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SHORT COMMUNICATION

Flavonoid Glycosides from the Fruit of *Rhus parviflora* and Inhibition of Cyclin Dependent Kinases by Hyperin

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Abstract Chrysoeriol-7-*O*-*β*-D-glucopyranoside (**1**), luteolin-7-*O*-*β*-D-glucopyranoside (**2**), quercetin-3-*O*-*β*-D-glucopyranoside (**3**), quercetin-3-*O*-*β*-D-galactopyranoside (**4**), and quercetin-3-*O*-*α*-L-rhamnopyranoside (**5**) were isolated for the first time from the fruits of *Rhus parviflora*. The chemical structures of the compounds were determined using nuclear magnetic resonance, fast atom bombardment mass spectrometry, and infrared spectroscopy. Compound **4** (hyperin) inhibited cyclin dependent kinases (CDK2 and CDK5) *in vitro* with IC_{50} values of 21.02 and 10.28 μM, respectively.

Keywords cyclin-dependent kinase (CDK) 2 · CDK 5 · hyperin · isoquercetin · luteoloside · quercetrin · *Rhus parviflora* · thermopsoside

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The plants belonging to genus *Rhus* are commonly known as Sumac. It is one of the important genera of the Anacardiaceae family, comprising over 250 species (Tianlu and Brach, 2008). *Rhus parviflora* Roxb. is indigenous to Nepal, Bhutan, India, and Sri Lanka at altitudes of 700–1,100 m (Press et al., 2000). *R. parviflora* is a sub-deciduous shrub with irregular crenate, trifoliate leaves, and small rounded drupes. Its wild edible fruit is known as 'Satibayer' in Nepal (Bajracharya, 1980). In Sanskrit language it is known as 'Tintidikah' and used in Ayurveda (traditional medicinal system of southern Asia) to cure neurological and stomach disorders (GOI, 2006) as well as to treat muscular inflammation (Manandhar, 1995). Previous investigations on the chemical constituents of the *R. parviflora* fruits found echinulin, trimethyl citrate, citric acid 2-methyl ester (Talapatra et al., 1993; 2001) and biflavonoids (Shrestha et al., 2012). The objective of the present study was to search for potential bioactive constituents.

The dried and powdered fruits of *R. parviflora* were extracted with 80% methanol (MeOH) and the concentrated extract was successively partitioned with ethyl acetate (EtOAc), *n*-butyl alcohol (*n*-BuOH), and H_2O. The EtOAc fraction was further purified by silica gel, octadecyl silica gel (ODS), and Sephadex LH-20 column chromatography, leading to the isolation of five flavonoid glycosides for the first time from *R. parviflora* fruit. The chemical structures of the compounds were determined using spectroscopic methods. The compounds were investigated for their inhibitory effect on cyclin-dependent kinases (CDK2 and CDK5).

CDK2 is responsible for cell cycle progression and also plays a role in apoptosis (de Azevedo et al., 2002). Gillardon et al. (2005) reported that CDK5 activity in the central nervous system was deregulated in Alzheimer's disease (AD), mouse models of amyotrophic lateral sclerosis (ALS), and Parkinson's disease. The exact relationship between cell cycle-related CDKs and CDK5

has yet to be elucidated, however, up-regulation CDK in an amyotrophic lateral sclerosis (ALS) model is alleviated by expression of neurofilament H, which is a proposed phosphorylation sink for CDK5 (Nguyen et al., 2003). Furthermore, CDK2 is known to regulate p53 (Price et al., 1995). Deregulation of CDK5 is caused by the accumulation of a truncated fragment of p35, p25, which is produced and accumulates in the brains of patients with AD (Patrick et al., 1999). The administration of CDK inhibitors (e.g., roscovitine and flavopiridol) and expression of dominant-negative kinase mutants prevented neuronal cell loss, and the neuroprotective effectiveness correlated with CDK5 inhibition (Weishaupt et al., 2003).

R. parviflora fruits were collected from Salyan (28°22'31"N, 82°9'42"E), Nepal in February 2010, and were identified by Prof. Damodar Prasad Joshi, Central Department of Environmental Science, Tribhuvan University, Nepal. The voucher specimen (KHU100309) is deposited in the Natural Products Chemistry Laboratory, Kyung Hee University, Yongin, Korea.

