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Antiviral Activity of 3,4′-Dihydroxyflavone on Influenza A Virus

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Influenza virus infection causes thousands of deaths and millions of hospitalizations worldwide every year and the emergence of resistance to anti-influenza drugs has prompted scientists to seek new natural antiviral materials. In this study, we screened 13 different flavonoids from various flavonoid groups to identify the most potent antiviral flavonoid against human influenza A/PR/8/34 (H1N1). The 3-hydroxyl group flavonoids, including 3,2′-dihydroxyflavone (3,2′-DHF) and 3,4′-dihydroxyflavone (3,4′-DHF), showed potent anti-influenza activity. They inhibited viral neuraminidase activity and viral adsorption onto cells. To confirm the anti-influenza activity of these flavonoids, we used an *in vivo* mouse model. In mice infected with human influenza, oral administration of 3,4′-DHF significantly decreased virus titers and pathological changes in the lung and reduced body weight loss and death. Our data suggest that 3-hydroxyl group flavonoids, particularly 3,4′-DHF, have potent antiviral activity against human influenza A/PR/8/34 (H1N1) *in vitro* and *in vivo*. Further clinical studies are needed to investigate the therapeutic and prophylactic potential of the 3-hydroxyl group flavonoids in treating influenza pandemics.

Keywords: flavonoid, 3-hydroxyl group flavonoids, 3,4′-dihydroxyflavone, influenza virus

Introduction

Influenza viruses that belong to the family Orthomyxoviridae have caused significant morbidity and mortality in humans through epidemics or pandemics (Palese, 2004). Influenza virus infection remains a serious health problem, particularly for young children and elderly people, and imposes significant economic costs worldwide (Neumann *et al.*, 2005). The main strategies for dealing with influenza involve annual immunization and antiviral drugs. Even though annual vaccination is the core strategy for preventing influenza infections, the development of effective antiviral drugs is necessary to provide additional preventive and therapeutic benefits (Hayden, 2006). At present, two classes of anti-influenza agents are available for influenza management. One class comprises M2 ion channel blockers (amantadine and rimantadine); the other comprises neuraminidase inhibitors (oseltamivir and zanamivir). Administration of these drugs immediately after infection can stop the infection. However, the isolation of viable mutant viruses that are resistant to these drugs has raised concerns (Carr *et al.*, 2002). Since 2005, the recommendations of the Centers for Disease Control and Prevention (CDC) for the use of adamantanes (amantadine and rimantadine) in the control of influenza virus infections have changed because of the dramatic rise in resistance to adamantanes. Recent data collected by the World Health Organization (WHO) revealed a sudden increase in oseltamivir-resistant influenza A/H1N1 viruses worldwide (Bouvier *et al.*, 2008; Hurt *et al.*, 2009). The efficacy of current drugs is limited, and improved therapies are needed. Anti-influenza drug development has become a high priority because of the risk of a new pandemic. Several novel agents that may be effective against influenza virus, specifically the avian flu virus strain, are currently under development. Natural antiviral nutrients are of special interest because they are usually available and can be used as part of the diet to combat diseases, including influenza infection.

Plant-derived flavonoids found in fruits, leaves, and vegetables have been the focus of many studies because of their beneficial health effects in several disease models (Williamson and Manach, 2005). Flavonoids, part of a group of natural substances with variable polyphenolic structures, are highly diversified natural plant pigments. Flavonoids are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea, and wine (Middleton, 1998). As natural substances, flavonoids were used for their beneficial effects on health long before they were isolated as effective compounds (Roberts, 2001). Several studies have suggested that flavonoids play a protective role in the prevention of cancer, coronary heart diseases, bone loss, and many other age-related diseases (Havsteen, 2002). Moreover, the various antiviral activities of flavonoids against influenza virus have been studied (Liu *et al.*, 2008).

