Bioorganic & Medicinal Chemistry Letters 20 (2010) 971–974

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Inhibition of neuraminidase activity by polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis*

Young Bae Ryu [a,†], Jang Hoon Kim [a,c,†], Su-Jin Park [a], Jong Sun Chang [a], Mun-Chual Rho [a], Ki-Hwan Bae [c], Ki Hun Park [b,*], Woo Song Lee [a,*]

[a] *Bioindustry Technology Research Center and AI Control Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongeup 580-185, Republic of Korea*
[b] *Division of Applied Life Science (BK 21 Program), EB-NCRC, Institute of Agriculture and Life Science, Graduate School of Gyeongsang National University, Jinju 660-701, Republic of Korea*
[c] *College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea*

ARTICLE INFO

Article history:
Received 13 October 2009
Revised 2 December 2009
Accepted 14 December 2009
Available online 4 January 2010

Keywords:
Anti-influenza
Coumestan
Glycyrrhiza uralensis
Neuraminidase

ABSTRACT

We isolated 18 polyphenols with neuraminidase inhibitory activity from methanol extracts of the roots of *Glycyrrhiza uralensis*. These polyphenols consisted of four chalcones (**1**–**4**), nine flavonoids (**5**–**13**), four coumarins (**14**–**17**), and one phenylbenzofuran (**18**). When we tested the effects of these individual compounds and analogs thereof on neuraminidase activation, we found that isoliquiritigenin (**1**, IC_{50} = 9.0 μM) and glycyrol (**14**, IC_{50} = 3.1 μM) had strong inhibitory activity. Structure–activity analysis showed that the furan rings of the polyphenols were essential for neuraminidase inhibitory activity, and that this activity was enhanced by the apioside group on the chalcone and flavanone backbone. In addition, the presence of a five-membered ring between C-4 and C-2′ in coumestan was critical for neuraminidase inhibition. All neuraminidase inhibitors screened were found to be reversible noncompetitive inhibitors.

Every year, influenza epidemics cause numerous deaths and millions of hospitalizations, but the most frightening effects are seen when new strains of the virus emerge, causing worldwide outbreaks of infection. Recent reports of direct avian-to-human transmission of influenza make the prospect of a new pandemic particularly alarming.[1] Influenza viruses contain two surface antigens, hemagglutinin (HA) and neuraminidase (NA). NA is an enzyme involved in the release of progeny virus from infected cells, cleaving sugars that bind to mature viral particles. Specifically, NA has been found to cleave the α-ketosidic bond that links a terminal neuraminic-acid residue to an adjacent oligosaccharide moiety. NA is also important for the trafficking of virus in the mucus layer of the respiratory tract, allowing the viruses access to underlying epithelial cells.[2] 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.[3] NA, which plays an important role in viral proliferation, is stably present in both influenza viruses A and B, Neuraminidase inhibitors (NAIs), such as zanamivir and oseltamivir, have been used for the treatment and prophylaxis of influenza viruses A and B.[4] The long-term efficacy of these drugs is, however, often limited by toxicity and the almost inevitable selection of drug-resistant viral mutants.[5]

Natural plants, especially *Glycyrrhiza*, are plentiful sources of flavonoid derivatives. Many flavonoids have been reported to show activity against human immunodeficiency virus (HIV), hepatitis B virus,[6] and influenza virus.[7] We have established an influenza virus NA activity assay to screen for and identify new influenza virus NA inhibitors from *Glycyrrhiza* species. *Glycyrrhiza uralensis*, a member of the Leguminosae family, is a traditional medicinal herb grown in various parts of the world. This plant has long been used to treat fever, liver ailments, dyspepsia, constipation, gastric ulcers, sore throats, asthma, and bronchitis. In addition, the roots are used as flavoring and sweetening agents in tobacco, chewing gum, candies, and beverages.[8] The major bioactive components of *G. uralensis* roots are flavonoids and pentacyclic triterpene saponins, including liquiritin, isoliquiritigenin, liquiritin apioside, glycyrrhizin, and glycyrrhizic acid.[9] Moreover, the constituents of *G. uralensis* have been found to exhibit anticancer, anti-diabetic, anti-inflammatory, anti-malarial, anti-bacterial, antioxidant, and estrogenic properties.[10] *G. uralensis* has also been reported to have antiviral properties. For example, isolated glycyrrhizin and derivatives thereof have shown activity against a variety of viruses, including herpes simplex type 1 (HSV-1);[11] hepatitis A, B, and C viruses (HAV, HBV, and HCV);[12] HIV-1;[13] and influenza virus,[14] but isolated polyphenols from the plant have not been reported to show such activity. Using bioassay-guided

