the H_2O extract from the untreated *hamcho* juice due to significant increases in extract yield with addition of some carbohydrates by enzymatic hydrolysis. Thus, the EtOH extract from the viscozyme-treated *hamcho* cake has antioxidant potential as radical scavenger. We therefore successively fractionated the EtOH extract with three solvents, Et_2O, EtOAc, and *n*-BuOH, to find the major principles for the strong antioxidant activity.

The radical scavenging activities of the Et_2O, EtOAc, and *n*-BuOH fractions from the EtOH extract of the viscozyme-treated *hamcho* cake against DPPH, superoxide, and hydroxyl radicals are shown in Table 3. All solvent fractions exhibited concentration-dependent radical scavenging activity against three reactive radicals (data not shown), and the IC_{50} values are listed in Table 3. The Et_2O fr. had potent DPPH (IC_{50}=154.92 μg/mL), superoxide (IC_{50}=47.74 μg/mL), and hydroxyl (IC_{50}=424.24 μg/mL) radical scavenging activities, although its hydroxyl radical scavenging activity was less than EtOAc fr. (IC_{50}=392.18 μg/mL), which contained small amounts of flavonoid glycosides (data not shown). However, two other solvent fractions (*n*-BuOH and H_2O frs.) did not exert significant radical scavenging activities. Based on these results, we further isolated and identified major antioxidant compounds in the Et_2O fr.

Isolation and identification of phenolic compounds The EtOH extract of viscozyme-treated *hamcho* cake was successively partitioned with Et_2O, EtOAc, and *n*-BuOH, The Et_2O fr. with strong antioxidant activity was further stepwise subjected to silica gel, ODS-A, and Sephadex LH-20 column chromatography. Five phenolic compounds were isolated in pure state, as previously described. The five phenolic compounds were assigned structures on the basis of UV, ^{1}H- & ^{13}C-NMR, and FABMS spectral data compared with, published data. One phenolic acid (Comp. **1**: procatechuic acid), two hydroxycinnamic acids (Comp. **2**: ferulic acid, Comp. **3**: caffeic acid), and two flavonoid aglycones (Comp. **4**: isorhamnetin, Comp. **5**: quercetin) were isolated and identified from *hamcho*, although their glycosides and derivatives have already been reported in *hamcho* (16-18, 20) and other plants (4, 29). The detailed data of 5 phenolic compounds for the UV, ^{1}H- & ^{13}C-NMR, and FABMS spectra data for the 5 compounds given in Table 4.

Phenolic acids, such as ferulic and coumaric acids, are found abundantly in cell walls linked to hemicelluloses in different forms in the cell walls of plants (30). Additionally, phenolic glycosides, including quercetin-3-*O*-β-D-glucoside, isorhamnetin-3-*O*-β-D-glucoside, and 3-caffeoyl-4-dihydrocaffeoylquinic acid, have already been found in *hamcho* (16, 20). Five phenolic aglycones were only found in the EtOH extract from the viscozyme-treated *hamcho* cake. Based On these results, our study demonstrate that viscozyme treatment liberation of procatechuic and ferulic acids by enzymatic hydrolysis of cell walls in *hamcho*, and also somewhat further converts the phenolic glycosides into their corresponding aglycones. Therefore, the antioxidant activity of the EtOH extract from the viscozyme-treated *hamcho* cake was stronger than that of the EtOH extract of the untreated *hamcho* cake, as shown in Table 2, because phenolic aglycones are generally known to have stronger antioxidant activity than their corresponding glycosides (3, 4). Thus, viscozyme treatment is an appropriate and effective method for producing high quality *hamcho* extract with higher yield and phenolic content and strong antioxidant activity.

