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ARTICLE

Isolation and Identification of Sea Buckthorn (*Hippophae rhamnoides*) Phenolics with Antioxidant Activity and α-Glucosidase Inhibitory Effect

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This study was performed to evaluate the antioxidant and α-glucosidase inhibitory effects from the extract, fractions, and isolated compounds of sea buckthorn leaves. Six compounds, kaempferol-3-*O*-β-D-(6″-*O*-coumaryl) glycoside, 1-feruloyl-β-D-glucopyranoside, isorhamnetin-3-*O*-glucoside, quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside, and isorhamnetin-3-*O*-rutinoside, were isolated from sea buckthorn leaf extracts. The butanol fraction (EC_{50} = 1.81 μg/mL) along with quercetin 3-*O*-β-D-glucopyranoside (EC_{50} = 1.86 μg/mL) had a higher DPPH radical-scavenging activity and showed stronger reducing power (OD_{700} = 1.83 and 1.78, respectively). The butanol fraction (477 mg GAE/g) contained the highest amount of phenolic compounds and also the most powerful α-glucosidase inhibitory effect (86%) at 5 μg/mL. The results indicate that sea buckthorn leaf extracts could potentially be used for food additives and the development of useful natural compounds.

KEYWORDS: Antioxidant activity; α-glucosidase inhibitory effect; *Hippophae rhamnoides*; kaempferol-3-*O*-β-D-(6″-*O*-coumaryl) glycoside; quercetin 3-*O*-β-D-glucopyranoside

INTRODUCTION

Some studies have revealed that fruits and vegetables are protective against certain forms of cancer (*1*). The dietary intake of flavonoids from fruits and vegetables has also been shown to be inversely related to coronary heart disease mortality (*2*). A consequence of peroxidation of human low-density lipoprotein (LDL) by reactive oxygen species (ROS) could result in these atherosclerotic diseases (*3*). Numerous theoretical premises and experimental studies on the phenolic compounds can perform protective functions that inhibit the oxidative damage of LDL (*4*). Antioxidant compounds may function as free radical scavengers, complexing agents for pro-oxidant metals, reducing agents, and quenchers of singlet oxygen formation (*5*). Because of their antioxidant characters, antioxidants are important in the prevention of human diseases. However, synthetic antioxidants (e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)) can cause lung damage (BHT) or promote the action of some carcinogens (BHA) (*6*). Therefore, research on natural antioxidants has been shown to play an important role in disease prevention.

Interest in glucosidase inhibitors is growing because the number of diabetes mellitus (DM) cases has been increasing worldwide in recent years. DM is a metabolic disorder characterized by elevated blood glucose levels with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both (*7*). One of the strategies to monitor blood glucose for type-2 DM is to either inhibit or reduce production of glucose from the small intestine (*8*). Acting as a key enzyme for carbohydrate digestion, intestinal α-glucosidase is one of the glucosidases located at the epithelium of the small intestine. α-Glucosidase has been recognized as a therapeutic target for modulation of postprandial hyperglycemia, which is the earliest metabolic abnormality to occur in type-2 DM (*9*). Several natural α-glucosidase inhibitors including acarbose, voglibose, and miglitol are clinically used (*10*). Thus, natural products of great structural diversity are still a good source for searching for such inhibitors, thereby motivating us to explore biologically active compounds from sea buckthorn (*Hippophae rhamnoides*).

Sea buckthorn is a deciduous shrub cultivated mainly in Europe and Asia for use in various life-saving drugs and health tonics (*11*). For example, extracts of sea buckthorn branches and leaves were historically administered to humans and animals to treat gastrointestinal distress in Mongolia (*12*). Sea buckthorn berries have high levels of vitamin C, vitamin E, carotenoids, carbohydrates, proteins, organic acids, dietary minerals, β-sitosterol, and polyphenolic acids (*13*). Extracts of sea buckthorn berries have antioxidative (*14*), antitumoric (*15*), anticarcinogenic (*16*), chemoprotective (*17*), and nutritional effects (*18*).

Many studies have shown that this medicinal plant possesses antioxidant and α-glucosidase inhibitory activities and protects the human body against both cellular oxidative reaction and diabetes (*19*, *30*). However, a detailed pharmacological screening of sea buckthorn leaf extract, fractions, and isolated bioactive compounds has not been reported. The aim of the present study was to isolate and identify of sea buckthorn phenolic compound and to investigate the biological activities *in vitro*, which included antioxidant and α-glucosidase inhibitory

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activity from sea buckthorn leaf extract, fractions, and isolated compounds.