* Corresponding authors. Tel.: +82 63 570 5170; fax: +82 63 570 5239 (W.S.L.); tel.: +82 55 751 5472 (K.H.P.).
E-mail addresses: khpark@gsnu.ac.kr (K.H. Park), wslee@kribb.re.kr (W.S. Lee).
† Both authors contributed equally to the work.

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doi:10.1016/j.bmcl.2009.12.106

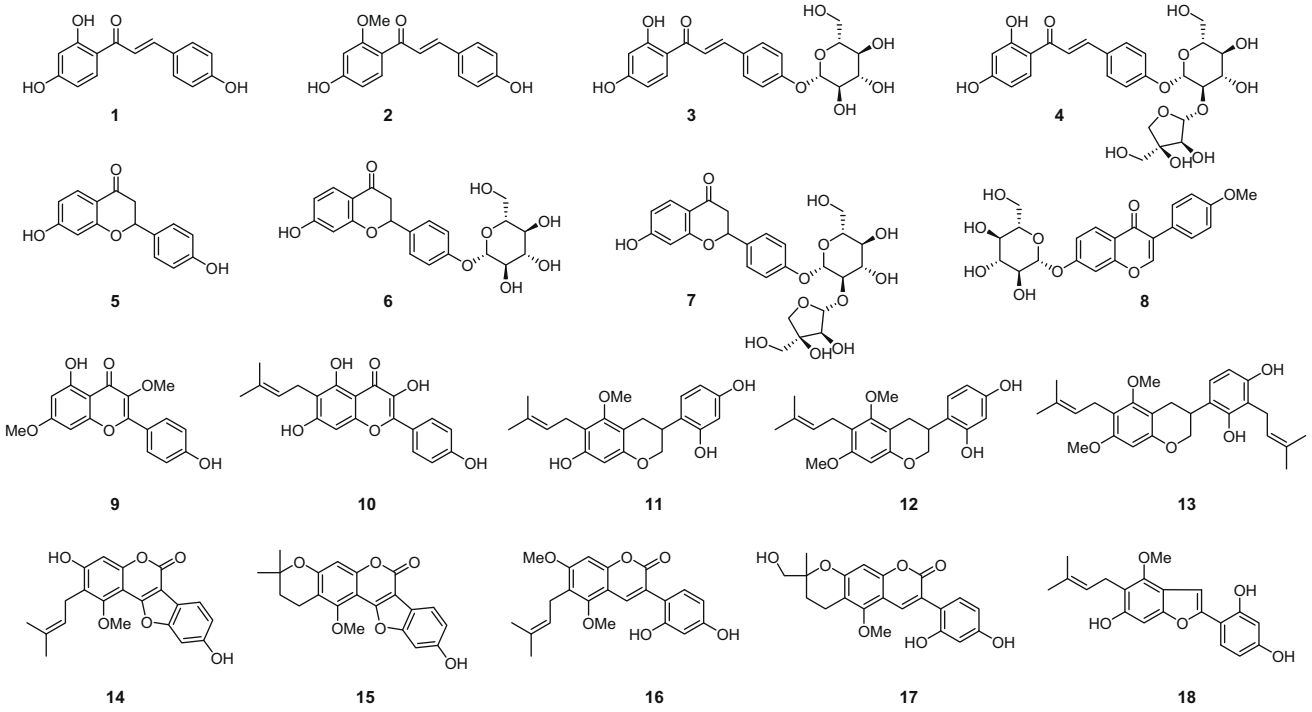

Figure 1. Chemical structures of compounds **1**–**18** isolated from the roots of *Glycyrrhiza uralensis*.

methods, we isolated 18 polyphenols from the roots of *G. uralensis* and evaluated their NA inhibitory capacities by assessment of structure-activity relationships. We also measured the kinetics of NA inhibition by NA inhibitors.

