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RESEARCH ARTICLE

Inhibitory Activity of Honeysuckle Extracts against Influenza A Virus *In Vitro* and *In Vivo*

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Abstract

Honeysuckle has been used in the treatment of influenza virus infection for thousands of years in China. However, its main active components and the functional mechanisms remain to be elucidated. Here, four honeysuckle extracts, including acids extract, flavonoids extract, total extract and acids-flavonoids mixture, were prepared to clarify the main active antiviral components. The cytopathic effect reduction assay showed that all the four extracts inhibited the replication of influenza viruses H1N1, H3N2 and the oseltamivir-resistant mutant strain H1N1-H275Y. The acids-flavonoids mixture had the strongest inhibitory effects *in vitro* with EC_{50} values of 3.8, 4.1, and > 20 μg/mL against H1N1, H3N2 and H1N1-H275Y, respectively, showing competitive antiviral activity with oseltamivir and ribavirin. Honeysuckle acids extract also showed the most significant antiviral activity *in vivo*. Oral administration of the acids extract at a dosage of 600 mg/kg/d effectively alleviated viral pneumonia, maintained body weight and improved the survival rate to 30% of the mice infected with a lethal dose of H1N1. The results of time-of-drug addition experiment and neuraminidase (NA) inhibition assay showed that honeysuckle extracts had a broad-spectrum inhibitory effect against influenza virus NAs. The flavonoid extract showed the strongest inhibitory effect on the NA of influenza virus H7N9 with an IC_{50} of 24.7 μg/mL. These results suggested that these extracts might exert their antiviral activity by suppressing the release of influenza viruses. Briefly, our findings demonstrate that acids and flavonoids extracts of honeysuckle are the major antiviral active components, and the acids extract has the potential to be developed into an antiviral agent against influenza virus, especially for oseltamivir-resistant viruses.

Keywords Honeysuckle extract · Antiviral activity · Influenza A virus (IAV) · Oseltamivir-resistant

Introduction

Influenza A virus is a considerable threat to human health and life, and can cause seasonal and widespread epidemics or pandemics with high levels of morbidity and mortality (Taubenberger and Kash 2010; Zhang *et al.* 2012). The circulating strains of seasonal Influenza viruses A/FM/1/47 (H1N1) and A/Beijing/32/92 (H3N2) are the primary causes of pandemic infections. In addition, the highly pathogenic avian A/Vietnam/1203/2004 (H5N1) virus and the recently reported A/Anhui/1/2013 (H7N9) virus have shown potential to cause severe influenza pandemics, with estimated mortality rates of 60% and 30%, respectively (Chen *et al.* 2004; Gao *et al.* 2013; Zhang *et al.* 2013). Vaccination may be helpful in controlling influenza infection. However, vaccine shortcomings, such as time lag, limited efficacy, side effects and storage difficulty,

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have restricted their use (Levine and Sztein 2004; Cinatl *et al.* 2007; Hampson 2008; Rohrs *et al.* 2014). Antiviral drugs are the most effective option for treating influenza infection.

Currently, there are three classes of anti-influenza virus drugs with different antiviral mechanisms, including M2 ion channel blockers (amantadine and rimantadine), viral RNA synthesis inhibitors (ribavirin and T-705) and neuraminidase (NA) inhibitors (oseltamivir and zanamivir) (Hay *et al.* 1985; De Clercq 2006; Furuta *et al.* 2013). However, because of the frequent emergence of drug-resistant variants, M2 ion channel blockers are no longer used in clinic (Hayden 2006; Ison 2011; Lee and Yen 2012). In addition, ribavirin causes toxic side effects and has a limited therapeutic effect. More importantly, NA inhibitor-resistant influenza viruses have emerged (Thorlund *et al.* 2011; Leang *et al.* 2013), limiting the future utility of NA inhibitors.

