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Flavonoid Glycosides as Acetylcholinesterase Inhibitors from the Whole Plants of *Persicaria thunbergii*

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Abstracts – The *n*-BuOH soluble fraction of the whole plant of *Persicaria thungergii* showed acetylcholinesterase inhibitory activity. Four flavonoid glycosides and a flavonoid were isolated from this fraction, and identified as quercitrin (**1**), luteolin-4'-*O*-*β*-D-glucopyranoside (**2**), quercetin (**3**), quercetin-3-*O*-glucuronide (**4**), and isorahmnetin-3-*O*-glucuronid (**5**), by chromatographed and spectral data, respectively. All isolated compounds were showed acetylcholinesterase inhibitory activity, with IC_{50} values of 243.1, 10.5, 39.1, 8.2 and 23.2 μM, respectively.
Keywords – *Persicaria thunbergii*, Flavonoids, Acetylcholinesterase inhibitory activity

Introduction

Dementia is one of the major diseases in aged people and is predicted to increase to 115.4 million people worldwide by 2050. Alzheimer's disease is the single most common form of dementia.[1] Alzheimer's disease is a naturally progressive neurodegeneration and an aging-associated disorder which is characterized by the deficits in the cholinergic system and the depositions of beta amyloid in the form of amyloid plaques and hyperphosphorylated tau in neurofibrillary tangles.[2] Oxidative stress and inflammation have been considered to be important factors of Alzheimer's disease.[3,4] However, major causes and progression mechanisms of the disease are not clearly clarified, which is one of the reasons for the difficulty in developing prevention techniques or treatment agents for Alzheimer's disease. Cholinergic hypothesis has been suggested as one of important causes of Alzheimer's disease from etiological research, which decreasing cholinergic function in the brain causes memory and learning impairments in Alzheimer's disease.[5,6] To date, this hypothesis has been widely accepted and acetylcholiensterase inhibitors are the most effective therapeutic agents for Alzheimer's disease.[7-9]

As part of continuing investigation to find acetylcholiensterase inhibitors from plants, we found that the *n*-BuOH soluble fraction of *Persicaria thunbergii* (= *Polygonum thunbergii*) showed inhibitory activity against acetylcholinesterase. *Persicaria thubergii* is distributed throughout Korea, China, Japan, and Russia.[10] It has been used in Chinese traditional medicine as analgesic and antidiarrheal agents.[11] Flavonoid compounds have been reported to be present in the plant and anticancer activity have been also reported from this plant. [12-14]

In the present study, repeated chromatography of the *n*-BuOH soluble fraction of MeOH extracts from the whole plant of *Persicaria thunbergii* led to the purification of five compounds. The structure elucidation of isolates and their acetylcholesterase inhibitory activity are described herein.

Experimental

General experimental procedures – UV/Vis determinations were carried out using a V-530 spectrophotometer (JASCO, Tokyo, Japan). The MS spectrum was measured using an API 3200 LC/MS/MS system (AB Sciex, USA). NMR spectra were recorded on an AVANCE 600 (Bruker, Rheinstetten, Germany). The chemical shifts were represented as parts per million (ppm) referenced to the residual solvent signal. Column chromatography was carried out using a Kieselgel 60, 63 - 200 μm and 40 - 63 μm (Merck,

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Darmstadt, Germany) and YMC gel ODS-A, 150 μm (YMC, Kyoto, Japan). TLC was performed on glass backed Kieselgel 60 F254 and RP F254s plates. All other chemicals and reagents used were of analytical grade. Electric eel acetylcholinesterase, acetylthiocholine iodide, and 5-5'-thiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (St. Louis, Mo.).

Plant material – The whole plant of *Persicaria thunbergii* was collected from Gonjicheon in Chuncheon, Kangwon Province, Korea (October, 2012). A voucher specimen (KNUH-W-12-10-1) was deposited in the Herbarium of College of Pharmacy, Kangwon National University, Korea.

