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Neuraminidase inhibitory activities of flavonols isolated from *Rhodiola rosea* roots and their in vitro anti-influenza viral activities

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ABSTRACT

Five flavonols (**3**, **5**, and **9**–**11**) were isolated from *Rhodiola rosea*, and compared with commercially available flavonoids (**1**, **2**, **4**, **6**–**8**, and **12**–**14**) to facilitate analysis of their structure–activity relationship (SAR). All compounds (**1**–**14**) showed neuraminidase inhibitory activities with IC_{50} values ranging from 0.8 to 56.9 μM. The in vitro anti-influenza virus activities of flavonoids **1**–**6**, **8**–**12**, and **14** were evaluated using two influenza viral strains, H1N1 (A/PR/8/34) and H9N2 (A/Chicken/Korea/MS96/96), testing their ability to reduce virus-induced cytopathic effect (CPE) in MDCK cells. We found that the activity of these compounds ranged from 30.2 to 99.1 μM against H1N1- and 18.5 to 133.6 μM against H9N2-induced CPE. Of compounds **1**–**14**, gossypetin (**6**) exhibited the most potent inhibitory activity, with IC_{50} values of 0.8 and 2.6 μM on neuraminidases from *Clostridium perfringens* and recombinant influenza virus A (rvH1N1), respectively. In contrast, kaempferol (**3**) exhibited the highest activity against two influenza viruses, H1N1 and H9N2 with EC_{50} values of 30.2 and 18.5 μM, respectively. Activity depended on the position and number of hydroxy groups on the flavonoids backbone. In kinetic studies, all isolated compounds behaved as noncompetitive inhibitors.

1. Introduction

The influenza virus is a highly infective agent that causes acute pulmonary diseases. In more serious cases, influenza causes pneumonia, which can be particularly fatal in young children, the elderly, and patients with cardiopulmonary diseases. In addition, influenza spreads around the world in seasonal epidemics, killing numerous people in pandemic years.[1] The influenza virus is an RNA virus of the family *Orthomyxoviridae*, which includes influenza viruses A, B, and C, and two other genera. Among these, influenza viruses A and B are responsible for the epidemic spread of influenza. The influenza virus contains eight pieces of segmented RNA, with hemagglutinin (HA) and neuraminidase (NA) as surface antigens. HA is responsible for most antigenic variations and contains binding sites for sialic acid residues on the surface of target cells, mediating the binding of the virus to target cells and the subsequent entry of the viral genome.[2] NA is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles. Specifically, NA cleaves the α-ketosidic bond that links a terminal neuraminic-acid residue to the adjacent oligosaccharide moiety, NA is therefore essential for the movement of the virus to and from sites of infection in the respiratory tract.[3,4] To develop new agents to treat viral disease, significant attention has been devoted to compounds that inhibit viral adsorption to epithelial cells, viral intrusion into cells, transcription and replication of viral genomes, viral protein expression, and progeny virus release from cells.[5]

To date, four antiviral agents have been approved by the FDA to treat influenza virus infection: amantadine, rimantadine, zanamivir, and oseltamivir. Amantadine and rimantadine block the M2 ion channel, which is essential for viral proliferation, thereby interfering with viral uncoating inside cells. Both the these M2 inhibitors, however, are effective only against influenza virus A and are associated with several toxic effects in the digestive and autonomic nervous systems, as well as with the emergence of drug-resistant variants during their 40 years of use.[6] Zanamivir and oseltamivir inhibit viral NA, which plays an important role in viral proliferation and is stably present in both influenza viruses A and B, and both agents have been used for the treatment and prophylaxis of influenza viruses A and B.[7] Although zanamivir has high antiviral activity, its bioavailability is low and it is excreted rapidly by the kidneys, while nausea and vomiting are frequent among adults receiving oseltamivir.[8]

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To develop novel neuraminidase inhibitors, pterocarpans and flavanones have been isolated by bioassay-guided fractionation of the methanolic extracts of the root of *Sophora flavescens*; these compounds showed potent inhibitory activity against NA from *Clostridium perfringens*.[9] Also, we studied flavanone from *Cudrania tricuspidata* exhibited NA inhibitory activities.[10] In addition, we found that ethyl acetate extracts of the root of *Rhodiola rosea* exhibited significant NA inhibitory activities with IC_{50} of 1.25 μg/mL. *R. rosea* is a perennial herbaceous plant in the Crassulaceae family that grows in cold regions and can be used for food and medicine. In folk remedies, *R. rosea* has been used as a sedative, an antipyretic, and an astringent. It is also prescribed as a powder or decoction for the treatment of diabetes, anemia, cholesystitis, fatigue, and nervous prostration and for postpartum care.[11] Many ingredients can be isolated from the roots and stems of *R. rosea*, including flavonoids,[12] phenylpropanoid and phenylethanol derivatives,[13] and aliphatic glycosides.[14] In this study, we describe the isolation, structure characterization, and antiviral activities of flavonoids **1**–**14** on neuraminidase and of **1**–**6**, **8**–**12**, and **14** on two kinds of influenza virus, H1N1 and H9N2, in MDCK cells.