Previously, 3-hydroxyl group flavonoids were reported to have anti-apoptotic and anti-oxidant activities. For example, in human cancer cells, treatment with 3-hydroxyl group flavonoids increased apoptosis (Kim *et al.*, 2008, 2012; Lee *et al.*, 2011b) and decreases reactive oxygen species generation (Lee *et al.*, 2007b, 2007c). Furthermore, we reported

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that the hydroxylation patterns of the flavonoid B ring play a critical role in the cellular function of 3-hydroxyl group flavonoids (Lee *et al.*, 2007a, 2011a). In this study, we tested the efficacy of the 3-hydroxyl group flavonoids against influenza A virus.

Materials and Methods

Virus, cells, and reagents

Influenza virus A/Puerto Rico/8/34 (A/PR/8/34) was kindly provided by the Immunogenetics Laboratory, Department of Animal Biotechnology, Konkuk University. Madin-Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (ATCC CCL-3) and maintained in Minimum Essential Media (MEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA) and 100 U/ml penicillin/streptomycin (Gibco). Before virus infection, MDCK cells were washed with PBS and cultured in virus growth medium (MEM without FBS) supplemented with 10% bovine serum albumin (Sigma-Aldrich, USA), 100 U/ml penicillin/streptomycin, and 2 μg/ml of trypsin TPCK (Gibco).

Flavonoid compounds, 3-hydroxyflavone (3-HF), 3,2′-dihydroxyflavone (3,2′-DHF), 3,3′-dihydroxyflavone (3,3′-DHF), 3,4′-dihydroxyflavone (3,4′-DHF), 4-hydroxyflavone (4-HF), 4′-hydroxy-5-methoxyflavone (4′-H5-MF), 4′-hydroxy-6-methoxyflavone (4′-H6-MF), 4′-hydroxy-7-methoxyflavone (4′-H7-MF), 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone (5,7-D,3′,4′,5′-TMF), 6,4′-dihydroxyflavone (6,4′-DHF), 7,8,4′-trihydroxyflavone (7,8,4′-THF), 3,5,7-trihydroxy-4′-methoxyflavone (diosmetin), and 3,5,7-3′,4′-pentahydroxyflavone (quercetin), were purchased from INDOFINE Chemical Company and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Tamiflu (oseltamivir) was kindly provided by the Immunogenetics Laboratory, Department of Animal Biotechnology, Konkuk University.

Cell viability and antiviral assays

MDCK cells were seeded in a 96-well plate at 2×10^4 cells/well for the determination of cell viability. When the cells reached confluency 24 h after seeding, they were washed twice with PBS and treated with the indicated concentrations of flavonoid. After incubation at 37°C for 48 h in a 5% CO_2 incubator, cell viability was determined using an MTT assay kit (Sigma-Aldrich) and an xMark^{TM} spectrophotometer (Bio-Rad, USA) to measure absorbance at 490 nm. For the antiviral assay, cells were infected with A/PR/8/34 virus at 100 $TCID_{50}$ when they reached 80–90% confluence. Two hours post-infection, the virus-containing medium was replaced with virus-free growth medium containing flavonoids. Cell viability was determined 48 h post-infection by MTT assay using a spectrophotometer to measure absorbance at 490 nm.

Viral yield reduction assay

MDCK cells were seeded in 6-well plates at a density of 3×10^5 cells/well, and the medium was removed 24 h after seeding when the cells were confluent. Cells were then washed twice with PBS and infected with the virus, which was diluted in virus growth media, at 100 $TCID_{50}$. Two hours post-infection, the virus-containing medium was removed, and the cells were treated with flavonoids diluted in virus growth medium. The hemagglutination (HA) assay was carried out 48 h after infection with virus in 96-well round-bottom plates containing 25 μl of PBS per well. HA titers were calculated as hemagglutination units/50 μl (HAU/50 μl).

