



HPLC analysis of Phenolic Substances and Anti-Alzheimer's Activity of Korean *Quercus* Species

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Abstract – This study aimed to establish the quantitative method to analyze the content of peroxynitrite-scavengers belonging to polyphenols in six Korean *Quercus* species (*Quercus mongolica*, *Q. dentata*, *Q. acutissima*, *Q. aliena*, *Q. serrata*, and *Q. variabilis*) by HPLC. The twelve peroxynitrite-scavengers, flavanols (catechins: (+)-catechin, (–)-epicatechin, and (–)-epigallocatechin), flavonols (kaempferol and quercetin), flavonol glycosides (astragalol glycosides (astragalol 6"-gallate and isoquercitrin 6"-gallate), gallic acid and its dimer (ellagic acid) were analyzed by HPLC. Further, anti-Alzheimer's activity was assayed in a passive avoidance test using mice by measuring the retention latency (sec), the concentration of acetylcholine (ACh), and acetylcholinesterase (AChE) activity. Simultaneous analysis of the extracts of the six *Quercus* leaves was achieved on a Capcell C18 column (5 µm, 250 mm × 4.6 mm i.d.) with a gradient elution of 0.05% HAc and 0.05% HAc in CH₃CN. In the extract of *Q. mongolica* leaves, the content of gallic acid (32.53 mg/g), (+)-catechin (28.78 mg/g), (–)-epicatechin (22.03 mg/g), astragalol 6"-gallate (20.94 mg/g), and isoquercitrin 6"-gallate (44.11 mg/g) and peroxynitrite-scavenging activity (IC₅₀, 0.831 µg/ml) were high. This extract delayed the retention latency and inhibited acetylcholinesterase activity in scopolamine-induced memory impairment of mice, suggesting that it has anti-Alzheimer's activity.

Keywords – *Quercus* species, *Quercus mongolica*, Fagaceae, Quantitative, HPLC, Passive avoidance test

Introduction

The Fagaceae plant, consisting of eight genus and more than 900 species, is distributed worldwide. In Korea, six *Quercus* species of the Fagaceae plants, *Quercus mongolica* (shingalnamu), *Q. dentata* (tukalnamu), *Q. acutissima* (sangsurinamu), *Q. aliena* (galchamnamu), *Q. serrata* (jolchamnamu), and *Q. variabilis* (gulchamnamu), are very popularly distributed. Each name in the parenthesis is Korean name. Since they bear nuts as fruit, in Korea they are usually called the six-nut-brothers.¹ The six plants are different species one another, although they have apparently similar morphology.

Flavanols, also called catechins, and procyanidins have been isolated from the stem bark of *Q. robur*,² and flavonoids including (+)-catechin and (–)-epicatechin have

been isolated from the leaves of *Q. caucheri*.³ In particular, the catechins such as (+)-catechin, (–)-epicatechin, (+)-gallocatechin, and (–)-epigallocatechin together with their 3-*O*-gallates are known to be contained mainly in green tea. These substances have preventive effects against cancer, cardiovascular disease, and neurological disorders.⁴

Peroxynitrite, a reactive nitrogen species, is formed from the combination of a nitric oxide radical and a superoxide anion radical. It nitrates tyrosine residues that exist in proteins, causes not only cytotoxicity and neurotoxicity but also atherosclerosis, diabetes mellitus, and hypercholesterolemia. It is known that peroxynitrite scavengers are particularly effective in the prevention of diabetic complications such as nephropathy, neuropathy, retinopathy, and cardiovascular dysfunction.⁵ Anti-Alzheimer's activities of certain antioxidants⁶ and peroxynitrite-scavengers⁷ have been reported.

On the other hand, Alzheimer's disease is a major part of dementia. This disease causes cognitive deficit, memory impairment, and behavioral disturbance. Furthermore, it is

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known that lower level of acetylcholine (ACh) concentration and β -amyloid ($A\beta$) aggregation mainly contributes to the pathogenicity of Alzheimer disease.⁸ Memory loss in Alzheimer's disease is usually attributed to a low concentration of acetylcholine in the brain. In these patients, the concentration of acetylcholine is lowered by a high activity of cholinesterase hydrolyzing ACh.⁹

Recently, it is known that oxidative stress causes neurodegeneration *via* $A\beta$ aggregation induced by amyloidogenic process of β -amyloid precursor protein (β -APP). Therefore, it was highly documented that natural products possessing antioxidant- and anti-cholinesterase activities are effective against Alzheimer's disease. Therefore, the experimental results on the HPLC analytical study of phenolic substances in the six *Quercus* species and passive avoidance test are described in this communication.