Starting with a methanol extract of *G. uralensis* roots, we purified compounds with NA inhibitory activity by activity-guided fractionation using an assay measuring effects on rvH1N1 (A/Bervig_Mission/1/18) neuraminidase.[15] Repeated column chromatography of the extract yielded four chalcones (**1**–**4**), nine flavonoids (**5**–**13**), four coumarins (**14**–**17**), and one phenylbenzofuran (**18**). By comparing the physical and spectral data[16–22] of these isolated polyphenols with those of known compounds, we identified these 18 isolates as isoliquiritigenin (**1**), 2′-methoxyisoliquiritigenin (**2**), isoliquiritin (**3**), isoliquiritin apioside (**4**), liquiritigenin (**5**), liquiritin (**6**), liquiritin apioside (**7**), ononin (**8**), kumatakenin (**9**), licoflavonol (**10**), glyasperin C (**11**), glyasperin D (**12**), licorisoflavan A (**13**), glycyrol (**14**), isoglycyrol (**15**), glycyrin (**16**), licopyranocoumarin (**17**), and licocoumarone (**18**) (Fig. 1).

To clarify the structure–activity relationships (SARs) in the isolated polyphenols (**1**–**18**) with respect to NA inhibitory activity, we examined polyphenol effects on the release of 4-methylumbelliferone from 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid.[23] Oseltamivir, a known NA inhibitor, was used as a positive control (IC_{50} = 1.59 nM). All of the polyphenols (**1**–**18**) showed a dose-dependent inhibitory effect on NA, with maximal activities observed at 200 μM (Fig. 2). The polyphenols were found to be reversible inhibitors because an increase in concentration rapidly reduced enzyme activity. All inhibitors showed a similar relationship between enzyme activity and enzyme concentration [e.g., see Fig. 3A for isoliquiritigenin (**1**) data].

When we compared chalcones with chalcone glycosides, we found that the activities of chalcones were higher than those of their corresponding glycosides (Table 1). Methylation of the 2-hydroxyl groups diminished inhibitory activity, whereas an increase in the number of hydroxyl groups at the 2 and 4′ positions of chalcones increased inhibitory activity [isoliquiritigenin (**1**, IC_{50} = 9.0 μM) > methoxyisoliquiritigenin (**2**, IC_{50} = 24.3 μM) > isoliquiritin (**3**, IC_{50} = 124.0 μM)]. Interestingly, isoliquiritin apioside (**4**), with glycoside group substitutions at the C-4′ position, exhibited the next highest activity (IC_{50} value = 12.9 μM) of the chalcone derivatives. Thus, apiose substitution