Traditional Chinese medicines have been regarded as sources of therapeutic agents with a long history (Koehn and Carter 2005; Cai *et al.* 2015), and have many advantages, such as multiple target effects, abundant availability and few toxic side effects. Honeysuckle (HS, *Lonicera japonica*) is the dry bud or early flower of *Lonicera japonica*, a well-known Chinese herbal medicine that has been used for the effective treatment of influenza virus infection for thousands of years (Zhou *et al.* 2015). To clarify the main antiviral components of honeysuckle and the underlying mechanisms of their action, we assessed the inhibitory activity of the total extract, organic acids extract, flavonoids extract and acids-flavonoids mixture of honeysuckle against IAVs (H1N1 and H3N2) *in vitro* and *in vivo*. We also investigated their inhibitory effects of these honeysuckle compounds on the oseltamivir-resistant strain H1N1-H275Y. Moreover, we studied the inhibitory effect of these extracts at different stages of the influenza virus life cycle and sought to determine their potential targets. Our findings demonstrate the potential utility of honeysuckle acids extract as an alternative antiviral agent for attenuating influenza virus, especially the oseltamivir-resistant mutant strain.

Materials and Methods

Cells and Virus

Madin-Darby canine kidney cells (MDCK) and human cervical cancer cells (HeLa) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDCK and HeLa cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) and RPMI-1640 medium supplemented with 10% (V/V) fetal bovine serum (FBS) respectively, and were maintained in an atmosphere containing 5% CO_2 at 37 °C.

Influenza viruses A/FM/1/47 (H1N1), A/Beijing/32/92 (H3N2), H1N1 oseltamivir-resistant mutant strain possessing a histidine-to-tyrosine substitution at position 275 (H275Y) of NA were conserved at the Department of Microbiology, School of Life Science and Technology, China Pharmaceutical University. Influenza A viruses were propagated in 10-day-old embryonated chicken eggs. All these viruses were stored at − 80 °C (Jung *et al.* 2010).

Reagents

Oseltamivir carboxylate (OC) were purchased from MedChemExpress (Shanghai, China). Ribavirin (RBV) was obtained from Sichuan Baili Pharmaceutical Co., Ltd. (Chengdu, China). Both of them were dissolved in double-distilled water to a 10 mg/mL concentration for use as stock solutions. 2′-(4-methyl-umbelliferyl)-α-D-N-acetyl-neuraminic acid sodium salt hydrate (MUNANA), 2-(N-morpholino) ethanesulfonic acid (MES) and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co., St. (Louis, MO, USA). DMEM, RPMI-1640 and FBS were purchased from Gibco Co. (NY, USA).

Sample Preparations

Honeysuckle was provided by Anhui Qimen Huangshan Yaogu Science and Technology Co., Ltd. The four honeysuckle extracts were prepared at the Department of Pharmaceutics, China Pharmaceutical University.

The Honeysuckle Flavonoids Extract

100 g honeysuckle was added with 1200 mL 70% ethanol and then refluxed at 85 °C for 2 h. After filtration, 900 mL 70% ethanol was added to the residue and refluxed for 2 h. After the second filtration, the filtrates from the two refluxed-steps were combined and evaporated under reduced pressure. The obtained extractum (150 mL) was mixed with petroleum ether (3–4 times the volume of obtained extractum), and the petroleum ether layer was discarded. The resultant concentrated solution was mixed with ethyl acetate (3–4 times the volume of the concentrated solution) and the ethyl acetate layer was collected. The ethyl acetate extraction step repeated twice. The ethyl acetate layers were combined and evaporated to dryness under reduced pressure.

The Honeysuckle Acids Extract

The water layer from the initial flavonoids extraction step was adjusted to pH 2 with 1 mol/L dilute hydrochloric acid. The acids extract was obtained through precipitation with ethyl acetate (4 times volume) twice. Then the extract was mixed and evaporated to dryness under reduced pressure.

The Honeysuckle Acids-Flavonoids Combination

The acids-flavonoids mixture was prepared by mixing 0.85 g acids extract and 0.75 g flavonoids extract.