Experimental

Instruments and reagents – The Varian HPLC system used in the present study consisted of Prostar 210 pumps, Prostar 325 UV-Vis detector, and a Shiseido Capcell PAK C18 column (5 μ m, 4.6 \times 250 mm, Japan). MetaTherm temperature controller was used to maintain a constant temperature of the HPLC column. The solvents used as the mobile phase, H₂O and MeOH together with acetic acid for acidifying, were purchased from J.T.Baker (Phillipsburg, NJ, USA). Collected data were processed using a Varian Star Workstation. Ten standard compounds, (–)-epigallocatechin, (+)-catechin, (–)-epicatechin, gallic acid, ellagic acid, isoquercitrin, astragalin, quercitrin, quercetin, and kaempferol, were purchased from Sigma Co. (St. Louis, MO, USA). The two compounds, astragalin 6"-gallate and isoquercitrin 6"-gallate, that have been isolated from *Euphorbia supina* were used as standards.¹⁰

Plant material – The leaves of six *Quercus* species, *Q. mongolica*, *Q. dentata*, *Q. acutissima*, *Q. aliena*, *Q. serrata*, and *Q. variabilis*, were collected in June 2014 in the mountain area in Wonju, Korea. Each collected set of leaves was dried and pulverized for extraction. The six plants were identified by Byong-Min Song, Department of Forestry Science, Sangji University, Korea. Voucher specimens were deposited in the Laboratory of Natural Product Chemistry, Department of Pharmaceutical Engineering, Sangji University, Korea.

Extraction – 5 g of dried, crushed, and lyophilized plant materials (leaves) was soaked in 250 ml 80% MeOH and extracted using ultrasonication for 5 hours at 60 °C. After cooling, the extract solution was filtered using filter paper and evaporated on a vacuum rotary evaporator. The extract

was dried using a freeze dryer, and weighed to measure the yield of extract.

Preparation of standard- and sample solutions – Standard stock solutions (1,000 μ g/ml) were prepared by dissolving each standard compound in MeOH, and stored 4 °C or below. Working standard solutions were prepared by serial dilution of standard stock solutions. The regression equation was determined by plotting the peak area (y) versus six concentrations (x , μ g/ml). To prepare the sample solution, each lyophilized extract was sufficiently dissolved in MeOH using ultrasonication. The solutions were filtered using a disposable syringe filter (0.50 μ m, Dismic-25JP, Advantec, Japan) prior to injection into HPLC system.

HPLC analytical method – Two solvents, solvent A (H₂O with 0.05% acetic acid, v/v) and solvent B (CH₃CN with 0.05% acetic acid, v/v) were used in this experiment. The linear gradient elution of the solvents was programmed as follows: 0 – 35 min (15 \rightarrow 65% B), 35 – 40 min (65% B), 40 – 42 min (65 \rightarrow 100% B), 42 – 46 min (100% B), 46 – 49 min (100 \rightarrow 15% B), and 49 – 55 min (15% B). The flow rate and column temperature were fixed at 1.0 ml/min and 40 °C, respectively. The detection wavelength was fixed at 254 nm and monitored over 40 minutes for each sample.

Validation of the HPLC method – The validation of the HPLC method was performed following ICH guideline (International Conference on Harmonization) in terms of linearity, sensitivity, precision, and accuracy. Linearity was evaluated by calculating the R^2 value of each regression equation. Sensitivity was evaluated by calculating the values of LOD (limit-of-detection) and LOQ (limit-of-quantification). The LOD and LOQ values were determined by the signal-to-noise (S/N) method, where an S/N ratio of 3 was used for LOD and 10 for LOQ.

Accuracy and precision of the method were investigated by the intermediate evaluation method measuring the intra-day and inter-day variability. The intra-day variability was examined by injecting sample solutions repetitively onto a HPLC system on the same day, and the inter-day variability was assessed by testing five times a day four consecutive days. Relative standard deviation (RSD) was determined by calculating the retention times and peak area produced from five different experiments. RSD values were considered as the measure of accuracy and precision. To assess accuracy, recovery tests were carried out by adding a standard compound to the sample solution. Recovery rates (%) were determined by calculating the rate of the spiked extract solution versus the non-spiked extract solution.

Peroxy-nitrite-scavenging assay – The assay to assess peroxy-nitrite-scavenging activity was performed by modif-

ying the method described by the Kooy *et al.*,¹¹ which monitors highly fluorescent rhodamine 123 rapidly formed from non-fluorescent DHR 123. The rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μ M DTPA. The final concentration of DHR 123 was 5 μ M. The buffer solution was prepared and stored in an ice bath prior to use. Plant extracts were dissolved in 10% DMSO to prepare concentrations of 0.08, 0.4, 2, and 10 μ g/ml.