Extraction and isolation – The air dried *Persicaria thunbergii* plant was cut into small pieces and extracted with MeOH (6.2 kg, 50 L × 2) for one week at room temperature. The MeOH extract (900 g) was suspended in water and then successively partitioned with *n*-hexane, $CHCl_3$, *n*-BuOH and water soluble residue. Each fraction was evaporated *in vacuo* to yield the residues of *n*-hexane fraction (fr.) (92 g), $CHCl_3$ fr. (4.2 g), and *n*-BuOH fr. (58 g). Among the fractions, the *n*-BuOH soluble fraction showed a 56.8% inhibition rate against acetylcholinesterase at 100 μg/mL. To isolate active compounds from the *n*-BuOH soluble fraction, various column chromatography separations were performed. The *n*-BuOH soluble fraction (56 g) was applied to silica gel column chromatography (1 kg, 63 - 200 μm, 15 × 50 cm) using isocratic elution with EtOAc : MeOH : H_2O (8 : 1 : 0.5) in order to divide the fraction into four fractions (Fr. 1 – Fr. 4). Fr. 2 (6.9 g) was re-chromatographed on silica gel (500 g, 63 - 200 μm, 10 × 50 cm) using isocratic elution with benzene : EtOAc : MeOH (9 : 2 : 1) to yield five sub-fractions (Fr. 2-1 – Fr. 2-5). Fr. 2-1-3 (1.2 g) was re-chromatographed on silica gel (90 g, 40 - 63 μm, 3 × 50 cm) using isocratic elution with benzene : EtOAc : MeOH (7 : 2 : 1) to give ten sub-fractions (Fr. 2-1-3-1 – Fr. 2-1-3-10). Fr. 2-1-3-8 (150 mg) and Fr. 2-1-3-9 (280 mg) were further purified by Sephadex LH20 (40 g, Pharmacia, 3 × 20 cm, 40% MeOH) to give compound **1** (12 mg) and **2** (4.5 mg), respectively. Fr. 3 (5.0 g) and Fr. 4 (10.2 g) were re-chromatographed on ODS (500 g, YMC gel, 150 μm, 15 × 50 cm) using isocratic elution with MeOH : H_2O (40 : 60) to yield six sub-fractions (Fr. 3-1 – Fr. 3-6). Fr. 3-3 (1.5 g) was re-chromatographed on silica gel (100 g, 40 - 63 μm, 3 × 50 cm) using isocratic elution with $CHCl_3$: MeOH (9 : 1) to give compound **3** (128.2 mg). Fr. 3-4 (2 g) was re-chromatographed on ODS flash column (130 g, Redisep®, Teledyne Isco) using isocratic elution with MeOH : H_2O (40 : 60) to give eight sub-fractions (Fr. 3-4-1 – Fr. 3-4-8).

Table 1. ^{13}C-NMR data of compounds **1** - **5** (150 MHz, CD_3OD)

No.	1	2	3	4	5
2	157.16	164.11	146.59	157.62	157.99
3	134.81	103.67	135.82	134.00	134.05
4	178.22	182.42	175.91	177.81	177.82
5	161.81	161.85	161.09	161.58	161.58
6	98.52	98.86	97.83	98.55	98.55
7	164.83	164.90	164.17	164.58	164.64
8	93.38	93.68	93.01	93.39	93.40
9	157.87	158.09	156.82	157.00	157.03
10	104.41	103.67	103.12	104.23	104.19
1'	121.45	125.86	122.74	121.44	121.48
2'	114.97	113.47	114.59	114.64	114.48
3'	145.04	147.29	144.81	144.54	148.46
4'	148.42	148.60	147.36	148.50	144.51
5'	115.52	116.61	114.83	115.82	115.91
6'	121.57	118.40	120.27	122.16	122.08
1"	102.15	101.83		102.88	103.39
2"	70.63	73.38		73.99	73.92
3"	70.73	76.14		76.17	75.92
4"	71.86	69.89		71.44	71.32
5"	70.51	77.07		75.63	75.68
6"	16.25	61.01		170.74	169.27
OCH_3					51.45

Chemical shifts are represented parts per million (δ)

Fr. 3-4-2 (450 mg) was purified by re-crystallization (30% MeOH) to give compound **4** (400 mg). Fr. 3-4-5 (300 mg) was purified by Sephadex LH20 (40 g, Pharmacia, 3 × 20 cm, 40% MeOH) to give compound **5** (100 mg).

Compound (1) – LC-ESI MS (negative mode) *m/z* : 447 $[M – H]^+$; UV λ_{max} (MeOH, nm) : 226 (sh), 256, 300, 347; ^1H-NMR (CD_3OD, 600 MHz) δ_H: 7.33 (1H, d, $J = 2.0$ Hz, H-2'), 7.30 (1H, dd, $J = 2.0$, 8.3 Hz, H-6'), 6.90 (1H, d, $J = 8.3$ Hz, H-5'), 6.36 (1H, d, $J = 2.0$ Hz, H-8), 6.19 (1H, d, $J = 2.0$ Hz, H-6), 5.34 (1H, br s, anomeric H), 0.93 (3H, d, $J = 6.1$ Hz, rhamnosyl methyl); ^{13}C-NMR (CD_3OD, 150 MHz) δ_C : see Table 1.

