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7,8-dihydroxyflavone and daphnetin have virucidal and neuraminidase inhibition activities against influenza A virus in vitro

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Abstract
Influenza A virus remains a major global health concern, underscoring the need for novel antiviral agents. This study investigated the antiviral potential of two natural compounds, 7,8-dihydroxyflavone (DHF) and daphnetin (DAP), against influenza A viruses in vitro. Following in vitro cytotoxicity and antioxidative activity assessments, the antiviral effects of DHF and DAP were evaluated, with a particular focus on their direct viral inhibition. DHF and DAP demonstrated complete virucidal activity influenza A virus at concentrations of 50 μM and 100 μM, respectively. However, neither compound inhibited influenza surface protein hemagglutination (HA), suggesting that their virucidal effects are independent of HA receptor binding. Both compounds exhibited neuraminidase (NA) inhibition, with DAP showing stronger activity compared to DHF. Furthermore, DHF and DAP suppressed influenza virus replication in cells, as evidenced by a reduction in green fluorescence protein (GFP) reporter expression in virus-infected cells. Growth kinetics analysis revealed that both compounds significantly reduced viral replication when applied to cells before or after viral infection. These findings demonstrate that DHF and DAP exhibit multifaceted antiviral activity, including direct virucidal action, NA inhibition, and suppression of viral replication. Our results suggest that DHF and DAP are promising candidates for the development of novel influenza therapeutics.

Keywords Antiviral agents, Influenza virus, 7,8-Dihydroxyflavone, Daphnetin, Neuraminidase, Virucidal activity

Introduction

Influenza virus, a member of the *Orthomyxoviridae* family, is an enveloped virus with a segmented RNA genome consisting of 7–8 segments [1, 2]. Influenza viruses are classified into four genetic types: A, B, C, and D. Among these, influenza A and B viruses cause seasonal epidemics in humans, whereas C and D viruses rarely infect humans. Influenza A viruses are further divided based on their surface glycoproteins, HA and NA, with 18 HA and 11 NA subtypes identified thus far, resulting in numerous possible subtypes through combinations of different HA and NA subtypes [3]. The frequent mutations and genetic reassortment of RNA genes contribute to the high genetic diversity and variability of the influenza virus. Influenza infections cause significant public health and economic burdens, leading to 3–5 million severe respiratory illnesses and 290,000–650,000 deaths annually worldwide [4]. In addition to seasonal influenza, influenza A viruses have been responsible for periodic pandemics occurring every 10–40 years, causing severe

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global health crises [5]. The high mutation rate of influenza viruses significantly reduces the efficacy of vaccines, making it challenging to predict and prevent future outbreaks. Moreover, influenza pandemics often result in substantial mortality and socio-economic disruptions before an effective vaccine is developed.

Given the inconsistent protective efficacy of influenza vaccines and the emergence of variant strains, antiviral agents play a crucial role in reducing the morbidity and mortality associated with influenza virus infections [6]. Currently approved influenza antiviral drugs include M2 ion channel inhibitors, NA inhibitors, and viral RNA polymerase inhibitors [7]. M2 ion channel inhibitors, such as amantadine and rimantadine, block the M2 proton channel, which is responsible for the acidification of the viral interior—a critical step in the influenza A virus replication cycle [8]. NA inhibitors, including zanamivir, laninamivir, oseltamivir, and peramivir, prevent the release of progeny virions by inhibiting the enzymatic activity of the viral NA protein [9]. In addition, favipiravir, pimodivir, and baloxavir marboxil act as polymerase inhibitors, targeting polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA), respectively [10]. Additionally, research on antiviral agents targeting the HA protein of the influenza virus is actively ongoing [11, 12]. However, the emergence of drug-resistant influenza virus strains remains a significant challenge in maintaining the long-term efficacy of antiviral treatments [13, 14]. These resistant strains often arise due to amino acid substitutions at the binding sites in the viral proteins targeted by existing drugs, which can reduce drug binding affinity and efficacy [15, 16]. To address this issue, novel antiviral agents with different mechanisms of action or combination therapies involving drugs with distinct targets have been proposed.

Recent research has focused on developing antiviral agents derived from plant-based natural compounds, which have demonstrated various biological activities, including anti-inflammatory, antioxidant, and anticancer properties [17, 18]. Some plant-derived compounds and have also exhibited antiviral activity against a range of viruses [19–22]. In this study, we investigated the antiviral activities of 7,8-dihydroxyflavone (DHF), a flavonoid isolated from *Lepisorus ussuriensis* (Regel & Maack) Ching (*L. ussuriensis*) [23], and daphnetin (DAP), a coumarin isolated from *Daphne jejudoensis* (*D. jejudoensis*) [24]. While DHF has been actively investigated for its potential as a drug candidate for neuropsychiatric disorders [25], no studies have reported the antiviral activity of DHF thus far. Research on the antiviral properties of DAP is very limited, with only one recent study indicating that DAP inhibits SARS-CoV-2 infection by downregulating angiotensin-converting enzyme 2 (ACE2) expression in host cells [26]. However, no studies have examined the antiviral effects of these compounds against influenza virus. Our study is the first to report the antiviral activities of DHF and DAP against influenza A/H1N1 and A/H5N2 viruses, demonstrating their virucidal and NA inhibition activity and suggesting their potential as novel antiviral agents for influenza treatment. Influenza A/H1N1 was selected as it is a major causative agent of seasonal influenza epidemics, while A/H5N2, an avian influenza virus, was chosen due to its potential risk of human infection. The inclusion of both subtypes allows for a comprehensive evaluation of the antiviral efficacy of DHF and DAP against influenza A viruses with different pathogenic profiles.

Materials and methods

Cell culture, influenza virus, and reagents

Madin-Darby canine kidney (MDCK) cells were used for influenza A virus experiments due to their high susceptibility to viral infection and widespread use in influenza research. MDCK cells express both α-2,3- and α-2,6-linked sialic acid receptors, allowing efficient viral entry and replication, making them suitable for evaluating antiviral activity. MDCK cells were purchased from the American Type Culture Collection (ATCC) and maintained in Minimum Essential Medium (MEM) (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and Zellshield antibiotic (Minerva Biolabs, Berlin, Germany) under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were passaged every 2–3 days for use in experiments. The influenza A viruses, A/Puerto Rico/8/34 (A/PR8, H1N1) and mouse-adapted A/aquatic bird/Korea/w81/05 (A/MA81, H5N2), and A/PR8-GFP reporter virus [27] were propagated in MDCK cells. The viruses were inoculated onto MDCK cells at a multiplicity of infection (MOI) of 0.01, and the supernatants were collected and clarified by centrifugation and stored in aliquots at -80 °C before use. Oseltamivir phosphate (OP) was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Isolation and structural identification of DHF and DAP

DHF from *L. ussuriensis* and DAP from *D. jejudoensis* were isolated using column chromatography, and their structures were identified through NMR spectroscopy analysis. Dried and powdered *L. ussuriensis* was extracted three times with methanol (MeOH) for 3 h under reflux. The combined extracts were filtered through filter paper, and the filtrate was concentrated under reduced pressure to yield a MeOH extract. The MeOH extract was suspended in distilled water and successively partitioned with *n*-hexane, chloroform, ethyl acetate (EtOAc), and *n*-butanol. Among them, the EtOAc fraction was subjected to silica gel column chromatography using a chloroform: methanol solvents to obtain 17 subfractions.