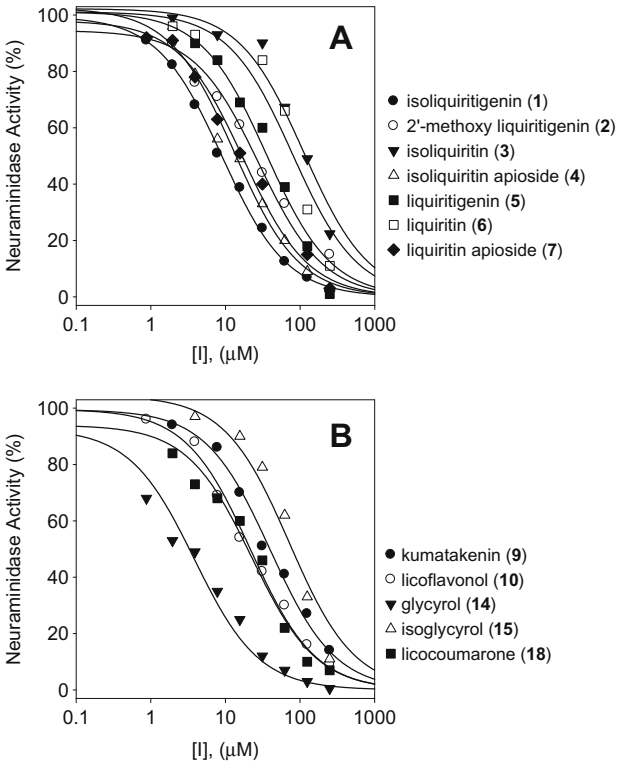

Figure 2. (A) Effects of compounds **1**–**6** on neuraminidase hydrolysis of neuraminic acid (compound **1**, ●; compound **2**, ○; compound **3**, ▼; compound **4**, △; compound **5**, ■; compound **6**, □; and compound **7**, ♦). (B) Effects of compounds **9**, **10**, **14**, **15**, and **18** on neuraminidase hydrolysis of neuraminic acid (compound **9**, ●; compound **10**, ○; compound **14**, ▼; compound **15**, △; and compound **18**, ■).

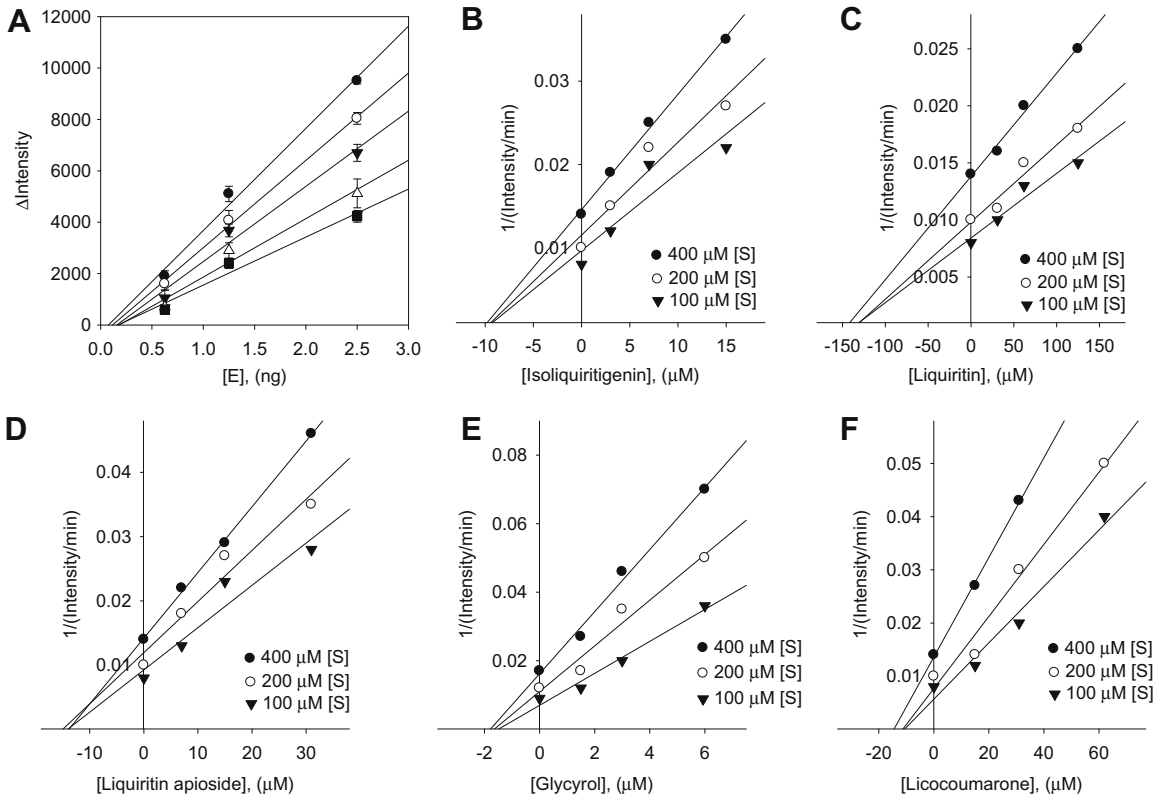

Figure 3. (A) NA hydrolytic activity in the presence of isoliquiritigenin (**1**) [0 μM (●), 2.5 μM (○), 5.0 μM (▼), 10.0 μM (△), and 20.0 μM (■)]. (B–F) Dixon plots of NA inhibition by compounds **1** (B), **6** (C), **7** (D), **14** (E), and **18** (F). The graphical symbols are substrate concentrations (100 μM, ▼; 200 μM, ○; and 400 μM, ●).