Hemagglutination inhibition (HI) assay

The hemagglutination inhibition assay was used to examine the mechanical effect of the flavonoids on the agglutination of chicken red blood cells (RBCs) by influenza A virus. Different concentration of flavonoids diluted in PBS were mixed with an equal volume of A/PR/34/8 influenza virus in a 96-well round-bottom plate and incubated for 1 h at 37°C in a 5% CO_2 incubator. Subsequently, chicken RBCs were added to each well and incubated for 30 min at room temperature to observe hemagglutination.

Neuraminidase inhibition (NAI) assay

The neuraminidase inhibition assay was used to test the effect of the flavonoids on the neuraminidase activity of the virus. Different concentration of flavonoids diluted in PBS were mixed with an equal volume of influenza A/PR/8/34 virus solution and incubated for 1 h at 37°C in a 5% CO_2 incubator. An equal volume of the substrate solution (2′-(4-methylumbelliferyl)-α-d-*N*-acetylneuraminic acid sodium (4-MU-NANA); Sigma-Aldrich) was then added, and the mixture was incubated at 37°C for 2 h under protection from light. The reaction was stopped by the addition of stop solution. The fluorescence of 4-methylumbelliferone was then measured with a fluorescence spectrophotometer (excitation 365 nm, emission 460 nm).

Reverse transcription-PCR

Confluent MDCK cells grown in 6-well plates were infected with influenza A/PR/8/34 virus at 100 $TCID_{50}$. At 24 h post-infection, cells were harvested and collected by centrifugation (1,500 × *g* for 5 min). Cell pellets were washed twice with PBS, and total RNA was isolated from the pellets using an easy-BLUE Total RNA Extraction kit (iNtRON Biotechnology, Korea). First-strand cDNA was synthesized from 3 μg of total RNA. PCR was performed using primers for hemagglutinin, (F)-GAAAGCTCATGGCCCAACCA and (R)-TCCCAGGGGTGTTTGACACT, and neuraminidase, (F)-TGCTTG-GTCAGCAAGTGCAT and (R)-GGTTGTCACC GAAAACCCCA. The amplification conditions were as follows: 95°C for 10 min, 95°C for 30 sec, 55°C 30 sec, and 72°C 1 min, 72°C, 4°C (30 cycles). The expression of a cellular housekeeping gene, *GADPH*, was used as a control.

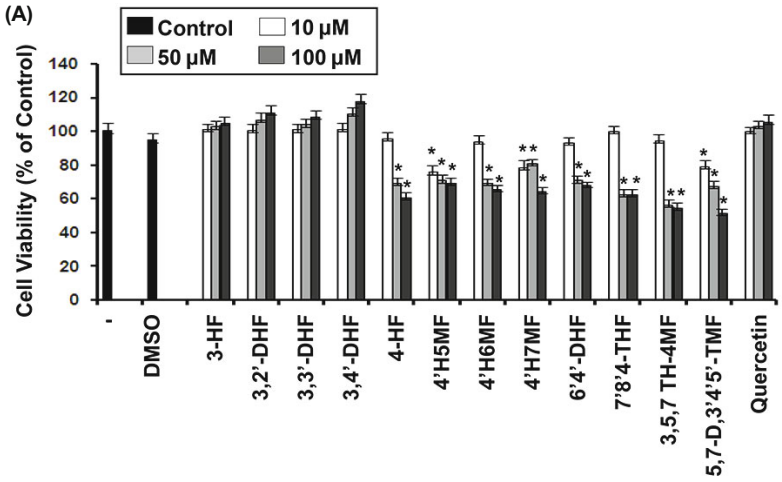
In vivo antiviral assay

Weight loss, survival rate, and lung viral titers were evaluated in six-week-old female C57BL/6 mice (Koatek) infected with influenza PR8 strain (A/PR/8/34, H1N1). Anesthetized mice were challenged by intranasal inoculation with 4.25 $\log_{10}EID_{50}$/ml (5 MLD_{50}/mice) in 30 μl of sterile