The final intensity was measured with or without treatment with 10 μ M peroxyxynitrite in 0.3N NaOH. The fluorescence intensity was measured at the excitation and emission wavelengths of 480 nm and 530 nm using the microplate reader FL (Bio-Tek Instruments Inc., Winooski, VT, USA). Peroxyxynitrite-scavenging activity was determined by subtracting the background fluorescence from the final fluorescence intensity, which was measured by detection of DHR 123 oxidation. L-Penicillamine was used as a positive control, and the data is expressed as the mean \pm SEM.

Animals and treatment – The 5 week-old ICR male mice purchased from SamtcoBiokorea Co. were adapted in a constant condition (temperature 22 °C, dampness 40 – 60%, light/dark cycle 12 h). Mice were divided into the five groups (each 10 mice): untreated group (n=5), control group (n=5), QM 50 group (n=7) treated with *Q. mongolica* extract (50 mg/kg dose), QM 100 group (n=7) treated with the same extract (100 mg/kg dose) and the positive control group (n=5) treated with donepezil (4 mg/kg). The two groups, QM 50 and QM 100, were orally administered to mice for 4 weeks at the 50 mg/kg and 100 mg/kg dose. Passive avoidance test were performed 30 min after the intraperitoneal injection of scopolamine (5 mg/kg dose).

Passive avoidance test – The apparatus used for the passive avoidance test was a shuttle box (50 \times 15 \times 40 cm, electric grid floor, Ugo, Italy) consisting of two compartments (25 \times 15 cm) divided by a connecting guillotine door (10 \times 10 cm).¹² Each compartment was designed so that it can be illuminated by an electric light bulb (20 W). This experiment was carried out under less than 60 dB noise and dark illumination. The animal was placed on the lighted compartment (1,500 lux) and then the guillotine door was opened. The door was designed so that it was automatically closed when a mouse entered the dark compartment. Foot-shock was given to the animal in the dark compartment with 3 mA electricity for 3 second using a stainless grid positioned on the bottom of dark compartment. This training trial was repeated five times so that the animal can learn the relationship between the foot-

shock and the compartment.

Anti-cholinesterase activity – After adding 2.6 ml of 0.1 M phosphate buffer (pH 8.0), 0.1 ml of 10 mM Ellman's solution and 0.2 ml enzyme source in the substrate of acetylcholine iodide, the absorbance was measured at 410 nm. Then, the anti-cholinesterase activity was calculated from the absorbance change at 410 nm for 2 min after adding 0.02 ml of 75 mM acetylcholine iodide.¹³ The activity was expressed as the unit of nmol/mg protein/min.

Acetylcholine concentration – 0.05 ml brain homogenate was mixed with 0.05 ml 1% hydroxylamine, and then 0.5 ml FeCl₃ (10% in 0.1N HCl) was added. The concentration of acetylcholine was determined by measuring the absorbance of the solution at 540 nm (Hestrin, 1949).¹⁴ The concentration was expressed as the unit of μ mol/mg protein.

Measurement of body and brain weight – The animal was fasted for 12 h after the test, and weighed for measurement of body weight. Further, mice were anesthetized with CO₂, the abdomen was opened, and the blood was collected from abdominal aorta. Then, the brains of mice were disclosed from the mice were washed, dried, and weighed.

Result and Discussion

We have reported the quantification of polyphenols with peroxyxynitrite-scavenging activity in various plant extracts. Such compounds were found to mainly belong to flavonoids, phenylpropanoids, and caffeoylquinic acids. Since the leaf extracts of the *Quercus* species showed potent peroxyxynitrite-scavenging activity during our preliminary study, we attempted to establish the HPLC analytical method to quantify such peroxyxynitrite scavengers contained in the leaves of the *Quercus* species through the validation experiment.

In the present study, we were concerned with the quantification of phenolic substances mainly flavonoids, since some phenolic substances have been qualitatively identified from the *Quercus* species.¹ The twelve phenolic substances, flavanols, flavonols and their glycosides, acylated flavonol glycosides, and gallic acid and its dimer (ellagic acid), were used as standard compounds for quantification, as shown in Fig. 1.