An enzyme-based assay was performed to determine inhibition of CDK2 and CDK5 (Cell Signaling Technology, USA) following the method of Jeon et al. (2005) using Dot-Blot apparatus (Bio-Dot; Bio-Rad Laboratories, USA) at range of concentrations between 0 to 100 μM. Briefly, the kinase dilution buffer was made by diluting 2 ng of CDK2 (cyclin E) or CDK5/p25 in 1× kinase buffer [5 mM Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM, ethylene glycol tetraacetic acid (EGTA), 0.1 mM dithiothreitol (DTT)] and pretreated at 37°C for 10 min. Subsequenly, 10 μM ATP and 0.25 μg of Rb-C fusion protein were added, and the mixture was kept at 37°C for 30 min. The reaction mixture was separated on 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane and incubated with phosphorylation-dependent antibody (α-pRbSer780). Alsterpaullone (Sigma-Aldrich, Korea) was used as positive control, and the intensity of the negative control (DMSO) was regarded as 100% for quantitative analysis. The extents of chemilminescence were imaged by ChemiDoc™ XRS plus system (Bio-Rad) and quantified using ImageLab™ software (Bio-Rad).

Dried and powdered fruits (6 kg) of *R. parviflora* were extracted at room temperature with 80% aqueous methanol (MeOH, 25 L × 3) for 24 h, which resulted in a yellowish brown concentrated extract (1440 g). The MeOH extract was successively partitioned with water (6 L), EtOAc (6 L ×3) and *n*-BuOH (6 L ×3), yielding concentrated extract in EtOAc (RPE, 48 g), *n*-BuOH (RPB, 173 g), and H_2O (RPW, 1219 g) fractions. The concentrated *R. parviflora* EtOAc fraction (RPE, 48 g) was subjected to a silica gel (SiO_2) column chromatography (c.c.) (ϕ 14×12 cm) and eluted with *n*-hexane-EtOAc (10:1 → 3:1 → 1:1, 20 L of each), and $CHCl_3$-MeOH (6:1 → 1:1, 10 L of each), resulting in 22 fractions (RPE-1 to RPE-22). Fraction RPE-17 [2 g, (elution volume/total volume) V_e/V_t 0.56–0.60] was subjected to a Sephadex LH-20 c.c. (ϕ 2.5×45 cm), and eluted with MeOH-H_2O (1:1, 4 L) to provide 30 fractions (RPE-17-1 to RPE-17-30), with isolation of compound **1** at RPE-17-21 [44.0 mg, V_e/V_t 0.40–0.42, TLC (RP-18 F_{254}s) R_f 0.62 in MeOH-H_2O (2:1)]. Fraction RPE-18 (2 g, V_e/V_t 0.60–0.61) was further purified by Sephadex LH-20 c.c. (ϕ 2.5×45 cm), and eluted with MeOH-H_2O (1:1, 2 L), resulting in 16 fractions (RPE-18-1 to RPE-18-16). Fraction RPE-18-8 (90 mg, V_e/V_t 0.09–0.16) was subjected to the SiO_2 c.c. (ϕ 2×10 cm), and eluted with $CHCl_3$-MeOH (6:1, 300 mL → 3:1, 600 mL) resulting in 11 fractions (RPE-18-8-1 to RPE-18-8-11), with isolation of compound **2** at RPE-18-8-6 [7.5 mg, V_e/V_t 0.13-0.21, TLC (SiO_2 F_{254}) R_f 0.55 in $CHCl_3$-MeOH (5:1)]. The fraction RPE-18-9 (239 mg, V_e/V_t 0.16–0.24) was subjected to the SiO_2 c.c. (ϕ 3×10 cm), and eluted with $CHCl_3$-MeOH (7:1, 200 mL → 6:1, 700 mL → 5:1, 700 mL → 4:1, 500 mL → 1:1, 500 mL) resulting in nine fractions (RPE-18-9-1 to RPE-18-9-9), with isolation of compound **3** at RPE-18-9-4 [21.9 mg, V_e/V_t 0.04–0.06, TLC (SiO_2 F_{254}) R_f 0.57 in $CHCl_3$-MeOH-H_2O (6:4:1)]. The fraction RPE-18-10 (510 mg, V_e/V_t 0.24–0.39) was subjected to the Sephadex LH-20 c.c. (ϕ 2.5×40 cm), and eluted with MeOH-H_2O (1:3, 15 L) to provide 18 fractions (RPE-18-10-1 to RPE-18-10-18), with isolation of compound **4** at RPE-18-10-4 [45.7 mg, V_e/V_t 0.17–0.19, TLC (RP-18 F_{254}s) R_f 0.55 in MeOH-H_2O (2:1)]. Fraction RPE-18-10-11~13 [81 mg, V_e/V_t 0.36–0.77] was subjected to the SiO_2 c.c. (ϕ 2.2×10 cm), and eluted with $CHCl_3$-MeOH-EtOH-H_2O (34:3:3:2, 3 L) to provide 14 fractions (RPE-18-10-11~13-1 to RPE-18-10-11~13-14), with isolation of compound **5** at RPE-18-10-11~13-6 [34 mg, V_e/V_t 014–0.29, TLC (RP-18 F_{254}) R_f 0.62 in MeOH-EtOH-H_2O (9:1:1)].