Antioxidant activity of phenolic compounds The antioxidant activities of the 5 phenolic compounds isolated

Table 4. ^{1}H- & ^{13}C-NMR and FABMS spectra, data of five phenolic compounds from viscozyme-treated *hamcho* cake[1)]

NMR	Compound				
	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5
UV(λ_{max})	259, 293	234, 323	237, 323	254, 366	254, 367
^{1}H-NMR					
H-2	7.43 (1H, d, J=1.5 Hz)	7.17 (1H, d, J=2.0 Hz)	7.03 (1H, d, J=1.5 Hz)		
H-5	6.76 (1H, d, J=8.5 Hz)	6.81 (1H, d, J=8.0 Hz)	6.77 (1H, d, J=8.5 Hz)		
H-6	7.39 (1H, dd, J=1.5, 8.5 Hz)	7.06 (1H, dd, J=2.0, 8.0 Hz)	6.92 (1H, dd, J=1.5, 8.5 Hz)	6.19 (1H, d, J=2.0 Hz)	6.19 (1H, d, J=2.0 Hz)
H-7		7.58 (1H, d, J=16.0 Hz)	7.52 (1H, d, J=15.5 Hz)		
H-8		6.32 (1H, d, J=16.0 Hz)	6.22 (1H, d, J=15.5 Hz)	6.47 (1H, d, J=2.0 Hz)	6.40 (1H, d, J=2.0 Hz)
H-2'				7.75 (1H, d, J=2.0 Hz)	7.67 (1H, d, J=2.0 Hz)
H-5'				6.94 (1H, d, J=9.0 Hz)	6.89 (1H, d, J=2.0 Hz)
H-6'				7.68 (1H, dd, J=2.0, 8.5 Hz)	7.53 (1H, dd, J=2.0, 8.5 Hz)
OCH_3		3.89(3H, s)		3.84(3H, s)	
^{13}C-NMR					
C-1	126.70	128.00	128.08		
C-2	115.66	111.82	115.19	146.59	147.76
C-3	145.88	146.85	146.72	135.79	136.27
C-4	150.50	149.51	146.93	175.85	176.20
C-5	117.93	116.60	116.62	160.66	160.55
C-6	123.62	124.08	122.89	98.19	98.95
C-7		150.59	149.48	163.94	163.13
C-8		116.35	116.31	93.57	94.46
C-9				156.14	155.93
C-10				102.98	104.85
C-1'				121.94	122.01
C-2'				111.69	115.56
C-3'				148.79	145.25
C-4'				147.34	148.09
C-5'				115.51	115.77
C-6'				121.69	120.24
OCH_3		56.58		55.75	
COOH	172.93	171.33	171.61		
FABMS $[M+H]^+$	155	195	181	317	303

[1)]Spectra were measured in CD_3OD (Comp. 1-3) and DMSO (Comp. 4, 5).

Table 5. DPPH, superoxide, and hydroxyl radical scavenging activities of five phenolic compounds isolated from the EtOH extract of viscozyme-treated *hamcho* cake

Phenolic compound	Radical scavenging activity (IC_{50}, μM)[1)]		
	DPPH radical	Superoxide radical	Hydroxyl radical
Procatechuic acid	38.25±2.15^{a}	18.59±2.58^{d}	1,320.58±362.45^{a}
Ferulic acid	38.65±1.93^{a}	>5,000	672.27±121.11^{d}
Caffeic acid	30.64±1.38^{b}	8.64±1.68^{e}	1,645.20±218.32^{a}
Quercetin	27.38±1.48^{c}	48.37±5.68^{a}	960.08±36.34^{c}
Isorhamnetin	38.67±2.01^{a}	35.21±2.18^{b}	1,097.02±18.65^{b}
(+)-Catechin[2)]	26.51±1.02^{bc}	25.64±2.72^{c}	750.27±14.24^{e}

[1)]IC_{50} represents the concentration of a compound required for 50% inhibition of DPPH, superoxide, and hydroxyl radicals. Values are mean±SD of triplicate analyses. Values with the same superscript in the column are not significantly different at $p<0.05$.
[2)](+)-Catechin was used as a positive reference.

