Neuroprotective Constituents from *Hedyotis diffusa*

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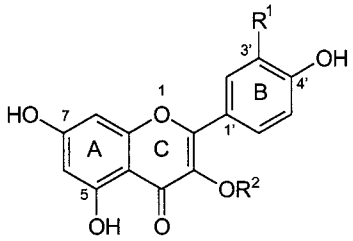
In a bioassay-guided search for neuroprotective compounds from medicinal plants, a MeOH extract of whole plants of *Hedoytis diffusa* yielded five flavonol glycosides, kaempferol 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside (**1**), quercetin 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside (**2**), quercetin 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-glucopyranoside (**3**), kaempferol 3-*O*-(2-*O*-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside (**4**), and quercetin 3-*O*-(2-*O*-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside (**5**), and four *O*-acylated iridoid glycosides (**6**−**9**). Compounds **1** and **2** are previously unreported natural products, and all nine compounds exhibited significant neuroprotective activity in primary cultures of rat cortical cells damaged by L-glutamate.

Glutamate is thought to be the major excitatory neurotransmitter in the central nervous system (CNS), where it is involved in neuronal survival, synaptogenesis, plasticity, learning, and memory.[1,2] Evidence is accumulating that glutamate and related excitatatory amino acid analogues cause a specific pattern of neurodegeneration in primary cultures of brain neurons.[3] Glutamate-induced neurotoxicity has been postulated to contribute to the neuronal injury and death that underlie many CNS disorders, such as seizures,[4] Alzheimer's disease,[5] Parkinson's disease,[6] ischemia,[7] and spinal cord trauma.[8] Therefore, glutamate-insulted primary cultures of rat cortical neurons were employed as a screening system to find neuroprotective compounds from natural sources.[9] *Hedyotis diffusa* extract showed significant neuroprotective activity from deleterious effects of glutamate in an in vitro assay system, and it prompted us to identify the active constituents of this plant using a bioactivity-guided isolation method.

Hedyotis diffusa Willd. (Rubiaceae), an annual herb distributed in northeastern Asia, is known in oriental folk medicine to have anticancer, antimicrobial, and antiinflammatory activities and is used to treat pneumonia in children, appendicitis, pelvitis, and some tumors in Korea and China.[10] Previous phytochemical studies revealed the presence of acylated iridoid glycosides[11,12] and anthraquinones,[13] but to the best of our knowledge, there have been no prior studies on its neuroprotective constituents. Herein we report the isolation of four known acylated iridoid glycosides and two new and three known flavonoid glycosides. The structural features required for neuroprotective properties are discussed.

Results and Discussion

A MeOH extract of air-dried whole plants of *H. diffusa* was evaporated in vacuo, suspended in H_2O, and successively partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH. The *n*-BuOH fraction showed neuroprotective activity (5 *μ*g/mL, 52.2%), and it was subjected to column chromatography over silica gel to yield 10 fractions. The active fractions were further separated by repetitive silica gel, Sephadex LH-20, MCI gel column chromatography, and ODS HPLC. Two new acylated flavonol glycosides (**1** and **2**) were isolated together with three known flavonoid glycosides **3**−**5** and acylated iridoid glycosides.