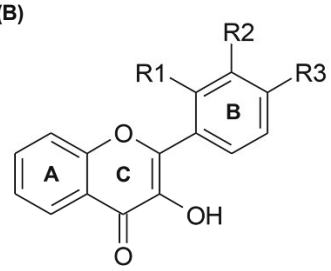
PBS. Two hours post-infection, 13 mice per group were administered the following compounds: (1) Tamiflu: 10 mg/kg per 200 µl (oral administration) daily for 5 days or (2) flavonoids (3,2′-DHF or 3,4′-DHF): 1 mg/kg per 30 µl (oral administration) daily for 5 days. On day 7 post-infection, three mice per group were euthanized, and the lungs were analyzed. Lung tissues were excised and homogenized using a homogenizer (TissueLyser; Qiagen, USA). Debris was pelleted by centrifugation (2,000 × *g*, 5 min). Thereafter, 10-fold serial dilutions of the supernatant from individual lung homogenates were injected into the allantoic sac of 10-day-old embryonated eggs, in triplicate for each dilution. The eggs were incubated at 37°C for 48 h, and the allantoic fluid was then harvested. The amount of virus in the fluid was determined by the HA assay, and the EID_{50}/ml virus titer in the fluid was calculated using the method of Reed and Muench, as described previously (Marjuki *et al.*, 2009; Smee *et al.*, 2010). Body weight, survival, and clinical signs were also monitored daily for 14 days.

Statistical analysis

The results were expressed as the mean ± SE. Each value represents the mean of at least three independent experiments in each group. The statistical significance of the difference between two cell populations was determined using the two-tailed Student's *t*-test (Origin software; OriginLab). *P* values equal to or less than 0.05 and 0.01 were considered significant.



(B)



Name	R1	R2	R3
3-HF	-	-	-
3,2'-DHF	OH	-	-
3,3'-DHF	-	OH	-
3,4'-DHF	-	-	OH

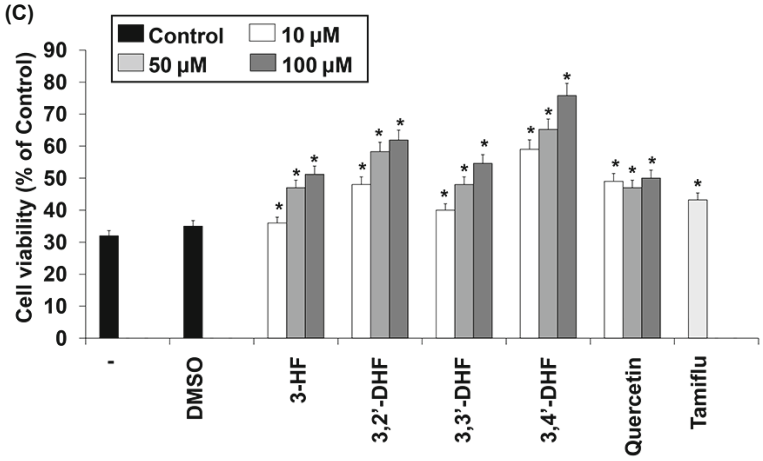


Fig. 1. Screening for antiviral flavonoids with cell viability and antiviral assays. (A) The viability of MDCK cells was determined after treatment with various flavonoids. Cells were treated with the indicated concentrations of flavonoids for 48 h, and the MTT assay was performed to access the cytotoxic effect of the indicated flavonoids (**P*<0.05). (B) Structure of the four 3-hydroxyl group flavonoids showing the positions of the different free hydroxyl groups located on the B ring. (C) MDCK cells were infected with A/PR/8/34 virus at 100 $TCID_{50}$. Two hours post-infection, the medium was replaced with virus-free growth medium containing flavonoids. The MTT assay was performed 48 h after infection (**P*<0.05).