2. Results and discussion

The isolation of constituents **3**, **5**, and **9**–**11** from *R. rosea* was guided by their NA inhibitory and anti-influenza virus activities. Commercially available flavone derivatives (**1**, **2**, **4**, **6**–**8**, and **12**–**14**) were prepared to investigate the best pharmacophore in the flavonoid scaffold, as shown in Figure 1. Based on spectroscopic data, including 2D NMR, and LC/MS fragmentation patterns, the isolated flavonol compounds were easily identified as kaempferol (**3**), herbacetin (**5**), rhodiolinin (**9**), rhodionin (**10**), and rhodiosin (**11**) (Table 1), all of which were compared with previous literature values.[15–17]

The biological activities of flavonoid derivatives **1**–**14** were assessed against NAs from *C. perfringens* and recombinant influenza virus A (rvH1N1) and confirmed using oseltamivir (Tamiflu) as a positive control. The IC_{50} value of oseltamivir with respect to influenza virus NA inhibition was 1.59 nM. We found that the IC_{50} values of the flavonoid derivatives **1**–**14** ranged from 0.8 to 56.9 μM against *C. perfringens* NA and from 2.2 to 56.5 μM against rvH1N1 NA (Table 2) and that all were dose dependent. The NA inhibitory activity of these compounds was ascertained by measuring the concentration required to inhibit 50% of the enzyme activity (IC_{50}) (Fig. 2).

To explore the structure–activity relationship (SAR) of isolated kaempferol (**3**), which has four hydroxy groups and showed IC_{50} values against *C. perfringens* and rvH1N1 NAs of 8.0 and 11.2 μM, respectively, we examined the optimal position and number of hydroxy groups on a flavone backbone and compared these results with those of various glycoside compounds. To assess the effect of the position of hydroxy groups, we evaluated the activity of luteolin (**2**), which has 3′ and 4′ dihydroxy groups. We found that luteolin had greater activity against bacterial neuraminidase (IC_{50} = 4.3 μM) than its analog **3** containing 3,4′-dihydroxy groups (IC_{50} = 8.0 μM). In contrast, apigenin (**1**), which has one fewer hydroxy moiety, has lower activity. Quercetin (**4**), which has more hydroxy substitutions, was equivalent in activity to herbacetin (**5**), which has 7,8-dihydroxy moieties. Interestingly, gossypetin (**6**), which is more hydrophilic, has a 10- or 4-fold increased potency compared with **3** against both *C. perfringens* and rvH1N1 NAs, with IC_{50} values of 0.8 and 2.6 μM, respectively, but showed a similar activity as **4** against rvH1N1 NA. Vicinal 7,8-hydroxy-protected rhodiolinin (**9**) showed an attenuated potency compared with herbacetin (**5**). Subsequently, we compared the activities of glycoside flavonoids **7**, **8**, and **10**–**14** with those of aglycone compounds **1**, **3**, and **4**–**6**. We found that glycosides showed 3- or 50-fold lower activities than the aglycone flavonoids.

We also assessed the activity of these compounds in vitro against two types of influenza virus, H1N1 (A/PR/8/34) and H9N2 (A/Chicken/Korea/MS96/96), using the CPE reduction assay in MDCK cells. The EtOAc soluble extract of *R. rosea* showed anti-influenza virus activities with EC_{50} values of 102.1 μg/mL against H1N1 and 145.4 μg/mL against H9N2. The water soluble extract of *R. rosea* also showed anti-influenza virus activities with EC_{50} values of 78.5 μg/mL against H1N1 and 139.7 μg/mL against H9N2. All tested flavone derivatives (**1**–**6**, **8**–**12**, and **14**) exhibited dose-dependent anti-influenza virus activities (SI values $\geqslant$ 1.5; SI = CC_{50}/EC_{50}) (Table 3).

The antiviral activities of the flavone derivatives **1**–**6**, **8**–**12**, and **14** had EC_{50} values of 30.2–99.1 μM against H1N1 (SI value = 1.96 to >9.93) and 18.5–103.1 μM against H9N2 (SI value = 1.60 to 16.22). Interestingly, flavonols **3**, **5**, and **9**–**11** had greater activity against H9N2 than against H1N1. All reference flavonoids apart from apigenin (**1**) showed more potent activity against H1N1 than against H9N2. Kaempferol (**3**) was the most effective, with EC_{50} values of 30.2 μM (SI value >9.93) against H1N1 and 18.5 μM (SI value >16.22) against H9N2. Although **3** showed fourfold less activity than oseltamivir (EC_{50} value = 8.3 μM against H1N1 and 6.25 μM against H9N2), kaempferol (**3**) showed high SI values, >9.93 and 16.22, suggesting that this compound may be a potent antiviral agent against influenza virus with no toxicity to host MDCK cells (CC_{50} value, >300 μM).

When we tested the anti-influenza virus activities of kaempferol (**3**) and oseltamivir by IFA, we observe green fluorescence in early-virus infected but not in mock-infected MDCK cells (Fig. 3B). Treatment of cells with 50 μM kaempferol (**3**) or oseltamivir, however, reduced the number of fluorescence-positive cells (Fig. 3C and D).

Replacement of the hydroxy group in **3** with a glycoside group yielded compounds **8** and **14**, both of which reduced anti-influenza activities, with **8** having an EC_{50} of 40.0 μM against H1N1 and 61.9 μM against H9N2; and **14** having an EC_{50} of 40.1 μM against H1N1 and 72.9 μM against H9N2. The compounds apigenin (**1**) and luteolin (**2**), which have one or two fewer hydroxyl moiety groups are substituted on the flavones backbone, had similar antiviral activities, with EC_{50} values of 30.2–43.1 μM against H1N1. Interestingly, the herbacetin derivative **9**, which had similar activity, was structurally related to the active constituent **5**, with the only difference between **3** and **5** being in the formation of protected hydroxy groups between C-7 and C-8 in **9**.

We used Lineweaver–Burk and Dixon plots to analyze kinetic mode and inhibition constant (K_i) for flavonoids **1**–**14**. The results for flavonoids **2**–**4**, **6**, and **9**–**10** are illustrated in Figure 4. Lineweaver–Burk plots (Fig. 4A–D) showed that, for compounds **2**–**4** and **6**, the V_{max} values were inversely related to concentration, without changing K_m. These inhibitors gave a family of straight lines, all of which intercepted on the *x*-axis. The three lines obtained from the uninhibited enzyme and two concentrations of compounds **9** and **10** intersected on second quadrant at a non-zero point (Fig. 4E and F). These results indicate that compounds **2**–**4**, **6**, and **9**–**10** are noncompetitive inhibitors of both NAs. A summary of the K_i values for compounds **1**–**14** showed that these results agreed with those with respect to the NAs (Table 2).

