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Isolation of Flavonoid Glycosides with Cholinesterase Inhibition Activity and Quantification from *Stachys japonica*

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Abstract – The three flavone glycosides, 4'-*O*-methylisoscutellarein 7-*O*-(6'''-*O*-acetyl)-β-D-allopyranosyl(1 → 2)-β-D-glucopyranoside (**1**), isoscutellarein 7-*O*-(6'''-*O*-acetyl)-β-D-allopyranosyl(1 → 2)-β-D-glucopyranoside (**3**), and isoscutellarein 7-*O*-β-D-allopyranosyl(1 → 2)-β-D-glucopyranoside (**4**) in addition to a flavonol glycoside, kaempferol 3-*O*-β-D-glucopyranoside (astragalin, **2**), were isolated from *Stachys japonica* (Lamiaceae). In cholinesterase inhibition assay, compound **1** significantly inhibited aceylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities (IC_{50}s, 39.94 μg/ml for AChE and 86.98 μg/ml for BChE). The content of isolated compounds were evaluated in this plant extract by HPLC analysis. Our experimental results suggest that the flavonoid glycosides of *S. japonica* could prevent the memory impairment of Alzheimer's disease.
Keywords – *Stachys japonica*, Lamiaceae, isoscutellarein glycoside, cholinesterase, Alzheimer's disease

Introduction

Alzheimer's disease can be caused by the loss of acetylcholine which is a neurotransmitter responsible for memory or cognition (Balkis et al., 2015). Many researchers attempt to search for therapeutic agents capable of inhibiting acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) from natural sources to develop anti-Alzheimer's drugs.[1]

In Korea, *Stachys japonica* (Lamiaceae) is used to treat the Alzheimer's disease,[2] though *S. sieboldii* is done for the same purposes.[3] It is also said that *S. japonica* is effective against mainly CNS disease like insomnia, anxiety, neurosis, and hypertension.[3] Phytochemical and pharmacological studies of *S. sieboldii*have demonstrated the presence of phenylethanoid glycosides[4] and its anti-Alzheimer's activities.[5] Flavonoid glycosides or other phenolic substances were known from some Stachys species.[6,7]

However, the components of *S. japonica* have not been elucidated. In the present phytochemical research, the four flavonoid glycosides including 4'-*O*-methylisoscutellarein glycoside (**1**), isoscutellarein glycoside (**3** and **4**) and a astragalin glycoside (**2**) were isolated. In the present study, the NMR data of compound **1** was assigned for the first time, though it has been identified by LC-MS method.[8,9] See comment in PubMed Commons below

We attempted to find natural glycosides with the anti-cholinesterase activity, since a lot of substances exist in the form of glycosides rather than of their aglycones. The aglycones and their glycosides were also tested for the cholinesterase inhibition activity, because glycosides are often hydrolyzed in the gastrointestinal tract.[10] Furthermore, the four compounds were quantitatively analyzed using an HPLC method.

Experimental

Instruments and reagents – The melting point (mp) was measured using an Electrothermal digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK).UV spectra were measured on a UV-160A UV-visible recording spectrophotometer. IR spectra were recorded with a KBr disk method on a JASCO 4200 FT-IR spectrometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were taken on a Bruker AM-600 spectro-

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meter using an internal standard tetramethylsilane (TMS). The ion exchange resin used for column chromatography was Diaion HP-20 (Mitsubishi Chemical Co.).

Plant material – The herbs of *S. japonica* (Lamiaceae) was collected in the field of Hoengsung-gun, Gangwon-do, Korea. Thisplant was dried cut before extraction. The plant was identified by Prof. Byong-Min Song (Department of Forest Science, Sangji University, Korea). The voucher specimen (natchem #54) was deposited in the laboratory of Natural Product Chemistry, Department of Pharmaceutical Engineering, Sangji University, Korea.