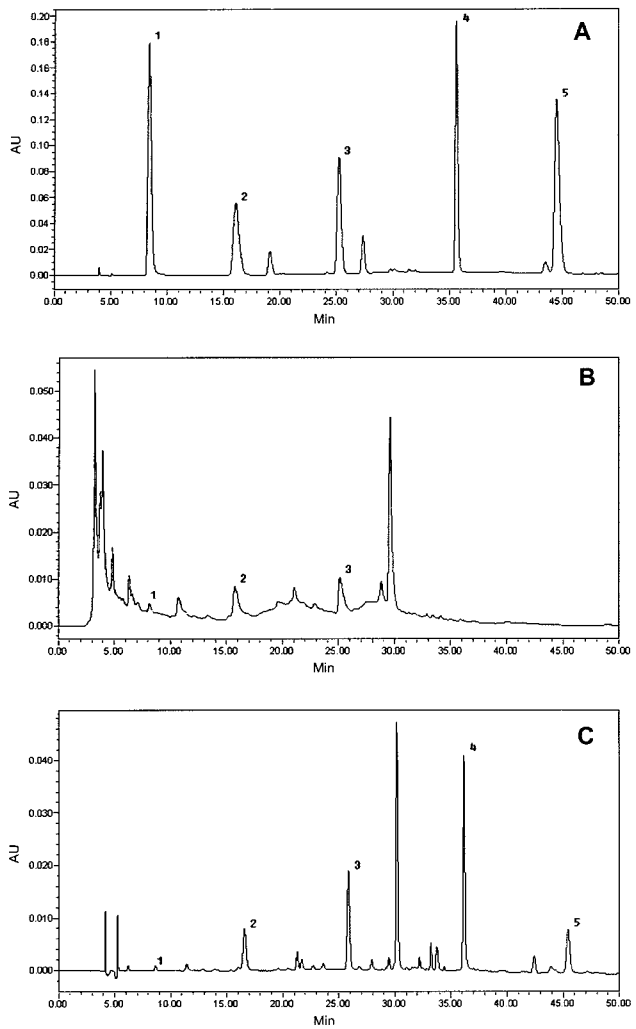

Fig. 2. HPLC chromatograms of five phenolic standards (A), and the water (B) and ethanol extract (C) from viscozyme-treated *hamcho* cake. 1, Procatechuic acid; 2, caffeic acid; 3, ferulic acid; 4, quercetin; 5, isorhamnetin.

from viscozyme-treated *hamcho* cake are shown in Table 5. Most of phenolic compounds exhibited strong dose-dependent radical scavenging activity (data not shown). All phenolic compounds effectively scavenged DPPH radical, although their activity was less than that of (+)-catechin, a well-known natural antioxidant ($p<0.05$). Meanwhile, all except ferulic acid greatly inhibited the generation of superoxide anion, and caffeic (IC_{50}=8.64 μM,) and procatechuic (IC_{50}=18.59 μM) acids was especially porent in scavenging enzymatically generated superoxide anion. Their activity was stronger than that of an well-known superoxide anion scavenger, (+)-catechin (IC_{50}= 25.64 μM), a well-known superoxied anion scavenger ($p<$ 0.05) (31). Moreover, quercetin and isorhamnetin also exhibited considerable superoxide radical scavenging activity with IC_{50} values of 48.37 and 35.21 μM, respectively. Finally, most of phenolic constituents effectively scavenged hydroxyl radical induced by Fe(II)/H_2O_2, and ferulic acid in particular exhibited the strongest hydroxyl radical scavenging activity with IC_{50} value of 672.27 μM.

Table 6. Levels of phenolic compounds in water and ethanol extracts from viscozyme-treated *hamcho* juice and cake[1)]

Phenolic compound	Content (mg%, dry base of *hamcho*)	
	Water extract	Ethanol extract
Procatechuic acid	8.87±1.29 (0.02)	1.54±0.34 (0.03)
Caffeic acid	34.71±2.78 (0.82)	6.87±1.54 (0.13)
Ferulic acid	39.30±3.56 (0.93)	8.45±2.15 (0.17)
Quercetin	ND	12.63±2.54 (0.25)
Isorhamnetin	ND	6.65±0.87 (0.13)

[1)]Values are mean±SD of duplicate analyses. Dry base (%) of *hamcho* extracts. ND: not detected.