1 R^1 = H R^2 = gal $\xrightarrow{2}$ glc $\xrightarrow{6}$ feruloyl

2 R^1 = OH R^2 = gal $\xrightarrow{2}$ glc $\xrightarrow{6}$ feruloyl

3 R^1 = OH R^2 = glc $\xrightarrow{2}$ glc $\xrightarrow{6}$ feruloyl

4 R^1 = H R^2 = gal $\xrightarrow{2}$ glc

5 R^1 = OH R^2 = gal $\xrightarrow{2}$ glc

Compound **1** gave a positive FABMS molecular ion at *m*/*z* 787 $[M + H]^+$ and an aglycone peak at *m*/*z* 287. The molecular formula was determined as $C_{37}H_{38}O_{19}$ by HR-FABMS. Its ^{13}C NMR spectrum showed 37 carbon signals. The UV and ^1H NMR spectra were suggestive of flavonol glycosides.[14] The ^1H NMR spectrum revealed H-3′,5′ and H-2′,6′ protons at *δ* 6.85 (2H, d, $J = 8.8$ Hz) and 8.03 (2H, d, $J = 8.8$ Hz), respectively, suggesting a *para*-substituted B ring. In addition, a 5,7-dihydroxy-substituted A ring was evident from the two broad singlets at *δ* 6.10 and 6.27 for H-6 and H-8, respectively. These data together indicate that the aglycone moiety is kaempferol, and the ^{13}C NMR spectrum of **1** was comparable to that of kaempferol itself.[15] The fragment at *m*/*z* 449 [aglycone + hexose + H]$^+$ suggested the existence of an acyl moiety (194 amu) since it indicated loss of hexose and acyl moieties from the molecule. The presence of a *trans* feruloyl moiety was deduced from the ^1H NMR spectrum, which displayed three additional 1,2,4-trisubstituted aromatic protons (*δ* 7.10, 6.86, and 6.70), a pair of double bond protons (*δ* 6.19 and 7.35), and a methoxy proton (*δ* 3.73) and consistent with the ^{13}C NMR.[16] A disaccharide unit was suggested by the pair of anomeric carbon resonances at *δ* 98.4 and 104.5. Together the anomeric protons (*δ* 5.56 and 4.63) and anomeric carbon signals suggested the *β*-configuration of the glycoside bonds. The HMBC experiment showed a long-range correlation between C-3 (*δ* 132.9) and the anomeric proton (*δ* 5.56), revealing the site of glycosidation to be the

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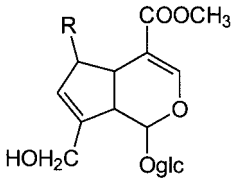
3-OH of kaempferol. Further evidence for 3-*O*-glycosylation was provided by the upfield shift of C-3 (ca. 2.7 ppm) and downfield shift of C-2 (ca. 9.4 ppm) by comparison with kaempferol. The ^{13}C NMR chemical shifts of the carbohydrate moiety of **1** were very similar to those of kaempferol 3-*O*-[2-*O*-(6-*O*-caffeoyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside isolated from *Brainea insignis*,[17] indicating that the sugar part of **1** is a [2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside. The sites of sugar and acyl linkages were further supported by the glycosylation shift (ca. 9 ppm) of C-2″ in galactose and acylation shift (ca. 2.5 ppm) of C-6‴ in glucose, respectively. These data established **1** to be kaempferol 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside.

Compound **2** gave the molecular formula $C_{37}H_{38}O_{20}$ based on HRFABMS. The UV spectrum indicated that **2** also had a flavonol skeleton.[14] The positive FABMS gave the molecular peak at *m*/*z* 803 $[M + H]^+$ and aglycone peak at *m*/*z* 303, implying an aglycone with an additional hydroxyl group compared with **1**. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1** except for the B ring. The ^1H NMR signals at *δ* 7.67, 7.51, and 6.85 suggested a 1,2,4-trisubstituted B ring. Therefore, the aglycone of **2** is quercetin, and this was confirmed by the ^{13}C NMR spectrum.[15] Other resonance signals in the ^1H and ^{13}C NMR spectra were in good agreement with **1**, suggesting that ferulic acid is esterified to the sugar moiety as [2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside. The site of glycosidation was revealed to be C-3 of quercetin by HMBC experiment, which showed a long-range correlation between C-3 (*δ* 133.0) and the anomeric proton (*δ* 5.63). The structure of **2** is therefore quercetin 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-galactopyranoside.