Table 1. Selectivity index for the antiviral activity of 3-hydroxyl flavonoids
The 50% effective concentration (EC_{50}) was determined by the percentage of inhibition of virus replication as a function of the compound concentration from the dose-response assessments. The 50% cytotoxic concentration (CC_{50}) was calculated as the concentration required for the decrease of cell viability by 50% compared to that for the controls. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} (SI= CC_{50}/EC_{50}).

Flavonoids	CC_{50} (μM)	EC_{50} (μM)	SI
3-HF	255	180	1.41
3,2′-DHF	300	95	3.15
3,3′-DHF	270	155	1.74
3,4′-DHF	255	45	5.6

Results

Antiviral effect of the 3-hydroxyl group flavonoids

To screen for antiviral flavonoids, we first assessed cell viability after treating cells with the flavonoids listed in Fig. 1A. The 3-hydroxyl group flavonoids (3-HF, 3,2′-DHF, 3,3′-DHF, and 3,4′-DHF) and quercetin increased the cell viability of MDCK cells (Fig. 1A). We then tested the antiviral effect the 3-hydroxyl group flavonoids, which have free hydroxyl groups positioned at different locations on the B ring (Fig. 1B). Among the 3-hydroxyl group flavonoids, 3,2′-DHF and 3,4′-DHF exhibited more potent antiviral effects than did the other 3-hydroxyl group flavonoids and quercetin (Fig. 1C and Table 1). The antiviral effect of the 3-hydroxyl group flavonoids was quantified using the selectivity index (SI), which was calculated using the 50% cytotoxic concentration (CC_{50}) and the 50% effective concentration (EC_{50}) values (Table 1). The results indicate that the 3-hydroxyl group flavonoids 3,2′-DHF and 3,4′-DHF may be effective natural antiviral compounds.

Inhibitory effect of 3,2′-DHF and 3,4′-DHF on virus adsorption onto RBCs

Next, we performed HA assays after treating cells with 3-hydroxyl group flavonoids. Treatment with 3,2′-DHF and 3,4′-DHF decreased the virus titer even at low concentration (10 μM) (Fig. 2A). The addition of a high concentration of 3,4′-DHF suppressed HA almost completely. We also investigated whether the 3-hydroxyl group flavonoids directly interfered with viral adsorption onto RBCs using the HI assay. Importantly, 3,2′-DHF and 3,4′-DHF inhibited viral adsorption onto RBCs, suggesting that the flavonoids directly interfered with the binding of influenza virus hemagglutinin to sialic acid receptors on MDCK cells.

Inhibitory effect of 3,4′-DHF treatment on viral neuraminidase activity and viral mRNA expression

Because neuraminidase plays a key role in the release of newly made virus particles by cleaving sialic acid moieties on target cell receptors (Smee *et al.*, 2010), we analyzed the effect of 3,4′-DHF on viral neuraminidase activity. Treatment with 3,4′-DHF suppressed viral neuraminidase activity. When 3,4′-DHF was added at a high concentration (100 μM), its neuraminidase-inhibitory effect was similar to that Tamiflu, which was used as a positive control (Fig. 3A). Our results suggest that 3,4′-DHF, like Tamiflu, inhibits virus release by inhibiting neuraminidase activity. We also analyzed the effect of 3,4′-DHF on viral mRNA synthesis, which usually occurs at the middle stage of the viral life cycle (Gil *et al.*, 2000). Twenty-four hours after viral infection, we quantified viral mRNA expression by quantitative RT-PCR (Fig. 3B) and found that 3,4′-DHF treatment blocked the synthesis of influenza hemagglutinin and neuraminidase mRNA.