Du and co-workers suggested that the presence of more OH groups in the B-ring of flavonoids reduced their NA inhibitory effect.[18] However, we found that more hydroxyl groups in the A- or B-ring on flavones[10] and flavonols is essential for showing their potent inhibitory activity on NAs from *C. perfringens* and rvH1N1. Also, glycosides resulted in a significant loss of potency, suggesting

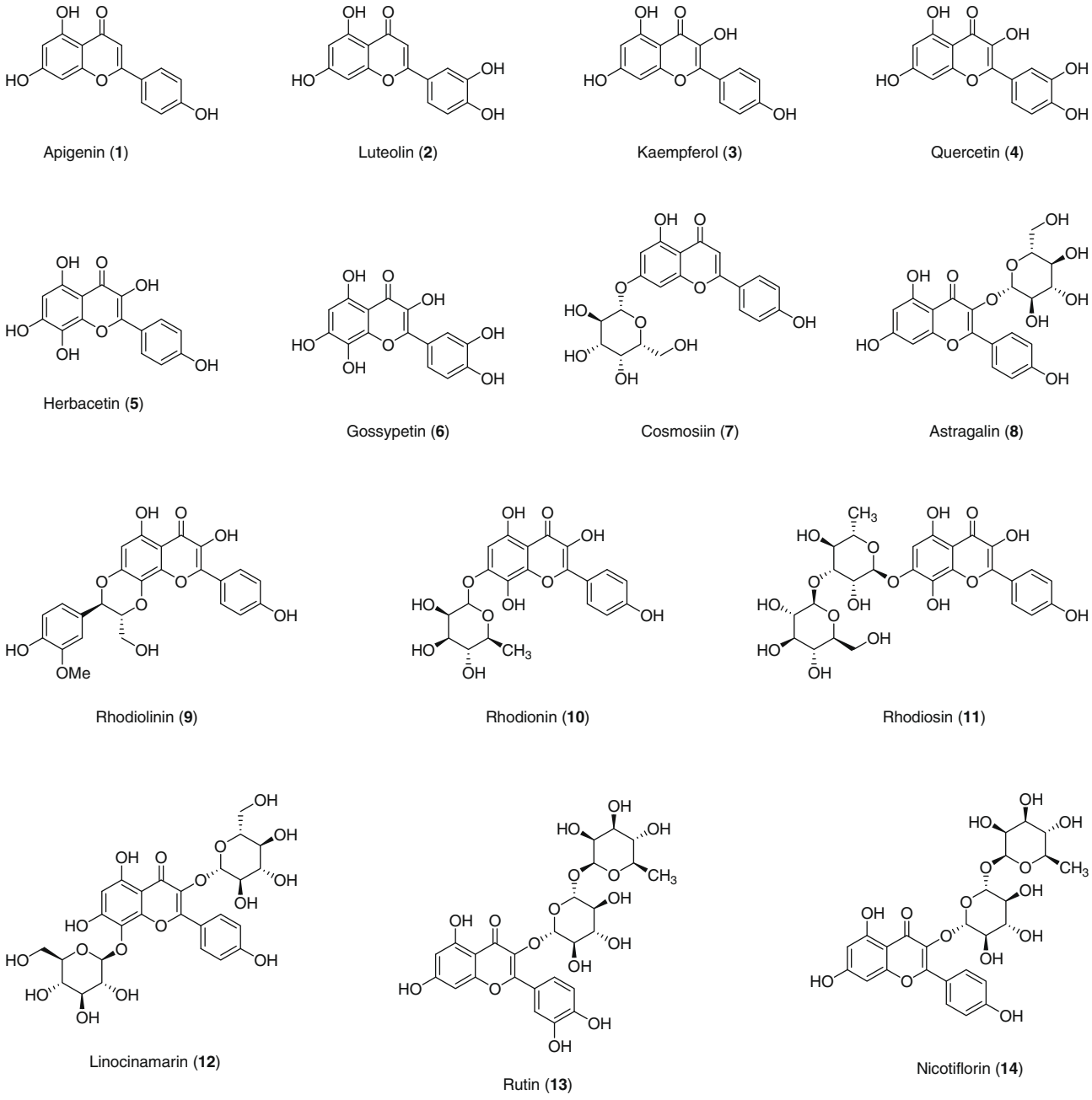

Figure 1. Chemical structures of isolated flavonols **3**, **5**, **9**–**11** from *R. rosea* and **1**, **2**, **4**, **6**–**8**, and **12**–**14**.

that binding to the active site of the target enzyme may be interrupted by a bulky sugar moiety.[19] Glycosides, however, are main component in natural sources such as plants and microbials and have many advantages in drug development. For example, sugar moieties attaching to natural compounds play a critical role in the biological activity with increasing in vivo solubility. These compounds remain in blood for a long time and bind specifically to target enzymes.[20] Glycosylation is also involved, in that drug molecules like aglycone are released from blood. Representatively, esperamicin and calicheamicinγ1I may act as enediyne antibiotics,[21] doxorubicin as an anti-cancer agent,[22] and digoxin, vancomycin, and lincomycin[23] as glycoside-drugs.