Extraction and fractionation – The plant material (1.95 kg) was extracted with aqueous MeOH (80% MeOH) three times under reflux. The extracted solution was filtered and evaporated under reduced pressure on a rotatory evaporator to give 203 g of an aq. MeOH extract. For fractionation, this extract (193 g) was suspended in H_2O, and partitioned with $CHCl_3$ three times in a separating funnel. Concentration of the $CHCl_3$-soluble portion yielded a $CHCl_3$ fraction (9.8 g). The residual H_2O-soluble portion was further fractionated with BuOH three times. The BuOH-soluble portion was dried*in vacuo* on a rotatory evaporator to give aBuOH fraction (23 g). Further fractionation of the BuOH fraction was performed overdiaion HP-20 column to remove unnecessary sugars or inorganic substances. The BuOH fraction (23 g) was passed into diaion HP-20 column with H_2O (2 L), 20% MeOH, and then 80% MeOH, respectively. Since we observed high content of the glycosides in the 80% MeOH fraction (11.0 g), this fraction was chosen for successive isolation.

Isolation – For isolation of flavonoid glycosides, the 80% MeOH fraction (8.0 g) was subjected to silica gel column chromatography (200 g SiO_2, Ø 4.5 × 33 cm) using the eluent of $CHCl_3$-MeOH-H_2O (7:3:1, lower phase) and collected by each 60 ml. The aliquots were grouped into four sub-fractions (SJ-#1, SJ-#2, SJ-#3, and SJ-#4) after checking TLC. For purification, in the separate columns, these four sub-fractions were further subjected to silica gel column (80 g SiO_2, Ø3.0 × 33 cm) chromatography using the solvent $CHCl_3$-MeOH-H_2O (7:2:1, lower phase). From the fractions of SJ-#1, SJ-#2, SJ-#3, and SJ-#4, the isolated compounds, **1** (32 mg), **2** (28 mg), **3** (85 mg), and **4** (39 mg), were obtained. Physical and spectroscopic data of the isolated compounds were described as below.

Table 1. ^{1}H-NMR data of compounds **1**, **3**, and **4** isolated from *S. japonica* (600 MHz, DMSO-d_6)

Position	**1**	**3**	**4**
Aglycone-3	6.84 (1H, s)	6.84 (1H, s)	6.83 (1H, s)
5-OH	12.32 (1H, s)	12.36 (1H, s)	12.40 (1H, s)
6	6.71 (1H, s)	6.71 (1H, s)	6.66 (1H, s)
2'	8.11 (1H, d, J = 9.0 Hz)	8.00 (1H, d, J = 9.0 Hz)	8.00 (1H, d, *J* = 9.0 Hz)
3'	7.15 (1H, d, J = 9.0 Hz)	6.96 (1H, d, J = 9.0 Hz)	6.97 (1H, d, *J* = 9.0 Hz)
5'	7.15 (1H, d, J = 9.0 Hz)	6.96 (1H, d, J = 9.0 Hz)	6.97 (1H, d, *J* = 9.0 Hz)
6'	8.11 (1H, d, J = 9.0 Hz)	8.00 (1H, d, J = 9.0 Hz)	8.00 (1H, d, *J* = 9.0 Hz)
OMe	3.89 (3H, s)	-	-
Glucose-1	5.09 (1H, d, J = 7.8 Hz)	5.08 (1H, d, J = 7.8 Hz)	5.11 (1H, d, J = 7.8 Hz)
2	3.60 (1H, m)	3.60 (1H, m)	3.60 (1H, m)
3	3.48 (1H, m)	3.49 (1H, m)	3.49 (1H, m)
4	3.27 (1H, m)	3.27 (1H, m)	3.27 (1H, m)
5	3.55 (1H, m)	3.55 (1H, m)	3.54 (1H, m)
6	3.52 (1H, m)	3.51 (1H, m)	3.49 (1H, m)
	3.76 (1H, m)	3.76 (1H, m)	3.74 (1H, m)
Allose-1	4.93 (1H, d, J = 7.8 Hz)	4.92 (1H, d, J = 7.8 Hz)	4.92 (1H, d, J = 7.8 Hz)
2	3.27 (1H, m)	3.27 (1H, m)	3.27 (1H, m)
3	3.93 (1H, m)	3.93 (1H, m)	3.23 (1H, m)
4	3.43 (1H, m)	3.43 (1H, m)	3.34 (1H, m)
5	3.89 (1H, m)	3.88 (1H, m)	3.64 (1H, m)
6a	4.06 (2H, m)	4.05 (2H, m)	3.41 (1H, m)
6b	-		3.55 (1H, m)
OAc	1.89 (3H, s)	1.89 (3H, s)	-