Compounds **3**−**5** were identified as quercetin 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl)-*β*-D-glucopyranosyl]-*β*-D-glucopyranoside (**3**),[16] kaempferol 3-*O*-(2-*O*-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside (**4**),[17] and quercetin 3-*O*-(2-*O*-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside (**5**),[18] respectively.

Four additional compounds were identified as iridoid glycosides. Their structures were determined as 6-*O*-*Z*-*p*-methoxycinnamoyl scandoside methyl ester (**6**), 6-*O*-*E*-*p*-methoxycinnamoyl scandoside methyl ester (**7**), 6-*O*-*Z*-*p*-coumaroyl scandoside methyl ester (**8**), and 6-*O*-*E*-*p*-coumaroyl scandoside methyl ester (**9**). The identification of these known compounds was carried out by comparing their physical and spectral data with previously reported values.[11,12]



6 R = 6-*O*-*Z*-*p*-methoxycinnamoyl

7 R = 6-*O*-*E*-*p*-methoxycinnamoyl

8 R = 6-*O*-*Z*-*p*-coumaroyl

9 R = 6-*O*-*E*-*p*-coumaroyl

The glycosides were all tested for their neuroprotective activities using primary cultures of rat cortical cells (Table 1). Activities were evaluated by assessing the viability of cortical cells after treatment with glutamate. All attenuated glutamate-induced neurotoxicity at 0.1−10 *μ*M levels.

Table 1. Neuroprotective Activities of Compounds **1**−**9** on Primary Cultures of Rat Cortical Cells Injured by Glutamate[a]

	cell viability (%)[b]		
compound	0.1 *μ*M	1 *μ*M	10 *μ*M
1	25.2 ± 3.21*,[c]	66.9 ± 5.8***	25.2 ± 3.6*
2	62.7 ± 1.1***	72.9 ± 3.3***	75.0 ± 0.2***
3	62.9 ± 2.9***	71.5 ± 1.6***	73.7 ± 2.7***
4	4.6 ± 3.6	27.0 ± 4.9 *	25.2 ± 3.6*
5	48.2 ± 0.7**	66.0 ± 1.6***	54.0 ± 2.9***
6	28.8 ± 4.6*	54.6 ± 2.9**	23.7 ± 4.4*
7	65.4 ± 4.1***	71.8 ± 2.8***	52.8 ± 3.9**
8	6.9 ± 0.9	25.7 ± 2.0*	6.3 ± 3.5
9	14.5 ± 1.0	62.2 ± 4.0***	26.8 ± 3.5*

[a] Cortical cell cultures were washed with DMEM and incubated with test compounds for 1 h. The cultures were then exposed to 50 *μ*M glutamate for 24 h in the presence of test molecules. After 24 h incubation, the cultures were assessed for the extent of neuronal damage (treatment throughout). The values shown are the mean ± SEM of three experiments. [b] Cell viability was determined by LDH assay. LDH released from control and glutamate-treated cultures were 110.9 ± 8.3 and 197.6± 10.2 mU/mL, respectively. Cell viability was calculated as 100 × (LDH released from glutamate-treated − LDH released from glutamate + test compound)/(LDH released from glutamate-treated − LDH released from control). [c] Results differ significantly from the glutamate-treated cells, *p < 0.05, **p < 0.01, ***p < 0.001; ANOVA test.

Among them, compounds **2**, **3**, and **7** significantly preserved the viability of glutamate-treated rat cortical cells, as assessed by the LDH assay.

Table 1 reveals several patterns in structure/activity of flavonoid glycosides (**1**−**5**). Compounds **2** and **3**, which possess both a di-OH B ring and an acyl substituent in the glycone moiety, show the strongest activity. The neuroprotective activities were reduced by loss of either feature as exemplified by **1** and **5**, respectively. Compound **4**, which has neither, exhibits the weakest activity. These cumulative results indicate that the presence of both a di-OH in the B ring and an acyl substituent might be crucial for the biological response, whereas replacement of galactose with glucose in the sugar part does not result in a change of activity.