The Total Extract Liquid of Honeysuckle

Crushed honeysuckle was mixed with 10 times volume of 60% ethanol and refluxed in water bath at 40 °C for 1.5 h. After filtration, the filtrate was collected. The same procedure was repeated twice with the remaining residue. The overall filtrates were mixed and kept at 4 °C for 24 h. The filtrates were evaporated under reduced pressure, and then dissolved in distilled water and lyophilized to get purified total extract.

After conversion, 1 g flavonoid fraction is equivalent to 33.3 g honeysuckle; 1 g organic acid fraction is equivalent to 28.6 g honeysuckle; 1 g flavonoid-acid mixture is equivalent to 30.8 g honeysuckle; 1 g total extract is equivalent to 3.3 g honeysuckle.

Each honeysuckle extract was dissolved in dimethyl sulfoxide (DMSO) or in 0.5% CMC-Na solution to a 100 mg/mL concentration as stock solution for *in vitro* or *in vivo* studies, respectively.

Cytotoxic Activity of Honeysuckle Extracts on MDCK or HeLa Cells

MDCK or HeLa cells (5×10^3 cells/well) were cultured in 96-well plates for 24 h. Then, the medium was replaced with the maintenance medium (DMEM/RPMI-1640 with 1% FBS) containing honeysuckle extracts at final concentrations of 0, 25, 50, 100, 200 and 400 μg/mL. After incubation for 48 h, the cell viability was measured by MTT assays as described previously (Tian *et al.* 2011). In brief, 10 μL of MTT solution (5 mg/mL) was added to each well. After incubation at 37 °C for 4 h, the MTT solution was replaced with DMSO (150 μL/well). The plate was shaken gently for 10 min to dissolve the precipitate of formazan. Absorbance at 490 nm was determined with a microplate reader. The cell viability was calculated using the following equation: cell viability (%) = (mean optical density of test—mean optical density of background)/(mean optical density of cell controls—mean optical density of background) × 100. The half maximal cytotoxic concentration (CC_{50}) was calculated by regression analysis in GraphPad Prism 5.

$TCID_{50}$ Determination of IAV

MDCK or HeLa cells (5×10^3 cells/well) were grown in 96-well plates for 24 h. The virus stock was serial two fold diluted by serum-free medium. Then, 100 μL of the serial virus dilution was added to each well at 37 °C for 2 h. The cells were incubated in the maintenance medium at 37 °C for 48 h. The cell viability was measured by MTT assay. The $TCID_{50}$ (50% tissue culture infectious dose) was determined using the Reed-Müench method.

Cytopathic Effect (CPE) Reduction Assay

MDCK or HeLa cells (5×10^3 cells/well) were cultured in 96-well plates for 24 h and incubated with 100 $TCID_{50}$ of influenza H1N1, H3N2 and H1N1-H275Y viruses at 37 °C for 2 h. Then, the cells were incubated with the maintenance medium in the presence of various concentrations of honeysuckle extracts (1, 2.5, 5, 10, and 20 μg/mL). 2 μg/mL oseltamivir and ribavirin were used as positive controls and 0.5% CMC-Na solution was used as placebo. After 48 h incubation, the CPE was observed with microscopy, and the viability of cells was measured by MTT assay. The inhibition rate of the test drugs was calculated using the following equation (Ding *et al.* 2014): inhibition rate (%) = (mean optical density of test—mean optical density of virus controls) / (mean optical density of cell controls—mean optical density of virus controls) × 100. The cell control group was MDCK cells treated with DMSO, and the virus control group was MDCK cells only infected with virus (the placebo group). The EC_{50} values were determined using regression analysis, and the selectivity index (SI) was defined as the ratio of CC_{50} to EC_{50}.