In summary, we have assessed the NA and influenza virus inhibitory activities of 14 flavonoids (**1**–**14**), including the isolated flavonols (**3**, **5**, and **9**–**11**) from *R. rosea*. These flavonoids were compared to determine the optimal position and number of hydroxy groups on the flavones backbone to exhibit significant and specific NA inhibitory activities. Especially, compound **3** was shown to be effective against influenza viruses by a CPE reduction assay. Our result, therefore, provided information relating the chemical structure of flavonoids to their inhibition of NA activity and viral replication. Also, these findings suggest that constituents and extracts of *R. rosea* may be considered as potential therapeutic agent in the treatment of influenza virus infections.

3. Materials and methods

3.1. Plant material

Commercial herb samples of *R. rosea* dried root were purchased from the Korea *Rhodioila rosea* association.

Table 1
^{13}C NMR of compounds **9**–**11** at 125 MHz (ppm)[a]

Position	Compound		
	9	**10**	**11**
2	148.2	148.7	148.7
3	137.5	137.3	137.3
4	177.5	177.8	177.8
4a	105.6	106.0	105.7
5	154.2	153.8	153.6
6	99.3	99.2	100.5
7	150.6	151.5	151.0
8	126.2	128.4	128.5
8a	145.6	146.1	145.9
1′	123.7	123.8	123.8
2′	131.0	131.1	131.1
3′	116.3	116.3	116.3
4′	160.7	160.7	160.7
5′	116.3	116.3	116.3
6′	131.0	131.1	131.1
Rhm-1[b]	128.7 (1″)	100.9	99.1
Rhm-2	112.1 (2″)	71.7	71.1
Rhm-3	149.2 (3″)	72.0	82.4
Rhm-4	148.6 (4″)	73.7	72.5
Rhm-5	121.8 (5″)	71.2	70.8
Rhm-6	121.8 (6″)	18.0	18.2
Glc-1[c]	78.9 (7″)		106.1
Glc-2	79.7 (8″)		77.8
Glc-3	61.9 (9″)		75.4
Glc-4			71.2
Glc-5			77.9
Glc-6			62.4
OCH_3	56.5		

[a] The chemical shifts of compounds **9**–**11** were determined in *methanol-d_4*.
[b] Rhm = rhamnose.
[c] Glc = glucose.

Table 2
Inhibitory effects of compounds **1**–**14** on NAs from *C. perfringens* and recombinant influenza virus A (rvH1N1)

Compound	*C. perfringens* IC_{50}[a] (μM)	rvH1N1 IC_{50}[a] (μM)	Inhibition type (K_i, μM)
1	17.4 ± 0.5	33.4 ± 7.0	Noncompetitive (37.1 ± 2.1)
2	4.3 ± 0.1	11.0 ± 0.7	Noncompetitive (19.6 ± 3.7)
3	8.0 ± 1.0	11.2 ± 1.0	Noncompetitive (21.1 ± 1.1)
4	1.7 ± 0.6	2.2 ± 0.3	Noncompetitive (3.8 ± 0.6)
5	1.4 ± 0.2	8.9 ± 1.4	Noncompetitive (13.7 ± 3.4)
6	0.8 ± 0.1	2.6 ± 1.1	Noncompetitive (7.7 ± 1.5)
7	39.3 ± 3.3	46.9 ± 1.0	Noncompetitive (53.8 ± 0.6)
8	29.4 ± 1.2	38.4 ± 6.3	Noncompetitive (33.0 ± 5.2)
9	6.1 ± 2.2	10.3 ± 0.2	Noncompetitive (14.1 ± 1.1)
10	40.6 ± 3.7	32.2 ± 3.2	Noncompetitive (59.4 ± 2.0)
11	56.9 ± 8.6	56.5 ± 0.5	Noncompetitive (64.1 ± 10.0)
12	39.1 ± 5.5	44.2 ± 3.9	Noncompetitive (63.8 ± 4.6)
13	30.9 ± 2.5	34.4 ± 5.0	Noncompetitive (33.8 ± 3.3)
14	55.5 ± 2.2	31.7 ± 3.0	Noncompetitive (32.7 ± 7.1)
Oseltamivir[b]	—	1.59 nM	NT[c]

[a] All compounds were examined in a set of duplicated experiment; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss.
[b] Oseltamivir was used as a positive control.
[c] NT = not tested.

3.2. General apparatus and chemicals

C. perfringens NA (EC 3.2.1.18, N2876) and 2′-(4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (M8639) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant influenza A virus H1N1 (rvH1N1) neuraminidase was from R&D systems, Inc. (4858-NM). Compounds **1**, **2**, and **4** were purchased from Sigma–Aldrich (St. Louis, MO) and compounds **6**–**8** and **12**–**14** from Chromadex Inc. All purifications were monitored by TLC (E. Merk Co., Darmstadt, Germany) using commercially available glass plates pre-coated with silica gel (E. Merck Co.), and visualized under UV at 254 nm or stained with 10% H_2SO_4. Column chromatography was performed on 230–400 mesh silica gel (Kieselgel 60, Merck, Germany). Melting points (mp) were measured on a Thomas Scientific capillary melting point apparatus (Electrothermal 9300, UK) and are uncorrected. ^{1}H and ^{13}C NMR data were all obtained on a JNM-ECA 500 (^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz) spectrometer (Jeol, Japan) in $CDCl_3$ or CD_3OD with TMS as an internal standard. Enzyme activity and kinetic analysis were assessed by LS-55 luminescence spectrometer (Perkin–Elmer Limited, Beaconsfield, Buckinghamshire, United Kingdom) and FLx 800 (BioTeck Instrument Inc., USA).