Table 2. ^{13}C-NMR data of compounds **1**, **3**, and **4** isolated from *S. japonica* (150 MHz, DMSO-d_6)

Position	1	3	4
Isoscutellarein 2	164.1	164.1	164.6
3	103.0	103.1	103.1
4	182.9	182.8	182.8
5	152.7	152.7	152.9
6	100.0	100.0	99.3
7	151.1	151.0	151.7
8	128.0	128.0	127.7
9	144.3	144.2	144.8
10	106.1	106.1	105.7
1'	123.4	121.7	121.7
2'	129.0	129.1	129.1
3'	115.1	116.4	116.5
4'	163.0	161.9	161.9
5'	115.1	116.4	116.5
6'	129.0	129.1	129.1
OMe	56.1	-	
Glc 1	100.6	100.6	100.2
2	83.0	83.1	81.8
3	77.7	77.7	77.6
4	69.8	69.8	69.8
5	76.1	76.1	76.2
6	61.1	61.1	61.1
All 1	103.9	103.2	102.2
2	72.0	72.0	71.5
3	71.3	71.3	72.0
4	67.4	67.3	67.7
5	72.0	72.0	75.0
6	64.0	64.0	61.5
CO<u>C</u>H$_3$	20.9	20.9	-
<u>C</u>OCH$_3$	170.8	170.8	-

4'-*O*-Methylisoscutellarein 7-O-(6'''-*O*-acetyl)-β-D-allopyranosyl(1→2)-β-D-glucopyranoside] (1) – Mp 247 ºC, yellowish powder; UV λ_{max} nm (log ε): (MeOH) 280 (4.29), 307 (4.37), 326 (4.28); (MeOH + NaOH) 319 (4.16), 373 (4.00); (MeOH + $AlCl_3$) 284 (4.23), 323 (4.41), 346 (4.34); (MeOH + $AlCl_3$+ HCl) 283 (4.24), 322 (4.36), 347 (4.25); (MeOH + NaOAc) 280 (4.33), 307 (4.40), 326 (4.33); (MeOH + NaOAc + H_3BO_3) 280 (4.30), 307 (4.37), 326 (4.28); IR ν_{max} (KBr) cm^{-1}: 3400 (O-H, broad), 2937 (C-H), 1734 (C=O), 1658 (α,β-unsaturated ketone), 1606, 1505, 1440 (aromatic C=C), 1221 (C-O), 1087, 1031 (glycosidic C-O); HR-FAB-MS (m/z): 665.1685 [M-H]$^-$ (calculated,m/z665.1718); ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): Table 1 and Table 2.

Kaempferol 3-*O*-β-D-glucopyranoside (astragalin, 2) – Mp 230 - 233 ºC, UV λ_{max} (MeOH) nm: 267, 300 (sh), 352; IR ν_{max} (KBr) cm^{-1}: 3620 – 3000 (broad, OH), 1655 (α,β-unsaturated ketone), 1606, 1562, 1506 (aromatic C=C), 1360, 1291 (aromatic C-O), 1179, 1056, 1011 (glycosidic C-O); ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): Literature.[11]

Isoscutellarein 7-O-(6'''-*O*-acetyl)-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (3) – Mp 235 ºC, orange yellow powder; UV λ_{max} (MeOH) nm (log ε): (MeOH) 278 (4.29), 307 (4.15), 328 (sh, 4.28); (MeOH + NaOH) 278 (sh, 4.29), 378 (4.41); (MeOH + $AlCl_3$) 282 (4.15), 323 (4.28), 350 (4.28); (MeOH + $AlCl_3$+ HCl) 282 (4.13), 323 (4.26), 350 (4.25); (MeOH + NaOAc) 277 (4.26), 307 (4.29), 328 (sh, 4.25); (MeOH + NaOAc + H_3BO_3) 278 (4.28), 307 (4.32), 328 (4.27); IR ν_{max} (KBr) cm^{-1}: 3374 (O-H, broad), 2937 (C-H), 1723 (C=O), 1659 (α,β-unsaturated ketone), 1608, 1582 m 1449 (aromatic C=C), 1220 (C-O), 1087, 1032 (glycosidic C-O); HR-FAB-MS (m/z): 651.1538 [M-H]$^-$ (calculated m/z651.1561); ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): Table 1 and Table 2.

