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Structure–activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities

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ABSTRACT

Flavonoids are polyphenolic compounds that widely exist in plant kingdom, and the structure–activity relationship (SAR) of 25 flavonoids was studied on neuraminidase (NA) activity of influenza virus. Three typical influenza virus strains A/PR/8/34 (H1N1), A/Jinan/15/90 (H3N2), and B/Jiangshu/10/2003 were used as the source of NAs, the average of IC_{50}s of these compounds on these NAs was used in the SAR analysis. The order of potency for NA inhibition was as follows: aurones > flavon(ol)es > isoflavones > flavanon(ol)es and flavan(ol)es. The SAR analysis of flavonoids on influenza virus NAs revealed that for good inhibitory effect, the 4′-OH, 7-OH, C4=O, and C2=C3 functionalities were essential, and the presence of a glycosylation group greatly reduced NA inhibition. The in vitro anti-viral activities of eight flavonoids were evaluated using a cytopathic effect (CPE) reduction method, the assay results confirmed the SAR as influenza virus neuraminidase inhibitors. The findings of this study provide important information for the exploitation and utilization of flavonoids as NA inhibitors for influenza treatment.

1. Introduction

Flavonoids widely exist in the plant kingdom. They are important constituents of many traditional herb medicines, vegetables, and fruits,[1,2] and have been paid more attention due to their various pharmacological activities such as anti-cancer,[3–5] anti-inflammatory,[6,7] anti-bacterial,[8,9] anti-oxidation,[10] and anti-viral activities.[11]

Neuraminidase (NA) is one of the two glycoproteins on the surface of influenza virus, which takes charge of catalyzing the cleavage of neuraminic acid residues to facilitate the movement of the virus to and from sites of infection in the respiratory tract. Because of the importance of this enzyme in the pathogenesis of influenza virus infection and the close correspondence of the conserved residues of the active sites from NAs of all influenza A and B viruses, the enzyme has been regarded as a drug target for drugs for the treatment of influenza.[12]

In recent years, several flavonoids have been reported that they had anti-influenza virus activity by inhibiting NA activities.[13,14] However, the structure–activity relationship (SAR) of flavonoids on NA inhibitory effect and their in vitro anti-influenza viral activities have not been studied to elucidate the structural features of flavonoids on NA inhibitory effect and to provide new experimental data for the future exploitation and utilization of flavonoids as anti-influenza medicines.

2. Results

2.1. Activities on NAs

In the present study, 25 compounds (**1**–**25**) from different subgroups of flavonoids were investigated for their inhibitory effect on NAs, which were from A/PR/8/34 (H1N1), A/Jinan/15/90 (H3N2), and B/Jiangsu/10/2003, and their molecular structures are shown in Figure 1. Their compound names are listed in Table 1 according to their subgroup classification. The activity of flavonoids examined in this study was expressed as 50% inhibitory concentration (IC_{50}). The IC_{50}s of flavonoids on NAs from different types or subtypes of influenza viruses were different because of the slight difference in their active sites,[15] so the average of IC_{50}s on these NAs was employed in the SAR analysis. Most of the tested flavonoids showed significant activities with $IC_{50} < 100$ μM (Table 1). According to their average of inhibitory effect, these flavonoids are classified into three categories. The flavonoids with $IC_{50} < 40$ μM are good inhibitors, and this group includes apigenin (**1**), luteolin (2), dinatin (3), daidzein (**18**) sulfuretin (**22**), 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**), and 2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (**25**). The flavonoids with IC_{50} ranging from 40 to 80 μM are