Compound **1**: Yellow powder (MeOH); m.p. 176–180°C; $[\alpha]_D^{25}$ = −35° (*c* 0.65, EtOH); IR_δ (KBr, cm^{-1}) 3303, 2890, 1680, 1618, 1520; negative FAB/MS *m/z*: 461 $[M-H]^-$; ^1H-NMR (400 MHz, pyridine-d_5, δ_H) 7.62 (1H, dd, *J*=8.4, 2.0 Hz, H-6'), 7.60 (1H, d, *J*=2.0 Hz, H-2'), 7.26 (1H, d, *J*=8.4 Hz, H-5'), 7.12 (1H, d, *J*=2.0 Hz, H-8), 6.85 (2H, d, *J*=2.0 Hz, H-6), 6.73 (1H, s, H-3), 5.79 (1H, d, *J*=7.2 Hz, H-1"), 3.82 (1H, s, 3'-OCH_3). ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C), 182.67 (C-4), 164.75 (C-2), 163.86 (C-7), 162.36 (C-5), 157.71 (C-9), 152.41 (C-4'), 148.83 (C-3'), 122.12 (C-6'), 121.24 (C-1'), 116.82 (C-5'), 110.20 (C-2'), 106.40 (C-10), 104.09 (C-3), 101.60 (C-1"), 100.51 (C-6), 95.31 (C-8), 78.97 (C-5"), 78.24 (C-3"), 74.61 (C-2"), 70.96 (C-4"), 62.14 (C-6"), 56.44 (3'-OCH_3).

Compound **2**: Yellow powder (MeOH); m.p. 258–260°C; $[\alpha]_D^{25}$ = −48° (*c* 0.15, EtOH); IR_ν (KBr, cm^{-1}) 3400, 1640; negative FAB/MS *m/z*: 447 $[M-H]^-$; ^1H-NMR (400 MHz, pyridine-d_5, δ_H) 7.86 (1H, d, *J*=2.0 Hz, H-2'), 7.49 (1H, br. d, *J*=8.0, 2.0 Hz, H-6'), 7.26 (1H, d, *J*=8.0 Hz, H-5'), 6.96 (1H, br. s, H-8), 6.88 (1H, s, H-3), 6.80 (1H, br. s, H-6), 5.77 (1H, d, *J*=7.2 Hz, H-1"). ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C), 182.08 (C-4), 165.13 (C-2), 163.76 (C-7), 162.36 (C-5), 157.68 (C-9), 151.76 (C-4'), 147.65 (C-3'), 122.13 (C-1'), 119.57 (C-6'), 116.77 (C-5'), 114.56 (C-2'), 106.47 (C-10), 103.81 (C-3), 101.66 (C-6), 100.51 (C-1"), 95.23 (C-8), 79.14 (C-2"), 78.40 (C-5"), 74.93 (C-3"), 71.09 (C-4"), 62.30 (C-6").