Isoscutellarein 7-O-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside (4) – Mp 239 ºC, orange yellow powder, UV λ_{max} nm (log ε): (MeOH) 279 (4.06), 305 (4.09), 328 (4.07); (MeOH + NaOH) 274 (4.06), 377 (4.10); (MeOH + $AlCl_3$) 274 (4.07), 324 (4.07), 349 (4.08); (MeOH + $AlCl_3$ + HCl) 274 (4.07), 324 (4.07), 349 (4.08); (MeOH + NaOAc) 276 (4.09), 307 (4.08), 326 (4.05); (MeOH + NaOAc + H_3BO_3) 276 (4.10), 307 (4.09), 326 (4.07); IR ν_{max} (KBr) cm^{-1}: 3425 (O-H, broad), 2931 (C-H), 1660 (α,β-unsaturated ketone), 1607, 1508, 1454 (aromatic C=C), 1223 (C-O), 1084, 1030 (glycosidic C-O); ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): Table 1 and Table 2.

Hydrolysis of compounds 2 and 3 – Compound **3** (60 mg) dissolved in 5% H_2SO_4 in MeOH-H_2O (60:40) was heated under reflux for 5 h. The cooled reaction mixture was partitioned with EtOAc. The aglycone (**3a**) was obtained from the concentration of the EtOAc-soluble portion to be identified as isoscutellarein by the interpretation of spectroscopic data (Teles et al., 2015). Acid hydrolysis of compound **2** was performed in the same way with compound **3**. The aglycone (**2a**) of **2** was produced by the concentration of the EtOAc-soluble portion and identified as kaempferol by direct comparison with authentic specimen.

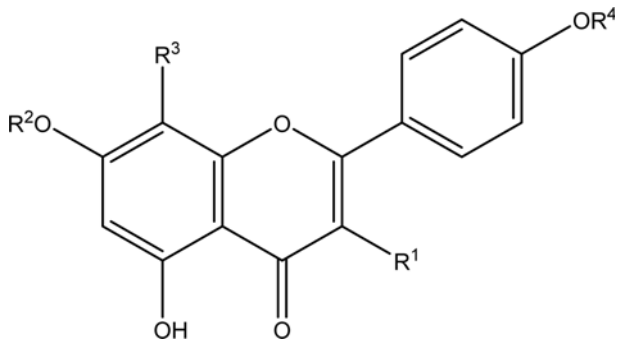
Kaempferol (2a) – Mp 277 - 279 ºC, UV λ_{max} (MeOH) nm: 267, 364; IR ν_{max} (KBr) cm^{-1}: 3350 (broad, OH), 1667 (α,β-unsaturated ketone), 1620, 1575, 1510,

(Aromatic C=C), 1375, 1245, 1175 (C-O); ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): Literature.[12]

Isoscutellarein (3a) – Mp 257 - 259 ºC, yellow powder, IR ν_{max} (KBr) cm^{-1}: 3367 (O-H, broad), 3028, 2954 (C-H), 1661 (α,β-unsaturated ketone), 1610, 1582, 1509, 1445 (aromatic C=C), 1243, 1178 (C-O), 831; UV λ_{max} nm (log ε): (MeOH) 292 (4.26), 329 (4.21); (MeOH + NaOH) 295 (4.11), 377 (4.33); (MeOH + $AlCl_3$) 308 (4.26), 363 (4.14); (MeOH + $AlCl_3$ + HCl) 305 (4.20), 358 (4.17); (MeOH + NaOAc) 293 (4.28), 346 (sh, 4.05); (MeOH + NaOAc + $AlCl_3$) 295 (4.30), 342 (sh, 4.11); ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): Literature.[13]

Cholinesterase inhibition assay – Cholinesterase activity was assayed modifying the method described by Ellman et al..[14] The inhibitory activities of AChE and BChE serving for ACh and BCh as the substrate, respectively, were measured. In brief, the solution added with 100 mM sodium phosphate buffer (pH 8.0) 140 μl, each sample 20 μl, and AChE (0.36 U) or BChE (0.36 U) 20 μl, respectively, in 96 well plates, was incubated for 15 min at room temperature, and then the reaction mixture was adjusted to 200 μl adding 10 μl DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] and the substrate, 10 μl of ACh or BCh, in 96 well plate. After 15 min, the yellow 5-thio-2-nitrobenzoate anion formed by the reaction between DTNB and thiocholine resulted from the hydrolysis of ACh or BCh was measured at 412 nm by a microplate reader VERSA max (Molecular Devices, CA, USA). The cholinesterase inhibition activity was expressed as the IC_{50} value (μg/ml) that is the concentration inhibiting the hydrolysis of ACh and BCh by 50%. The cholinesterase inhibition rate (%) was calculated by the following equation: Inhibition (%) = $[1 - (A_{samp} / A_{con}) / A_{std}] \times 100$, where A_{samp}, A_{con}, and A_{std} are the absorbance when added with the sample and enzyme, with the sample but without the enzyme, and without the sample, respectively.