MATERIALS AND METHODS

General. ^{1}H and ^{13}C NMR spectra were obtained using DPX 400 and AVANCE 600 (Bruker) NMR spectrometers. UV spectra were measured with a V-530 spectrophotometer (Jasco Co., Japan).

Chemicals. α-Tocopherol, BHA, BHT, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 4-nitrophenyl-α-D-glucopyranoside (*p*NPG), α-glucosidase (EC 3.2.1.20), linoleic acid, and Folin−Ciocalteu reagent were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were of analytical grade or better.

Extraction and Isolation of Bioactive Compounds. Sea buckthorn leaves were supplied by Samsung Herb Medicine Co. (Chuncheon, Korea). The samples were dried at room temperature and then ground into a powder using a blender. The air-dried, powdered sea buckthorn leaves (800 g) were extracted with methanol at room temperature. The solution was filtered, evaporated under reduced pressure, and lyophilized. The lyophilized sample was dissolved in small amounts of water and then was further partitioned with organic solvents to yield *n*-hexane, *n*-butanol (BuOH; water saturated), and aqueous fractions. The solvent fractions were assayed before separation of additional chromatographic subfractions.

The BuOH fraction (20 g) was separated on a silica gel column (400 g, 5 × 50 cm) by stepwise gradient elution with $CHCl_3$/MeOH (9:1, 7:1, 5:1, 2:1, and 1:1) to yield five subfractions (fractions 1−5). Fraction 4 was again separated on silica gel by elution with water-saturated EtOAc/MeOH (15:1) to give five subfractions (fractions 4-1−4-5). Compound **1** (44 mg) was separated from fraction 4-1 by silica gel column chromatography using water-saturated EtOAc/MeOH (15:1). Fraction 4-2 was again separated on silica gel by elution with $CHCl_3$/MeOH (7:1) to yield six subfractions (fractions 4-2-1−4-2-6). Fraction 4-2-4 was subjected to ODS gel chromatography by elution with 60% MeOH to yield two subfractions (fractions 4-2-4-1−4-2-4-2). Fraction 4-2-4-1 was recrystallized from 60% MeOH to give compound **2** (48.7 mg). Fraction 4-2-4-2 was then separated on silica gel by elution with $CHCl_3$/MeOH/AcOH (7:1:0.1) to give compound **3** (10 mg). Fraction 4-2-6 was subjected once more to ODS gel chromatography by elution with 40% MeOH to give compound **4** (36.4 mg). Fraction 4-4 was chromatographed on a 40 g silica gel RediSep column (Isco Co., Lincoln, NE) with $CHCl_3$/MeOH/H_2O (40:10:0.5) to yield two subfractions (fractions 4-4-1−4-4-5). Fraction 4-4-4 was purified by silica gel chromatography (43 g RediSep column, silica, 40% MeOH) to give compounds **5** (63.5 mg) and **6** (39 mg).

Scavenging of DPPH Free Radical. The free radical scavenging activity was measured by DPPH assay. The 5 mL assay mixture contained 3.98 mL of methanol, 20 μL of extract, and 1 mL of DPPH (0.15 mM in methanol). After incubation at room temperature for 30 min, the absorbance was measured at 517 nm using a spectrophotometer (V-530, Jasco Co., Japan). Ascorbic acid, BHA, BHT, and α-tocopherol were used as reference compounds. Data were processed using Excel, and the concentration of tested sample required to reduce the initial free radical concentration by 50% (EC_{50}) was calculated. Percent inhibition of DPPH was calculated by using the following equation: % inhibition = $(1 - Abs_{sample}/Abs_{control}) \times 100$, where Abs_{sample} is the absorbance of the experimental sample and $Abs_{control}$ is the absorbance of the control. The experiment was performed in triplicate.

Determination of Reducing Power. The reducing power of the sample was determined by using the Oyaizu method (*20*) with some modifications. Different concentrations of sample were mixed with 0.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 mL of 1% potassium ferricyanide (w/v), followed by incubation at 50 °C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid (w/v) was added to the mixture, followed by centrifugation at 650 rpm for 10 min. The upper layer (0.5 mL) was mixed with 0.5 mL of deionized water and 0.1 mL of 0.1% ferric chloride (w/v). The absorbance of the resultant solution was measured at 700 nm. Ascorbic acid, α-tocopherol, BHA, and BHT were used as reference compounds. The experiment was performed in triplicate.