at the C_G-2 position of isoliquiritin (**3**) greatly enhanced inhibitory activity, whereas glucose substitution at the C-4′ position of isoliquiritigenin (**1**) significantly decreased activity. Similarly, when we compared flavanones and flavanone glycosides, we found that the chalcone derivatives showed equal or better efficacies. Liquiritin substituted with an apiose group (**7**) demonstrated the most potent neuraminidase inhibitory activity of the isolated flavanones (**5**–**7**). Substitution of glucose at the C-4′ position in the flavanone backbone also diminished inhibitory activity [liquiritin (**5**, IC_{50} = 82.3 μM)].

Table 1
Inhibitory effects of isolated compounds **1**–**18** on neuraminidase activities

Compound	IC_{50} (μM)[a]	Inhibition type (K_i, μM)
1	9.0 ± 0.7	Noncompetitive (9.9 ± 0.7 μM)
2	24.3 ± 2.2	Noncompetitive (20.1 ± 2.5 μM)
3	124.0 ± 2.3	Noncompetitive (94.0 ± 10.1 μM)
4	12.9 ± 1.2	Noncompetitive (14.2 ± 2.0 μM)
5	46.8 ± 3.3	Noncompetitive (33.1 ± 10.7 μM)
6	82.3 ± 0.1	Noncompetitive (123.7 ± 0.7 μM)
7	18.2 ± 2.0	Noncompetitive (20.4 ± 0.1 μM)
8	30% at 200 μM	ND[b]
9	36.4 ± 6.9	Noncompetitive (90.3 ± 8.0 μM)
10	20.6 ± 0.9	Noncompetitive (33.0 ± 6.6 μM)
11	20% at 200 μM	ND
12	20% at 200 μM	ND
13	30% at 200 μM	ND
14	3.1 ± 1.0	Noncompetitive (2.9 ± 1.2 μM)
15	92.4 ± 0.7	Noncompetitive (77.5 ± 3.1 μM)
16	10% at 200 μM	ND
17	10% at 200 μM	ND
18	27.8 ± 0.7	Noncompetitive (12.1 ± 0.8 μM)

[a] All compounds were examined in a set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss; Oseltamivir was used as a positive control (IC_{50} value = 1.59 nM).
[b] Not determined.

These findings suggest that the apiose moiety may play a pivotal role in NA inhibition, by interacting with the neuraminidase hydrophilic site. This is similar to the reported mode of action of peramivir (BCX-1812), a five-membered sugar derivative shown to be a highly selective inhibitor of influenza A and B virus NAs and a potent inhibitor of influenza A and B virus replication in cell culture.[24]

A recent assessment of influenza virus NA inhibitory activity of naturally occurring flavonoids found that the order of potency for NA inhibition was aurones > flavon(ol)es > isoflavones > flavanon(ol)es and flavan(ol)es, and that the presence of a glycosylation group greatly reduced NA inhibition.[25] Our findings on nine flavonoids were similar, in that the NA inhibitory activities of flavones, kumatakenin (**9**, IC_{50} = 36.4 μM) and licoflavonol (**10**, IC_{50} = 20.6 μM) were stronger than those of derivatives. Substitution of a glycoside moiety at C-7 in isoflavone (**8**, $IC_{50} \geqslant 200$ μM) and at C-4′ in flavanone (**6**, IC_{50} = 82.3 μM) reduced activity. None of isoflavans (**11**–**13**), containing methoxy group substitutions, showed any inhibitory activity ($IC_{50} \geqslant 200$ μM).