Inhibitory Efficacy of Honeysuckle Extracts on Different Stages of Viral Life Cycle

To investigate the inhibitory effect of honeysuckle at different stages of the influenza virus life cycle, a time-of-drug-addition experiment was carried out as previously described (Yu *et al.* 2016). In the first protocol (pre-infection), MDCK cells were pre-incubated with several concentrations of honeysuckle extracts (1, 2.5, 5, 10, and 20 μg/mL) for 12 h before virus infection. In the second protocol (co-infection), virus and honeysuckle extracts were added to MDCK cells simultaneously and incubated for 2 h. In both protocols, the cells were incubated with the maintenance medium for 48 h after virus adsorption. In the third protocol (post-infection), after infected with the

influenza virus for 2 h, the maintenance medium containing honeysuckle extracts was added to MDCK cells. Cell viability was determined by MTT assay.

Therapeutic Efficacy Study in Mice

The 18–22 g six-week-old male ICR mice were obtained from Nantong University (Nantong, Jiangsu, China) and the experiment was performed as previously described (Ding *et al.* 2014). Ninety-eight mice were divided into seven groups: normal control group (uninfected), placebo group (infected mice with saline), four honeysuckle extracts-treated groups (600 mg/kg/d), and the ribavirin-treated group (positive control, 100 mg/kg/d). Mice were inoculated intranasally with $8 \times LD_{50}$ (50% mice lethal dose) mouse-adapted A/FM/1/47 (H1N1) virus suspended in 50 μL normal saline. After infection for 2 h, honeysuckle extracts, ribavirin or saline (placebo) were administered once daily for 5 days by oral gavage. Survival and weight were observed daily for 14 days. On day 5 and day 6 after infection, four mice per group were randomly selected and euthanized for lung pathological examinations. The lung index was calculated using the equation: Lung index = lung weight/body weight × 100. The lung injury score was evaluated according to the degree of congestion and edema of the lungs, the degree of bronchial necrosis, the degree of alveolar and bronchial epithelium injury, and the degree of inflammatory infiltration by the professional.

qRT-PCR Analysis

The viral load in the lung tissues was determined by quantitative real time RT-PCR (qRT-PCR). RNA was extracted from the lungs by homogenizing with Trizol (Invitrogen, USA) reagent, and subsequently reverse transcribes to cDNA. qRT-PCR was performed on an ABI 7500 Real-Time PCR System (ABI, USA). The GAPDH was used as endogenous control. The primer for H1N1 (FM1) detection: Forward: 5′'-GAGAAAGAAGTCCTTGT GC-3′ and Reverse: 5′-TCTATCATTCCAGTCCATCCC-3′.

Hematoxylin and Eosin Staining

The lungs of mice from each group were fixed in 4% paraformaldehyde and embedded in paraffin. Then, sections (5 μm) were stained with hematoxylin and eosin (HE). Histological examinations were performed using an Olympus microscope.

Neuraminidase (NA) Inhibition Assay

An NA inhibition assay was performed as previously described (Kim *et al.* 2013). Allantoic fluid containing the influenza viruses H1N1, H1N1-H275Y or H3N2 and the cell lysate of CHO expressing viral NAs, including A/Vietnam/1203/2004 (H5N1), A/Anhui/1/2013 (H7N9) and H7N9-R294K were used for the NA inhibition assay. First, the allantoic fluid or cell lysate was incubated with serial honeysuckle extracts (15.625, 31.25, 62.5, 125, 250 μg/mL) at 37 °C in a 96-well black OptiPlate for 5 min. Then, 20 μmol/L fluorescent substrate MUNANA was added and the plates were incubated at 37 °C for 30 min. Fluorescence was measured using an AFLx800 fluorescence microplate reader (Bio-Tek) at excitation and emission wavelengths of 360 and 450 nm respectively.