Compound **3**: Yellow powder (MeOH); m.p. 235–236°C;

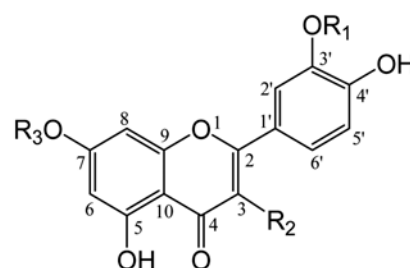
$[\alpha]_D^{25}$ = −17° (*c* 0.25, MeOH); IR_v (KBr, cm^{-1}) 3300, 1650; negative FAB/MS *m/z*: 463 $[M-H]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.84 (1H, d, *J*=2.0 Hz, H-2'), 7.56 (1H, dd, *J*=2.0, 8.6 Hz, H-6'), 6.85 (1H, d, *J*=8.6 Hz, H-5'), 6.36 (1H, br. s, H-8), 6.17 (1H, br. s, H-6), 5.22 (1H, d, *J*=7.6 Hz, H-1''). ^{13}C-NMR (100 MHz, CD_3OD, δ_C), 179.21 (C-4), 165.79 (C-7), 162.72 (C-5), 158.32 (C-9), 158.18 (C-2), 149.76 (C-4'), 145.65 (C-3'), 135.50 (C-3), 123.08 (C-1'), 122.92 (C-6'), 118.29 (C-5'), 115.87 (C-2'), 105.34 (C-10), 104.26 (C-1''), 99.80 (C-6), 94.65 (C-8), 78.14 (C-3''), 78.01 (C-5''), 75.63 (C-2''), 71.12 (C-4''), 62.10 (C-6'').

Compound **4**: Yellow powder (MeOH); m.p. 226–228°C; $[\alpha]_D^{25}$ = −83° (*c* 2.0, pyridine); IR_v (KBr, cm^{-1}) 3250, 1630, 1605, 1520, 1500; negative FAB/MS *m/z*: 463 $[M-H]^-$; ^{1}H-NMR (400 MHz, pyridine-d_5, δ_H) 8.42 (1H, d, *J*=1.2 Hz, H-2'), 8.06 (1H, dd, *J*=8.0, 1.2 Hz, H-6'), 7.23 (1H, d, *J*=8.0 Hz, H-5'), 6.65 (1H, br. s, H-6), 6.61 (1H, br. s, H-8), 6.00 (1H, d, *J*=7.2 Hz, H-1''). ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C), 178.70 (C-4), 165.84 (C-7), 162.55 (C-5), 157.74 (C-2), 157.44 (C-9), 150.61 (C-4'), 146.57 (C-3'), 135.50 (C-3), 122.90 (C-6'), 122.88 (C-5'), 122.63 (C-1'), 116.14 (C-2'), 105.31 (C-1''), 105.03 (C-10), 99.70 (C-6), 94.45 (C-8), 77.48 (C-3''), 75.29 (C-5''), 73.23 (C-2''), 69.67 (C-4''), 61.78 (C-6'').

Compound **5**: Yellow powder (MeOH); m.p. 178–182°C; $[\alpha]_D^{25}$ = −178° (*c* 0.1, MeOH); IR_v (KBr, cm^{-1}) 3228, 1655, 1614, 1549, 1450; negative FAB/MS *m/z*: 447 $[M-H]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.32 (1H, d, *J*=2.0 Hz, H-2'), 7.29 (1H, dd, *J*=8.4, 2.0 Hz, H-6'), 6.90 (1H, d, *J*=8.4 Hz, H-5'), 6.34 (1H, d, *J*=2.0 Hz, H-8), 6.17 (1H, d, *J*=2.0 Hz, H-6), 5.34 (1H, d, *J*=1.6 Hz, H-1''), 0.94 (3H, d, *J*=6.0 Hz, H-6''). ^{13}C-NMR (100 MHz, CD_3OD, δ_C), 179.50 (C-4), 165.76 (C-7), 163.10 (C-5), 159.25 (C-9), 158.43 (C-2), 149.71 (C-4'), 146.32 (C-3'), 136.19 (C-3), 122.94 (C-6'), 122.88 (C-1'), 116.94 (C-2'), 116.34 (C-5'), 105.86 (C-10), 103.49 (C-1''), 99.79 (C-6), 94.71 (C-8), 73.24 (C-4''), 72.09 (C-3''), 71.98 (C-5''), 71.87 (C-2''), 17.63 (C-6'').