HPLC analysis – As the two mobile solvents, 0.05% acetic acid in MeOH (solvent A) and H_2O (solvent B) were used. ACapcell Pak C18 column (5 μm, 4.6 mm × 250 mm, Shiseido, Japan) was used as a HPLC column. The programmed elution was performed in the following gradient elution: 0 – 20 min (20 → 65% B), 20 – 21 min (65 → 100% B), 21 – 25 min (100% B), 25 – 27 min (100 → 20% B), and 27 – 30 min (20% B), at the flow rate of 1.0 ml/min at the column temperature of 40 ºC. The detection wavelength was fixed at 254 nm. For the sensitivity for detection and quantification, the LOD (limit-of-detection) and LOQ (limit-of-quantification) were determined by anS/N (signal-to-noise) method, respectively.



1 : R^1 = H, R^2 = (6-*O*-acetyl)-β-D-allopyranosyl(1 → 2)-β-D-glucopyranosyl, R^3 = OH, R^4 =Me
2 : R^1 = *O*-β-D-glucopyranosyl, R^2 = R^3 = R^4 = H
2a : R^1 = OH, R^2 = R^3 = R^4 = H
3 : R^1 = R^4 = H, R^2 = (6-*O*-acetyl)-β-D-allopyranosyl(1 → 2)-β-D-glucopyranosyl, R^3 = OH
3a : R^1 = R^2 = R^4 = H, R^3 = OH
4 : R^1 = R^4 = H, R^2 = β-D-allopyranosyl(1 → 2)-β-D-glucopyranosyl, R^3 = OH

Fig. 1. Structure of flavonoid glycosides (**1** – **4**) isolated from *S. japonica* and their aglycones (**2a** and **3a**).

Result and Discussion

One flavonol glycoside and three flavone glycosides were isolated from the BuOH fraction obtained from 80% MeOH extract of *S. japonica*. By the interpretation of ^{1}H- and ^{13}C-NMR spectral data, compounds **1**, **3**, and **4** were identified as 4'-O-methylisoscutellarein 7-(6'''-*O*-acetyl)-*O*-β-D-allopyranosyl(1 → 2)-β-D-glucopyranoside] (**1**), isoscutellarein 7-*O*-(6'''-*O*-acetyl)-β-D-allopyranosyl(1 → 2)-β-D-glucopyranoside] (**3**), and isoscutellarein 7-*O*-β-D-allopyranosyl-(1 → 2)-β-D-glucopyranoside] (**4**). Meanwhile, the flavonol glycoside (**2**) was astragalin (kaempferol3-*O*-β-D-glucopyranoside) (Fig. 1).

The UV spectroscopic data of compounds **1**, **3** and **4** measured by the addition of shift reagent were noted in the experimental section. No significant shifts of absorption bands were observed by the addition of NaOAc and H_3BO_3, though considerable shifts due to 5-hydroxy-4-keto system were shown by the addition of $AlCl_3$. These results suggest that the three compounds are the flavone 7-*O*-glycosides with no *o*-dihydroxy group in the B-ring. IR spectra of the three compounds demonstrated the presence of hydroxy group, aliphatic C-H, α, β-unsaturated ketone, aromatic C=C, aromatic C-O, aliphatic C-O, and glycosidic C-O in the functional group region.