3.3. Extraction and isolation

The dried roots of *R. rosea* (1.0 kg) were extracted with 95% MeOH (10 L × 2) at room temperature for 10 days. The insoluble precipitate was removed by filtration and the solution was concentrated to dryness under reduced pressure. The residue (380 g) was suspended in H_2O and partitioned with *n*-hexane (10.3 g), EtOAc (59.8 g), and *n*-BuOH (182.7 g). The ethyl acetate-soluble fraction (59.8 g) was subjected to two rounds of silica gel column chromatography eluting with a mixture of chloroform and methanol, followed by purification through column chromatography on Sephadex LH-20. The ethyl acetate-soluble fraction was developed on a silica gel column (silica gel: Merck, Art 9385, column size: φ7 × 40 cm) eluted with 1.5 L of a mixture of chloroform:methanol (v/v, 9:1, 7:1, 5:1, 3:1, or 1:1) or 1.5 L of 100% methanol to yield three fractions. Fraction 1 (7.6 g, with chloroform:methanol = 9:1 and 7:1) was further divided into four sub-fractions, 1–1, 1–2, 1–3, and 1–4, by column chromatography on silica gel eluted with 500 mL of a chloroform/methanol mixture (v/v, 20:1, 15:1, 10:1 or 7:1, respectively). From sub-fractions 1–3 (3.7 g, with chloroform:methanol = 10:1–7:1), kaempferol (**3**) and rhodiolinin (**9**) were isolated through chromatography on Sephadex LH-20 column (GE Heathcare Co, Sweden), using $CHCl_3$/MeOH (1:1) and Lichroprep RP-18 (40–63 μm, Merck). Fraction 2 (25 g, chloroform/methanol = 5:1 and 3:1) was subjected to a second round of silica gel chromatography eluted with 500 mL of chloroform : methanol (v/v, 9:1, 5:1, 3:1, or 1:1), or 500 mL of 100% methanol, yielding five sub-fractions, 2–1, 2–2, 2–3, 2–4, and 2–5. Fractions 2–5 (5.3 g, chloroform/methanol = 9:1 to 5:1) was separated through chromatography on a Sephadex LH-20 column (GE Heathcare Co, $CHCl_3$/MeOH = 1:1) and Lichroprep RP-18 (40-63 μm, Merck) into herbacetin (**5**). Likewise, sub-fractions 2–3 (6.56 g, chloroform/methanol = 5:1 to 3:1) was separated by column chromatography on a Sephadex LH-20 column (GE Heathcare Co, $CHCl_3$/MeOH = 1:1) and Lichroprep RP-18 (40–63 μm, Merck) to yield compounds rhodionin (**10**) and rhodiosin (**11**). The structures of isolated compounds **9–11** were confirmed by spectroscopically and compared with previously reported values.[15–17]

Rhodiolinin (**9**): Yellow powder; $[\alpha]_D^{20}$ −57.5 (*c* 0.6, acetone); mp 235–237 °C; HREIMS *m/z* = 480.1059 (calcd for $C_{25}H_{20}O_{10}$ $[M]^+$, 480.1056); ^{1}H NMR (500 MHz, CD_3OD) δ 8.16 (d, *J* = 8.60 Hz, 2H, H-2′,6′), 7.02 (d, *J* = 1.40 Hz, 1H, H-2″), 6.92 (dd, *J* = 1.75 Hz, 8.05 Hz, 1H, H-6″), 6.90 (d, *J* = 8.9 Hz, 2H, H-3′,5′), 6.85 (d, *J* = 8.0 Hz, 1H, H-5″), 6.24 (s, 1H, H-6), 5.05 (d, *J* = 8.0 Hz, 1H, H-7″), 4.06 (m, 1H, H-8″), 3.88 (s, 3H, –OCH_3), 3.85 (m, 1H, H-9″), 3.53 (dd, *J* = 3.70 Hz, 12.6 Hz, 1H, H-9″). ^{13}C NMR (125 MHz, CD_3OD): see Table 1.

Rhodionin (**10**): Green amorphous powder; $[\alpha]_D^{20}$ −78.5 (*c* 0.7, acetone); mp 234–237 °C; HREIMS *m/z* = 448.1003 (calcd for $C_{21}H_{20}O_{11}$ $[M]^+$, 448.1006); ^{1}H NMR (500 MHz, CD_3OD) δ 8.20 (2H, d, *J* = 8.3 Hz, H-2′, 6′), 6.90 (2H, d, *J* = 8.6 Hz, H-3′, 5′), 6.64 (1H, s, H-6), 5.52 (1H, s, rham-1), 4.14 (1H, s, rham-2), 3.98 (1H, dd, *J* = 3.15 Hz, 9.45 Hz, rham-5), 3.69 (1H, m, rham-3), 3.49 (1H,

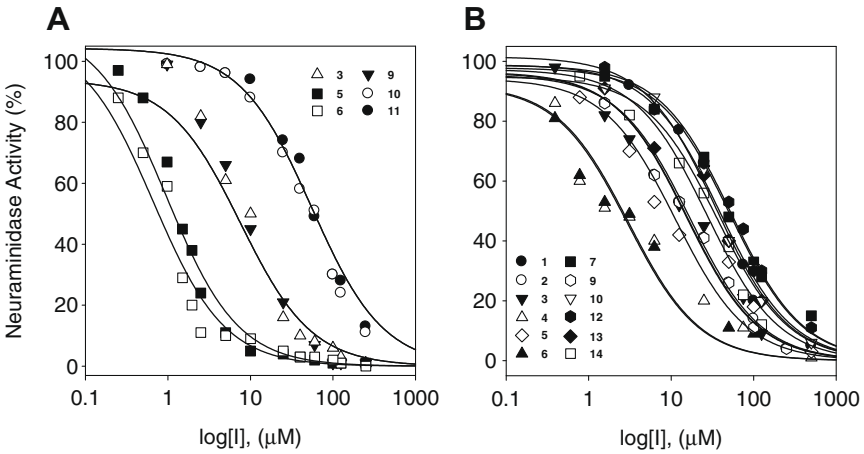

Figure 2. Effects of flavonoids on the two kinds of neuraminidases. (A) Effects of compounds **3**, **5**, **6**, and **9**–**11** on neuraminidase from *C. perfringens* for the hydrolysis of 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid at room temperature. (B) Effects of compounds **1**–**7**, **9**, **10**, and **12**–**14** on neuraminidase from rvH1N1 for the hydrolysis of 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid at room temperature.