To establish a reliable HPLC method, the method for the mobile phase, gradient elution, UV wavelength and column temperature was optimized through repetitive experimentation. The two solvents, 0.05% HAc in H₂O and 0.05% HAc in CH₃CN, were chosen, since they were highly resolvable and environmentally-friendly. The addition of HAc improved peak shapes, likely by inhibiting the

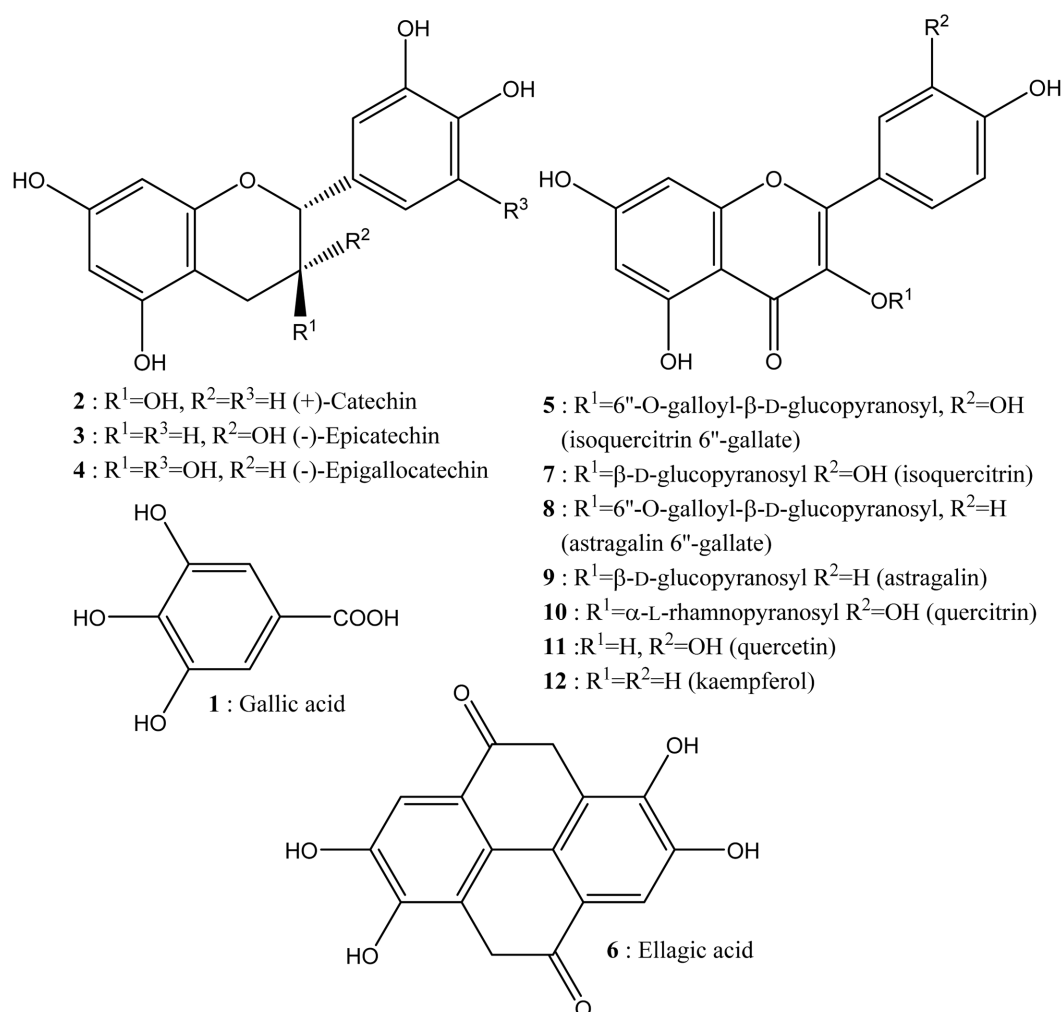


Fig. 1. Structure of the twelve phenolic compounds used for the analysis of *Quercus* species.

ionization of phenolic OHs. Gradient elution was employed to achieve better separation in a shorter time frame. Better separation and more constant retention times were shown when the column temperature was fixed at 40 °C.

The optimized HPLC method was validated by investigating linearity, sensitivity, precision, and accuracy, as shown in Tables 1 and 2. Linearity of each regression equation was established since the R^2 values were more than 0.999. The method was sufficiently precise and stable because the RSD values obtained from the intra-day and inter-day variability tests were over the ranges of 0.66–4.15% and 1.17–4.91%, respectively. Recovery rates (%) were between 92.71 and 104.69%, indicating that this analytical method is accurate.

The HPLC chromatograms of the twelve standard compounds and six extracts are shown in Fig. 2, and the contents of the components are in Table 3. The plant extract with higher extraction rates also exhibited higher total

amounts of polyphenols. Representatively, the extract of *Q. mongolica* exhibited 164.90 mg/g total amount of extract, and its extraction rate was the highest at 24.8%. Eleven compounds were observed in *Q. mongolica*, although (–)-epigallocatechin was not identified. In the *Q. mongolica* extract, the contents of isoquercitrin, astragaline, quercitrin, quercetin, kaempferol, and ellagic acid were relatively low, whereas the content of isoquercitrin 6''-gallate (44.11 mg/g) and astragaline 6''-gallate (20.94 mg/g) were high, indicating that isoquercitrin and astragaline mainly exist in the form of their 6''-O-gallates. In this extract, the content of (+)-catechin (28.78 mg/g) and (–)-epicatechin (22.03 mg/g) were also high, although (–)-epigallocatechin is not present. The content of gallic acid and its dimer, ellagic acid, were 32.53 mg/g and 3.37 mg/g, respectively. In particular, isoquercitrin 6''-gallate and astragaline 6''-gallate were not observed in *Q. dentata*, whereas the flavanols were not observed in *Q. variabilis*. A class of flavanols mainly