Determination of Total Phenol Content. Total phenolic content was measured using the Folin−Ciocalteu assay (*21*). Briefly, 0.1 mL of sample and 50 μL of 2 N Folin−Ciocalteu reagent were added to a 5 mL volumetric flask. The solutions were mixed and allowed to stand for 3−5 min at room temperature. Next, 0.3 mL of a 20% sodium carbonate solution was added. Solutions were mixed and kept aside for 15 min. Finally, 1 mL of distilled water was added and the absorbance measured at 725 nm. The total phenolic content of the sample was determined by comparing the optical density of the sample with those of different concentrations of gallic acid, a standard phenolic compound. Analysis for each sample was performed in triplicate, and values for total phenolic content are expressed in milligrams of gallic acid equivalent (GAE) per gram of samples.

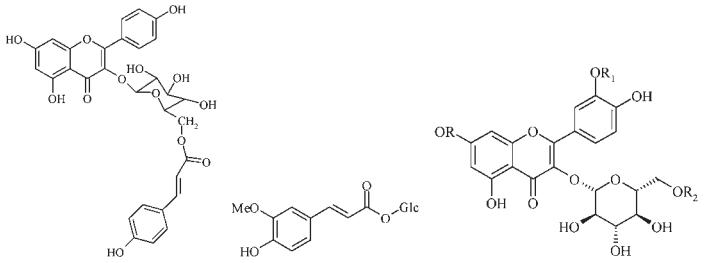
Determination of Antilipid Peroxidative Activity. Antilipid peroxidative activity was determined by using the FTC method in a linoleic acid emulsion (*22*) with some modifications. The reaction medium contained 0.02 mL of sample (10 mg/mL), 0.2 mL of 2.51% linoleic acid in ethanol, 0.4 mL of 0.04 M potassium phosphate buffer (pH 7.0), and 0.38 mL of distilled water. The solution (1 mL) was mixed and incubated for 24 h at 70 °C in darkness. The same reaction medium without sample was used as the control sample. Synthetic antioxidants (BHA and α-tocopherol) at identical concentrations were used for comparison. A 0.05 mL aliquot of the mixture was diluted with 2.85 mL of 75% ethanol, followed by the addition of 0.05 mL of 30% ammonium thiocyanate (w/v) and 0.05 mL of 20 mM of ferrous chloride in 3.5% HCl. The absorbance of the red color of the test solution was measured at 500 nm. The experiment was performed in triplicate.

The TBA assay is a measure of antioxidant activity based upon the reaction of TBA with malonaldehyde, an aldehyde product produced by lipid peroxidation. The sample solution was prepared and incubated as described above. One milliliter of 20% trichloroacetic acid (TCA) and 1 mL of 0.67% TBA solution were added to 0.5 mL of the sample mixture prepared for the FTC method. This mixture was placed in a heating block (95 °C) for 10 min and was centrifuged at 3000 rpm for 20 min after cooling to room temperature. Antioxidant activity was based on the absorbance of the supernatant at 532 nm after the FTC method. The experiment was performed in triplicate.

Inhibition of α-Glucosidase. α-Glucosidase (50 μL, 0.5 U/mL) and 0.2 M potassium phosphate buffer (pH 6.8, 50 μL) were mixed with test sample (50 μL; 10, 50, and 100 ppm). After incubation at 37 °C for 15 min, 3 mM *p*NPG (100 μL) was added. The reaction was incubated again at 37 °C for 10 min and then stopped by the addition of 0.1 M Na_2CO_3 (750 μL). The absorption of 4-nitrophenol was measured at 405 nm. The reaction mixture without sample was used as a control, and the mixture without substrate was used as a blank. The experiment was performed in triplicate. The percent inhibition of α-glucosidase was calculated as $[1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$, where Abs_{sample} represents the absorbance of the experimental sample, Abs_{blank} represents the absorbance of the blank, and $Abs_{control}$ represents the absorbance of the control. The experiment was performed in triplicate.