From subfraction 11, recrystallization with MeOH was performed to yield a pure DHF. ^{1}H-NMR (500 MHz, DMSO): δ_H 8.16–7.50 (B-ring). 7.56 (1H, d, J = 5.5 Hz, H-5), 6.96 (1H, d, J = 5.5 Hz, H-6), 6.88 (1H, s, H-3); ^{13}C-NMR (125 MHz, DMSO): δ_C 176.8 (C = O), 161.7 (C-2), 150.6 (C-8), 146.6 (C-9), 133.1 (C-4'), 131.4 (C-2',6'), 129.0 (C-3',5'), 126.3 (C-6), 116.5 (C-5), 115.0 (C-7), 106.0 (C-3). Dried and pulverized leaves of *D. jejudoensis* were extracted three times with MeOH for 3 h under reflux. The combined extracts were filtered through filter paper and then concentrated under reduced pressure to yield a crude MeOH extract. The MeOH extract was suspended in distilled water and successively partitioned with *n*-hexane, methylene chloride (MC), EtOAc, and *n*-butanol. Among them, the MC fraction was subjected to silica gel column chromatography using a gradient elution system of EtOAc: MeOH to afford 22 subfractions. From subfraction 7, recrystallization with MeOH was carried out to obtain a pure DAP. ^{1}H-NMR (500 MHz, DMSO): δ_H 9.0–10.0 (OH), 7.89 (1H, d, J = 9 Hz, H-5), 7.70 (1H, d, J = 9 Hz, H-6), 6.78 (1H, d, J = 9 Hz, H-3), 6.16 (1H, d, J = 9 Hz, H-2); ^{13}C-NMR (125 MHz, DMSO): δ_C 160.4 (C = O), 149.6 (C-4'), 145.0 (C-8'), 143.7 (C-O), 132.1 (C-7), 118.8 (C-6), 112.4 (C-5), 112.0 (C-3), 111.2 (C-2).

In vitro cytotoxicity assay of DHF and DAP

The in vitro cytotoxicity of DHF and DAP was evaluated using the MTT assay kit (Sigma-Aldrich, St. Louis, MO, USA). MDCK cells were seeded in 96-well plates at a density of 10^4 cells/well and incubated for 24 h. The cells were then treated with DHF and DAP at concentrations ranging from 6.3 to 200 μM and incubated for another 24 h. Subsequently, 10 μL of MTT reagent was added to the wells, and the plate was incubated at 37 °C for 3 h. After incubation, 100 μL of DMSO was added to the wells and incubated at room temperature (RT) for 30 min. The absorbance at 570 nm was measured to determine the cell viability relative to the DMSO-treated control.

Antioxidant activity assay of DHF and DAP

The antioxidant activity of DHF, DAP, L-ascorbic acid (Sigma-Aldrich), and gallic acid (Thermo Scientific, Waltham, MA, USA) was assessed using the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical-scavenging assay. A 20 μL aliquot of the diluted compounds was mixed with 180 μL of 100 μM DPPH reagent (Dojindo Laboratories, Kumamoto, Japan) and incubated for 30 min at RT. The absorbance was measured at 514 nm. The DPPH radical-scavenging activity was calculated using the formula: DPPH radical-scavenging activity (%) = [1 − (absorbance of sample/absorbance of control)] × 100%.

Hemagglutination Inhibition (HI) activity of DHF and DAP

A hemagglutination assay was performed to determine the hemagglutination units (HAU) of influenza A virus. In V-shaped 96-well plates, 50 μL of influenza virus was mixed with 50 μL of 1% chicken red blood cells (cRBC) (Innovative Research, Novi, MI, USA) and incubated at 4 °C for 1 h for allow hemagglutination. The HAU was determined as the highest dilution of the virus at which complete hemagglutination occurred. For the HI assay, 25 μL of diluted DHF or DAP was mixed with 25 μL of influenza virus (4 HAU) and incubated at 37 °C for 30 min. Subsequently, 50 μL of 1% cRBC was added, and the mixture was incubated at 4 °C for 1 h. The inhibitory concentration at which hemagglutination was prevented was determined.

Plaque assay for influenza virus Titration

The virucidal activity of DHF and DAP was assessed through a viral plaque assay. DHF and DAP were two-fold serially diluted and mixed with 10^4 plaque-forming units (PFU) of influenza A virus. The mixtures were incubated at 37 °C for 30 min before being applied to MDCK cell monolayers grown in 6-well plates. The cells were incubated at RT for 45 min for virus adsorption, then were washed twice with PBS. An overlay medium consisting of 1% low-melting agarose (Lonza, Rockland, ME, USA) and 2.5% trypsin (Gibco) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) was added to the wells. After solidification of the overlay media, the plates were incubated for 2–3 days until plaque formation. Plaques were fixed with 4% formaldehyde, stained with crystal violet, and counted for viral titration.

Neuraminidase Inhibition (NI) activity of DHF and DAP

The NA enzyme activity of the influenza virus was evaluated using an enzyme-linked lectin assay, which detects the release of sialic acid from fetuin by viral NA. 96-well plates were coated with 100 μL/well of 50 μg/mL fetuin (Sigma-Aldrich) and incubated at 4 °C for 24 h to allow protein binding. Serially diluted influenza virus samples were then added to the fetuin-coated wells and incubated at 37 °C for 1 h, enabling the viral NA to cleave sialic acid from fetuin. After washing, 100 μL of peroxidase-conjugated lectin (Sigma-Aldrich), which specifically binds to exposed galactose residues generated by NA activity, was added and incubated at RT for 1 h. The wells were washed again before adding 100 μL of TMB substrate solution (Thermo Scientific), which reacts with the peroxidase enzyme to produce a colorimetric signal. After 5 min, the reaction was stopped by adding 50 μL of 2 N sulfuric acid, and absorbance was measured at 450 nm. To assess NI activity, two-fold serially diluted DHF, DAP, and OP were pre-incubated with A/PR8 (H1N1) virus (OD_{450} = 1) at 37 °C for 2 h before being transferred to

the fetuin-coated plates. NA enzymatic activity was then measured using the same protocol as described above. A reduction in the absorbance indicated inhibition of NA activity due to reduced sialic acid cleavage and subsequent lectin binding.

Fluorescence analysis using A/PR8-GFP reporter virus

To evaluate the impact of the compounds on the replication dynamics of the influenza virus, the A/PR8-GFP reporter virus, which harbors the GFP gene within the NS segment, was employed. MDCK cells seeded in 96-well plates were infected with 100 PFU of A/PR8-GFP per well in the presence of the compounds. At 24 h post-infection, the cells were washed with DPBS and subsequently stained with Hoechst 33,342 for 15 min to visualize the nuclei of the cells. GFP expression was then analyzed using a Cytation 1 fluorescence microscope (BioTek, Houston, TX, USA) to assess viral replication.

Statistical analysis

All experiments were performed at least three times, and results were expressed as mean ± standard deviation (SD). Statistical significance between experimental groups was analyzed using *Student's t-test* (one-tailed analysis) in GraphPad Prism 9.0 software. Differences were considered statistically significant at $P < 0.05$, with significance levels indicated as follows: ***; $P < 0.001$, **; for $P < 0.01$, and *; for $P < 0.05$. Nonlinear regression analysis was carried out using GraphPad Prism 9.0 software.