Its activity was greater than that of (+)-catechin, a well-known hydroxyl radical scavenger ($p<0.05$) (32). In addition, quercetin (IC_{50}=960.08 μM) and isorhamnetin (IC_{50}=1,097 μM) also exhibited moderate hydroxyl radical scavenging activity, but procatechuic (IC_{50}=1,320.58 μM) and caffeic (IC_{50}=1,645.20 μM) acids were less active than other phenolic compounds. Thus, these results support earlier reports that the DPPH, superoxide, and hydroxyl radical scavenging activities of phenolic acids increase as the number of phenolic hydroxyl groups, and a free catechol group is essential for high superoxide and hydroxyl radical scavenging activity (33, 34). Furthermore, the superoxide and hydroxyl radical scavenging activities of phenolic compounds were also reported to decrease when a methoxyl group adjacent to the 4-hydroxyl group *para*-substituted on an aromatic ring (35, 36). However, our results differed from this in the hydroxyl radical scavenging activity of phenolic compounds. This discrepancy is probably due to the different *in vitro* assay used in each study. Thus, the strong antioxidant activity of the EtOH extract from viscozyme-treated *hamcho* cake could be ascribed to considerable radical scavenging properties of phenolic compounds. This report first demonstrated that the EtOH extract from viscozyme-treated *hamcho* cake contains antioxidant phenolics that act as reactive oxygen radical scavengers.

Quantification of phenolic compounds The levels of the 5 phenolic compounds in the H_2O and EtOH extracts from viscozyme-treated *hamcho* juice and cake, respectively, were determined by HPLC; typical HPLC chromatograms are shown in Fig. 2. Three (procatechuic, caffeic, and ferulic acids) and 5 phenolics (procatechuic acid, caffeic acid, ferulic acid, quercetin, and isorhamnetin), as well as other unknown compounds were detected in the H_2O and defatted EtOH extracts of *hamcho* juice and cake, respectively, by comparison of retention time of standard phenolic compounds. As shown in Table 6, levels of procatechuic, caffeic, and ferulic acids in viscozyme-treated *hamcho* juice were 8.87, 34.71, and 39.30 mg%, while those of procatechuic, caffeic and ferulic acids, quercetin, and isorhamnetin in viscozyme-treated *hamcho* cake were 1.54, 6.87, 8.45, 7.46, 12.63, and 6.65 mg% (dry base of *hamcho*), respectively. Thus, levels of procatechuic, caffeic, and ferulic acids in viscozyme-treated *hamcho* juice were higher than those in viscozyme-

treated *hamcho* cake. However, quercetin and isorhamnetin were barely detectable in the viscozyme-treated *hamcho* juice. In contrast, quercetin and isorhamnetin were detected in considerable amounts in the viscozyme-treated *hamcho* cake, along with procatedchuic, caffeic, and ferulic acids. Thus, these results suggest that quercetin and isorhamnetin may be the primary constituents responsible for strong antioxidant activity of the EtOH extract from viscozyme-treated *hamcho* cake.

In conclusion, we prepared several H_2O and EtOH extracts from *hamcho* juice and cake by enzymatic treatments. Among them, the EtOH extract from viscozyme-treated *hamcho* cake produced higher yield and phenolic content, and significant radical scavenging activity against DPPH, superoxide, and hydroxyl radicals. Five phenolic compounds, procatechuic, caffeic, and ferulic acids, quercetin, and isorhamnetin, were isolated and identified from the EtOH extract of viscozyme-treated *hamcho* cake, and most of phenolic compounds had considerable DPPH, superoxide, and hydroxyl radical scavenging activities. In particular, quercetin and isorhamnetin were found to be major principles for strong antioxidant activity of the EtOH extract of viscozyme-treated *hamcho* cake. Thus, these results suggest that the EtOH extract of viscozyme-treated *hamcho* cake may be a promising source of natural antioxidants, which play important physiological roles in inhibiting oxidative stress-mediated degenerative diseases. Further study is required to isolate and identify other antioxidant compounds from *hamcho* cake, and further determine their antioxidant activity *in vivo*.