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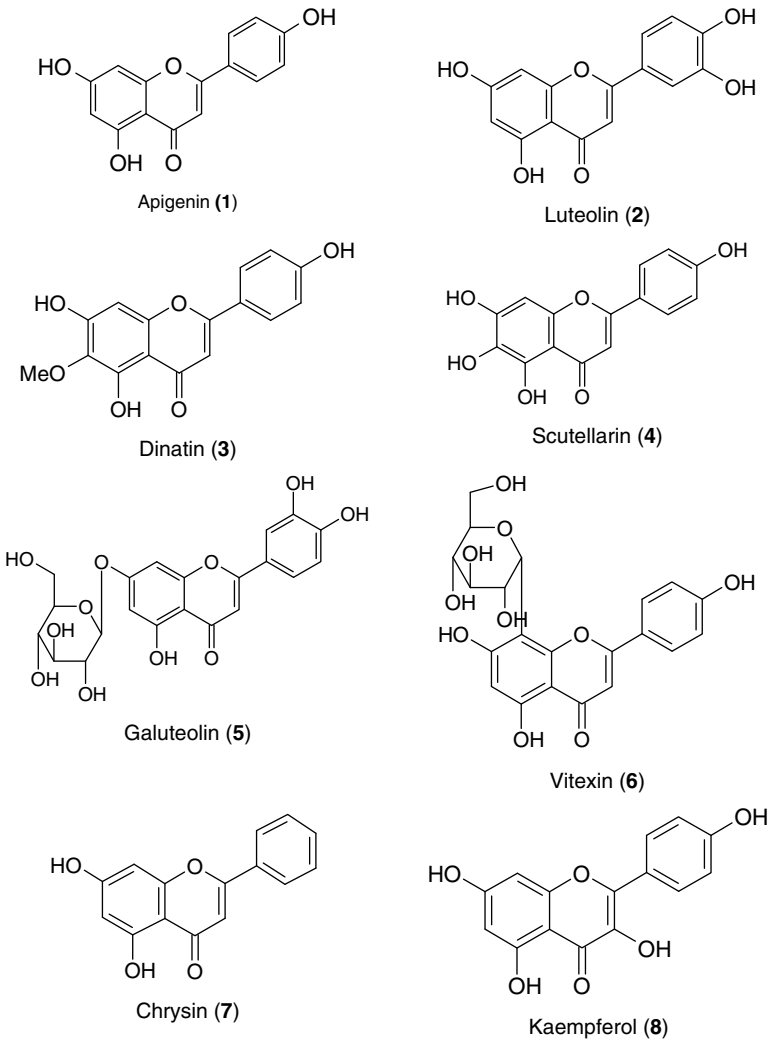


Figure 1. Molecular structures of the 25 flavonoids studied.

moderate agents, whereas those with IC_{50} > 80 μM are weak inhibitors (Table 1).

2.2. In vitro anti-influenza virus activity

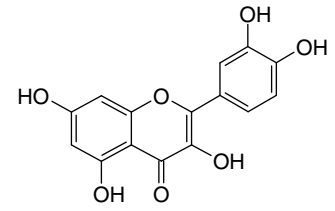
The in vitro anti-influenza virus activities of eight effective flavonoids were evaluated by the influenza virus A/Jinan/15/90 (H3N2) induced CPE reduction assay in MDCK cells (Table 2). The assay results showed that they all possessed in vitro anti-influenza virus activity, but only apigenin (**1**), luteolin (**2**), dinatin (**3**) and 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**) displayed significant anti-influenza virus activities with IC_{50}s 4.74 μM to 24.70 μM, while galuteolin (**5**), daidzein (**18**), sulfuretin (**22**), and 2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (**25**) displayed relatively weak activities.

3. Discussion

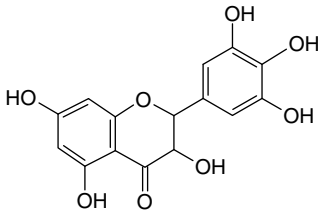
Although the anti-influenza virus activities of several flavonoids have been reported in the past few years,[13,14,16] the mechanism(s) for their action and SAR have not been fully understood. The difficulties of the SAR analysis exist in their structural diversity. Flavonoids are based on the structure of a phenylbenzopyrone and differ from one another in terms of hydroxyl or glycosylated substituents, the position of the benzenoid substituent, the degree of unsaturation, and the type of sugar attached.