Table 1. Linearity and limit of detection and quantification (LOD and LOQ) of the analytes

Compound	Equation of the linear regression ^a	Linear range (µg/mL)	R ² ^b	t _R	LOD ^c (µg/mL)	LOQ ^d (µg/mL)
Gallic acid (1)	y = 321.92x + 78.01	3.13-100.0	0.9998	3.41	0.13	0.44
(-)-Epigallocatechin (2)	y = 44.059x + 24.48	15.6-500.0	0.9996	4.21	2.17	7.23
(+)-Catechin (3)	y = 43.089x + 30.85	15.6-500.0	0.9995	5.21	2.07	6.90
(-)-Epicatechin (4)	y = 60.405x + 43.58	15.6-500.0	0.9996	6.21	1.26	4.21
Isoquercitrin 6"-gallate (5)	y = 166.69x + 73.31	3.13-100.0	0.9998	8.25	0.28	0.93
Ellagic acid (6)	y = 1651.8x + 94.63	0.78-25.00	0.9991	8.94	0.03	0.10
Isoquercitrin (7)	y = 472.75x + 75.62	1.56-50.00	0.9997	9.19	0.09	0.31
Astragalin 6"-gallate (8)	y = 255.79x + 59.05	3.13-100.0	0.9998	10.17	0.24	0.80
Astragalin (9)	y = 459.93x + 56.94	1.56-50.00	0.9997	10.72	0.14	0.50
Quercitrin (10)	y = 456.52x + 72.11	1.56-50.00	0.9998	10.93	0.11	0.35
Quercetin (11)	y = 663.43x + 79.10	1.56-50.00	0.9996	16.31	0.06	0.21
Kaempferol (12)	y = 460.36x + 63.79	1.56-50.00	0.9997	19.89	0.12	0.41

^ay, peak area at 254 nm; x, concentration of the standard (µg/ml); ^bR², correlation coefficient for 6 data points in the calibration curves (n = 3);

^cLOD, limit of detection (S/N = 3); ^dLOQ, limit of quantification (S/N = 10).

Table 2. Recovery and precision data of each analyte

Compounds	Recovery test					Precision test				
	Initial conc. (µg/ml)	Amount added (µg)	Concentration after addition (µg/ml)		Recovery (%)	RSD (%)	Intra-day variability RSD (%)		Inter-day variability RSD (%)	
			Expect	Real			t _R	Area	t _R	Area
Gallic acid	16.26	12.50	28.76	27.17	94.45	1.39	0.43	2.69	0.92	4.76
(-)-Epigallocatechin	9.240	15.63	24.87	23.06	92.71	2.81	0.41	4.15	0.89	6.12
(+)-Catechin	14.39	15.63	30.02	28.29	94.22	1.97	0.21	3.97	0.75	4.91
(-)-Epicatechin	11.02	15.63	26.64	24.81	93.12	2.45	0.19	3.24	0.63	4.28
Isoquercitrin 6"-gallate	22.06	25.00	47.05	45.81	97.35	0.57	0.15	2.54	0.67	4.21
Ellagic acid	1.685	1.560	3.245	3.397	104.69	2.01	0.14	1.54	0.51	2.92
Isoquercitrin	4.475	3.130	7.605	7.773	102.21	1.79	0.11	1.37	0.39	2.86
Astragalin 6"-gallate	10.47	12.50	22.97	22.62	98.47	0.51	0.09	0.66	0.41	1.17
Astragalin	1.260	1.560	2.820	2.712	96.18	1.53	0.08	0.76	0.25	1.23
Quercitrin	0.625	0.780	1.405	1.344	95.65	2.16	0.06	0.92	0.31	1.59
Quercetin	0.100	0.780	0.880	0.891	101.28	2.31	0.06	1.32	0.69	1.74
Kaempferol	0.105	0.780	0.885	0.904	102.15	2.49	0.08	1.45	0.78	2.18

Recovery tests were performed on the extract of *Q. mongolica* spiked with each standard compound except for (-)-epigallocatechin. The tests of (-)-epigallocatechin were performed on the extract of *Q. dentata*. Relative standard deviation (RSD) values of precision tests were calculated for both retention time (t_R) and peak area of three independent experiments.

include (+)-catechin, (+)-gallocatechin, (-)-epicatechin, and (-)-epigallocatechin together with their 3-*O*-gallates. However, their 3-*O*-gallates and (+)-gallocatechin were not identified (data not shown).