Compound (2) – LC-ESI MS (negative mode) *m/z* : 447 $[M – H]^+$; UV λ_{max} (MeOH, nm) : 218, 268, 334; ^1H-NMR (CD_3OD, 600 MHz) δ_H: 7.45 (1H, dd, $J = 2.1$, 8.4 Hz, H-6'), 7.44 (1H, d, $J = 2.1$ Hz, H-2'), 7.42 (1H, d, $J = 8.4$ Hz, H-5'), 6.60 (1H, s, H-3), 6.45 (1H, d, $J = 1.9$ Hz, H-8), 6.21 (1H, d, $J = 1.9$ Hz, H-6), 4.93 (1H, d, $J = 7.5$ Hz, anomeric H); ^{13}C-NMR (CD_3OD, 150 MHz) δ_C: see Table 1.

Compound (3) – LC-ESI MS (negative mode) *m/z* : 301 $[M – H]^+$; UV λ_{max} (MeOH, nm) : 223 (sh), 255, 294, 369; ^1H-NMR (CD_3OD, 600 MHz) δ_H: 7.72 (1H, d,

$J = 2.1$ Hz, H-2'), 7.62 (1H, dd, $J = 2.1$, 8.7 Hz, H-6'), 6.88 (1H, d, $J = 8.7$ Hz, H-5'), 6.38 (1H, d, $J = 2.2$ Hz, H-8), 6.17 (1H, d, $J = 2.2$ Hz, H-6); ^{13}C-NMR (CD_3OD, 150 MHz) δ_C: see Table 1.

Compound (4) – LC-ESI MS (negative mode) *m/z* : 477 $[M - H]^+$; UV λ_{max} (MeOH, nm) : 218, 256, 298, 351; ^{1}H-NMR (CD_3OD, 600 MHz) δ_H: 7.63 (1H, dd, $J = 2.2$, 8.9 Hz, H-6'), 7.62 (1H, s, H-2'), 6.84 (1H, d, $J = 8.9$ Hz, H-5'), 6.37 (1H, d, $J = 1.7$ Hz, H-8), 6.19 (1H, d, $J = 1.7$ Hz, H-6), 5.32 (1H, d, $J = 7.8$ Hz, anomeric H); ^{13}C (CD_3OD, 150 MHz) δ_C: see Table 1.

Compound (5) – LC-ESI MS (negative mode) *m/z* : 491$[M - H]^+$; UV λ_{max} (MeOH, nm) : 241 (sh), 265 (sh), 294, 344; ^{1}H-NMR (CD_3OD, 600 MHz) δ_H: 7.58 (1H, s, H-2'), 7.57 (1H, dd, $J = 2.1$, 8.1 Hz, H-6'), 6.83 (1H, d, $J = 8.1$ Hz, H-5'), 6.37 (1H, d, $J = 1.9$ Hz, H-8), 6.19 (1H, d, $J = 1.9$ Hz, H-6), 5.22 (1H, d, $J = 7.8$ Hz, anomeric H), 3.65 (3H, s, $OC\underline{H_3}$); ^{13}C (CD_3OD, 150 MHz) δ_C: see Table 1.

Acid hydrolysis of compounds 4 and 5 – Compound **4** and **5** (5 mg) were each heated with aqueous 10% HCl (2 mL) in a sealed vial at 95 °C for 3 h. The aglycone was extracted with EtOAc and then the aqueous layer was neutralized with *N, N*-dioctylmethylamine (10% in $CHCl_3$) and dried. The sugars were identified as glucuronic acid by comparison with authentic samples on TLC in $CHCl_3$: MeOH : H_2O (20 : 10 : 1); detection was accomplished with *p*-anisidine-phthalic acid.

Determination of acetylcholinesterase inhibitory activity – The acetylcholinesterase inhibition assay was measured according to the method of Ellman *et al.*[15] with slight modification. Briefly, tested compounds were dissolved in DMSO. The reaction mixture had a final volume of 1 mL and contained sodium phosphate buffer (100 mM, pH 8.0), up to 10 μL of the tested sample solution, and 20 μL of acetylcholinesterase (5 U/mL), which were mixed and incubated for 10 min at 37 °C. The reactions were started with the addition of 40 μL of 10 mM dithionitrobenzoic acid (DTNB) and 10 μL of 75 mM acetylthiocholine iodide (ATCI) as a substrate. The hydrolysis was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm for 6 min using a spectrophotometer.[16]

A control reaction was carried out using water instead of compounds.

$$\text{Inhibition activity (\%)} = [1 - (\Delta A_{sample} / \Delta A_{control})] \times 100$$

ΔA_{sample} is the absorbance of the tested compounds and $\Delta A_{control}$ is the absorbance of the control.