3.1. Relationship between chemical structures and NA activities

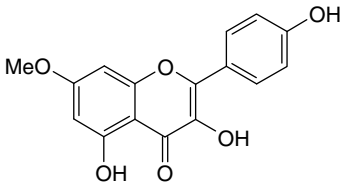
Apigenin (**1**), luteolin (**2**), dinatin (**3**), and scutellarin (**4**) belong to the flavone class of compounds, which have three OH groups at the C4′, C5, and C7 positions. Apigenin (**1**) has a single B-ring OH group (4′ position) while luteolin (**2**) has two B-ring OH groups, dinatin (**3**) has a A-ring OCH3 group (6 position) and two A-ring OH groups, while scutellarin (**4**) has three A-ring OH groups. Luteolin (**2**) has slightly reduced inhibition compared with apigenin (**1**), hence it seems that more OH groups in the B-ring will reduce the effect. The inhibitory effect of scutellarin (**4**) became moderate due to its 6-OH group in the A-ring, while the inhibitory effect of dinatin (**3**) almost did not change compared with apigenin (**1**), it indicated that 6-OH group in the A-ring was not favorable to effect while modified 6-OH group did not obviously affect the effect. Galuteolin (**5**), the luteolin-7-*O*-glucoside, is a flavone with glycosylation at the 7-OH position. Its inhibitory effect became moderate. This may be due to the presence of the bulky glycosylation causing steric hindrance or to the modification of the 7-OH group. Vitexin (**6**), the apigenin-8-*C*-glucoside, is also flavone with glycosylation at the C8 position in A-ring, and its effect also became moderate which indicated that glycosylation in C8 position also decreased



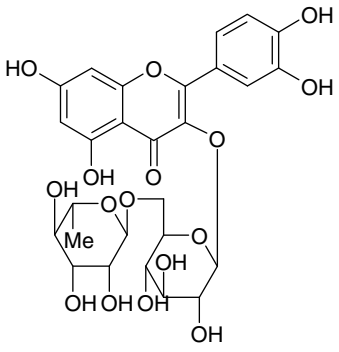
Quercetin (**9**)



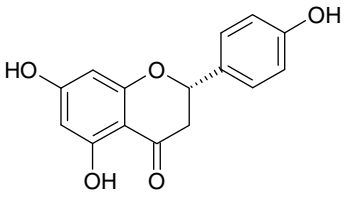
Myricetin (**10**)



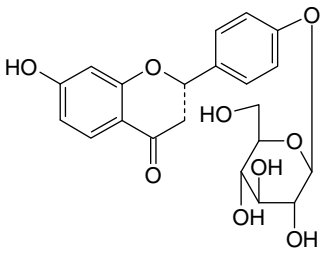
Rhamnocitrin (**11**)



Rutin (**12**)



Naringenin (**13**)



Liquiritin (**14**)

Fig. 1 (*continued*)

the effect. While chrysin (**7**), which is 5,7-dihydroxyflavone, displayed weaker activity than apigenin (**1**) because of its lack of 4′-OH. For good flavonoids, such as apigenin (**1**), luteolin (**2**), dinatin (**3**), and daidzein (**18**), it seems that 4′-OH, 7-OH, C2═C3, and C4═O functionalities are important.

Kaempferol (**8**), quercetin (**9**), and myricetin (**10**) belong to the flavonols class of compounds which have an OH group at the C3 position. Quercetin (**9**) and myricetin (**10**) have lower effect compared with kaempferol (**8**). It confirms that more OH groups in the B-ring will decrease the effect of the 4′-OH group, which is one of the active groups in flavonoids, and reduce the inhibition. Compared with apigenin (**1**), kaempferol (**8**) has lower activity, it seems that the presence of the 3-OH group in the C-ring or glycosylation of this group will reduce the inhibitory effect (Table 1). Compared with kaempferol, rhamnocitrin (**11**) has lower effect because of the modification of the 7-OH group, thus the 7-OH group is essential for the effect.