By comparing our extensive spectroscopic data with those described in the literatures, the compounds were determined to be chrysoeriol-7-*O*-*β*-D-glucopyranoside (**1**, thermopsoside), luteolin-7-*O*-*β*-D-glucopyranoside (**2**, luteoloside), quercetin-3-*O*-*β*-D-glucopyranoside (**3**, isoquercetin), quercetin-3-*O*-*β*-D-galactopyranoside (**4**, hyperin), and quercetin-3-*O*-*α*-L-rhamnopyranoside (**5**, quercetrin), Fig. 1.

Compounds **1-5** had 1,2,4-trisubstituted catechol B-rings and the typical phloroglucinol ring A with 1,2,3,5-tetrasubstitution. The gHMBC spectrum of compound **1** had cross peaks between the anomer proton at δ_H 5.79 (1H, d, *J*=7.2 Hz, H-1'') and C-7 (δ_C 163.86) and between the oxygenated methyl signal at 3.82 (3'-OCH_3) and C-3' carbon at (δ_C 148.83). Sugar moiety signals were observed at δ_C 101.60 (C-1''), 78.97 (C-5''), 78.24 (C-3''), 74.61 (C-2''), 70.96 (C-4''), and 62.14 (C-6''), suggesting the presence of *β*-glucopyranosyl unit. Compound **1** was finally identified as chrysoeriol-7-*O*-*β*-D-glucopyranoside (thermopsoside) by comparing the spectroscopic data with the literature (Kattaeve and Nikonov, 1973). The gHMBC spectrum of compound **2** had a cross peak of the anomer proton signal at δ_H 5.77 (1H, d, *J*=7.2 Hz, H-1'') with C-7 (δ_C 163.76). Sugar moiety signals were observed at δ_C 100.51 (C-1''), 79.14 (C-2''), 78.40 (C-5''), 74.93 (C-3''), 71.09 (C-4''), and 62.30 (C-6''), suggesting the presence of a *β*-glucopyranosyl unit. These data led to identifying compound **2** as luteolin-7-*O*-*β*-D-glucopyranoside (luteoloside), which was confirmed by comparing our spectroscopic data with the literature (Boersma et al., 2002). The gHMBC spectrum of compound **3** had a cross peak between the anomer proton signal at δ_H 5.22 (1H, d, *J*=7.6 Hz, H-1'') and C-3 (δ_C 135.50). Sugar moiety signals were present at δ_C 104.26 (C-1''), 78.14 (C-3''), 78.01 (C-5''), 75.63 (C-2''), 71.12 (C-4''), and 62.10 (C-6''), suggesting the presence of *β*-glucopyranosyl unit. Compound **3** was identified as quercetin-3-*O*-*β*-D-glucopyranoside (isoquercetin) by comparing the spectroscopic data with those of the literature (Cui et al., 2012). Compound **4** was almost identical to compound **3** except for a monosaccharide moiety. An anomeric proton signal of compound **4** at δ_H 6.00 (1H, d, *J*=7.2 Hz, H-1'') had a cross peak with C-3 (δ_C 135.50) in the gHMBC spectrum. Sugar moiety signals were present at δ_C 105.31 (C-1''), 77.48 (C-3''), 75.29 (C-5''), 73.23 (C-2''), 69.67 (C-4''), and 61.78 (C-6''), indicating the presence of *β*-galactopyranosyl unit. Compound **4** was identified as quercetin-3-*O*-*β*-D-galactopyranoside (hyperin) by comparing the physical and spectroscopic data with those of the literature (Cui et al., 2012). The range of coupling constants (*J*=7.2 Hz to 7.6 Hz) of anomer protons in compounds **1-4** revealed that all compounds had a *β* configuration for the sugar moiety. For compound **5**, proton ^{13}C-NMR signals at δ_H 5.34 (1H, d, *J*=1.6 Hz, H-1'') and sugar moieties at δ_C 103.49 (C-1'') 73.24 (C-4''), 72.09 (C-3''), 71.98 (C-5''), 71.87 (C-2''), and 17.63 (C-6'') indicated the presence of an *α*-rhamnopyranosyl unit. The anomer proton was correlated with C-3 (δ_C 136.19) in the gHMBC spectrum. The coupling constant *J*=1.6 Hz of the anomer proton confirmed the *α* configuration of the sugar moiety. Therefore,



	R_1	R_2	R_3
1	CH_3	H	*β*-D-Glc
2	H	H	*β*-D-Glc
3	H	*O*-*β*-D-Glc	H
4	H	*O*-*β*-D-Gal	H
5	H	*O*-*α*-L-Rha	H

Fig. 1. Chemical structures of compounds **1-5** isolated from the fruits of *Rhus parviflora*.