Measurements were performed twice, and the concentration of each test sample giving 50% activity inhibition (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity. Berberine was used as a reference compound.

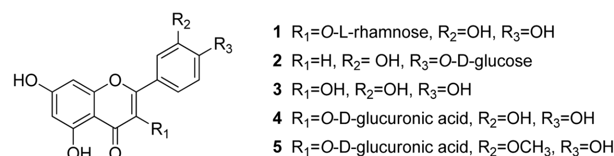

Fig. 1. The structures of compounds **1** - **5**.

Results and Discussion

Compounds **1**, **2**, and **3** were identified as quercitrin,[17] luteolin-4'-*O*-*β*-D-glucopyranoside,[18] and quercetin,[19] respectively, by comparing their physico-chemical data with those of literature values.

The ^{1}H-NMR spectrum of **4** exhibited signals due to the H-6 and H-8 position of flavonoid at δ 6.19 (1H, d, $J = 1.7$ Hz) and 6.37 (1H, d, $J = 1.7$ Hz), respectively, and H-2' position at δ 7.62 (1H, s), H-5' position at δ 6.84 (1H, d, $J = 8.9$ Hz), and H-6' position at δ 7.63 (1H, dd, $J = 2.2$, 8.9 Hz). A doublet ($J = 7.8$ Hz) at δ 5.32 showed **4** is a flavonoid glycoside and sugar configuration is *β* form.[20] Acid hydrolysis of **4** afforded glucuronic acid as sugar moiety. In the ^{13}C-NMR spectrum of **4**, C-2 signal presented at δ 157.62, which suggested that sugar moiety of **4** is attached to the C-3 position of flavonol skeleton.[21] These data allowed us to identify **4** as quercetin-3-*O*-*β*-D-glucuronide.[22] ^{1}H- and ^{13}C-NMR spectra of compound **5** was very similar to those of compound **4** excepting the presence of methoxyl signals at δ_H 3.65 and δ_C 51.45, respectively. Based on these data and on values previously reported in the literature,[23] compound **5** was identified as isorhamnetin-3-*O*-*β*-D-glucuronide. Among the isolated compounds, compound **1** and **3** were previously reported by Lee *et al.*,[12] but compounds **2**, **4**, and **5** were isolated for the first time in this plant.

Alkaloids,[24-26] coumarins,[27,28] xanthones,[29] terpenoids,[30] phenolic amides,[31] and flavonoids[32-34] have been isolated from plant sources as acetylcholinesterase inhibitors. To determine the inhibitory activity of each compound, all isolates were tested for their inhibitory activity against acetylcholinesterase (Table 2).

All of the tested compounds exhibited acetylcholiesterase inhibitory activity with IC_{50} values of 243.1 (**1**), 10.5 (**2**), 39.1 (**3**), 8.2 (**4**), and 23.2 (**5**) μM, respectively. Quercitrin (**1**) and quercetin (**3**) were previously reported as acetylcholinesterase inhibitors with similar IC_{50} values.[35,36] Fan

Table 2. Acetylcholinesterase inhibitory activity of compounds **1** - **5**

Tested compounds	IC_{50}[1)] (μg/ml)	IC_{50} (μM)
1	108.9	243.1
2	4.7	10.5
3	11.8	39.1
4	3.9	8.2
5	11.4	23.2
Berberine[2)]	1.5	4.5

[1)] The inhibitory activity dose that reduced 50% of acetylcholinesterase activity and expressed as mean of two different experiments.
[2)] A positive control

et al.[33] reported that flavones have stronger inhibitory effect than flavonols, and that for flavonoids, the structure elements required for acetylcholesterase inhibition are not only the presence of a 4'-methoxyl group but also the presence of a 7-*O*-sugar whereas quercetin-3-*O*-*β*-D-glucuronide (**4**) showed slightly higher inhibitory effect than luteolin-4'-*O*-*β*-D-glucopyranoside (**2**) in the present study. This might be considered because D-glucuronide accelerates the interaction between the acetylcholinesterase and the tested molecule compared to D-glucose.[37,38] These results strongly suggest that the extracts of whole plant of *Persicaria thubergii* might be a valuable candidates for the development of preventative agents for Alzheimer's disease.

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