Flavanones have a single bond between the C-2 and C-3 positions in the C-ring. Naringenin (**13**), liquiritin (**14**), and hesperidin (**15**) have very weak activity. Catechin (**16**) and epicatechin (**17**) belong to flavanols class of compounds which lack the C(2)═C(3) bond and C(4)═O in the C-ring, their activities are also very weak. These results confirm that the presence of C(4)═O and C(2)═C(3) bonds are very important in exhibiting their activities.

In the isoflavone class, the B-ring is connected at the C3 position to the C-ring. Daidzein (**18**) has good effect because it possesses 7-OH, 4′-OH, C(2)═C(3) double bond, and C(4)═O, while genistein (**19**) displayed reduced effect compared with daidzein (**18**) because it has a 5-OH group. The results indicated that 5-OH is not good for the effect. Formononetin (**20**), which is methoxyl daidzein (**18**), has a weak effect. The results confirm that 4′-OH group is very important for the effect. The observation of the activities and structures of genistein (**19**) and apigenin (**1**) shows that the connection of the C-ring at the C3 position is not favorable for the activity. Sophoricoside (**21**), the genistein-4′-*O*-glucoside, also exerts very weak effect because of the hindrance or to the modification of 4′-OH group, it confirms that 4′-OH group is favorable for activity.

In the aurone class, C-ring is a 5-ring, and its position is different from the other classes of compounds. The comparison of sulfurenin (**22**) and 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**) shows that 3′-OH slightly reduces the effect. 2-((*E*)-Phenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**24**) displayed obviously reduced effect compared with 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**) because of its lack of 4′-OH group, it also confirms that 4′-OH group is essential. 2-((*E*)-4′-Hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (**25**) has slightly decreased effect compared with 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihy-drobenzofuran-3-one (**23**) because of the presence of 4-OH group, which is the same group as 5-OH in other flavonoid classes, the results confirm that 5-OH group in flavonoids disfavor the effect.

In summary, among the 25 flavonoids (**1**–**25**) examined in this study, the most effective inhibitors are apigenin (**1**), luteolin (**2**), dinatin (**3**), daidzein (**18**), sulfuretin (**22**), 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**), and 2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-