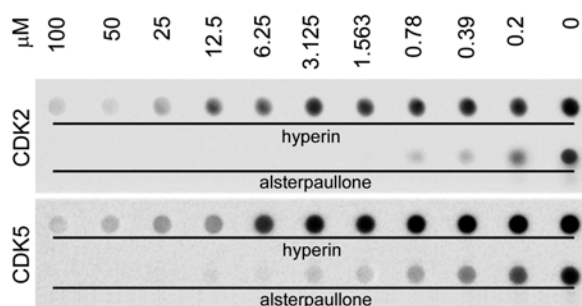


Fig. 2. CDK inhibition by compound **4** at various concentrations. Rb-C (retinoblastoma protein C-terminus) fusion proteins treated with compound **4** were incubated with CDK enzymes, and Rb phosphorylation in the dot-blot was visualized with phosphorylation-dependent antibody α-pRbSer780). Alsterpaullone was used as positive control, and the intensity of the negative control (DMSO) was regarded as 100% for quantitative analysis. Each panel is the representation of three independent experiments.

compound **5** was identified as quercetin-3-*O*-*α*-L-rhamnopyranoside (quercitrin) by comparing physical and spectroscopic data with the literature (Jung et al., 2007).

Compounds **1-5** were isolated for first time from *R. parviflora* fruit. Only compound **4** markedly inhibited CDK2 and CDK5 at IC_{50} values of 21.02 and 10.28 μM (Fig. 2) *in vitro*, respectively. In comparison with a well known positive control alsterpaullone, which showed IC_{50} values of 0.094 μM for CDK2 and 0.33 μM for CDK5, which were highly comparable with literature IC_{50} values of 0.20 and 0.040 μM (Leost et al., 2000); the inhibition of CDK2 is marginal but that of CDK5 is of significance. The aglycons of compounds **3** and **4** are the same but they have different sugar moieties (glucopyranose and galactopyranose, respectively). Compound **3** lacks significant calcium inhibitory activity (Ma et al., 2009), but compound **4** has been reported for marked activity to lower free intracellular calcium concentration (Chen and Ma 1999). Bei et al. (2009) attributed the ability of 100 μg/mL hyperin against hypoxia-reoxygen-induced injury to its ability to scavenge free radicals and preventing their formation. Furthermore, hyperin markedly prevented global cerebral ischemia and reperfusion-induced elevation in mitochondrial malondialdehyde generation at the same dose levels, at which point it decreases the cerebral infarct size in rats (Chen and Ma, 1999; Gupta et al., 2003). Furthermore, protection of neonatal rat neurons by hyperin in anoxia-reoxygenation injury may be related to inhibition of Ca^{2+} overload, NO release, and lipid peroxidation (Zhou and Chen, 2010). The CDK5/p25 formation is postulated to occur *via* intracellular calcium increase following calpain activation (Camins et al., 2006; Zhang et al., 2011), and inhibition of the Ca^{2+}-calpain-p25/Cdk5 pathway may be responsible for CDK5 inhibition. Compound **4** in comparison with N_6-isopentenyladenine (IC_{50} value of 80 μM) and indolylmethylene-indolinone 8a (IC_{50} value of 25 μM), (Knockaert et al., 2002) showed remarkable CDK5/p25 inhibition capacity. Although, the inhibitory capacity of Compound **4** was moderate in comparison with those of roscovitine with an IC_{50} value of 0.16 μM (Meijer et al., 1997) and flavopiridol with an IC_{50} value of 0.17 μM, (Losiewicz et al., 1994), as it is one of the dietary flavonoid glycosides that reach the central nervous system (Juergenliemak et al., 2003), the inhibitory activity was of significance. The CDK5/p25-specific inhibitor precisely targets neurons, and its activation is regarded as an early event in neurodegeneration. Therefore, compound **4** could be a potential drug target for preventing neurodegeneration.

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