Statistical Analysis

All assays were performed in triplicate. Values in the texts and figures are presented as the mean ± SD. Data were analyzed statistically by an un-paired, two-tailed Student's *t*-test. The significance was defined as $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

Antiviral Activity of Honeysuckle Extracts against H1N1 and H3N2 *In Vitro*

The cytotoxicity of the honeysuckle extracts in MDCK and Hela cells was examined. As shown in Fig. 1A, upon treatments with the four honeysuckle extracts at 25 μg/mL, cell viability remained at least 95% at 48 h post-infection, indicating that the treatments induced limited cytotoxicity at concentrations less of 25 μg/mL. In addition, the CC_{50} and EC_{50} values of the four honeysuckle extracts on the influenza viruses were determined (Table 1). The total extract induced the lowest cytotoxicity, with a CC_{50} value of 350.0 μg/mL in MDCK cells. The acids-flavonoids mixture showed the most effective antiviral activity against H1N1 and H3N2 with EC_{50} values of 3.8 μg/mL and 4.1 μg/mL, respectively.

To investigate whether these honeysuckle extracts can inhibit the replication of the H1N1 and H3N2 influenza viruses, a CPE reduction assay was performed in MDCK cells. The CPE reduction was confirmed by direct microscopic observation. Each honeysuckle treatment cell group had a considerably lower CPE than the placebo group (H1N1 infected cells treated with DMSO) (Fig. 1B). All four honeysuckle treatment protected MDCK cells from influenza virus infection in a dose-