Table 3
In vitro anti-influenza virus activities of the EtOAc and H_2O extracts and flavonoids **1**–**6**, **8**–**12**, and **14** against A/PR/8/34 (H1N1) and A/Chicken/Korea/MS96/96 (H9N2) in MDCK cells using the CPE reduction assay

Extract or compound	A/PR/8/34 (H1N1)			A/Chicken/Korea/MS96/96 (H9N2)		
	CC_{50}[a] (μM)	EC_{50}[b] (μM)	SI[c]	CC_{50}[a] (μM)	EC_{50}[b] (μM)	SI[c]
Tamiflu	>300	8.3	>36.14	>300	6.25	>48
H_2O extract	>500 μg/mL	78.5 μg/mL	>6.37	>500 μg/mL	139.7 μg/mL	>3.58
EtOAc extract	478.4 μg/mL	102.1 μg/mL	4.69	464.3 μg/mL	145.4 μg/mL	3.19
Apigenin (**1**)	>300	60.4	>4.97	>300	42.7	>7.03
Luteolin (**2**)	164.9	84.0	1.96	164.9	103.1	1.60
Kaempferol (**3**)	>300	30.2	>9.93	>300	18.5	>16.22
Quercetin (**4**)	253.8	43.1	5.89	253.8	133.6	1.90
Herbacetin (**5**)	293.7	35.0	8.39	293.7	23.0	12.77
Gossypetin (**6**)	283.0	43.0	6.58	283.0	36.3	7.80
Cosmosiin (**8**)	>300	40.0	>7.50	>300	61.9	>4.85
Rhodiolinin (**9**)	>300	41.7	>7.19	>300	29.3	>10.24
Rhodionin (**10**)	>300	74.8	>4.01	>300	49.6	>6.05
Rhodiosin (**11**)	297.3	81.9	3.63	297.3	35.1	8.47
Linocinamarin (**12**)	>300	99.1	>3.03	>300	100	>3
Nicotiflorin (**14**)	>300	40.1	>7.48	>300	72.9	>4.12

[a] CC_{50}: mean (50%) value of cytotoxic concentration.
[b] EC_{50}: mean (50%) value of effective concentration.
[c] SI: selective index, CC_{50}/IC_{50}.

t, J = 9.6 Hz, rham-4), 1.26 (3H, d, J = 6.0 Hz, rham-CH_3). ^{13}C NMR (125 MHz, CD_3OD): see Table 1.

Rhodiosin (**11**): Green amorphous powder; $[\alpha]_D^{20}$ −42.3 (*c* 0.3, acetone) mp 195–199 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 8.20 (2H, d, J = 8.85 Hz, H-2′, 6′), 6.91 (2H, d, J = 9.15 Hz, H-3′, 5′), 6.64 (1H, s, H-6), 5.56 (1H, s, rham-1), 4.67 (1H, d, J = 7.45 Hz, glc-1), 4.42 (1H, d, J = 1.15 Hz, rham-2), 4.15–3.30 (9H, glycoside proton), 1.28 (3H, d, J = 6.0 Hz, rham-CH_3). ^{13}C NMR (CD_3OD, 125 MHz): see Table 1.

3.4. Cells and viruses

Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC CCL-3; Manassas, VA, USA) and grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The influenza strains A/PR/8/34 (H1N1) (ATCC VR-1469) and A/Chicken/Korea/MS96/96 (H9N2) were propagated in MDCK cells in the presence of 10 μg/mL trypsin (1:250; GIBCO Invitrogen Corporation, California).

3.5. Neuraminidase (*Clostridium perfringens*) inhibition assay

The enzyme assay was performed as previously reported with slight modifications.[24] In general, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (SIGMA, M8639) 0.125 mM in 50 mM sodium acetate buffer (pH 5.0) was used as a substrate. Neuraminidase 0.1 U/mL in acetate buffer was used as the enzyme source. The isolated compounds were dissolved in MeOH and diluted to appropriate concentrations in acetate buffer. Enzyme (15 μL) was added to 15 μL of sample solution mixed with buffer (510 μL) in a cuvette, and 60 μL of substrate was added at 37 °C. 4-Methylumbelliferone was immediately quantified by fluorometrically by LS-55 luminescence. The excitation wavelength was 365 nm, with the excitation slits set at 2.5 nm, and the emission wavelength was 450 nm, with the emission slits set at 20 nm. For the determination of enzyme activity (fitting experimental data to the logistic curve by Eq. 1), we used a time-driven protocol with initial velocity recorded over a range of concentrations and the data analyzed using a nonlinear regression program [Sigma Plot (SPCC Inc., Chicago, IL)].