The iridoid glycosides possess acyl moieties but differ in the stereochemistry of the double bond and the existence of a *p*-methoxy substituent in the aromatic ring. The viabilities of sample-treated cortical cells were in increasing order **8** < **9** ≅ **6** < **7**. This result demonstrates that iridoids with an *E*-acyl moiety are more active than the *Z* form. Thus, **9** (*E*) shows stronger activity than **8** (*Z*), and the same is true for **7** (*E*) versus **6** (*Z*). A methoxy group in the aromatic ring of the acyl moiety seems to be important in producing neuroprotection against glutamate-induced toxicity. Compounds having *p*-methoxycinnamoyl acyl moieties (**6** and **7**) exhibit more potent activities than their coumaroyl counterparts (**8** and **9**). This structure−activity study demonstrates that a *p*-methoxy group in the aromatic ring and a *trans* double bond in the acyl moiety of acylated iridoid glycosides play an essential neuroprotective role against glutamate-induced damage.

Experimental Section

General Experimental Procedures. Melting points were determined with a Buchi B-540 instrument. UV spectra were recorded in MeOH using a Shimadzu UV-1601 PC spectrophotometer. IR spectra were recorded in KBr using a Perkin-Elmer 1710 spectrometer. ^1H NMR spectra were recorded on a Bruker AMX 500 spectrometer (500 MHz) and ^{13}C NMR on a JEOL GSX 400 spectrometer (400 MHz) or JEOL LA 300 spectrometer (300 MHz). The FABMS were measured on a

JEOL JMS-AX505WA mass spectrometer and HRFABMS on a JEOL JMS-HX 110/110A mass spectrometer. Optical rotations were measured on a JASCO DIP 1000 digital polarimeter. Column chromatography was performed over Si gel 60 (230−400 mesh, Merck), MCI gel (CHP 20P 75−100 μm, Mitsubishi Chemical Co., Japan), or Sephadex LH-20 (25−100 μm, Pharmacia). HPLC separations were performed with a Hitachi HPLC system (L-7100 Pump, L-7420 UV−vis detector) on an ODS H80 column (4 μm, 250 × 10 mm, YMC).

Plant Material. Whole dried plants were purchased from Kyungdong oriental medicine market, Seoul, Korea, in 1998, and identified by Dr. Dae Suk Han, emeritus professor, College of Pharmacy, Seoul National University. A voucher specimen (SNUPH-0090) has been deposited in the herbarium of the College of Pharmacy, Seoul National University.

Extraction and Isolation. Whole plants (2.5 kg) were extracted three times with MeOH using an ultrasonic apparatus for 3 h, and the extract was concentrated in vacuo. The extract (62 g) was suspended in water and successively partitioned with *n*-hexane (19.8 g), $CHCl_3$ (7.4 g), and *n*-BuOH (15.0 g). Separation of neuroprotective principles was carried out by activity-guided fractionation. The *n*-BuOH extract, which showed neuroprotective activity, was fractionated by column chromatography over silica gel using a $CHCl_3$ and MeOH mixture of increasing polarity to give 10 fractions (100% $CHCl_3$ → 100% MeOH). Fractions 5, 7, and 10 showed significant neuroprotective activity. Fraction 5 was separated by semipreparative HPLC (YMC J'sphere, ODS-H80, 4 μm, 10 × 250 mm, 2 mL/min) in MeOH−H_2O (55:45) to obtain compounds **6** (24 mg, t_R 23.0 min) and **7** (8.9 mg, t_R 20.6 min), and fraction 7 by HPLC in MeOH−H_2O (45:55) yielded compounds **8** (11.9 mg, t_R 18.2 min) and **9** (38.6 mg, t_R 22.2 min) with monitoring at 230 nm. The last fraction was further chromatographed on MCI gel using H_2O−MeOH (gradient) as a solvent system, providing four subfractions (subfraction 10-1: 25% MeOH, 10-2: 50% MeOH, 10-3: 75% MeOH, 10-4: 100% MeOH). Subfraction 10-2 was purified by HPLC on an ODS column with 20% CH_3CN monitored at 254 nm to give compounds **4** (10.2 mg, t_R 13.2 min) and **5** (9.7 mg, t_R 10.4 min). Subfraction 10-3 was resolved by Sephadex LH-20 column chromatography using MeOH and then purified by HPLC on an ODS column in 25% CH_3CN with monitoring at 254 nm affording compounds **1** (19 mg, t_R 9.5 min) and the mixture of **2** and **3** (t_R 12.2 min). Re-HPLC of a mixture of **2** and **3** using 17% CH_3CN in H_2O provided compounds **2** (8.9 mg, t_R 64.5 min) and **3** (9.7 mg, t_R 68.2 min).