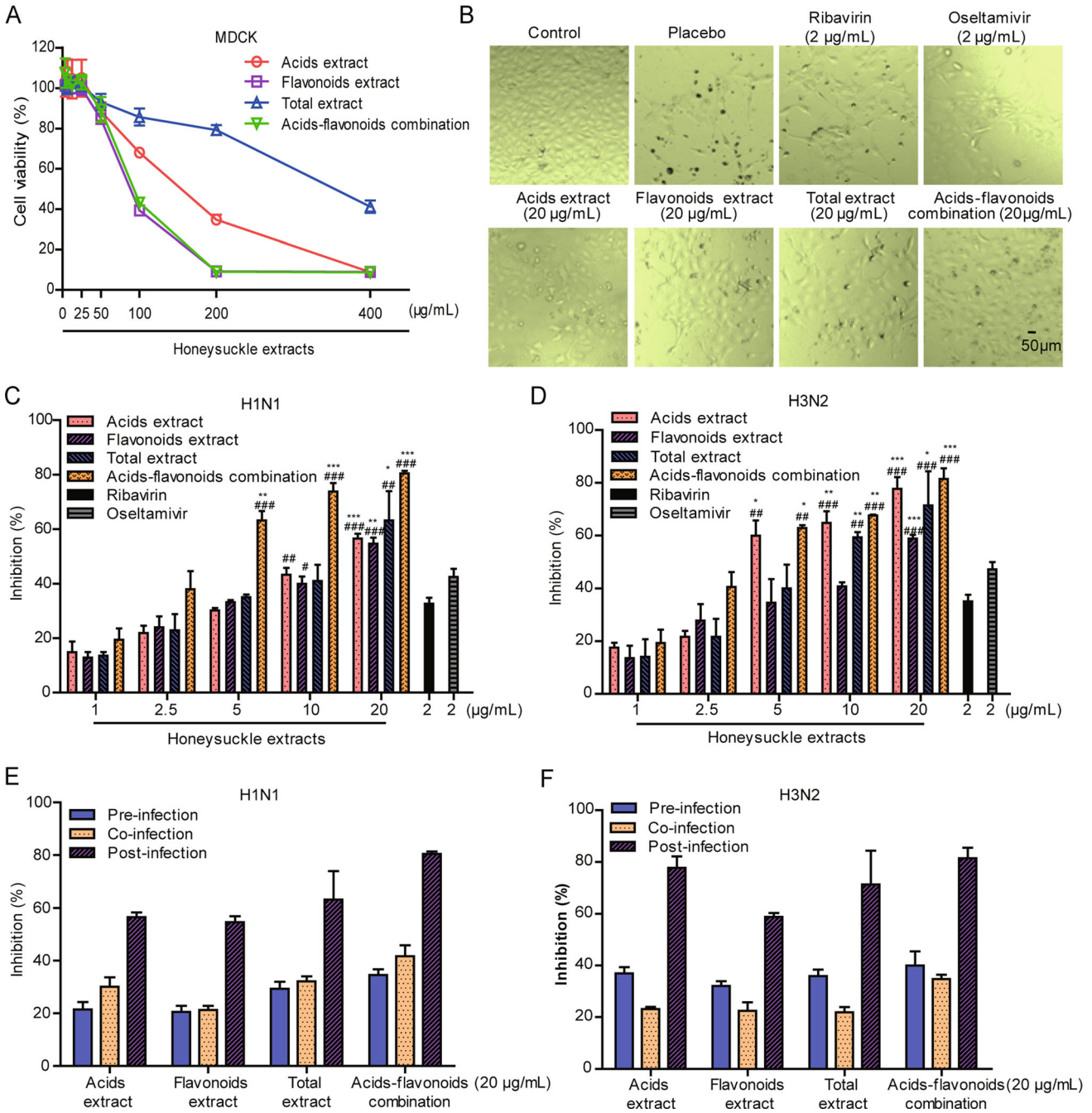

Fig. 1 Inhibitory effects of honeysuckle extracts on influenza viruses H1N1 and H3N2 in MDCK cells. **A** Cellular toxicity of honeysuckle extracts in MDCK cells. **B** Microscopic images for H1N1 virus-infected, 20 μg/mL honeysuckle extracts-treated, 2 μg/mL OC-treated, and 2 μg/mL ribavirin-treated MDCK cells at 48 h post-infection. **C** Inhibitory rates of honeysuckle extracts (1–20 μg/mL) against influenza virus A/FM/1/47 (H1N1), and **D** H3N2 in MDCK cells. 2 μg/mL oseltamivir carboxylate and ribavirin were used as positive controls. Inhibitory rates of honeysuckle extracts (20 μg/mL) against H1N1 (**E**) and H3N2 (**F**) influenza viruses with different infection protocols in MDCK cells. The results were presented as the mean ± S.D. of three independent experiments, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the oseltamivir-treated group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, compared with the ribavirin-treated group.

dependent manner while inducing low cytotoxicity (Fig. 1C, 1D). In addition, acids-flavonoids mixture exhibited stronger suppressive effects than the three extracts in MDCK cells, especially at a final concentration of 20 μg/mL, with inhibition rates of 80.4% and 81.4% against H1N1 and H3N2, respectively. At a concentration of 2.5 μg/mL, the acids-flavonoids mixture had an anti-influenza-virus activity effect against H1N1 and H3N2 similar to that of oseltamivir and ribavirin.

Table 1 Cytotoxicity and antiviral activity of honeysuckle extracts.

Compounds	CC_{50}[a]		H1N1		H3N2	
	MDCK	HeLa	EC_{50}[b]	SI[c]	EC_{50}	SI
Acids extract	139.8 ± 9.3	143.3 ± 6.0	15.6 ± 1.5	8.9	5.6 ± 0.6	24.9
Flavonoids extract	82.4 ± 0.8	76.9 ± 1.4	17.2 ± 3.8	4.8	14.1 ± 2.5	5.9
Total extract	350.0 ± 14.5	178.1 ± 2.7	14.3 ± 7.0	25.2	7.4 ± 1.0	48.6
Acids-flavonoids	88.3 ± 3.9	116.5 ± 2.9	3.8 ± 0.3	23.5	4.1 ± 0.5	21.8
Oseltamivir	> 500		2.6 ± 0.1	> 192	3.1 ± 0.1	> 161
Ribavirin	13.7		3.5 ± 0.1	3.9	ND[d]	ND

The results were the mean ± S.D. of three independent experiments.

[a]Concentration of drugs required to reduce the viability of normal MDCK cells by 50% (μg/mL).