We also found that the aromaticity of the five-membered ring B between C-4 and C-2′ in coumarin was important for the potency of these compounds acting against NA. Among the coumarin derivatives (**14**–**17**), the parent structure showed less inhibitory activity (**16** and **17**, $IC_{50} \geqslant 200$ μM), whereas compound **14,** which has a five-membered closed ring B, showed dramatically increased (IC_{50} = 3.1 μM) activity against NA. Comparison of the two coumestan inhibitors, **14** and **15**, showed that the presence of a free hydroxyl group and the 1,3-dihydroxyphenol motif at C-7 in the A-ring had a great effect on inhibitor potency, in that isoglycyrol (**15**), which did not contain the motif, was 30-fold less active (IC_{50} = 92.4 μM) than was glycyrol (**14**).

Interestingly, the phenylbenzofuran analog licocoumarone (**18**) displayed significant activity against NA, with an IC_{50} value of 27.8 μM. Licocoumarone (**18**), which is structurally related to the

flavones (**9** and **10**), has a C2–C3 double bond in the C ring. The phenylbenzofuran skeleton is noteworthy, in that such compounds usually do not act as NA inhibitors.

We also tested the effect of polyphenols (**1**–**18**) on the kinetics of NA hydrolysis of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU). The Dixon plots of 1/*V* versus [*I*] resulted in a family of straight lines passing through the same point on the horizontal axis, as illustrated for representative compounds **1**, **6**, **7**, **14**, and **18** (Fig. 3B–F, respectively). In these kinetic plots, the abscissa [*I*] is the reciprocal of the concentrations of compounds, whereas the ordinate 1/*V* is the reciprocal of the change in intensity over time, thus representing a reciprocal of NA activity. These findings indicate that compounds (**1**–**18**) are noncompetitive inhibitors of NA, with NA inhibitory activity decreasing with increasing concentration of the substrate. From these kinetic plots, we calculated that the NA inhibitors **1**–**7**, **9**, **10**, **14**, **15**, and **18** had inhibition constants (K_i) of 9.9, 20.1, 94.0, 14.2, 33.1, 123.7, 20.4, 90.3, 33.0, 2.9, 77.5, and 12.1 μM (Table 1), respectively.

In summary, we isolated four chalcones (**1**–**4**), nine flavonoids (**5**–**13**), four coumarins (**14**–**17**), and one phenylbenzofuran (**18**) by bioassay-guided fractionation from the roots of *G. uralensis* and evaluated activities against NA. Of these 18 derivatives, compounds **1** and **14,** exhibited the most potent NA inhibitory activities, with IC_{50} values of 9.0 μM and 3.1 μM, respectively. In a study of structure–activity relationships, we found that five-membered ring compounds showed higher NA inhibitory activity than did the other compounds, with the ring-closed coumarin, coumestan (**14**) being the most inhibitory.

Acknowledgments

This research was supported by a grant from Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry (No. 308025-05-1-SB010) and KRIBB Research Initiative Program, Republic of Korea.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.12.106.