The peroxy-nitrite-scavenging activities of the six extracts are shown in Table 4. The inhibitory rate (%) measured at the four concentrations (0.08, 0.4, 2, and 10 µg/ml) are shown together with their IC₅₀ values. Of the six extracts,

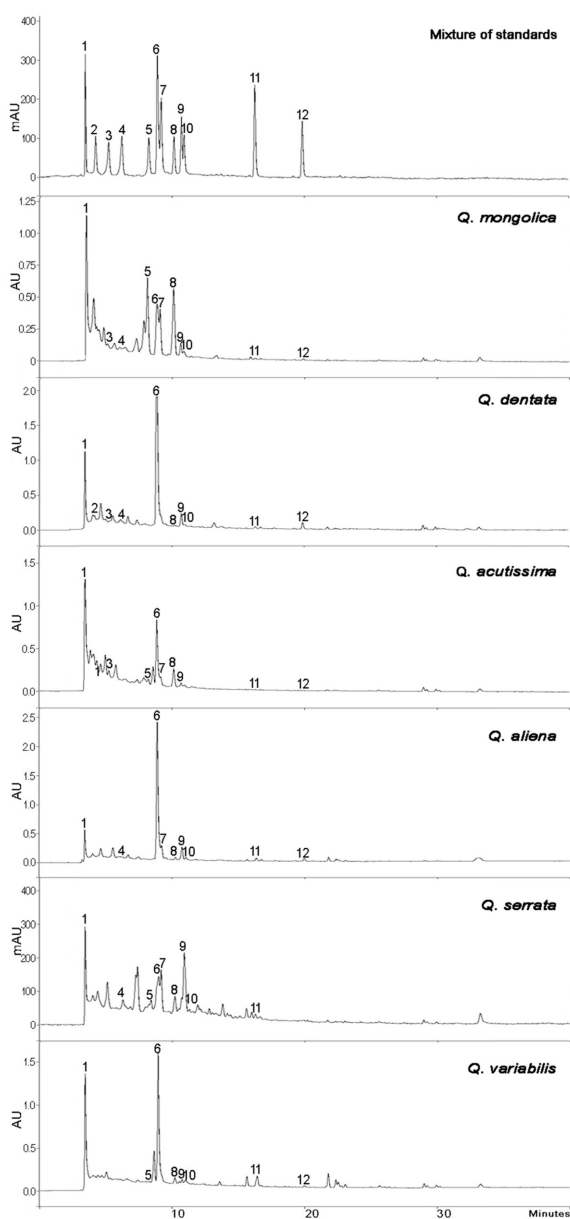
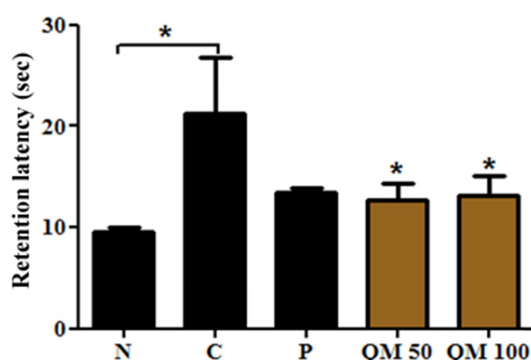
the 80% MeOH extract obtained from *Q. mongolica* exhibited the highest peroxy-nitrite-scavenging activity. Both the total amount of phenolic substances and peroxy-nitrite-scavenging activity were highest in *Q. mongolica*. The activities were in the following order: *Q. mongolica* (IC₅₀, 0.831 µg/ml) > *Q. acutissima* (1.316 µg/ml) > *Q. variabilis* (1.451 µg/ml) > *Q. aliena* 1.503 µg/ml > *Q. serrata* (1.672 µg/ml) > *Q. dentata* (1.727 µg/ml). Of the twelve phenolic

Table 3. Amount of compounds in the leaf extracts (80% MeOH) of six *Quercus* species

Plant material	Yield of extract (%)	Amount of compounds in the extract (mg/g)												Total
		1	2	3	4	5	6	7	8	9	10	11	12	
<i>Q. mongolica</i>	24.8	32.53	–	28.78	22.03	44.11	3.37	8.95	20.94	2.52	1.25	0.20	0.21	164.90
<i>Q. dentata</i>	14.6	23.25	18.48	27.39	28.90	–	14.95	–	0.59	3.99	1.22	0.44	1.94	121.14
<i>Q. acutissima</i>	23.6	50.31	–	49.05	–	10.96	6.07	6.19	12.40	6.68	–	0.07	0.12	141.85
<i>Q. aliena</i>	12.6	18.70	–	–	19.85	–	16.04	6.44	2.96	6.69	1.73	1.04	1.36	74.81
<i>Q. serrata</i>	18.0	10.06	–	–	20.01	2.91	1.56	3.15	2.44	1.65	6.34	0.19	–	48.33
<i>Q. variabilis</i>	9.80	37.94	–	–	–	6.26	7.42	–	2.63	0.97	1.37	2.07	0.28	58.95

The sign (–) indicates that the compound cannot be quantified (< LOQ) or not detected (< LOD) under 254 nm UV wavelength.