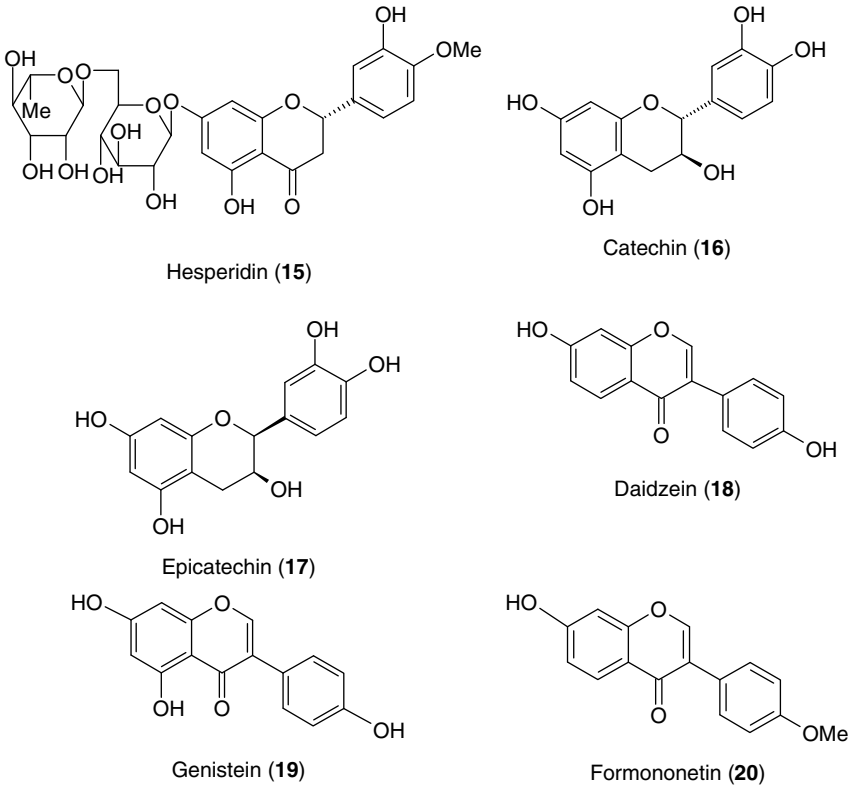


Fig. 1 (*continued*)

one (**25**). It is shown that for good inhibitory effects, the OH group at C-7 position (for aurones, at C-6 position) in the A-ring, C-4′ position in the B-ring, a double bond between C-2 and C-3 positions (for aurones, a double bond is between C-2 position and phenylidene), and C=O functionality at the C-4 position (for aurones, at the C-3 position) are essential. The presence of an OH group at the C-3 position or the C-5 position (for aurones, it is at C-4 position) will slightly reduce the inhibitory effect. Moreover, glycosylation groups at any position obviously decrease the effect. Addition of an OH group in the B-ring causes steric hindrance and disfavors the effect.

The order of potency of NA inhibitory effect in our study was as follows: aurones (sulfuretin (**22**), 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**), and 2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (**25**)) > flavones (apigenin (**1**) and luteolin (**2**)) > flavonols (kaempferol (**8**), quercetin (**9**), and myricetin (**10**)) > isoflavones (daidzein (**18**), genistein (**19**), and formononetin (**20**)) > flavanon(ol)es (naringenin (**13**) and liquiritin (**14**)) and flavan(ol)es (catechin (**16**) and epicatechin (**17**)).

3.2. Relationship between structures and in vitro anti-viral activities

The comparison of the activities of apigenin (**1**) and luteolin (**2**) in Table 2 indicated that 3′-OH can obviously decrease in vitro anti-viral activity of influenza virus A/Jinan/15/90 (H3N2). Similarly, the comparison of the activities of sulfuretin (**22**) and 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**) gave the same results. Galuteolin (**5**) exhibited much weaker activity than luteolin (**2**) because of the presence of glycosylation group, and it indicated that glycosylation groups significantly disfavor the effect. The contrast of the effect of daidzein (**18**) and apigenin (**1**) confirmed that the scaffold of isoflavones can remarkably reduce the effect. Compared with sulfuretin derivative (**23**), sulfuretin derivative (**25**) displayed lower activity because of the presence of 4-OH group (for flavones, it is 5-OH group). Thus, the SAR of flavonoids against influenza virus is in coincidence with the SAR of flavonoids as NA inhibitors.

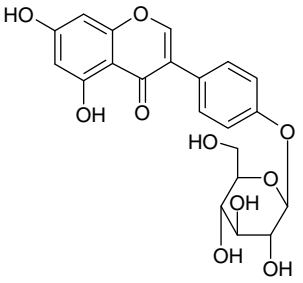
Interestingly, compared with reference compounds, apigenin (**1**), luteolin (**2**), dinatin (**3**), and 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**) all exerted higher in vitro anti-influenza virus activities than ribavirin, and showed weaker activities than oseltamivir carboxylic acid, while other flavonoids, such as galuteolin (**5**), daidzein (**18**), sulfuretin (**22**), and sulfuretin derivative (**25**), displayed weaker in vitro anti-influenza virus activities than ribavirin and oseltamivir carboxylic acid.