RESULTS AND DISCUSSION

Identification of Isolated Compounds and Quantitative Analysis of Individual Phenolic Compounds. We separated the BuOH fraction of sea buckthorn leaf extract by silica gel and ODS gel chromatography, yielding six compounds (**Figure 1**; **Table 1−3**). The ^{1}H and ^{13}C NMR spectra of Com-1 indicated the presence of kaempferol glycoside (**Tables 1** and **2**). Chemical shifts of H-6 and H-8 at δ_H 6.13 and 6.30 (each 1H, d, J = 1.96 Hz), respectively, and C-2 at δ_C 158.41 showed that sugar was attached to the C-3 position of Com-1 (*22*, *23*). Furthermore, two aromatic doublets at δ_H 6.82 and 7.30 (each 2H, J = 8.54 Hz) along with two olefinic doublets at δ_H 6.07 and 7.40 (each 1H, J = 15.94 Hz) suggest the presence of a coumaroyl moiety of Com-1. These results allowed us to establish kaempferol-3-*O*-β-D-(6″-*O*-coumaryl) glycoside as the structure of Com-1 by comparison of the spectral data with literature values (*24*). ^{1}H NMR spectrum of Com-2 (**Table 1**) showed three aromatic protons at δ_H 6.82 (1H, d, J = 8.23 Hz), 7.09 (1H, dd, J = 1.93, 8.23 Hz), and 7.19 (1H, d, J = 1.93 Hz), two olefinic protons at δ_H 6.40 and 7.72 (each 1H, J = 15.91 Hz), a methoxyl at δ_H 3.88 (3H, s), and an anomeric proton at δ_H 5.59 (1H, d, J = 7.85 Hz). ^{13}C NMR (**Table 2**) showed a carbonyl carbon at δ_C 167.75. These data showed that 1-feruloyl-β-D-glucopyranoside was the structure of Com-2 (*25*). The ^{1}H and ^{13}C NMR spectra of Com-3 and Com-6 showed ishorhamnetin



Compounds	R	R_1	R_2	Chemical name
Com-1				Kaempferol-3-*O*-β-$_D$-(6"-*O*-coumaryl)glycoside
Com-2				1-Feruloyl-β-$_D$-glucopyranoside
Com-3	H	CH_3	H	Isorhamnetin-3-*O*-glucoside
Com-4	H	H	H	Quercetin 3-*O*-β-$_D$-glucopyranoside
Com-5	Rham	H	H	Quercetin 3-*O*-β-$_D$-glucopyranosyl-7-*O*- α-$_L$-rhamnopyranoside
Com-6	H	CH_3	Rham	Isorhamnetin-3-*O*-rutinoside

Glc, glucose; Rham, rhamnose

Figure 1. Chemical structures of phenolic glucosides isolated from the leaf extracts of *Hippophae rhamnoides*.

Table 1. ^{1}H NMR Data for Compounds **1**, **2**, **3**, **5**, and **6** (400 MHz, MeOH-d_4)

	δ of ^{1}H (J, Hz)				
no.	Com-1	Com-2	Com-3	Com-5	Com-6
2		7.19 (d, 1.93)			
5		6.82 (d, 8.23)			
6	6.13 (d, 1.96)	7.09 (dd, 1.93, 8.23)	6.22 (br s)	6.46 (d, 2.07)	6.16 (d, 1.21)
7		6.40 (d, 15.91)			
8	6.30 (d, 1.96)	7.72 (d, 15.91)	6.42 (br s)	6.85 (d, 2.07)	6.35 (d, 1.21)
2′	7.99 (d, 8.88)		7.95 (d, 1.27)	7.96 (d, 2.00)	7.92 (d, 1.72)
3′	6.79 (d, 8.82)				
5′	6.79 (d, 8.82)		6.93 (d, 8.39)	6.93 (d, 8.48)	6.88 (d, 8.38)
6′	7.99 (d, 8.88)		7.61 (dd, 1.27, 8.39)	7.57 (dd, 2.00, 8.48)	7.58 (dd, 1.72, 8.38)
2‴	7.30 (d, 8.54)				
3‴	6.82 (d, 8.54)				
5‴	6.82 (d, 8.54)				
6‴	7.30 (d, 8.54)				
7‴	6.07 (d, 15.94)				
8‴	7.40 (d, 15.94)				
-OCH_3		3.88 (s)	3.97 (s)		3.92 (s)
5-OH				12.6 (s)	
glucosyl anomeric H	5.25 (d, 7.27)	5.59 (d, 7.85)	5.42 (d, 7.12)	5.58 (d, 7.52)	5.21 (d, 7.33)
rhamnosyl anomeric H				5.57 (s)	4.52 (s)
rhamnosyl CH_3				1.12 (d, 6.15)	