Compounds: **1** (gallic acid), **2** (–)-epigallocatechin, **3** (+)-catechin, **4** (–)-epicatechin, **5** (isoquercitrin 6"-gallate), **6** (ellagic acid), **7** (isoquercitrin), **8** (astragalol 6"-gallate), **9** (astragalol), **10** (quercitrin), **11** (quercetin), and **12** (kaempferol).

**Fig. 2.** HPLC chromatograms of mixed standards and the extracts of six *Quercus* species.**Fig. 3.** Effect of the extract of *Q. mongolica* leaves on the retention latency in passive avoidance test. QM 50 and QM 100 represent the group of mice treated with 50 and 100 mg/kg dose, respectively. The retention test was performed 24 h after the training trial. Normal group (N) of mice without any treatment (n = 5); Control group (C) was intraperitoneally injected with 1 mg/kg of scopolamine (n = 5); The positive control group (P) was injected with donepezil (x mg/kg); The two treatment groups, QM 50 and QM 100, were orally administered for 4 weeks before the training trial. Bars represent means \pm SEM of retention latency. * $p < 0.05$ vs. the C.

substances, potent activities of flavanols have been reported.¹⁵ Therefore, the extract of *Q. mongolica* leaves with the highest content of peroxynitrite scavengers will be used to prevent diabetic complications such as nephropathy, neuropathy, retinopathy, and cardiovascular disease that can be caused by the overproduction of peroxynitrites.⁵ In addition, it was established to reliably analyze the peroxynitrite scavengers in the Korean *Quercus* species by the validation experiments.

Since oxidative stress or increased formation of peroxynitrite is reported to result in cognitive impairment,¹⁶ we evaluated the *in vivo* anti-Alzheimer's activity of the extract of *Q. mongolica* leaves using passive avoidance test. As in the control group (Fig. 3), treatment of mice with scopolamine delayed the retention latency (sec) staying in the dark compartment compared to the normal group. This indicates that administration of scopolamine-induced memory

Table 4. Peroxynitrite-scavenging effect of the leaf extracts of six *Quercus* species

Extract	Concentration ($\mu\text{g/ml}$)				IC ₅₀ $\mu\text{g/ml}$
	0.08	0.4	2	10	
<i>Q. acutissima</i>	12.41 \pm 0.46 ^a	31.07 \pm 0.45	67.37 \pm 0.18	91.93 \pm 0.27	1.316 \pm 0.011
<i>Q. aliena</i>	16.76 \pm 2.67	32.84 \pm 3.54	59.19 \pm 2.00	88.37 \pm 0.12	1.503 \pm 0.117
<i>Q. dentata</i>	12.41 \pm 0.98	20.59 \pm 2.06	56.07 \pm 0.62	89.01 \pm 0.07	1.727 \pm 0.037
<i>Q. mongolica</i>	31.06 \pm 0.01	48.02 \pm 0.62	70.68 \pm 0.21	87.55 \pm 0.36	0.831 \pm 0.015
<i>Q. serrata</i>	7.81 \pm 1.04	24.23 \pm 1.21	57.10 \pm 0.65	87.63 \pm 0.25	1.672 \pm 0.012
<i>Q. variabilis</i>	15.49 \pm 1.19	24.29 \pm 0.36	63.59 \pm 0.07	89.35 \pm 0.15	1.451 \pm 0.001
L-penicillamine	–	38.92 \pm 0.07	73.74 \pm 0.86	91.37 \pm 0.07	0.910 \pm 0.015

^aValue represents the mean \pm SEM (n = 2).

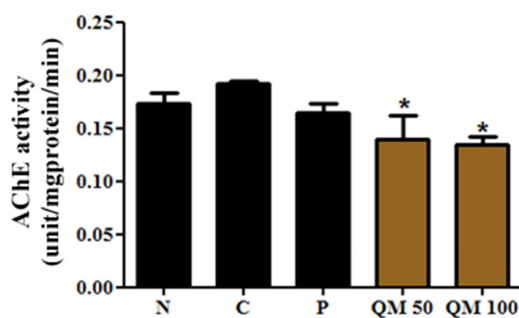


Fig. 4. Acetylcholinesterase inhibitory activity of the extract of *Q. mongolica* leaves at the 50 (QM 50) and 100 mg/kg (QM 100) dose. Acetylcholinesterase inhibitory activity of the extract of *Q. mongolica* leaves at the 50 (QM 50) and 100 mg/kg (QM 100) dose. * $p < 0.001$ vs. C.