As shown in Table 1, the singlet peaks of C-3 of **1**, **3**,

and **4** shown at δ 6.84, 6.84 and 6.83, respectively, suggest that the aglyconesare the flavone type. In particular, chemical shifts of C-5 and C-7 are shown in δ_C 149.5 and 150.8 ppm in the literature data (^{13}C-NMR) of isoscutellarein 5-*O*-glucoside. As shown in Table 2, the peaks of C-5 in **1**, **3**, and **4** were observed at δ_C 152.7, 152.7, and 152.9, and those of C-7 were shown in 151.1, 151.0, and 151.7. These results indicate that the sugar moiety is linked not to the C-5 position of the aglycone but to the C-7. The identification of 4'-*O*-methylisoscutellarein, the aglycone of compound **1**, and isoscutellarein, that of compounds **3** and **4**, was supported by literature data.[13,15]

The presence of the two sugars, D-glucopyranosyl and D-allopyranosyl, in the glycosides was identifiable in the course of comparison with the literature NMR data of the flavonoid glycosides **1**, **3**, **4**, isolated from other Stachys species.[6,7,16] In the ^{1}H-NMR spectrum of compound **1**, β-configuration of D-glucose can be identified from the coupling constant (d, J = 7.8 Hz) of the anomeric proton shown at δ 5.09. The β-configuration of D-allose was also observed from the coupling constant (d, J = 7.8 Hz) at δ 4.92. The anomeric proton of D-allose shown at δ 4.93 is long-range coupled to δ_C 83.1 in the HMBC spectrum, indicating that the second sugar is attached to the C-2 position in the first sugar. The presence of acetyl group at the C-6''' of D-allose was determined by the HMBC spectral interpretation.

The NMR data of compounds **1**, **3**, and **4** were successfully assigned, as shown in Table 1 and Table 2. Therefore, the three compounds were identified to be 4'-*O*-methylisoscutellarein 7-(6'''-*O*-acetyl)-*O*-β-D-allopyranosyl (1→2)-β-D-glucopyranoside] (**1**), isoscutellarein 7-*O*-(6'''-*O*-acetyl)-β-D-allopyranosyl(1→2)-β-D-glucopyranoside] (**3**), and isoscutellarein 7-*O*-β-D-allopyranosyl-(1→2)-β-D-glucopyranoside] (**4**) by comparing with literature data.[6,7,16] The four compounds were isolated for the first time from *S. japonica*.

Cholinesterases divided into AChE and BChE are key enzymes that play significant roles in cholinergic trans−mission by hydrolyzing ACh.[17] Cholinesterase inhibitors can treat Alzheimer's disease, since the inhibition of cholinesterase increases this neurotransmitter responsible for brain's memory. In our tests, the samples of the aq. MeOH extract, its two fractions ($CHCl_3$- and BuOH fractions), the four flavonoid glycosides isolated from the BuOH fraction, and the aglycones (**2a** and **3a**) obtained by hydrolysis of glycosides were assayed for cholinesterase inhibition activity. The aq. MeOH extract displayed the data of 86.09 ± 1.27, 172.88 ± 0.71 μg/ml as the IC_{50} value. The activities of $CHCl_3$- and BuOH fractions were similar each other.

Table 3. Cholinesterase inhibitory activities of the *S. japonica* extract, its fractions and isolated compounds

Samples	AChE[a]	BChE
	Mean ± SEM	Mean ± SEM
Aq. MeOH extract	86.09 ± 1.27	172.88 ± 0.71
$CHCl_3$ fraction	68.66 ± 1.32	133.96 ± 2.43
BuOH fraction	74.20 ± 2.20	117.94 ± 7.61
1	39.94 ± 0.76	86.98 ± 1.72
2	66.76 ± 2.82	> 200
3	65.40 ± 0.45	109.76 ± 2.79
4	59.55 ± 2.92	160.84 ± 3.26
2a	32.19 ± 0.82	52.34 ± 1.51
3a	58.19 ± 1.11	79.60 ± 0.28
Apigenin[b]	12.80 ± 0.37	9.18 ± 0.67

[a]The values (μg/ml) indicate 50% cholinesteraseinhibitory effects. These data represent the average values of three repeated experiments. [b]Positive control.