glycosides (**Tables 1** and **2**). The ^{1}H NMR spectrum of Com-3 showed an anomeric proton at δ_H 5.42 (1H, d, J = 7.12 Hz), whereas Com-6 showed two anomeric protons at δ_H 5.21 (1H, d, J = 7.52 Hz) and 4.52 (1H, s). From the ^{13}C NMR signals of the C-2 positions at δ_C 158.54 and 158.79, we found that the sugar units of two compounds were attached to the C-3 position. The terminal sugar of Com-6 was determined to be rhamnose by the low-field chemical shift of glucose C-6 methylene at δ_C 68.94 (*23*). These data allowed us to establish isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-rutinoside as the structures of Com-3 and Com-6, respectively, by comparison of spectral data with literature values (*26*, *29*). The ^{1}H and ^{13}C NMR spectra of Com-4 and Com-5 showed quercetin glycosides (**Tables 1**−**3**). The ^{1}H NMR spectrum of Com-4 showed H-6 and H-8 signals at δ_H 6.22 and 6.44 (each 1H, br s), respectively, and an anomeric proton at δ_H 5.42 (1H, d, J = 7.12 Hz), whereas Com-5 showed H-6 and H-8 signals at δ_H 6.46 and 6.85 (each 1H, d, J = 2.07 Hz), respectively, and two anomeric protons at δ_H 5.58 (1H, d, J = 7.12 Hz) and 5.57 (1H, s), respectively. The ^{13}C NMR spectra of the C-2 positions of Com-4 and Com-5 exhibited at δ_C 158.49 and 158.79, respectively. These data suggest that Com-4 had one sugar attachment site, whereas Com-5 had two sugar attachment sites (*22*, *23*). Confirmation of each sugar attachment site of Com-5 was performed by HMBC spectroscopy. These data allowed us to establish quercetin-3-*O*-β-D-glucoside and quercetin-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside as the structures of Com-4 and Com-5, respectively, by comparison of the spectral data with literature values (*27*, *28*). Although these isolated compounds were previously reported, Com-1 and Com-2 were isolated from sea buckthorn leaves for the first time.

The UPLC analyses for the quantitative determination of Com-1−6 in sea buckthorn leaves were carried out by peak assignment of the retention times and UV−vis spectra. Among these compounds, Com-1 showed the highest content in sea buckthorn leaves. The Com-1 content of the samples was 6.8 ± 0.3 mg/g of extract, whereas the other phenolic compounds (i.e., Com-2−6) ranged in content from 1.8 to 6.1 mg/g of extract (data not shown).

Table 2. ^{13}C NMR Data for Compounds **1**, **2**, **3**, **5**, and **6** (400 MHz, MeOH-d_4)

	δ of ^{13}C				
no.	Com-1	Com-2	Com-3	Com-5	Com-6
1		127.53			
2	158.41	111.83	158.54	156.34	158.79
3	135.23	149.37	135.32	133.62	135.92
4	179.44	150.93	177.99	177.94	179.66
5	162.98	114.72	161.7	161.23	163.3
6	99.99	124.4	100.06	98.66	100.38
7	165.92	148.27	165.11	161.94	166.37
8	94.85	116.54	94.85	94.95	95.38
9	159.35	167.75	158.63	157.17	159.19
10	105.62		105.67	106.03	106.07
1′	122.73	95.78	123.13	121.27	123.33
2′	132.25	74.04	114.38	113.8	114.96
3′	116.06	78.02	148.43	147.3	148.64
4′	161.55	71.09	150.89	149.98	151.21
5′	116.06	78.78	116.02	115.58	116.48
6′	132.25	62.35	123.83	122.64	124.38
1″	103.98		103.67		
2″	75.75		75.94		
3″	75.82		78.1		
4″	71.34		71.51		
5″	78.01		78.57		
6″	64.34		62.55		
1‴	127.11				
2‴	131.22				
3‴	116.8				
4‴	161.21				
5‴	116.8				
6‴	131.22				
7‴	146.58				
8‴	114.75				
9‴	168.83				
$-OCH_3$		56.47	56.78		57.17
rhmanosyl CH_3					18.32
Glc-1				101.91	104.96
-2				74.68	76.35
-3				76.76	77.72
-4				70.18	72.44
-5				77.86	78.55
-6				60.96	68.94
Rham-1				99.74	102.93
-2				70.43	72.47
-3				70.59	72.69
-4				71.95	74.26
-5				70.18	70.19
-6				18.28	

Total Phenolic Content and Antioxidant Activity. To investigate the antioxidant activities of sea buckthorn leaves, we extracted phenolic compounds using methanol and analyzed the total phenolic content (**Table 4**). The total phenolic content ranged from 48 to 477 mg GAE/g. Total phenolic contents were determined to be in the following order: BuOH fraction (477 mg GAE/g) > methanolic extract (190 mg GAE/g) > aqueous fraction (173 mg GAE/g) > hexane fraction (48 mg GAE/g). Polyphenolic constituents in the BuOH fractions of sea buckthorn leaves most likely therefore contributed to their high antioxidant activities (DPPH free radical scavenging activity and reducing power activity).