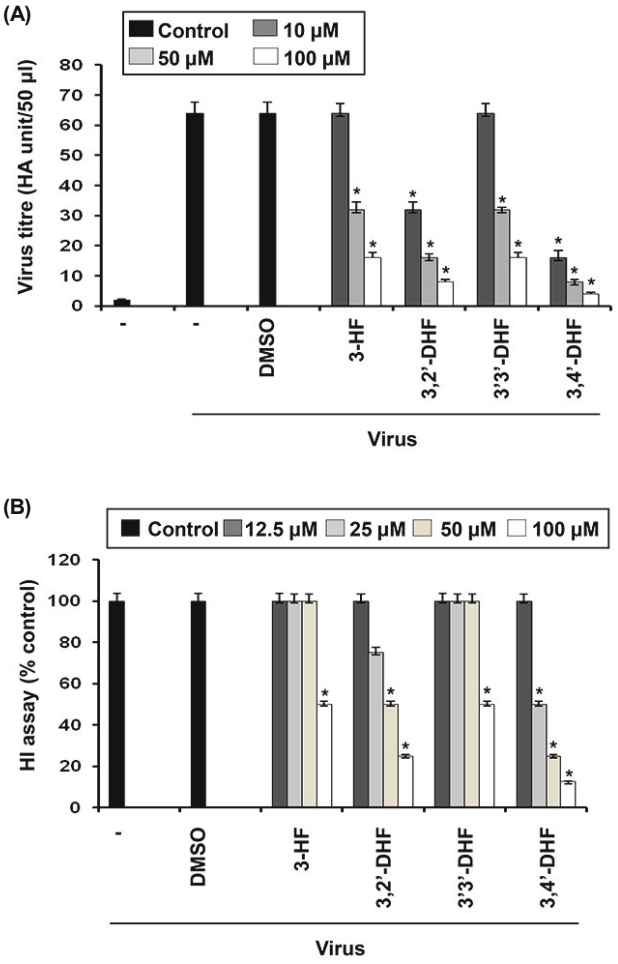


Fig. 2. Inhibitory effect of 3,2′-DHF and 3,4′-DHF on virus adsorption onto red blood cells (RBCs). (A) MDCK cells were infected with 100 $TCID_{50}$ A/PR/8/34 virus. The medium was changed 2 h after virus infection, and cells were treated with 10, 50, or 100 μM of the indicated 3-hydroxyl group flavonoid. The hemagglutination (HA) assay was performed 48 h after virus infection. HA titers were calculated as hemagglutination units/50 μl (HAU/50 μl) (*P<0.05). (B) For the hemagglutination inhibition (HI) assay, 3-hydroxyl group flavonoids diluted in 25 μl PBS (to concentrations of 25, 50, 100, or 200 μM) were mixed with an equal volume of influenza virus. A 1% suspension of chicken RBCs (50 μl) was then added to each well and incubated for 30 min at room temperature to induce hemagglutination (*P<0.05).

In vivo antiviral effect of 3,4′-DHF treatment

The *in vivo* antiviral effects of 3,4′-DHF on influenza virus infection were evaluated in a mouse model. After infection with A/PR/8/34 (H1N1) virus, mice were orally administered 1 mg/kg 3,2′-DHF or 3,4′-DHF daily for 5 days. As a control group, mice were orally administrated 10 mg/kg