Results

In vitro cytotoxicity of DHF and DAP

We evaluated the antiviral activity of DHF and DAP against influenza A viruses. DHF and DAP belong to a flavone and coumarin, respectively, that are both naturally occurring compounds in many plants (Fig. 1a). To assess the in vitro cytotoxicity of DHF and DAP, MDCK cells were treated with two-fold serial dilutions of the compounds for 24–48 h at 37 °C, and cell viability was determined using the MTT assay. DHF treatment to cells for 24 h did not show observable cell cytotoxicity at concentrations of 12.5–200 μM compared to the DMSO-treated control (Fig. 1b). Treatment of DHF to cells for 48 h demonstrated cell viability of 12.6% and 45.5% at 200 μM and 100 μM, respectively, showing a cell viability less than 80% compared to the DMSO-treated control (Fig. 1c). However, treatment of DHF to cells for 48 h at concentrations of 50 μM and less did not display cytotoxicity. Similar to DHF, treatment of DAP to cells for 24 h did not demonstrate cytotoxicity at concentrations of 12.5–200 μM (Fig. 1d). Treatment of DAP to cells for 48 h yielded cell viability of 42.5% but not cytotoxic at the concentrations of 100 μM and less (Fig. 1e). In all subsequent experiments, DHF and DAP were treated to cells at non-cytotoxic concentrations determined in the in vitro cytotoxicity tests.

Antioxidative activity of DHF and DAP

Plant-derived polyphenols are well-known for their antioxidative properties, which is also associated with antiviral effects [28, 29]. To assess the antioxidative activity of the compounds, DPPH radical-scavenging activity of DHF and DAP were measured. The DPPH radical-scavenging activity of DHF ranged from 5.9 to 51.6% at concentrations between 6.3 μM and 200 μM (Fig. 2a). On the other hand, DAP exhibited DPPH radical-scavenging activity ranging from 10.1 to 59.0% at the same concentrations (Fig. 2b), demonstrating slightly higher activity than DHF. L-ascorbic acid and gallic acid were used as positive controls in the assay. L-ascorbic acid exhibited DPPH radical-scavenging activity of 3.5–40.1% at concentrations between 6.3 and 200 μM (Fig. 2c). Gallic acid demonstrated DPPH radical-scavenging activity of 10.9–53.8% at the same concentrations (Fig. 2d). The results suggest that DHF and DAP have comparable antioxidative activities to well-known antioxidants such as L-ascorbic acid and gallic acid.

DHF and DAP exert virucidal activity against influenza A virus via an HA-independent mechanism

We next evaluated the potential of DHF and DAP to inactivate influenza A virus. To assess the virucidal effects of the compounds, 10^4 PFU of A/MA81 (H5N2) virus were incubated with varying concentrations of the compounds for 2 h at 37 °C. Following incubation, residual viral titers were quantified using a viral plaque assay (Fig. 3a). Treatment of DMSO to the virus resulted in the infectious viral titers of 6.9×10^3 PFU/mL, causing minimal reduction in the viral titers (Fig. 3b). In contrast, treatment of DHF to the virus completely inactivated the virus at 50–200 μM, with no detectable viral plaques. Treatment of 25 μM of DHF resulted in the viral titers of 5×10^2 PFU/mL, which corresponds to 92% reduction in viral titers compared to the DMSO-treated control, suggesting still robust virucidal activity. Treatment of DAP to the virus resulted in complete virucidal activity at 100–200 μM, producing no detectable viral plaques (Fig. 3c). Treatment of DAP to the virus led to significant decrease in viral titers by 94.3% and 65.6% at 50 μM and 25 μM, respectively, compared to the DMSO control. The results show that DHF and DAP are able to eliminate the viral infectivity when treated to the virus for 2 h at 37 °C, showing potent virucidal activity. One of the key mechanisms for inactivating the influenza virus is the inhibition of binding of influenza viral HA to cellular receptors, termed as HI activity. To test whether DHF and DAP exert HI activity, HI assay was performed using cRBC. As seen in HI assay results, DHF or DAP did not show HI activity when the

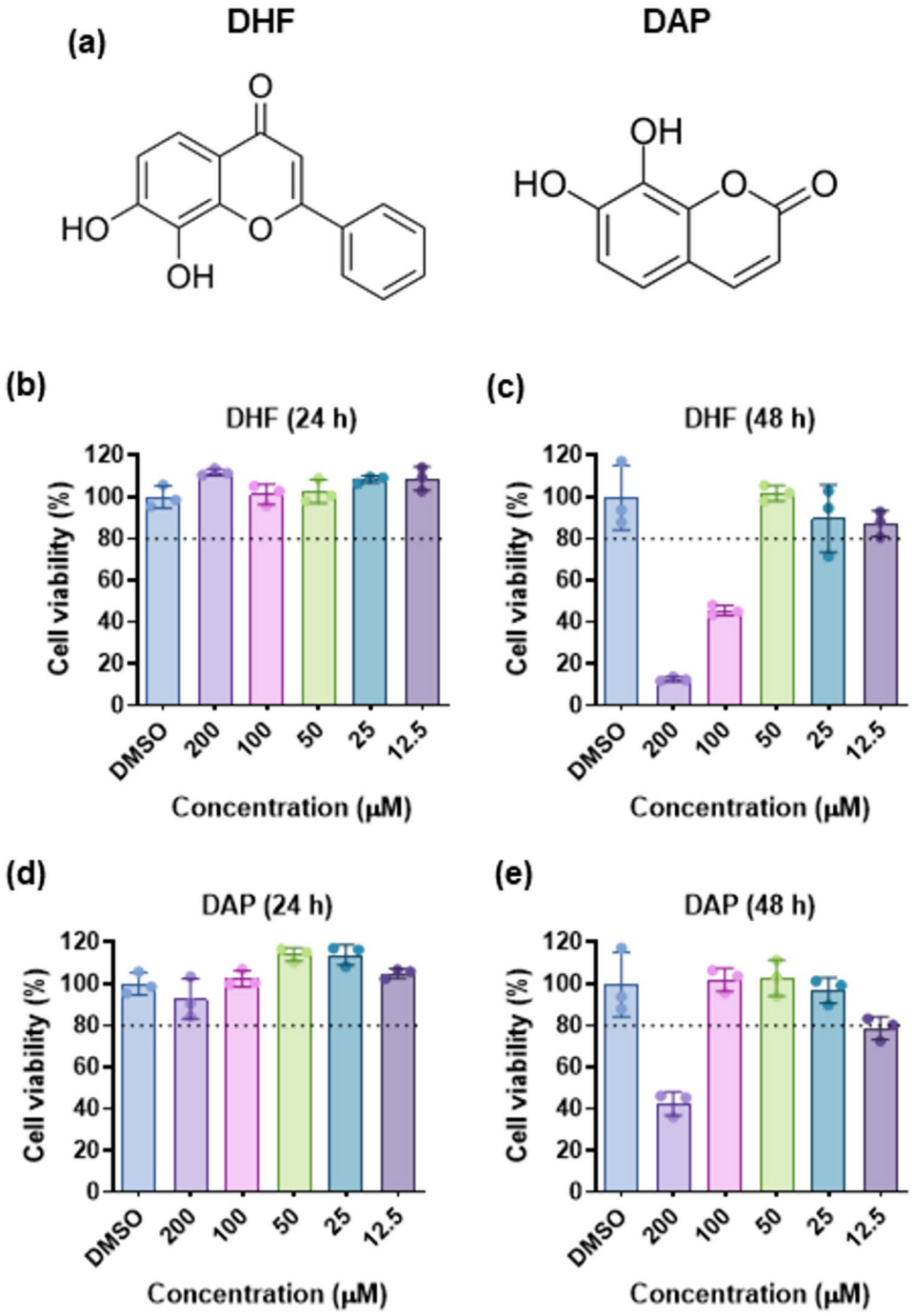

Fig. 1 In vitro cytotoxicity of DHF and DAP in MDCK cells. **a** Chemical structure of DHF and DAP. **b** and **c** Cell viability (%) of MDCK cells treated with DHF. MDCK cells grown in 96-well plates were treated with DHF at concentrations ranging from 12.5–200 μM for 24 h (**b**) or 48 h (**c**). **d** and **e** Cell viability (%) of MDCK cells treated with DAP for 24 h (**d**) or 48 h (**e**). Dashed lines indicate the cell viability of 80%

compounds were treated to A/MA81 (H5N2) or A/PR8 (H1N1) viruses even at the highest concentration of 200 μM (Fig. 3d and e). The results indicate that virucidal activity of DHF and DAP operates independently of HI activity.