The methanolic extract, the fractions, and isolated compounds of sea buckthorn leaves altogether displayed various free radical scavenging activities (**Table 5**). The BuOH fraction (EC_{50} = 1.81 μg/mL) along with Com-4 (EC_{50} = 1.86 μg/mL) had high free radical scavenging activities. The DPPH free radical scavenging activities of isolated compounds were in the following order: Com-4 (EC_{50} = 1.86 μg/mL) > Com-1 (EC_{50} = 5.32 μg/mL) > Com-2 (EC_{50} = 13.79 μg/mL) > Com-5 (EC_{50} = 59.83 μg/mL) > Com-6 (EC_{50} = 87.19 μg/mL) > Com-3 (EC_{50} = 159.20 μg/mL). Com-4 contains 2,3-double bonds in conjugation with a 4-oxo group in the C-ring as well as an *o*-dihydroxyl (catechol) group in the B-ring. Com-4 demonstrated higher free radical scavenging activity, consistent with Cai et al. (*31*), who studied the relationship between structure and free radical scavenging activity for

Table 3. ^{1}H and ^{13}C NMR Data for Compound **4** (400 MHz, DMSO-d_6)

no.	^{1}H	^{13}C
2		158.49
3		135.64
4		179.5
5		163.07
6	6.19 (d, 1.77)	99.95
7		166.17
8	6.38 (d, 1.77)	94.77
9		159.02
10		105.68
12		116.03
13		145.94
14		149.89
15		117.58
16		123.23
2′	7.71 (d, 1.89)	
5′	6.87 (d, 8.43)	123.09
6′	7.58 (dd, 1.89, 8.43)	104.34
7′		75.76
8′		78.14
9′		71.23
10′		78.42
1″		62.57
anomeric H	5.25 (d, 7.47)	

Table 4. Total Phenolic Content of the Leaf Extracts from *Hippophae rhamnoides*

sample	TPC[a] (mg GAE/g)
HR-ex (methanolic extract)	190 ± 3
HR-H (*n*-hexane fraction)	48 ± 6
HR-B (*n*-BuOH fraction)	477 ± 7
HB-W (aqueous fraction)	173 ± 12

[a] Total phenol content analyzed as gallic acid equivalent (GAE) mg/g of extract and fractions; values are the mean ± standard derivation of triplicates.

Table 5. DPPH Free Radical Scavenging Activity of Extract, Fractions, and Isolated Compounds from *Hippophae rhamnoides*

sample	EC_{50}[a] (μg/mL)
HR-ex (methanolic extract)	5.04 ± 0.47
HR-H (*n*-hexane fraction)	30.19 ± 0.36
HR-B (*n*-BuOH fraction)	1.81 ± 0.26
HB-W (aqueous fraction)	5.40 ± 0.65
Com-1 (kaempferol-3-*O*-β-D-(6″-*O*-coumaryl)glycoside)	5.32 ± 1.24
Com-2 (1-feruloyl-β-D-glucopyranoside)	13.79 ± 0.06
Com-3 (isorhamnetin-3-*O*-glucoside)	159.20 ± 12.33
Com-4 (quercetin 3-*O*-β-D-glucopyranoside)	1.86 ± 0.22
Com-5 (quercetin 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside)	59.83 ± 6.09
Com-6 (isorhamnetin-3-*O*-rutinoside)	87.19 ± 9.70
BHA (butylated hydroxyanisole)	4.17 ± 0.56
BHT (butylated hydroxytoluene)	44.88 ± 3.85
AsA (ascorbic acid)	1.21 ± 0.03
vitamin E (α-tocopherol)	3.29 ± 0.37

[a] Amount required for 50% reduction of DPPH after 30 min. Each value is the mean ± standard derivation of triplicate tests.

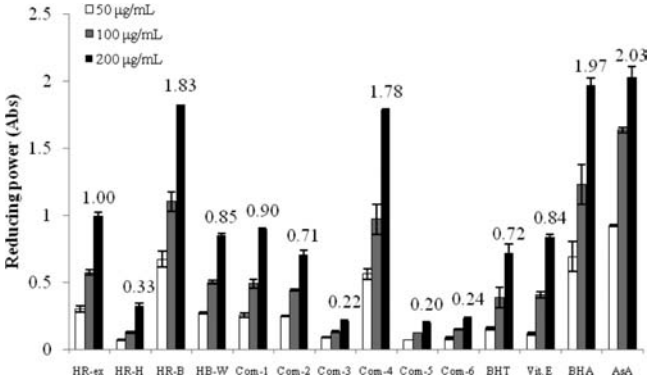


Figure 2. Reducing power of extract, fractions, and isolated compounds from *Hippophae rhamnoides*. For abbreviations, see **Table 5**.