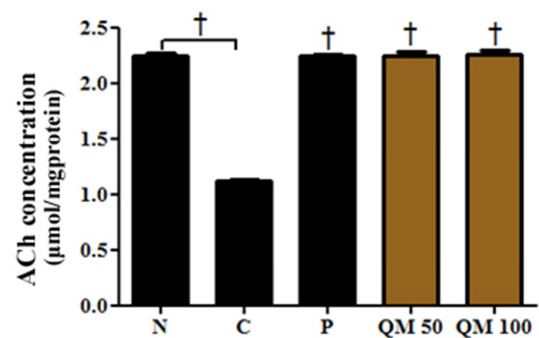


Fig. 5. Effect of the extract of *Q. mongolica* leaves on the concentration of acetylcholine in scopolamine-treated mice. Effect of the extract of *Q. mongolica* leaves on the concentration of acetylcholine in scopolamine-treated mice. † $p < 0.001$ vs. C.

impairment. On the contrary, treatments of mice with QM 50 (the extract of *Q. mongolica* leaves, 50 mg/kg dose) and QM 100 (100 mg/kg dose), and donepezil for the positive control considerably shortened the retention latency of the control group, suggesting that the QM extract is effective against the memory impairment.

As shown in Fig. 4, AChE activity in the control group was higher than in the normal group. The treatment of mice with QM reduced AChE activity. Furthermore, the concentration of AChE increased in the brain. Prevention of memory loss could be attributed to the increase of acetylcholine concentration by the treatment of QM.

The average body- and brain weights of mice were shown in Fig. 5. No change of body weight was observed among the five groups. Although no change of the brain weight was observed, it was statistically significant between the two groups of QM 50 and QM 100. Since the reduction of a brain weight is usually appeared in patients of Alzheimer's disease, QM extract would be beneficial for this disease.

It has been reported that oxidative stress and free radicals cause Alzheimer's disease *via* neurodegenerative toxicity.

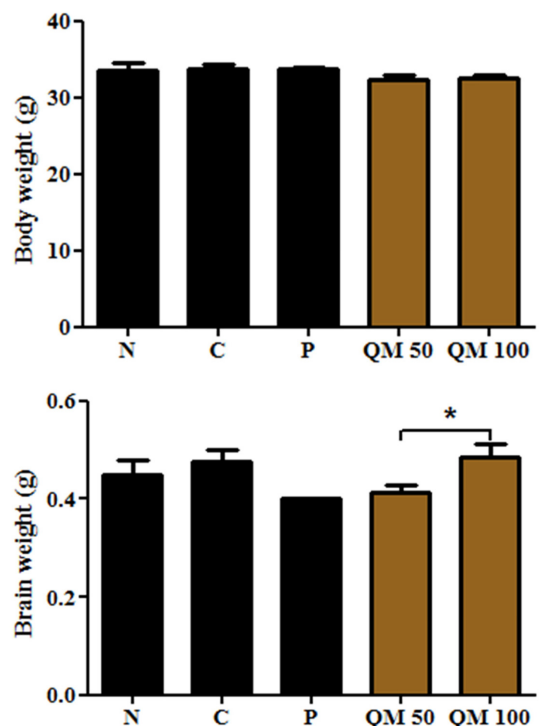


Fig. 6. Effect of the extract of *Q. mongolica* leaves on body and brain weight in scopolamine-treated mice.

Therefore, many natural phenolic substances are effective against Alzheimer's disease based on the reduction of acetylcholinesterase activity or oxidative stress.^{17,18} Furthermore, oxidative stress induces the accumulation of amyloid- β peptide (A β) causing neurotoxicity depending on amyloidogenic process of β -amyloid precursor protein.¹⁹ It was reported that many natural phenolics are active on Alzheimer's disease as in the examples of salvianolic acid B,²⁰ ellagic acid,²¹ curcumin,²² flavanone,²³ rutin, and galangin.²⁴

Therefore, it is concluded that the QM containing the phenolic substances, isoquercitrin 6"-gallate, astragalol 6"-gallate, (+)-catechin, (-)-epicatechin, gallic acid, may be active on the memory loss induced by the decrease of acetylcholine concentration in the brain. Furthermore, it may be beneficial to prevent neurodegenerative toxicity observed in Alzheimer's disease by inhibiting oxidative stress or peroxynitrite formation.

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