NI activity of DHF and DAP against influenza A viruses

We next examined whether DHF and DAP inhibit the enzymatic function of influenza viral NA. At the stage of the viral release from the infected cells, influenza NA protein cleaves the sialic acid residue bound to viral HA, thus facilitating viral release. Two influenza A virus strains, A/PR8 (H1N1) and A/MA81 (H5N21) viruses,

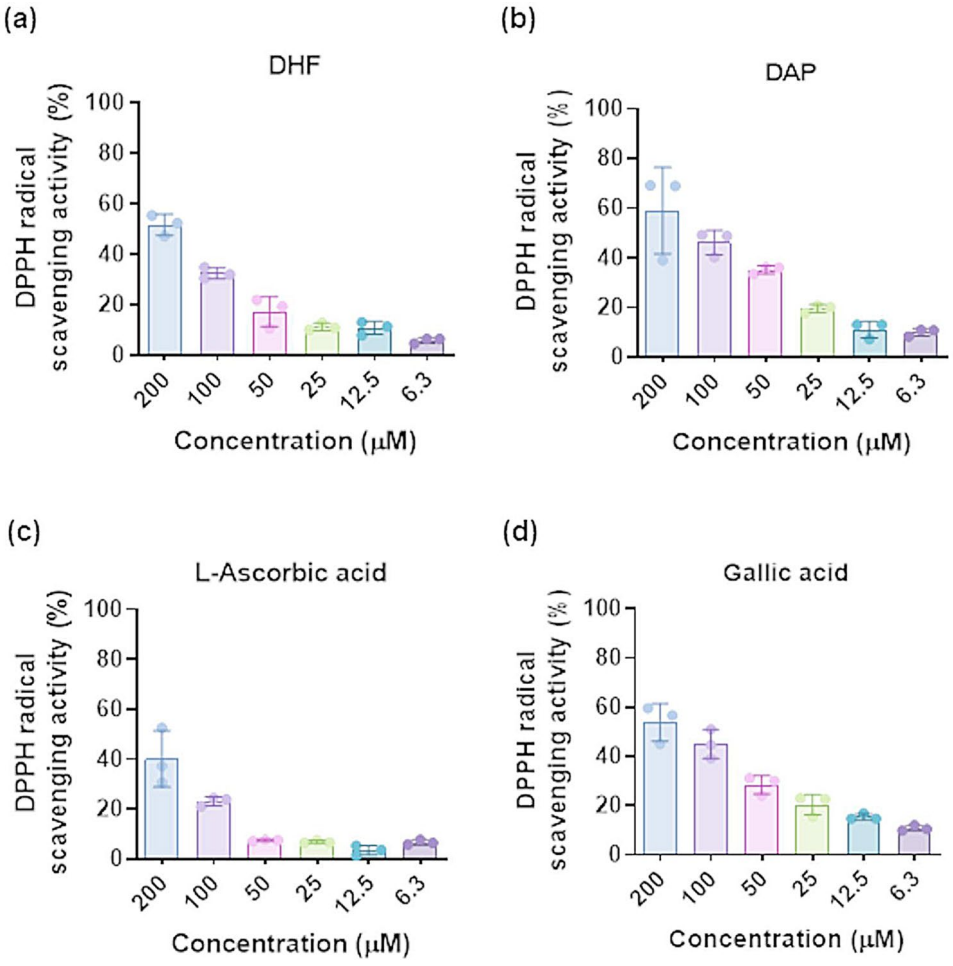

Fig. 2 Antioxidative activity of DHF and DAP. **a–d** Antioxidant activity of DHF (**a**), DAP (**b**), and positive control compounds, L-ascorbic acid (**c**) and gallic acid (**d**). Antioxidant activity of the compounds was evaluated using the DPPH radical-scavenging assay. Two-fold serial dilutions of the compounds were mixed with 100 μM DPPH reagent and incubated for 30 min. Absorbance was measured at 514 nm

were treated with two-fold serial dilutions of DHF and DAP at 37 °C for 2 h, and NI assay was performed. Against A/PR8 (H1N1) virus, DHF demonstrated NI activity ranging from 6.8 to 57.4% at concentrations of 6.3 μM to 200 μM, with an IC_{50} value of 116.8 μM (Fig. 4a). Against A/MA81 (H5N2) virus, DHF displayed NI activity ranging from 15.5 to 39.4%, at the same concentrations (Fig. 4b), showing relatively weaker NI activity than against A/PR8 (H1N1) virus. Against A/PR8 (H1N1) virus, DAP demonstrated NI activity ranging from 11.9 to 85.0% at the concentrations of 6.3 μM to 200 μM, with an IC_{50} value of 77.5 μM (Fig. 4c). Against A/MA81 (H5N2) virus, DAP showed NI activity ranging from 18.8 to 48.7% at the same concentrations (Fig. 4d). For comparison, NI activity of OP, a well-known NA inhibitor, was also measured against the two viruses. OP demonstrated potent NI activity, with IC_{50} values of 79.1 nM and 28.7 nM against A/PR8 (H1N1) and A/MA81 (H5N2) viruses, respectively (Fig. 4e and f). In summary, DAP showed higher NI activity than DHF, and the two compounds showed higher NI activity against A/PR8 (H1N1) virus than against A/MA81 (H5N2) virus, demonstrating varying degree of NI activity depending on the viral subtype.

Post-treatment of DHF and DAP suppresses influenza A virus replication in cells

To assess whether virucidal and NI activities of DHF and DAP translate into the inhibition of influenza virus replication in infected cells, we used the A/PR8-GFP reporter virus, which encodes GFP in the NS segment and expresses GFP in virus-infected cells, allowing for monitoring of viral replication through fluorescence analysis. MDCK cells grown in 96-well plates were infected with 8 MOI of A/PR8-GFP virus, followed by treatment with DHF and DAP. 9 h later, the cells were subjected to quantitative fluorescence imaging and intensity analysis. As shown in the fluorescence images, GFP expression was observed in cells infected with A/PR8-GFP virus, while uninfected cells did not express GFP (Fig. 5a). In the DMSO-treated control group, 40.9% of