many phenolic compounds. For Com-3 and Com-4, there were many differences in free radical scavenging activity produced by methylation of the 3′-hydroxyl group of the B-ring. The free radical scavenging activity was increased by the presence of a hydroxyl group at C-3 (*32*), but was decreased upon glycolation as compared to the data in a previous study (*33*) (isorhamnetin (EC_{50} = 5.7 μg/mL) vs isorhamnetin-3-*O*-glucoside (EC_{50} = 159.20 μg/mL) or isorhamnetin-3-*O*-rutinoside (EC_{50} = 87.19 μg/mL)). In addition, the BuOH fraction (EC_{50} = 1.81 μg/mL), which contained the highest level of phenolic compounds, likewise showed a free radical scavenging activity about 24.8-fold higher than that of the BHT (EC_{50} = 44.88 μg/mL) positive control (**Table 5**). These findings agreed with the model that phenolic compounds act as reducing agents, hydrogen donors, and singlet-oxygen quenchers during antioxidant mechanisms (*34*).

Reducing power was tested by colorimetric assay at 700 nm (**Figure 2**). Increasing the sample concentration resulted in the increase of ferric reducing antioxidant activity for all samples tested. The BuOH fraction (OD_{700} = 1.83) along with Com-4 (OD_{700} = 1.78) showed higher activity than α-tocopherol (OD_{700} = 0.84) or BHT (OD_{700} = 0.72), but did not exhibit higher antioxidant activity than that of BHA (OD_{700} = 1.97) and ascorbic acid (OD_{700} = 2.03). The hexane fraction, along with Com-3, Com-5, and Com-6, showed a moderate degree of reducing power. Therefore, the polyphenols present in the sea buckthorn leaves could act as reductor by donating electrons to free radicals and terminating the free radical mediated chain reaction.

The antioxidant activities of the extracts were measured by the FTC method, in comparison with the commercial antioxidants BHT and α-tocopherol. Low absorbance values measured via FTC method reflect high antioxidant activity. **Figure 3A** shows changes in absorbance for each sample during 24 h of incubation at 70 °C. Autoxidation of the linoleic acid emulsions of control, α-tocopherol, aqueous fraction, Com-1, Com-4, Com-5, and Com-6 was accompanied by a rapid increase in peroxide levels. In contrast, the significantly lower absorbances of methanolic extract, hexane fraction, BuOH fraction, and BHT indicated an increased antioxidant activity. Nonetheless, isolated compounds showed some degree of antilipid peroxidative activity. The order of increasing antioxidant activity for the isolated compounds was as follows: Com-2 > Com-4 > Com-6 > Com-5 > Com-1 (**Figure 3A**).

The FTC method measures the production of peroxide at the initial stages of linoleic acid oxidation, whereas the TBA method is used to estimate the production of secondary products such as aldehydes, ketones, etc. (*35*). During the oxidation of a linoleic acid emulsion, peroxides are gradually decomposed to lower molecular weight compounds such as malonaldehyde, which

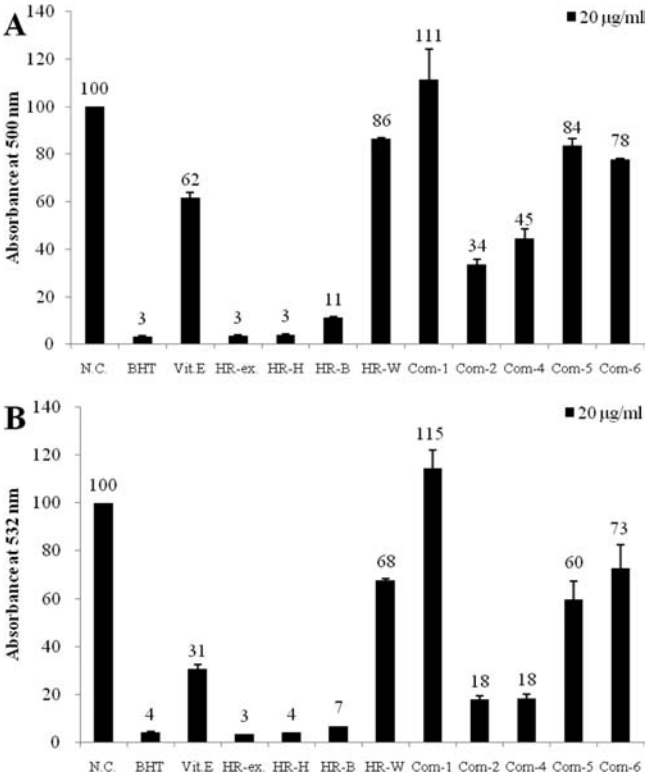


Figure 3. Antioxidative activity of extract, fractions, and isolated compounds from *Hippophae rhamnoides* as determined by the ferric thiocyanate (**A**) and thiobarbituric acid methods (**B**). N.C., negative control. For abbreviations, see **Table 5**.

can be measured by TBA method. The results obtained were similar to that of the FTC method (**Figure 3B**).