$$\text{Activity}\ (\%) = 100[1/(1 + ([I]/IC_{50}))] \quad (1)$$

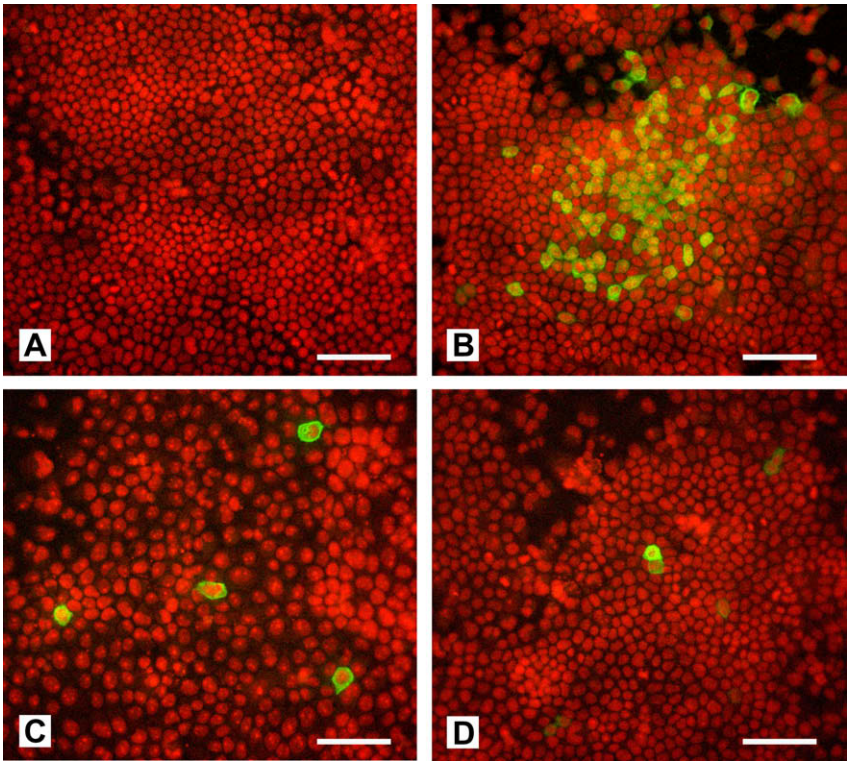

Figure 3. The antiviral effects of a representative compound **3** on influenza A/PR/8/34 in immunofluorescence assay. (A) Mock-inoculated MDCK cells show no positive fluorescence reaction in the cells. (B) Virus-infected MDCK cells without compound **3**. There are many positive cells (green). (C) Virus-infected MDCK cells with compound **3**. There are remarkably reduced positive cells. (D) Virus-infected MDCK cells with olseltamivir. Bar: A–D, 100 μm.

3.6. Neuraminidase (recombinant influenza A virus, rvH1N1) inhibition assay

This neuraminidase inhibitory assay was conducted using recombinant neuraminidase deduced from the 1918 Spanish flu virus NA (A/Bervig_Mission/1/18). NA inhibition activities were determined by Enzyme-Linked Immunosorbent Assay (ELISA). All samples were dissolved in MeOH at 5 mM and diluted. Fifty microliters of substrate, 800 μM 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate solution, was mixed with 80 μL of 50 mM Tris buffer (containing 5 mM $CaCl_2$ and 200 mM NaCl, pH 7.5) at room temperature. Twenty microliters of the sample solution and 50 μL of NA (0.05 pg/mL in the same Tris buffer) were added to a well in a plate. The mixture was recorded at excitation and emission wavelengths of 365 and 445 nm. The inhibition ratio was obtained using the equation:

$$\text{Activity } (\%) = [(S - S_0)/(C - C_0)] \times 100 \quad (2)$$

where C is the fluorescence of the control (enzyme, buffer, and substrate) after 20 min of incubation, C_0 is the fluorescence of the control at zero time, S is the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation, and S_0 is the fluorescence of the tested samples at zero time. To allow for the quenching effect of the samples, the sample solution was added to the reaction mixture C, and any reductions in fluorescence were assessed.

3.7. Cytopathic effect (CPE) inhibition assay

The CPE inhibition assays used in this study were performed as described previously.[25] In brief, virus at 100 $TCID_{50}$ (tissue culture infectious dose) were inoculated onto near confluent MDCK cell monolayers (1×10^5 cells/well) for 1 h with occasional rocking. The solution was removed and the cells replaced with EMEM containing 10 μg/mL trypsin and several compounds at different concentration. The cultures were incubated for 3–4 days at 35 °C under 5% CO_2 atmosphere until the cells in the infected, untreated control well showed complete viral CPE as observed by light microscopy. All compounds were assayed for virus inhibition in triplicate. After 3–4 days, 0.034% neutral red was added to each well and incubated for 2 h at 35 °C in the dark. The neutral red solution was removed and the cells were washed with PBS (pH 7.4). Destaining solution (containing 1% glacial acetic acid, 49% H_2O, and 50% ethanol) was added to each well. The plates were incubated in the dark for 15 min at room temperature. Absorbance was read at 540 nm using a microplate reader. The 50% effective concentration (EC_{50}) was calculated by regression analysis.

3.8. Cytotoxicity assay

MDCK cells were grown in 96-well plates at 1×10^5 cells/well for 24 h. The plates were replaced with media containing serially diluted compounds. After 48 h of incubation, the medium was removed and 5 μL MTT (3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide, SIGMA) solution was added to each well and incubated at 37 °C for 4 h. After removal of supernatant, 100 μL 0.04 M HCl–isopropanol was added to dissolve formazan crystals. Absorbance was measured at 540 nm with subtraction of the background measurement at 655 nm in a microplate reader. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis.