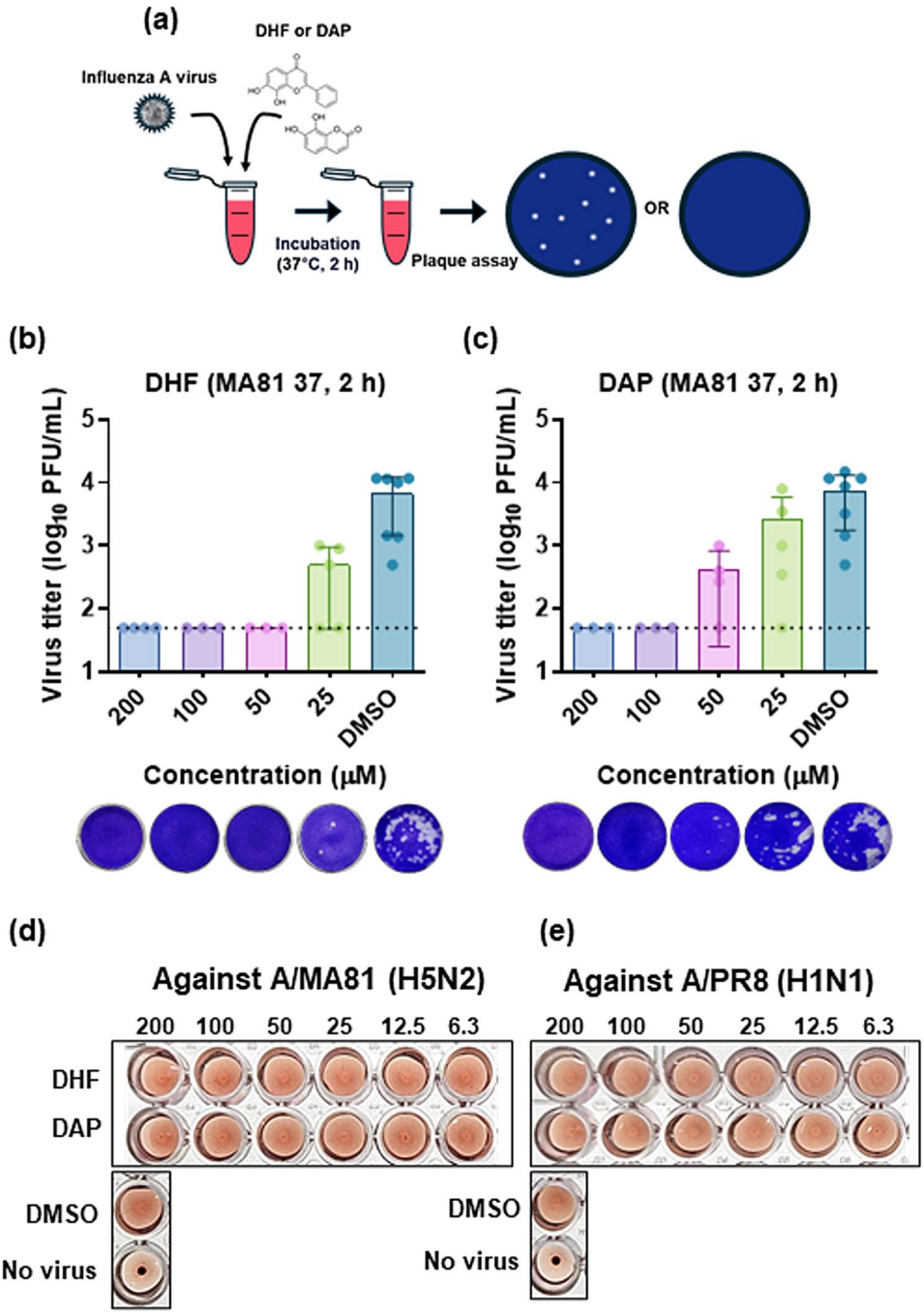

Fig. 3 Virucidal activity of DHF and DAP against influenza A virus. **a** Schematic representation of the virucidal assay, where 10^4 PFU of A/MA81 (H5N2) virus was incubated with DHF or DAP for 2 h at 37 °C, followed by viral titration via plaque assay. **b** and **c** Residual viral titers after treatment of DHF (**b**) or DAP (**c**) at various concentrations to the virus. Representative images of plaque assay at a 10^{-1} dilution are shown below at each indicated concentration. DMSO was used as a vehicle control. Dashed lines indicate the detection limit, 1.677. **d** and **e** HI assay results. 4 HAU of influenza A viruses was treated with various concentrations of DHF or DAP for 2 h at 37 °C, and the mixtures were subjected to HI assay. HI results using A/MA81 (H5N2) (**d**) and A/PR8 (H1N1) viruses (**e**) are shown. DMSO was used as a vehicle control

the cells expressed GFP, whereas treatment with DHF reduced the percentage of GFP-expressing cells to 30.7% and 19.6% at 25 μM and 50 μM, respectively (Fig. 5b), suggesting that DHF treatment suppressed viral replication in infected cells. Similarly, DAP treatment also reduced GFP expression compared to the DMSO-treated control. At concentrations of 50 μM and 100 μM, DAP treatment resulted in 35.1% and 34.4% of GFP-expressing cells, respectively (Fig. 5b), indicating a reduction in viral replication. The GFP intensity analysis followed a similar