Therefore, the findings of this study provide important information relating the chemical structure of flavonoids to their ability to inhibit the activity of NA and to inhibit the replication of influenza virus. This understanding would facilitate the design of chemical compounds with higher potency to serve as potential NA inhibitors, and provide information for the exploitation and utilization of flavonoids as NA inhibitors for influenza treatment.

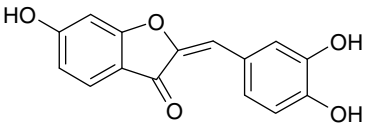
4. Materials and methods

4.1. Compounds

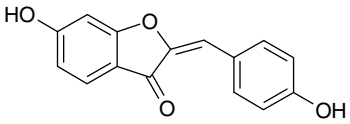
Apigenin (**1**), luteolin (**2**), dinatin (**3**), scutellarin (**4**), galuteolin (**5**), vitexin (**6**), chrysin (**7**), kaempferol (**8**), quercetin (**9**), myricetin (**10**), rhamnocitrin (**11**), rutin (**12**), naringenin (**13**), liquiritin (**14**), hesperidin (**15**), catechin (**16**), epicatechin (**17**), daidzein (**18**), genistein (**19**), formononetin (**20**), and sophoricoside (**21**) were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. Sulfuretin (**22**), 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**), 2-((*E*)-phenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**24**), and 2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (**25**) were provided by National Center for pharmaceutical Screening, Institute of Materia Medica, Chinese



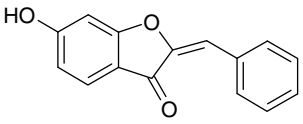
Sophoricoside (**21**)



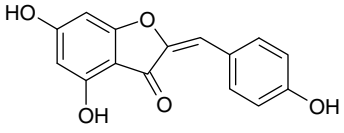
Sulphuretin (**22**)



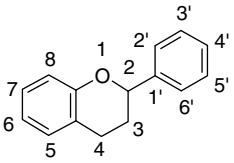
2-((E)-4'-hydroxyphenylidene)
-6-hydroxy-2,3-dihydrobenzofuran-3-one
(**23**)



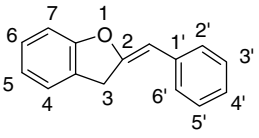
2-((E)- phenylidene)-6-hydroxy-2,3
-dihydrobenzofuran-3-one (**24**)



2-((E)-4'- hydroxyphenylidene)
-4,6-dihydroxy-2,3-dihydrobenzofuran
-3-one (**25**)



The numbering system (1)



The numbering system (2)

Fig. 1 (*continued*)

Academy of Medical Sciences. Oseltamivir carboxylic acid with 98% purity was purchased from Toronto Research Chemicals Inc. and ribavirin with 98% purity was provided by the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. The compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and used within 3 months of preparation.

4.2. Reagents

2′-(4-Methylumbelliferyl)-α-D-acetyl neuraminic acid (MUNANA) and 2-*N*-morpholino-ethanesulfonic acid (MES) were purchased from Sigma. $CaCl_2$ and NaOH were purchased from Beijing Chemical Reagents Company. Dulbecco's minimum essential medium (DMEM) and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from the Sigma Company. Trypsin–EDTA and trypsin (1:250) were purchased from the Gibco Company. Fetal bovine serum (FBS) was from Biofluids Inc.

4.3. Virus

Influenza viruses such as A/PR/8/34(H1N1), A/Jinan/15/90(H3N2), and B/Jiangsu/10/2003, which were used as the source of NAs, were kindly provided by China Centers for Disease Control.

4.4. NA activity assay

A standard fluorimetric assay was used to measure influenza virus NA activity with slight modifications.[17] The substrate MUNANA is cleaved by NA to yield a fluorescent product which can be quantified. The reaction mixture containing test flavonoids, and NA enzyme or virus suspension in MES buffer (32.5 mM) and calcium chloride (4 mM, pH 6.5) was incubated at 37 °C for 30–60 min (different incubation times for different viruses). After incubation, the reaction was terminated by adding NaOH (34 mM). Fluorescence was quantified with excitation wavelength at 360 nm and emission wavelength at 450 nm. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of NA inhibitor necessary to reduce NA activity by 50% relative to a reaction mixture containing virus but no inhibitor. The data are expressed as the mean of three to five independent experiments.