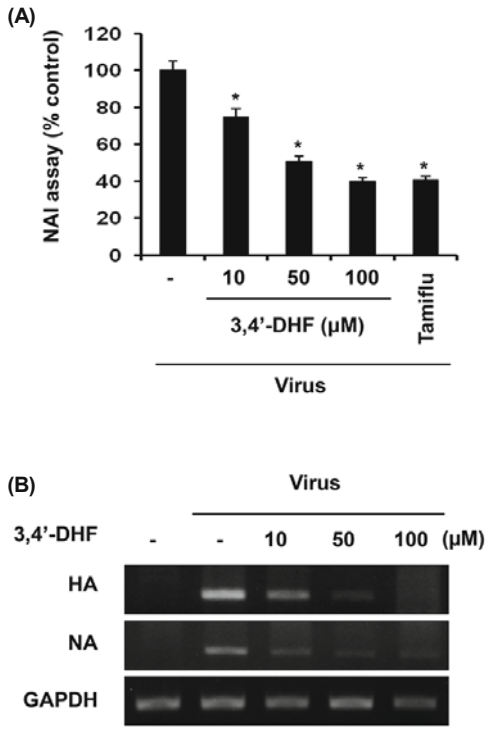


Fig. 3. Inhibitory effect of 3,4′-DHF on viral neuraminidase activity and viral mRNA expression. (A) The neuraminidase-inhibitory (NAI) activity of 3,4′-DHF against A/PR/8/34 virus was analyzed. (B) MDCK cells were infected with A/PR/8/34 virus (100 $TCID_{50}$). The cells were harvested 24 h after infection, and RT-PCR was performed with forward and reverse primers for hemagglutinin (HA) and neuraminidase (NA).

Tamiflu daily for 5 days. We compared the effects of the flavonoids with those of Tamiflu. The virus titers in the lung tissues were measured to evaluate the inhibition of influenza virus replication. Three mice per group were sacrificed on day 7 post-infection for determination of lung viral titers, which were reported as EID_{50}/ml for each lung (Table 2). Lung virus titers of the mice treated with 3,2′-DHF, 3,4′-DHF, or Tamiflu were 5.75, 5.25, and 5.52 $\log_{10}EID_{50}$/ml, respectively. These titers were lower than those of mice in the PBS control group (7.5 $\log_{10}EID_{50}$/ml). Compared to 3,2′-DHF and Tamiflu, 3,4′-DHF was more potent in decreasing the lung virus titer. The mice were also monitored daily for 14 days to assess body weight changes and survival. Body weight loss in mice treated with 3,2′-DHF or 3,4′-DHF was significantly reduced compared to body weight loss in the PBS control group (Fig. 4A). All mice in the PBS control group died by day 8 post-infection, whereas survival was prolonged in the flavonoid-treated groups of mice, in which the survival rates were 60% to 70% (Fig. 4B). All mice treated with Tamiflu survived. These results confirmed that the flavonoids were effective at treating infection with influenza virus.

Table 2. Determination of lung virus titers following influenza virus infection in 3,2′-DHF- or 3,4′-DHF-treated mice

Lung viral titers were evaluated in mice following virus infection. After virus infection, each group of mice was orally administered with the indicated antiviral material, and the lung virus titers were measured with the viral yield reduction assay. The EID_{50}/ml virus titer in the fluid was calculated using the method of Reed and Muench (Marjuki *et al.*, 2009; Smee *et al.*, 2010).

Treatment	Geometric mean titre ($\log_{10}EID_{50}$/ml)			
	PBS (w/o)	3,2′-DHF	3,4′-DHF	Tamiflu
A/PR/8/34	7.5 ± 0.21	5.75 ± 0.25*	5.25 ± 0.43*	5.52 ± 0.16**

*P<0.05 compared to PBS controls. **P<0.01 compared to PBS controls.

Discussion

Because the development and production of vaccines that prevent influenza virus takes a long time, antiviral drug discovery is a high priority for combatting the risk of a new pandemic (Williamson and Manach, 2005). The goal of our study was to find a novel antiviral flavonoid. Previously, we reported that 3,4′-DHF and other 3-hydroxyl group flavonoids had anti-apoptotic or anti-oxidant activity; the position of the hydroxyl (OH) substitution was a critical factor in determining the flavonoids' effects (Kim *et al.*, 2008, 2012; Lee *et al.*, 2007a, 2011b). In particular, 3,4′-DHF exhibited anti-apoptotic and anti-oxidant activities in keratinocytes and bovine embryo development (Lee *et al.*, 2011a, 2011b). Furthermore, we also showed a protective effect of 5,7,3′,4′-tetrahydroxy flavanone (eriodictyol) against UV-induced apoptosis (Lee *et al.*, 2007c, 2011a). In this study, many flavonoids, including 4-HF, 6,4′-DHF, 7,8,4-THF, 5,7-D,3′,4′,5′-