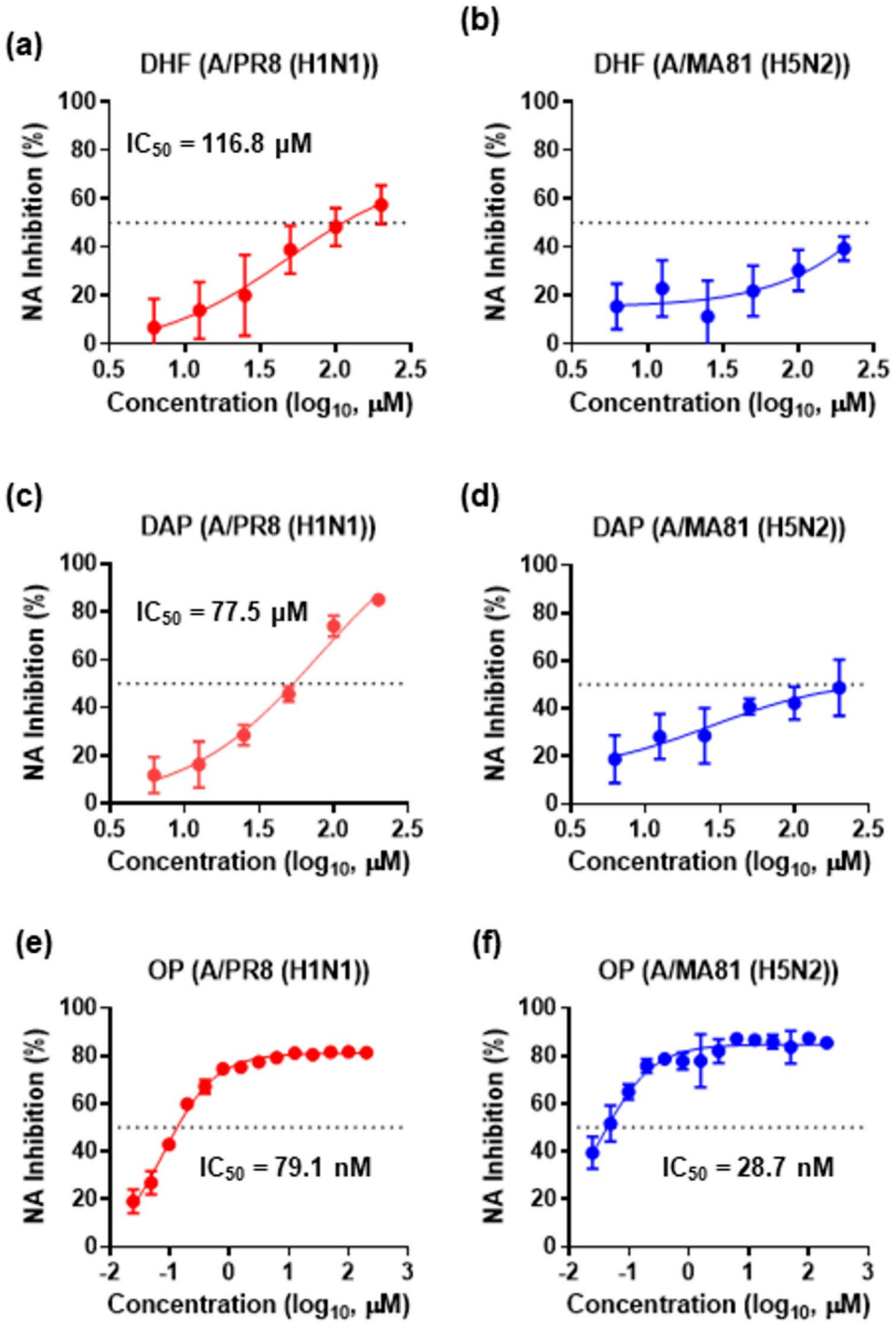

Fig. 4 NI activity of DHF and DAP. **a** and **b** NI activity of DHF against influenza A viruses. A predetermined titer of influenza virus (120 µL) corresponding to an OD_{450} of 1 in the NA assay was incubated with two-fold serial dilutions of DHF (120 µL) at 37 °C for 2 h. The mixtures were then transferred to 96-well plates pre-coated with fetuin and further incubated at 37 °C for 1 h. Following incubation, the wells were aspirated, and 100 µL of lectin was added, followed by incubation at RT for 1 h. After removing unbound lectin, 100 µL of TMB substrate was added to each well. The reaction was stopped after 5 min by adding 50 µL of 2 N H_2SO_4, and absorbance was measured at 450 nm. NA inhibition (%) by DHF against A/PR8 (H1N1) virus (**a**) and A/MA81 (H5N2) virus (**b**) are shown. **c** and **d** NI activity of DAP against influenza A viruses. NA inhibition (%) by DAP against A/PR8 (H1N1) (**c**) and A/MA81 (H5N2) viruses (**d**) are shown. **e** and **f** NI activity of OP against influenza A viruses. NA inhibition (%) by OP against A/PR8 (H1N1) (**e**) and A/MA81 (H5N2) viruses (**f**) are shown. Nonlinear regression analysis was performed using GraphPad Prism 9.0 software

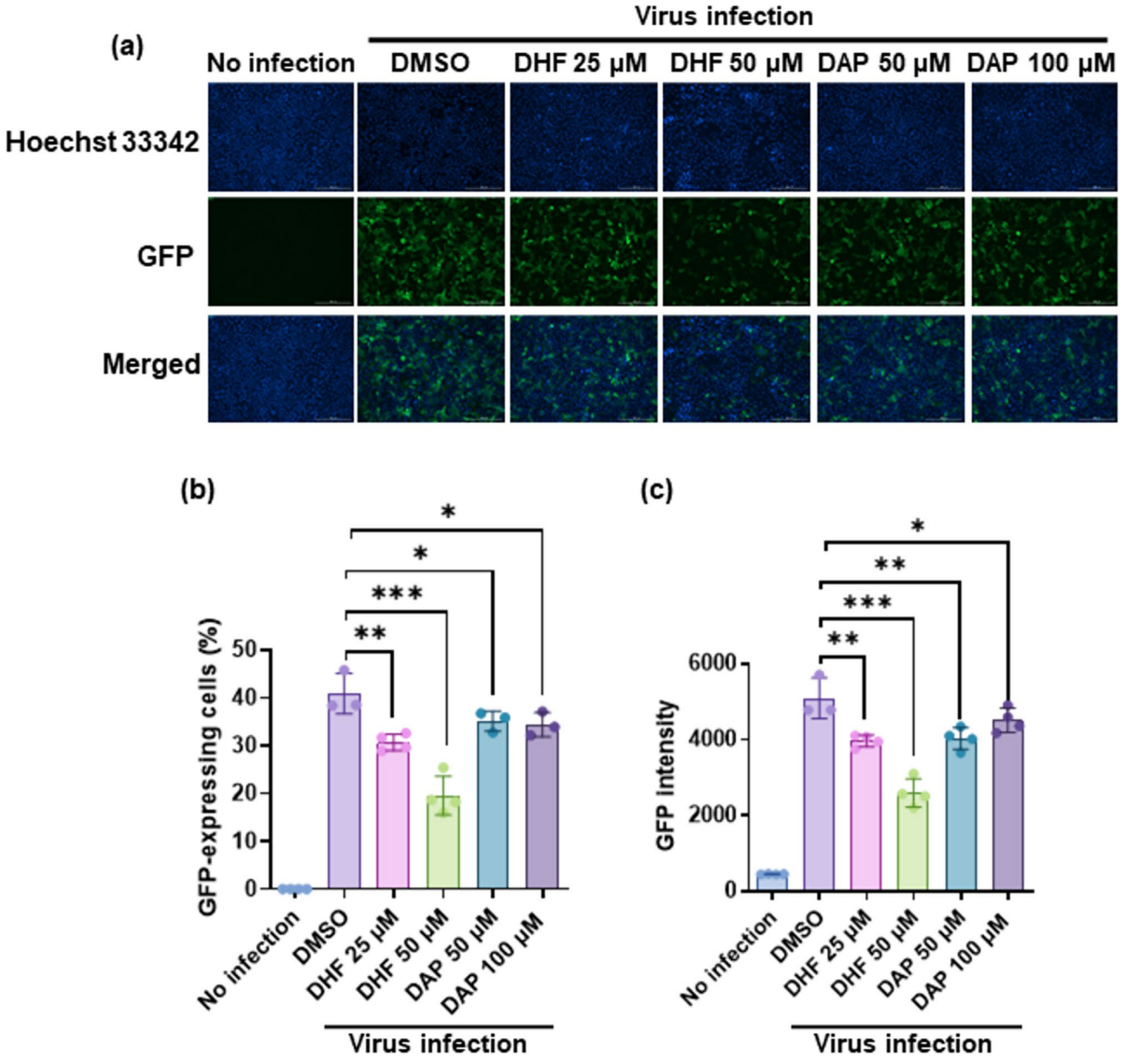

Fig. 5 Suppression of A/PR8-GFP reporter virus replication by DHF and DAP. **a** Fluorescence images of MDCK cells infected with the A/PR8-GFP virus in the presence of DHF or DAP. MDCK cells were infected with 8 MOI of A/PR8-GFP virus and the cells were treated with DHF (25 μM and 50 μM) or DAP (50 μM and 100 μM) for 9 h before obtaining the fluorescence images. Hoechst 33,342 was used to stain nuclei. **b** Quantification of GFP-expressing cells (%) by post-treatment of DHF or DAP. **c** GFP fluorescence intensity quantification by post-treatment of DHF or DAP. DMSO was used as a vehicle control

pattern to the GFP expression results. While the DMSO-treated control yielded a GFP intensity of 5090, DHF treatment resulted in GFP intensities of 3967 and 2592 at 25 μM and 50 μM, respectively (Fig. 5c). The relative GFP intensities by treatment of DHF were 77.9% and 50.1% at 25 μM and 50 μM, respectively compared to the DMSO control. DAP treatment also decreased GFP intensity, resulting in relative fluorescence intensities of 79.1% and 88.5% compared to the control (Fig. 5c). These results together demonstrate that DHF and DAP significantly inhibit influenza virus replication when administered post-infection.