4.5. Cytotoxicity in MDCK cells

Cell viability was determined by the slightly modified MTT method.[18] One hundred microliters of 3-fold serial dilutions of flavonoids in medium were added to each well of a 96-well plate containing a confluent cell monolayer in triplicate, blank medium was used as the control. After 3 days of incubation at 37 °C in a humidified CO_2 atmosphere (5% CO_2), 12 μL of MTT solution (5 mg/ml in phosphate buffered saline) was added to each well. The plate was further incubated at 37 °C for 3 h to allow formation of formazan product. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. After 15 min, the contents of the wells were homogenized on a microplate shaker. The optical densities (OD) were then measured with a microplate spectrophotometer at a wavelength of 540 nm.

Table 1
Names and classes of flavonoids in this study, and their inhibition on NAs of influenza A and B viruses

No.	Class	Compound name or chemical name	NA inhibitory effect [IC_{50} (μM)][a]				Inhibitory effect
			A/PR/8/34 (H1N1)	A/Jinan/15/90 (H3N2)	B/Jiangsu/10/2003	Average	
1	Flavones	Apigenin	31.6 ± 0.9	28.9 ± 0.7	45.7 ± 2.3	35.4 ± 9.0	Good
2		Luteolin	33.7 ± 0.7	32.6 ± 0.1	53.3 ± 5.1	39.9 ± 11.6	Good
3		Dinatin	46.3 ± 4.4	26.0 ± 0.5	33.2 ± 0.4	35.2 ± 10.3	Good
4		Scutellarin	50.6 ± 0.9	47.3 ± 1.3	59.9 ± 3.8	52.6 ± 6.5	Moderate
5		Galuteolin	47.4 ± 0.5	52.2 ± 1.8	58.6 ± 2.7	52.7 ± 5.6	Moderate
6		Vitexin	46.5 ± 0.6	45.1 ± 1.3	49.6 ± 3.1	47.1 ± 2.3	Moderate
7		Chrysin	45.7 ± 1.9	33.36 ± 3.8	52.9 ± 2.5	44.0 ± 9.9	Moderate
8	Flavonols	Kaempferol	58.6 ± 0.6	38.1 ± 0.3	46.4 ± 0.8	47.7 ± 10.3	Moderate
9		Quercetin	58.4 ± 3.8	87.6 ± 5.5	67.5 ± 2.6	71.2 ± 14.9	Moderate
10		Myricetin	82.6 ± 8.9	46.2 ± 3.9	75.4 ± 6.7	68.1 ± 19.3	Moderate
11		Rhamnocitrin	51.5 ± 6.1	83.9 ± 4.4	62.0 ± 7.2	65.8 ± 16.5	Moderate
12		Rutin	52.2 ± 1.6	87.7 ± 5.9	>100	>100	Weak
13	Flavanon(ol)es	Naringenin	>100	>100	>100	>100	Weak
14		Liquiritin	>100	>100	>100	>100	Weak
15		Hesperidin	>100	>100	>100	>100	Weak
16	Flavan(ol)es	Catechin	>100	>100	>100	>100	Weak
17		Epicatechin	>100	>100	>100	>100	Weak
18	Isoflavones	Daidzein	37.1 ± 0.6	26.6 ± 0.3	46.8 ± 1.9	36.8 ± 10.1	Good
19		Genistein	77.1 ± 5.1	134.4 ± 11.5	83.3 ± 9.0	98.3 ± 31.4	Weak
20		Formononetin	>100	>100	>100	>100	Weak
21		Sophoricoside	>100	>100	>100	>100	Weak
22	Aurones	Sulfuretin	29.6 ± 0.5	27.7 ± 0.8	51.2 ± 5.7	36.2 ± 13.1	Good
23		2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one	22.0 ± 0.7	22.1 ± 0.3	22.9 ± 0.5	22.3 ± 0.5	Good
24		2-((*E*)-phenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one	72.0 ± 3.5	73.3 ± 7.9	86.6 ± 6.1	77.3 ± 8.1	Moderate
25		2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one	25.6 ± 1.1	22.3 ± 0.6	25.4 ± 1.0	24.4 ± 1.9	Good
	Positive control	Oseltamivir carboxylic acid	0.015 ± 0.007	0.0032 ± 0.0019	0.217 ± 0.096	0.078 ± 0.120	