3.9. Immunofluorescence assay (IFA)

MDCK cells were grown on 8-well chamber slides (LAB-TEK, NUNC, USA). Cell monolayers were infected with influenza virus

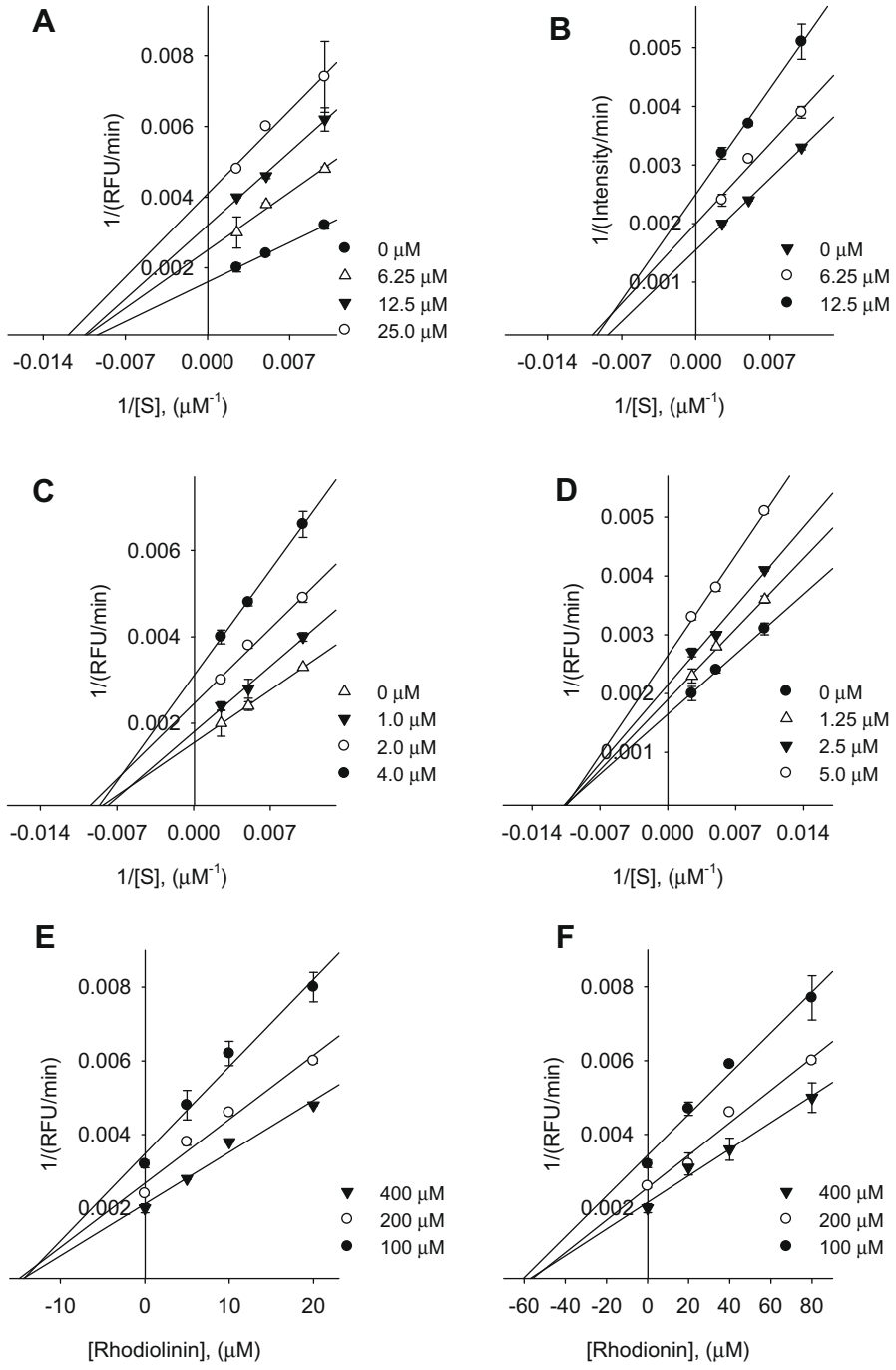

Figure 4. Graphical determination of the type of inhibition for compounds **2**–**4**, **6**, **9**, and **10**. (A–D) Lineweaver–Burk plots for the inhibition of compounds **2**–**4** and **6** on neuraminidase from rvH1N1 for the hydrolysis of substrate. Conditions were as follows: 200 mM substrate, 0.05 pg/mL units of neuraminidase, 50 mM Tris buffer (pH 7.5), at room temperature. In the presence of different concentrations of compounds for lines from bottom to top: (A) for compound **2**, 0, 6.25, 12.5, and 25.0 μM; (B) for compound **3**, 0, 6.25, and 12.5 μM; (C) for compound **4**, 0, 1.0, 2.0, and 4.0 μM; (D) for compound **6**, 0, 1.25, 2.5, and 5.0 μM. (E) and (F) Dixon plots for inhibition of compounds **9** and **10** on neuraminidase from rvH1N1 for the hydrolysis of substrate. In the presence of different concentrations of substrate for lines from bottom to top: 400, 200, and 100 μM.

(A/PR/8/34) at 100 $TCID_{50}$/0.1 mL for 1 h. The solution was removed and the cells replaced with EMEM containing 10 μg/mL trypsin and 50 μM of several compounds. The cultures were incubated for 24 h at 35 °C under 5% CO_2 atmosphere. The cells were rinsed carefully three times with PBS (pH 7.4) and fixed with 80% acetone solution for 15 min at room temperature (rt). After washing three times with PBS (pH 7.4), the cells were incubated with monoclonal antibodies against the influenza virus M1 protein (Santa Cruz, USA) diluted 1:50 in PBS (pH 7.4) at 37 °C for 1 h. After washing with PBS (pH 7.4), the cells were incubated with the secondary FITC-conjugated goat anti-mouse IgG antibody (Santa Cruz, USA) diluted 1:100 in PBS (pH 7.4) at 37 °C for 1 h. After washing with PBS (pH 7.4), the cells were stained with 500 nM propidium iodide solution for 10 min at rt. After washing three times with PBS (pH 8.0), the slides were mounted with antifade reagent (Molecular Probe, Eugene, USA) and observed in a fluorescence microscope (Leica, NY, USA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.036.

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