Influenza viral growth kinetics analysis for antiviral activity of DHF and DAP

To quantitatively assess influenza viral growth kinetics, DHF and DAP were administered to cells either before or after viral infection, and viral titers in the culture media were measured at various time points using a plaque assay (Fig. 6a). In the DMSO-treated cells, A/PR8 (H1N1) virus replicated rapidly, reaching approximately 4.8×10^6

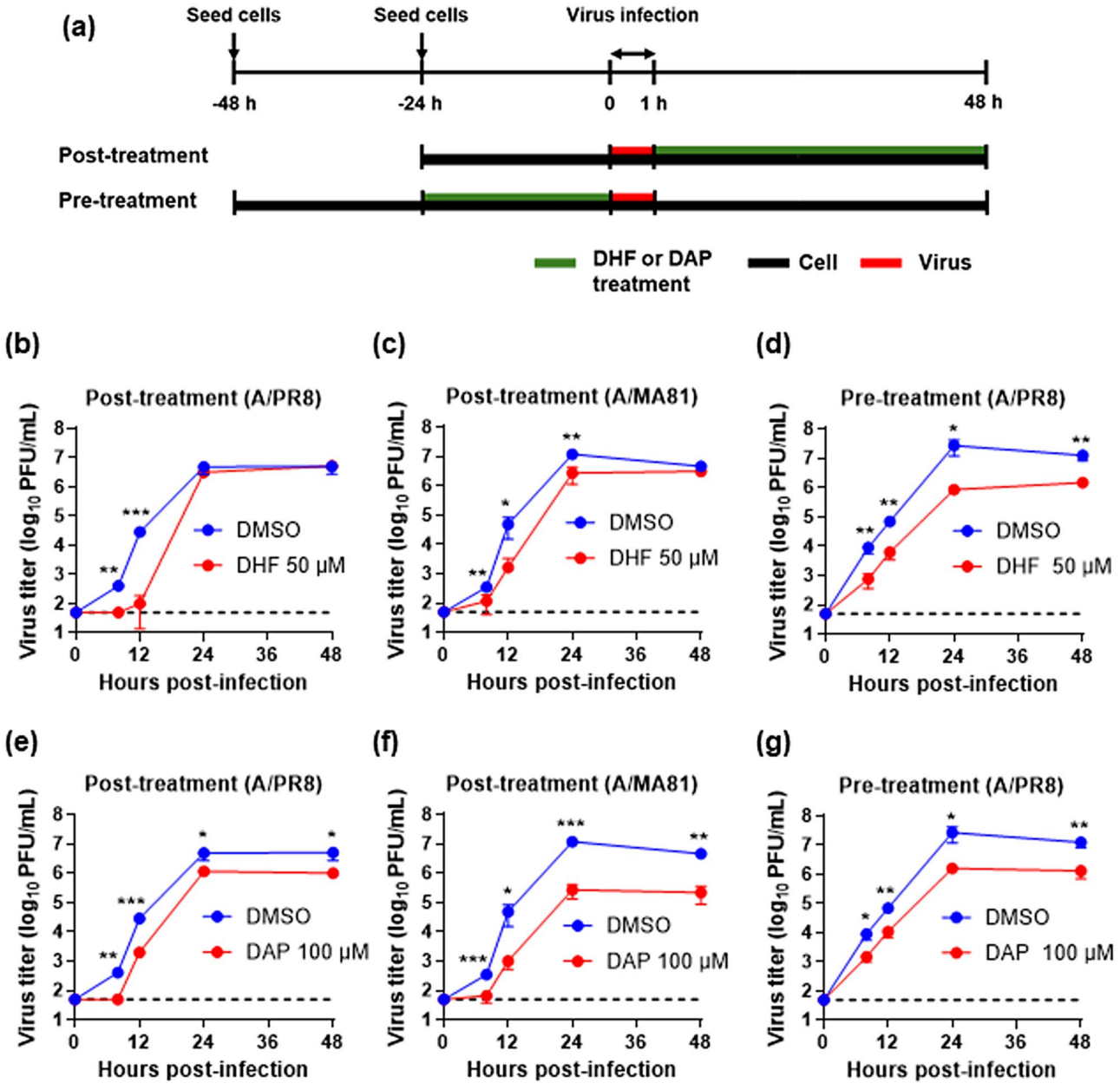

Fig. 6 Inhibitory effects of DHF and DAP on influenza virus growth kinetics. **a** Schematic representation of post-treatment and pre-treatment experiments. **b** and **c** Antiviral effects of DHF post-treatment to cells on influenza A virus growth kinetics. MDCK cells were infected with 0.01 MOI of influenza A viruses and the cells were then treated with 50 μM DHF. The viral titers of A/PR8 (H1N1) (**b**) and A/MA81 (H5N2) viruses (**c**) in the supernatants collected at different time-points were measured by plaque assay. **d** Antiviral effects of DHF pre-treatment to cells on influenza A virus growth kinetics. MDCK cells were treated with 50 μM DHF for 24 h, and the cells were infected with 0.01 MOI of A/PR8 (H1N1) virus. **e** and **f** Antiviral effects of 100 μM DAP post-treatment to cells on influenza A virus growth kinetics. The viral titers of A/PR8 (H1N1) (**e**) and A/MA81 (H5N2) viruses (**f**) in the supernatants collected at different time-points were measured by plaque assay. **g** Antiviral effects of 100 μM DAP pre-treatment to cells on influenza A virus growth kinetics. MDCK cells were treated with 100 μM DAP for 24 h, and the cells were infected with 0.01 MOI of A/PR8 (H1N1) virus. DMSO was used as a vehicle control. Dashed lines indicate the detection limit, 1.677

PFU/mL at 24 h post-infection (hpi), before plateauing at 48 hpi (Fig. 6b). In contrast, post-treatment with 50 μM DHF to A/PR8 (H1N1) virus-infected cells significantly reduced viral titers to 10^2 PFU/mL at 12 hpi, representing a 287.5-fold reduction compared to the DMSO control at the same time point (Fig. 6b). Similarly, in A/MA81 (H5N2) virus-infected cells, post-treatment with 50 μM DHF resulted in approximately 3-fold, 30-fold, and 4-fold reductions in viral titers at 8, 12, and 24 hpi, respectively (Fig. 6c). These findings suggest that DHF effectively inhibits viral replication, particularly during the early stages of infection. To further investigate its antiviral

activity, cells were pre-treated with 50 μM DHF for 24 h to allow cellular uptake, followed by washing and subsequent infection with A/PR8 (H1N1) virus. Viral titers in the culture media were then measured using a plaque assay. Pre-treatment with DHF resulted in 12-fold, 11-fold, 3-fold, and 8-fold reductions in viral titers at 8, 12, 24, and 48 hpi, respectively, compared to the control, demonstrating a significant and sustained suppression of viral replication across all time points (Fig. 6d). These results indicate that DHF can effectively inhibit viral replication even when administered prior to infection. Similarly, DAP exhibited antiviral effects under both pre- and post-treatment conditions. Post-treatment with 100 μM DAP to A/PR8 (H1N1) virus-infected cells led to 8-fold, 14-fold, 4-fold, and 5-fold reductions in viral titers at 8, 12, 24, and 48 hpi, respectively, compared to the control (Fig. 6e). In A/MA81 (H5N2) virus-infected cells, post-treatment with 100 μM DAP resulted in 5-fold, 48-fold, 44-fold, and 20-fold reductions in viral titers at 8, 12, 24, and 48 hpi, respectively (Fig. 6f). Furthermore, pre-treatment with 100 μM DAP significantly reduced viral titers at all time points compared to the control (Fig. 6g). These findings indicate that DAP effectively suppresses viral replication, regardless of whether it is administered before or after viral infection.