References and notes

1. Webby, R. J.; Webster, R. G. *Science* **2003**, *302*, 1519.
2. Klenk, H. D.; Rott, R. *Adv. Virus Res.* **1988**, *34*, 247.
3. De Clercq, E. *Nat. Rev. Drug Disc.* **2006**, *5*, 1015.
4. Zhang, J.; Yu, K. Q.; Zhu, W.; Jiang, H. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3009.
5. Nicholson, K. G.; Wood, J. M.; Zambon, M. *Lancet* **2003**, *362*, 1733.
6. (a) Jassim, S. A. A.; Naji, M. A. *J. Appl. Microbiol.* **2003**, *95*, 412; (b) Li, J.; Huang, H.; Zhou, W.; Feng, M.; Zhou, P. *Biol. Pharm. Bull.* **2008**, *31*, 743.
7. (a) Mori, S.; Miyake, S.; Kobe, T.; Nakaya, T.; Fuller, S. D.; Kato, N.; Kaihatsu, K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4249; (b) Ryu, Y. B.; Curtis-Long, M. J.; Kim, J. H.; Jeong, S. H.; Yang, M. S.; Lee, K. W.; Lee, W. S.; Park, K. H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6046.
8. Asada, T.; Li, W.; Toshikawa, T. *Phytochemistry* **2000**, *55*, 323.
9. Kamei, J.; Nakamura, R.; Ichiki, H.; Kubo, M. *Eur. J. Pharmacol.* **2003**, *469*, 159.
10. (a) Zore, G. B.; Winston, B. S.; Surwase, W. S.; Mesharm, V. D.; Sangle, S. S.; Kulkarni, Mohan.; Karuppayil, S. *Phytomedicine* **2008**, *15*, 29; (b) Liang, R. J. *Carbohydr. Polym.* **2008**, *73*, 558; (c) Dong, S. J.; Inoue, A.; Zhu, Y.; Tanji, M.; Kiyama, R. *Food Chem. Toxicol.* **2007**, *45*, 2470.
11. Pompei, R.; Flore, O.; Marccialis, M. A.; Pani, A.; Loddo, B. *Nature* **1979**, *281*, 689.
12. Sato, H.; Goto, W.; Yamamura, J.; Kurokawa, M.; Kageyama, S.; Takahara, T.; Watanabe, A.; Shiraki, K. *Antiviral Res.* **1996**, *30*, 171.
13. Ito, M.; Nakashima, H.; Baba, M.; Pauwels, R.; De Clercq, E.; Shigeta, S.; Yamamoto, N. *Antiviral Res.* **1987**, *7*, 127.
14. Utsunomiya, T.; Kobayashi, M.; Pollard, R. S.; Suzuki, F. *Antimicrob. Agents Chemother.* **1997**, *41*, 551.
15. This neuraminidase inhibitory assay was conducted using recombinant neuraminidase deduced from the 1918 Spanish flu virus NA (A/Bervig_Mission/1/18). Brifely, all samples were dissolved in MeOH at 5 mM and diluted. Fifty microliters of substrate (800 μM) were 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) were added to a well in a plate. The mixture was recorded at excitaion and emisstion wavelengths of 365 and 445 nm.[23]
16. Yahara, S.; Ogata, T.; Saijo, R.; Konish, R.; Yamahara, J.; Miyahara, K.; Nohara, T. *Chem. Pharm. Bull.* **1989**, *37*, 979.
17. Saitoh, T.; Kinoshita, T.; Shibata, S. *Chem. Pharm. Bull.* **1976**, *24*, 1242.
18. Demizu, S.; Kajiyama, K.; Takahashi, K.; Hiraga, Y.; Yamamoto, S.; Tamura, Y.; Okada, K.; Kinoshita, T. *Chem. Pharm. Bull.* **1988**, *36*, 3474.
19. Shiozawa, T.; Urata, S.; Kinoshita, T.; Saitho, T. *Chem. Pharm. Bull.* **1989**, *36*, 2239.
20. Kinoshita, T.; Saitoh, T.; Shibata, S. *Chem. Pharm. Bull.* **1978**, *26*, 135.
21. Hatano, T.; Yasuhara, T.; Fukuda, T.; Noro, T.; Okuda, T. *Chem. Pharm. Bull.* **1989**, *37*, 3005.
22. Lu, Z.; Fukai, T.; Nomura, T.; Zhang, R.-Y.; Lou, Z.-C. *Heterocycles* **1992**, *34*, 575.
23. Jeong, H. J.; Ryu, Y. B.; Park, S.-J.; Kim, J. H.; Kwon, H. J.; Kim, J. H.; Park, K. H.; Rho, M.-C.; Lee, W. S. *Bioorg. Med. Chem.* **2009**, *17*, 6816.
24. Sudhakar Babu, Y.; Chand, P.; Bantia, S.; Kotian, P.; Dehghani, A.; El-Kattan, Y.; Lin, T.-H.; Hutchison, T. L.; Elliott, A. J.; Parker, C. D.; Ananth, S. L.; Horn, L. L.; Laver, G. W.; Montgomery, J. A. *J. Med. Chem.* **2000**, *43*, 3482.
25. Liu, A.-L.; Wang, H.-D.; Lee, S. M.; Wang, Y.-T.; Du, G.-H. *Bioorg. Med. Chem.* **2008**, *16*, 7141.