Kaempferol 3-*O*-[2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside (1): dark yellow amorphous powder; mp 210−212 °C; $[\alpha]^{25}_D$ −0.026 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 267, 327 nm; IR (KBr) ν_{max} 3421 (OH), 1655 (α,β-unsaturated C=O), 1606, 1509 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.65 (1H, s, C-5, OH), 8.03 (2H, d, J = 8.8, H-2′,6′), 7.35 (1H, d, J = 15.9, H-γ), 7.10 (1H, d, J = 1.6, H-2‴′), 6.86 (1H, dd, J = 8.1, 1.6, H-6‴′), 6.85 (2H, d, J = 8.8, H-3′, 5′), 6.70 (d, J = 8.1, H-5‴′), 6.27 (1H, br s, H-8), 6.19 (1H, d, J = 15.9, H-β), 6.10 (1H, br s, H-6), 5.56 (1H, d, J = 7.5, H-1″), 4.63 (1H, J = 7.8, H-1‴), 3.73 (3H, s, OCH_3), 2.3−4.2 (m, sugar protons); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 177.4 (s, C-4), 166.5 (s, C-α), 164.1 (s, C-7), 161.2 (s, C-5), 159.9 (s, C-4′), 156.2 (s, C-2), 155.4 (s, C-9), 149.2 (s, C-4‴′), 147.8 (s, C-3‴′), 145.0 (d, C-γ), 132.9 (s, C-3), 131.0 (d, C-2′, C-6′), 125.4 (s, C-1‴′), 122.9 (d, C-6‴′), 120.8 (s, C-1′), 115.3 (d, C-5‴′), 115.2 (d, C-3′, C-5′), 113.9 (d, C-β), 110.9 (d, C-2‴′), 104.5 (d, C-1‴), 103.8 (s, C-10), 98.6 (d, C-6), 98.4 (d, C-1″), 93.6 (d, C-8), 81.1 (d, C-2″), 76.2 (d, C-5″), 75.8 (d, C-3‴), 74.5 (d, C-2‴), 74.0 (d, C-5‴), 73.3 (d, C-3″), 69.5 (d, C-4‴), 67.5 (d, C-4″), 63.3 (t, C-6‴), 59.8 (t, C-6″), 55.6 (q, OCH_3); FABMS (glycerol) *m*/*z* 787 $[M + H]^+$, 449 [(M − feruloyl glucose) + $H]^+$, 287 [aglycone + $H]^+$; HRFABMS *m*/*z* 809.1921 (calcd for $C_{37}H_{38}O_{19}Na$, 809.1905).