α-Glucosidase Inhibitory Effect. The inhibition of α-glucosidase was measured by using the substrate 4-nitrophenyl-α-D-glucopyranose. The BuOH fraction (86%) was the most effective inhibitor at a concentration of 5 μg/mL, followed by Com-1 (78%), methanolic extract (76%), and hexane fraction (70%) (**Figure 4**). The BuOH fraction showed an α-glucosidase inhibitory effect similar to that of the known α-glucosidase inhibitor acarbose (IC_{50} = 2.1 μg/mL), but the other samples showed effects lower than that of the positive control (not shown). Among the six isolated compounds, Com-1 had the highest α-glucosidase inhibitory effect, which was in the following order: Com-1 > Com-6 > Com-3 > Com-2 > Com-4 > Com-5. Although the 3′,4′-dihydroxylated double bond between C2 and C3 in the C-ring showed high α-glucosidase inhibitory activity (*32*), Com-4 and Com-5 showed low values of inhibitory rate due to glycolation. As seen in **Table 5**, the BuOH fraction contained the highest level of phenolic compounds and also the highest α-glucosidase inhibitory activity. In our previous paper, *Rhus verniciflua* methanolic extract along with the EtOAc and BuOH fractions had high phenolic contents and high α-glucosidase inhibitory activities (*32*). This suggests that high phenolic content in the BuOH fraction from sea buckthorn may contribute to the α-glucosidase inhibition.

In conclusion, six active components from sea buckthorn leaf extract were isolated (kaempferol-3-*O*-β-D-(6″-*O*-coumaryl) glycoside, 1-feruloyl-β-D-glucopyranoside, isorhamnetin-3-*O*-glucoside, quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside, and isorhamnetin-3-*O*-rutinoside). Among the six isolated compounds, quercetin 3-*O*-β-D-glucopyranoside showed the highest free radical scavenging activity and kaempferol-3-*O*-β-D-(6″-*O*-coumaryl) glycoside

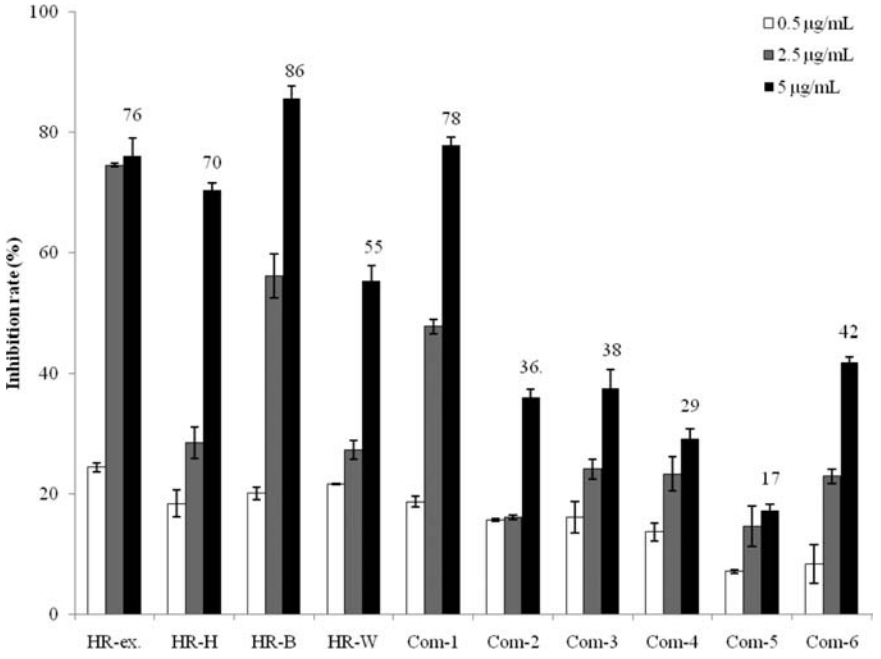


Figure 4. Dose-dependent changes in α-glucosidase inhibition for extract, fractions, and isolated compounds from *Hippophae rhamnoides*. For abbreviations, see **Table 5**.

had the highest α-glucosidase inhibitory activity. It is concluded that sea buckthorn leaf extracts could potentially be used for food additives and the development of useful natural compounds.

ABBREVIATIONS USED

DM, diabetes mellitus ; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FTC, ferric thiocyanate; HR, *Hippophae rhamnoides*; LDL, low-density lipoprotein; ROS, reactive oxygen species; TBA, thiobarbituric acid.

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