Acknowledgments

This work was supported by the RRC Program of Ministry of Commerce, Industry, and Energy (MOCIE). We are grateful to Dr. Sung-Hong Kim and Ms. Shin-Ae Chae, Korea Basic Science Institute, Daegu, Korea, for FABMS and NMR measurements.

References

1. Frei B. Nonenzymatic antioxidant defense systems. pp. 107-114. In: Natural Antioxidants in Human Health and Disease. Briviba K, Sies H (eds). Academic Press, London, UK (1994)
2. Beckman KB, Ames BN. Oxidant, antioxidant, and aging. pp. 201-246. In: Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Scandalios JG (ed). Cold Spring Harbor Laboratory Press, New York, NY, USA (1997)
3. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J. Nutr. 130: 2073S-2085S (2000)
4. Naczk M, Shahidi F. Phenolic compounds in plant foods: Chemistry and health benefits. Nutraceuticals Food. 8: 200-218 (2003)
5. Branen AL. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J. Am. Oil Chem. Soc. 52: 59-65 (1975)
6. Ito N, Fukushima S, Fukushima H. Carcinogenicity and modification of the carcinogenicity response by BHA and BHT, and other antioxidants. CRC Cr. Rev. Food Technol. 15: 109-116 (1985)
7. Faulkner DJ. Marine natural products. Nat. Prod. Rep. 1: 7-55 (2000)
8. Lee CM. A Pictorial Book of the Korean Flora. Hayngmunsa Press, Seoul, Korea. p. 990 (1985)
9. Min JG, Lee DS, Kim TJ, Park JH, Cho TY, Park DI. Chemical composition of *Salicornia herbacea* L. J. Food Sci. Nutr. 7: 105-107 (2002)
10. Bang MA, Kim HA, Cho YJ. Hypoglycemic and antioxidant effect of dietary *hamcho* powder in streptozotocin-induced diabetic rats. J. Korean Soc. Food Sci. Nutr. 31: 840-846 (2002)
11. Park SH, Ko SK, Choi JG, Chung SH. *Salicornia herbacea* prevents high fat diet-induced hyperglycemia and hyperlipidemia in ICR mice. Arch. Pharm. Res. 29: 256-264 (2006)
12. Han SK, Kim SM. Antioxidative effect of *Salicornia herbacea* L. grown in closed sea beach. J. Korean Soc. Food Sci. Nutr. 32: 207-210 (2003)
13. Han SK. Antioxidant effect of fermented *Salicornia herbacea* L. liquid with EM (effective microorganism) on pork. J. Korean Food Sci. Anim. Resour. 24: 298-302 (2004)
14. Cha JY, Jeon BS, Park JW, Kim BK, Jeong CY, Ryu JS, Choi CK, Cho YS. Hypocholesterolemic effect of yogurt supplemented *Salicornia herbacea* extract in cholesterol-fed rats. Korean J. Life Sci. 14: 24-28 (2004)
15. Lee JT, An BJ. Detection of physical activity of *Salicornia herbacea*. Korean J. Herbol. 17: 61-69 (2002)
16. Park SH, Kim KS. Isolation and identification of antioxidant flavonoids from *Salicornia Herbacea* L. J. Korean Soc. Appl. Biol. Chem. 47: 120-123 (2004)
17. Lee YS, Lee HS, Shin KH, Kim BK, Lee S. Constituents of the halophyte *Salicornia herbacea*. Arch. Pharm. Res. 27: 1034-1036 (2004)
18. Lee YS, Lee S, Lee HS, Kim BK, Ohuchi K, Shin KH. Inhibitory effect of isorhamnetin-3-*O*-β-D-glucoside from *Salicornia herbacea* on rat lens aldose reductase and sorbitol accumulation in streptozotocin-induced diabetic rat tissues. Biol. Pharm. Bull. 28: 916-918 (2005)