Discussion

Influenza virus infection remains a major global health threat, necessitating the development of novel antiviral agents with broad efficacy and minimal cytotoxicity. In this study, we investigated the antiviral potential of two naturally derived compounds, DHF and DAP, against influenza A viruses. Our findings demonstrate that both DHF and DAP exert significant antiviral activity through multiple mechanisms, including direct virucidal effect, NA inhibition, and suppression of viral replication in infected cells. These results highlight the therapeutic potential of these compounds as promising candidates for influenza treatment.

Before assessing the antiviral activity of DHF and DAP, in vitro cytotoxicity of the compounds was evaluated in MDCK cells using the MTT assay. Both compounds displayed minimal cytotoxicity at concentrations below 50 μM (DHF) and 100 μM (DAP) after 48 h of treatment, indicating that their antiviral effects are unlikely to be due to cellular toxicity. Given the established role of antioxidant properties in the antiviral activity of natural polyphenols, the antioxidant potential of DHF and DAP was examined using the DPPH radical-scavenging assay. Both compounds demonstrated significant radical-scavenging activity, consistent with previous reports [30, 31], with DAP showing slightly higher activity than DHF across the tested concentrations. These findings imply that the antioxidant capacity of DHF and DAP may contribute to their antiviral efficacy. This is supported by previous studies on epigallocatechin gallate (EGCG), a well-known polyphenol in green tea, which exhibits antiviral activity through multiple mechanisms. In addition to its strong antioxidant properties, EGCG can form reactive quinone intermediates upon oxidation, which covalently bind to nucleophilic residues on viral proteins. This cross-linking can disrupt the structural and functional integrity of viral proteins, ultimately inhibiting viral entry and replication [32, 33]. These observations suggest a mechanistic link between the antioxidant activity and virucidal effects of polyphenolic compounds such as DHF and DAP.

One of the key findings of this study is the potent virucidal activity of DHF and DAP against influenza A virus. When the virus was pre-incubated with DHF or DAP for 2 h at 37 °C, a significant reduction in the viral infectivity was observed. Treatment with 25 μM DHF resulted in a 92% reduction in the viral titers, while complete inactivation was achieved at 50–200 μM. Similarly, DAP exhibited dose-dependent virucidal activity, with 100–200 μM completely inactivating the virus. These results suggest that both compounds can directly neutralize influenza A virus, rendering it non-infectious before cellular entry. To determine whether DHF and DAP interfere with the viral HA receptor binding, a HI assay was performed. Interestingly, neither compound exhibited HI activity, even at the highest tested concentration of 200 μM. This suggests that the virucidal effects of DHF and DAP are independent of HA receptor binding inhibition. Instead, the mechanism of viral inactivation may involve direct disruption of the viral envelope or interaction with viral proteins essential for infectivity. This has been demonstrated by plant-derived ethyl pheophorbides and curcumin, which exhibit antiviral activity against influenza viruses without displaying HI activity [34, 35]. Further investigations are warranted to elucidate the exact mechanism of virucidal activity of DHF and DAP.

In addition to virucidal effects, DHF and DAP were evaluated for their ability to inhibit influenza NA, a key enzyme required for viral release from infected cells. Both compounds demonstrated dose-dependent NI activity, with DAP exhibiting greater inhibitory potency than DHF. Against A/PR8 (H1N1) virus, DAP achieved an IC_{50} of 77.5 μM, whereas DHF had a higher IC_{50} of 116.8 μM, indicating that DAP is a more potent NA inhibitor than DHF. The compounds were less effective against A/MA81 (H5N2) virus, suggesting strain-specific differences in NA susceptibility. The ability of DHF and DAP to inhibit NA function suggests that their antiviral effects extend beyond direct virucidal activity. NA inhibitors, such as oseltamivir and zanamivir, are widely used as first-line treatments for influenza [36], but increasing drug resistance has necessitated the search for alternative inhibitors [37]. Although the NI activity of DHF

and DAP was shown to be lower than that of OP, their observed NA inhibitory effects position them as potential candidates for further structure-based optimization to enhance their inhibitory potency.

Given their virucidal and NA inhibitory effects, we next examined whether DHF and DAP could suppress viral replication in infected cells. Using the A/PR8-GFP reporter virus, we monitored GFP expression as an indicator of viral replication. Post-treatment with 50 μM DHF resulted in a 52% reduction in GFP-expressing cells compared to the DMSO control, demonstrating a significant inhibitory effect on viral replication. DAP also reduced viral replication, albeit to a lesser extent than DHF at equivalent concentrations. Quantitative fluorescence intensity analysis further confirmed that DHF was more effective than DAP in suppressing viral replication at the tested concentrations.

A detailed growth kinetics analysis further supported the antiviral potential of DHF and DAP. Post-treatment with 50 μM DHF to A/PR8 (H1N1)-infected cells resulted in a significant reduction in viral titers at 12 hpi, with continued suppression through 48 hpi. Similarly, against A/MA81 (H5N2) virus, post-treatment with DHF led to a 30-fold reduction in viral titers at 12 hpi, indicating potent early-stage inhibition of viral replication. Pre-treatment studies revealed that DHF also exhibited prophylactic antiviral activity, significantly reducing viral titers across all time points when administered 24 h prior to infection. This suggests that DHF may enhance the cellular antiviral response, possibly by modulating host defense mechanisms or interfering with early viral entry steps. Likewise, DAP demonstrated antiviral activity in both pre- and post-treatment conditions, with persistent reductions in viral titers for A/PR8 (H1N1) and A/MA81 (H5N2). These findings highlight the broad-spectrum antiviral efficacy of DHF and DAP against different influenza A subtypes.

In summary, our study provides compelling evidence that DHF and DAP exhibit multifaceted antiviral activity against influenza A virus through direct virucidal action, neuraminidase inhibition, and suppression of viral replication in infected cells. Importantly, both compounds demonstrated low cytotoxicity at their effective antiviral concentrations, making them promising candidates for further development. Overall, our findings suggest that DHF and DAP represent promising natural antiviral agents with the potential to contribute to the development of new influenza therapeutics. Further research and development are warranted to explore their full clinical potential.

Abbreviations

DHF	7,8-dihydroxyflavone
DAP	Daphnetin
OP	Oseltamivir phosphate
HA	Hemagglutinin
NA	Neuraminidase
IC_{50}	Inhibitory concentration 50
GFP	Green fluorescence protein
ACE2	Angiotensin-converting enzyme 2
MDCK	Madin-Darby canine kidney
MEM	Minimum Essential Medium
A/PR8	A/Puerto Rico/8/34
A/MA81	Mouse-adapted A/aquatic bird/Korea/w81/05
MOI	Multiplicity of infection
RT	Room temperature
DPPH	2,2-diphenyl-1-picrylhydrazyl
HI assay	Hemagglutination inhibition assay
HAU	Hemagglutination units
cRBC	Chicken red blood cells
PFU	Plaque-forming units
DMEM	Dulbecco's Modified Eagle Medium
NI assay	Neuraminidase inhibition assay
hpi	Hours post-infection

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Author contributions
SK and JK performed the experiments. SL and YJ wrote the manuscript. YJ conceived the study. All authors approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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