[a] 50% inhibitory concentration, the average of three to five determinations.

Table 2
In vitro anti-influenza virus activities of some flavonoids against influenza virus A/Jinan/15/90 (H3N2) in MDCK cells using the CPE inhibition assay

Compound	CC_{50}[a] (μM)	MNCC[b] (μM)	IC_{50}[c] (μM)	SI[d]
Apigenin (**1**)	39.59	7.63	4.74	8.35
Luteolin (**2**)	12.44	7.20	6.82	1.82
Dinatin (**3**)	172.46	24.70	24.70	6.98
Galuteolin (**5**)	>1116	>1116	>1116	ND[e]
Daidzein (**18**)	>787	>787	>787	ND
Sulfuretin (**22**)	27.44	9.15	>9.15	ND
2-((*E*)-4′-Hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one (**23**)	151.53	87.47	15.71	9.64
2-((*E*)-4′-Hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (**25**)	>740.74	>740.74	>740.74	ND
Oseltamivir carboxylic acid	17.12	1.02	0.15	114.1
Ribavirin	>512	ND	25.62	>19.98

[a] CC_{50}: mean (50%) cytotoxic concentration.
[b] MNCC: maximal non-cytotoxic concentration.
[c] IC_{50}: mean (50%) inhibitory concentration.
[d] SI: selective index, CC_{50}/IC_{50}.
[e] ND: not determined.

The median cytotoxic concentration (CC_{50}) was calculated as the concentration of flavonoids that decreased the number of viable cells to 50% of the untreated control. The maximal non-cytotoxic concentration (MNCC) was defined as the maximal concentration of the sample that did not exert a cytotoxic effect and had more than 90% viable cells.

4.6. CPE reduction assay

The anti-viral activity of the NA inhibitors was measured by the cytopathic effect (CPE) inhibition assay with slight modification.[19] One hundred microliters of virus suspension (200 $TCID_{50}$/mL) was added to each well of a 96-well plate containing confluent cell monolayer. After being incubated at 37 °C for 2 h, the virus solution was removed, and 100 μL consecutive 3-fold serial dilutions of the active flavonoids and reference compound oseltamivir carboxylic acid were added to each well of the 96-well culture plates, using the MNCC as the highest concentration. An infection control without flavonoids was also included. The plates were incubated at 37 °C in a humidified CO_2 atmosphere (5% CO_2) for 24 h, then the CPE was observed. The virus-induced CPE was scored as follows: scores: 0 = 0% CPE, 1 = 0–25% CPE, 2 = 25–50% CPE, 3 = 50–75% CPE, and 4 = 75–100% CPE. The reduction in virus multiplication was calculated as a percentage of the virus control (% virus control = $CPE_{exp}/CPE_{virus\ control} \times 100$). The IC_{50} of the CPE with respect to virus control was estimated using the Reed–Muench method

and was expressed in μML. The selective index (SI) was calculated from the ratio CC_{50}/IC_{50}.

4.7. Statistical analysis

Statistical calculations were carried out with Microsoft Excel 2003. Results are expressed as the means ± SD of six independent experiments.

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