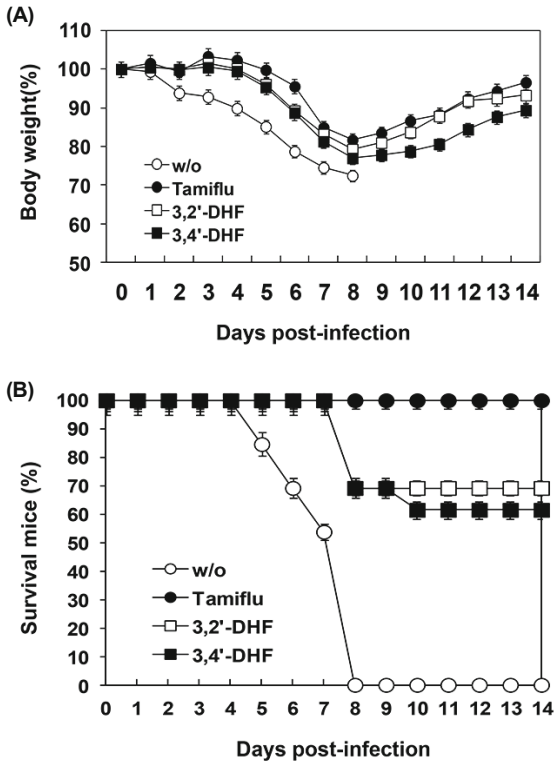


Fig. 4. The effects of 3,4′-DHF on the replication of influenza A virus in a mouse model. Weight loss and survival rate were evaluated in six-week-old female C57BL/6 mice infected with influenza A/PR/8/34 (H1N1) and subsequently treated with Tamiflu, 3,2′-DHF, or 3,4′-DHF daily for 5 days. Body weight (A) and survival (B) were monitored for 14 days.

TMF, diosmetin, 4′-H5-MF, 4′H6-MF, and 4′-H7MF, were cytotoxic in MDCK cells. However, quercetin and several 3-hydroxyl flavonoids increased cell viability. The antiviral effect of 3,2′-DHF and 3,4′-DHF was stronger than that of quercetin. Our previous study suggested that the hydroxyl group substitution at the 3-carbon of the C6C3C6 skeleton affected cell viability (Lee *et al.*, 2007c). Here, we showed that the 3-hydroxyl group flavonoids, particularly 3,2′-DHF and 3,4′-DHF, had potent antiviral activity, confirming that the hydroxyl group substitution pattern influences the regulation of cellular functions.

In this study, treatment with 3,4′-DHF inhibited HA and neuraminidase. A previous report demonstrated that some flavonoids inhibit the adsorption of influenza virus onto RBCs (Song *et al.*, 2005). These properties have also been attributed to other flavonoids, such as quercetin, kaempferol, and luteolin glycosides (Gil *et al.*, 2000; Cerda *et al.*, 2003). The inhibitory activity might be due to the inhibition of endosome acidification, which interferes with viral membrane fusion (Imanishi *et al.*, 2002). Furthermore, like Tamiflu, 3,4′-DHF also suppressed neuraminidase activity, indicating that 3,4′-DHF may be an effective natural antiviral compound. Lung viral titers were substantially reduced in 3,2′-DHF- and 3,4′-DHF-treated mice, as in Tamiflu-treated mice. These findings suggest that 3,2′-DHF and 3,4′-DHF can be developed as safe and effective natural antiviral compounds. In conclusion, our studies showed that the 3-hydroxyl group flavonoids, especially 3,4′-DHF, inhibited influenza A virus replication *in vitro* and *in vivo*. Development of 3-hydroxyl group flavonoids may lead to effective and safe anti-influenza agents that are effective against Tamiflu (oseltamivir)-resistant viruses and pandemic influenza viruses.

Acknowledgements

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