Quercetin 3-*O*-[2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside (2): dark yellow amorphous powder; mp 207−210 °C; $[\alpha]^{25}_D$ −0.058 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 251, 262, 332 nm; IR (KBr) ν_{max} 3401 (OH), 1655 (α,β-unsaturated C=O), 1608, 1508 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.66 (1H, s, C-5, OH), 7.67 (1H, dd, J = 8.5, 2.0, H-6′), 7.51 (1H, d, J = 2.0, H-2′), 7.34 (1H, d, J = 15.9, H-γ), 7.10 (1H, d, J = 1.3, H-2‴′), 6.86 (1H, dd, J = 8.1, 1.3, H-6‴′), 6.85 (1H, d, J = 8.5, H-5′), 6.70 (1H, d, J = 8.1, H-5‴′), 6.27 (1H, br s, H-8), 6.18 (1H, d, J = 15.9, H-β), 6.14 (1H, br s, H-6), 5.63 (1H, d, J = 7.5, H-1″), 4.63 (1H, J = 7.6, H-1‴), 3.74 (3H, s, OCH_3), 2.5−4.5 (m, sugar protons); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 177.4 (s, C-4), 166.6 (s, C-α), 163.9 (s, C-7), 161.2 (s, C-5), 159.9 (s, C-4′), 156.1 (s, C-9), 155.2 (s, C-2), 149.2 (s, C-4‴′), 147.8 (s, C-3‴′), 145.0 (d, C-γ), 144.9 (s, C-3′), 148.5 (s, C-4′), 133.0 (s, C-3), 125.4 (s, C-1‴′), 122.8 (d, C-6‴′), 122.2 (d, C-6′), 121.1 (s, C-1′), 115.9 (d, C-5′), 115.3 (d, C-2′), 115.3 (d, C-5‴′), 114.0 (d, C-β), 111.0 (d, C-2‴′), 104.7 (d, C-1‴), 103.8 (s, C-10), 98.5 (d, C-6), 98.2 (d, C-1″), 93.3 (d, C-8), 81.6 (d, C-2″), 76.3 (d, C-5″), 75.8 (d, C-3‴), 74.6 (d, C-2‴), 73.9 (d, C-5‴), 73.2 (d, C-3″), 69.4 (d, C-4‴), 67.5 (d, C-4″), 63.2 (t, C-6‴), 59.8 (t, C-6″), 55.6 (q, OCH_3); FABMS (glycerol) *m*/*z* 803 $[M + H]^+$, 303 [aglycone + $H]^+$; HRFABMS *m*/*z* 825.1871 (calcd for $C_{37}H_{38}O_{20}Na$ 825.1854).

Cell Culture. Primary cultures of mixed cortical cells were prepared from 17- to 19-day-old fetal Sprague-Dawley rats, as described previously.[9] In brief, the trypsin-dissociated cortical cells were plated on 15-mm dishes (Falcon, Lincoln Park, NJ) coated with poly-L-lysine at a density of 1 × 10^5 cells/dish. The cortical cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Gibco) with penicillin (100 IU/mL, Sigma, St. Louis, MO) and streptomycin (10 μg/mL, Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. After 3 days in culture, nonneuronal cell division was halted by the addition of 5-fluorodeoxyuridine (50 μM, Sigma). Cultures were allowed to mature for 2 weeks before they were used for experiments.

Neurotoxicity. All compounds and extracts were dissolved in DMSO (final culture concentration, 0.1%). Preliminary studies indicated that the solvent had no effect on cell viability at the concentration used. Cortical cell cultures were washed with DMEM and incubated with sample for 1 h. The cultures were then exposed to 50 μM glutamate for 24 h with samples. After 24 h incubation, the cultures were assessed for the extent of neuronal damage.

Assessment of Neurotoxicity. Neuronal integrity was assessed by spectrophotometric measurement of the efflux of LDH into the culture medium. To assess neuronal integrity, 30 μL of the medium was collected and assayed for LDH release by using the modified method of Choi and Koh.[19] Optical densities (OD) were read by using an automated spectrophotometric plate reader set at 450 nm. Data were expressed as percent cell viability relative to vehicle-treated control cultures. Cell viability was calculated as 100 × (OD of glutamate + sample treated − OD of glutamate-treated)/(OD of control − OD of glutamate-treated).

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