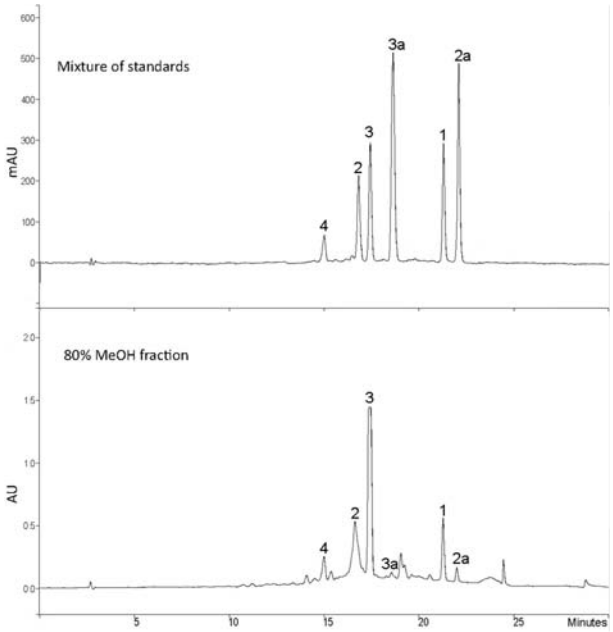


Fig. 2. HPLC chromatogram of standard compounds and 80% MeOH fraction of *S. japonica*.

As shown in Table 3, the IC_{50}s of AChE inhibition were observed in the range of 39.94 – 66.76 μg/ml whereas those of BChE were in the range of 52.34 – 160.84 μg/ml. Compound **1** possessing the aglycone of 4'-*O*-methylisoscutellarein was more active (IC_{50}, 39.94 μg/ml for AChE and 86.98 μg/ml for BChE) than other compounds with isoscutellarein. Astragalin (**3**) was less active than its aglycone (kaempferol). In this assay, apigenin which is known to have potent anti-cholinesterase activity[1,18]

Table 4. Linearity of standard curves and limits of detection and quantification for the standard compounds

Compound	t_R (min)	Calibration equation (linear model)[a]	Linear range (μg/ml)	R^{2} [b]	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
1	20.11	$y = 157.68x + 113.14$	7.81 - 250.0	0.999	1.18	3.95
2	16.92	$y = 378.45x + 106.78$	7.81 - 250.0	0.999	0.51	1.70
2a	22.10	$y = 957.20x + 158.03$	7.81 - 250.0	0.999	0.15	0.49
3	17.22	$y = 278.85x + 122.48$	7.81 - 250.0	0.999	0.63	2.12
3a	17.98	$y = 313.95x + 86.27$	7.81 - 250.0	0.999	0.68	2.27
4	14.83	$y = 95.75x + 55.73$	7.81 - 250.0	0.999	2.03	6.76

[a] y, peak area at 254nm; x, concentration of the standard (μg/ml); [b] R^2, correlation coefficient for 6 data points in the calibration curves (n = 4); [c] LOD, limit of detection (S/N = 3); [d] LOQ, limit of quantification (S/N = 10).

Table 5. Content of six compounds (mg/g) in the extract and fractions of *Stachys*

Fraction/ extract	Analytes						Total
	1	**2**	**2a**	**3**	**3a**	**4**	
80% MeOH fraction	55.96	50.94	2.43	153.54	4.45	53.70	321.03
BuOH fraction	26.76	24.36	1.16	73.43	2.13	25.68	153.54
MeOH extract	3.19	2.90	0.14	8.75	0.25	3.06	18.30
Dry plant material	0.33	0.30	0.01	0.91	0.03	0.32	1.90

The data was present as average of three determinations.

and anxiolytic activity[19] was used as the positive control.

Six compounds including the four isolated ones and the two aglycones were able to be identified on the HPLC chromatogram by direct comparison with standard ones (Fig. 2). As shown in Table 4, the regression equations which the linearity was verified by the R^2 value more than 0.999 were established as the HPLC analytical method. Limits of detection and quantification were also sufficiently low. In the HPLC chromatogram of the 80% MeOH fraction, compound **3** exhibited the highest peak of the tested compounds. The content of compound **3** was 154.54 mg/g 80% MeOH fraction (Table 5). However, the concentration of the two aglycones, **2a** and **3a**, were low.

In consequence, the four flavonoids present in the hydrophilic fraction (BuOH fraction) of the *S. japonica* extract may contribute to the prevention of memory impairment of Alzheimer's disease. Furthermore, compound **1** with apotent cholinesterase inhibition activity were identified as 4'-*O*-isoscutellarein 7-*O*-(6'''-*O*-acetyl)-β-D-allopyranosyl(1→2)-β-D-glucopyranoside and quantified in *S. japonica.*

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