19. Lee CH, Kim IH, Kim YE, Oh SW, Lee HJ. Determination of betaine from *Salicornia herbacea* L. J. Korean Soc. Food Sci. Nutr. 33: 1584-1587 (2004)
20. Chung YC, Chun HK, Yang JY, Kim JY, Han EH, Kho YH. Tungtungmadic acid, a novel antioxidant, from *Salicornia herbacea*. Arch. Pharm. Res. 28: 1122-1126 (2005)
21. Lee KY, Lee MH, Chang IY, Yoon SP, Lim DY, Jeon YJ. Macrophage activation by polysaccharide fraction isolated from *Salicornia herbacea*. J. Ethnopharmacol. 22: 145-149 (2005)
22. Jeong CY, Ryu JS, Choi CK, Jeon BS, Park JW, Kim BK, Shin GG, Bae DW, Cha JY. Supplemented effect of *Salicornia Herbacea* extracted powder on preparation and quality characteristics of fermented milk product. J. Life Sci. 14: 788-793 (2004)
23. Heo SJ, Jeon YJ. Antioxidant effect and protecting effect against cell damage by enzymatic hydrolysates from marine algae. Food Ind. Nutr. 10: 31-41 (2005)
24. Kim KR, Jang MJ, Choi SW, Woo MH, Choi JH. Effects of water extract from enzymic-treated *hamcho* (*Salicornia herbacea*) on lipid metabolism in rats fed high cholesterol diet. J. Korean Soc. Food Sci. Nutr. 35: 55-60 (2006)
25. Jang MJ, Choi SW, Woo MH, Choi JH. Antioxidant and anti-thrombus activities of water and methanol extracts from *Salicornia herbacea* prepared by enzymatic treatment. Ann. Nutr. Metab. 51: in press (2006)
26. Kwon YJ, Rhee SJ, Chu JW, Choi SW. Comparison of radical scavenging activity of extracts of mulberry juice and cake prepared from mulberry (*Morus* spp.) fruit. J. Food Sci. Nutr. 10: 111-117 (2005)
27. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16: 144-158 (1965)
28. SAS Institute, Inc. SAS User's Guide: Statistics. Cary, NC, USA (1985)
29. Lee HS. Tyrosinase inhibitors of *Pulsatilla cernua* root-derived materials. J. Agr. Food Chem. 50: 1400-1403 (2002)
30. Kato Y, Nevins DJ. Isolation and identification of 2-*O*-(5'-*O*-*trans*-feruoyl-β-L-arabinofuranosyl)-(13)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose as a component of Zea shoot cell walls. Carbohyd. Res. 137: 139-150 (1985)
31. Furuno K, Akasako T, Sugihara N. The contribution of the pyrogallol moiety to the superoxide radical scavenging activity of flavonoids. Biol. Pharm. Bull. 25: 19-23 (2002)
32. Ricardo da Silva JM, Darmon N, Fernandez Y, Mitjavila S. Oxygen

free radical scavenger capacity in aqueius models of different procyanidins from grape seeds. J. Agr. Food Chem. 39: 1549-1552 (1991)
33. Chimi H, Cillard J, Cillard P, Rahmani M. Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. J. Am. Oil Chem. Soc. 68: 307-312 (1991)
34. Natella F, Nardini M, Di Felice M, Scaccini C. Benzoic and cinnamic acid derivatives as antioxidants: structure-activity relation. J. Agr. Food Chem. 47: 1453-1459 (1999)
35. Husain RS, Cillard J, Cillard P. Hydroxyl radical scavenging activity of flavonoids. Phytochemistry 26: 2489-2491 (1987)
36. Toda S, Kumura M, Ohnishi M. Effect of phenolcarboxylic acids on superoxide anion and lipid peroxidation induced by superoxide anion. Planta Med. 57: 8-10 (1991)