[b]Concentration of drugs required to improve the viability of influenza virus-infected cells by 50% (μg/mL).

[c]Selectivity index, CC_{50}/EC_{50}.

[d]Not determined.

To clarify the possible anti-influenza mechanism of the four honeysuckle extracts treatments, their inhibitory effects on influenza virus replication were examined using three different infection protocols. As shown in Fig. 1E–1F, when 20 μg/mL honeysuckle extracts were added 2 h post-infection, the inhibition rates against H1N1 and H3N2 viruses were much higher than those observed after the 'pre-infection' or 'co-infection' treatment of infected cells, indicating that honeysuckle treatments mainly inhibited the replication and release of influenza virus but not viral adsorption or penetration.

Taken together, these results indicate that all four honeysuckle extracts treatments effectively inhibited the replication of H1N1 and H3N2 influenza viruses in MDCK cells, and the honeysuckle acids-flavonoids mixture had the strongest inhibitory effects *in vitro*.

Honeysuckle Extracts Effectively Inhibited the Replication of Oseltamivir-Resistant Mutant Influenza Virus H1N1-H275Y *In Vitro*

In addition to wild type influenza viruses, we also investigated the inhibitory effects of the honeysuckle extracts treatments on the oseltamivir-resistant mutant strain H1N1-H275Y *in vitro*. As shown in Fig. 2, four honeysuckle extracts obviously inhibited the replication of the influenza virus H1N1-H275Y in a dose-dependent manner in MDCK cells. The inhibition rate of the acids-flavonoids mixture against H1N1-H275Y virus was 47.8% at a concentration of 20 μg/mL, while the inhibition rates of oseltamivir and ribavirin were only 3.5% and 14.8%, respectively, showing that acids-flavonoids mixture can effectively inhibit the replication of the oseltamivir-resistant virus *in vitro*.

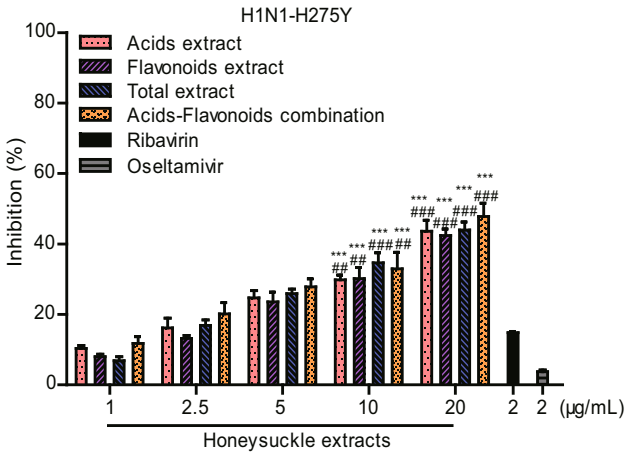

Fig. 2 Inhibitory effects of honeysuckle extracts on oseltamivir-resistant mutant influenza virus H1N1-H275Y in MDCK cells. 2 μg/mL oseltamivir carboxylate and ribavirin were used as positive controls. The results were presented as the mean ± S.D. of three independent experiments, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the oseltamivir-treated group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, compared with the ribavirin-treated group.

Therapeutic Efficacy of Honeysuckle Extracts against H1N1 *In Vivo*

The inhibitory efficacy of four honeysuckle extracts on influenza A virus *in vivo* was evaluated in an H1N1-infected mouse model. The experimental protocol is shown in Fig. 3A. On 'day 0', the mice were infected intranasally with a lethal dose of H1N1 virus. At 2 h post infection, drugs were delivered orally to mice once daily for 5 days (days 0–4).

As shown in Fig. 3B, acids extract-treated mice were obviously protected from death caused by influenza virus, whereas all the mice administered the placebo died within 8 days. Treatment of the mice with 600 mg/kg/d of honeysuckle acids extract, flavonoids extract, total extract

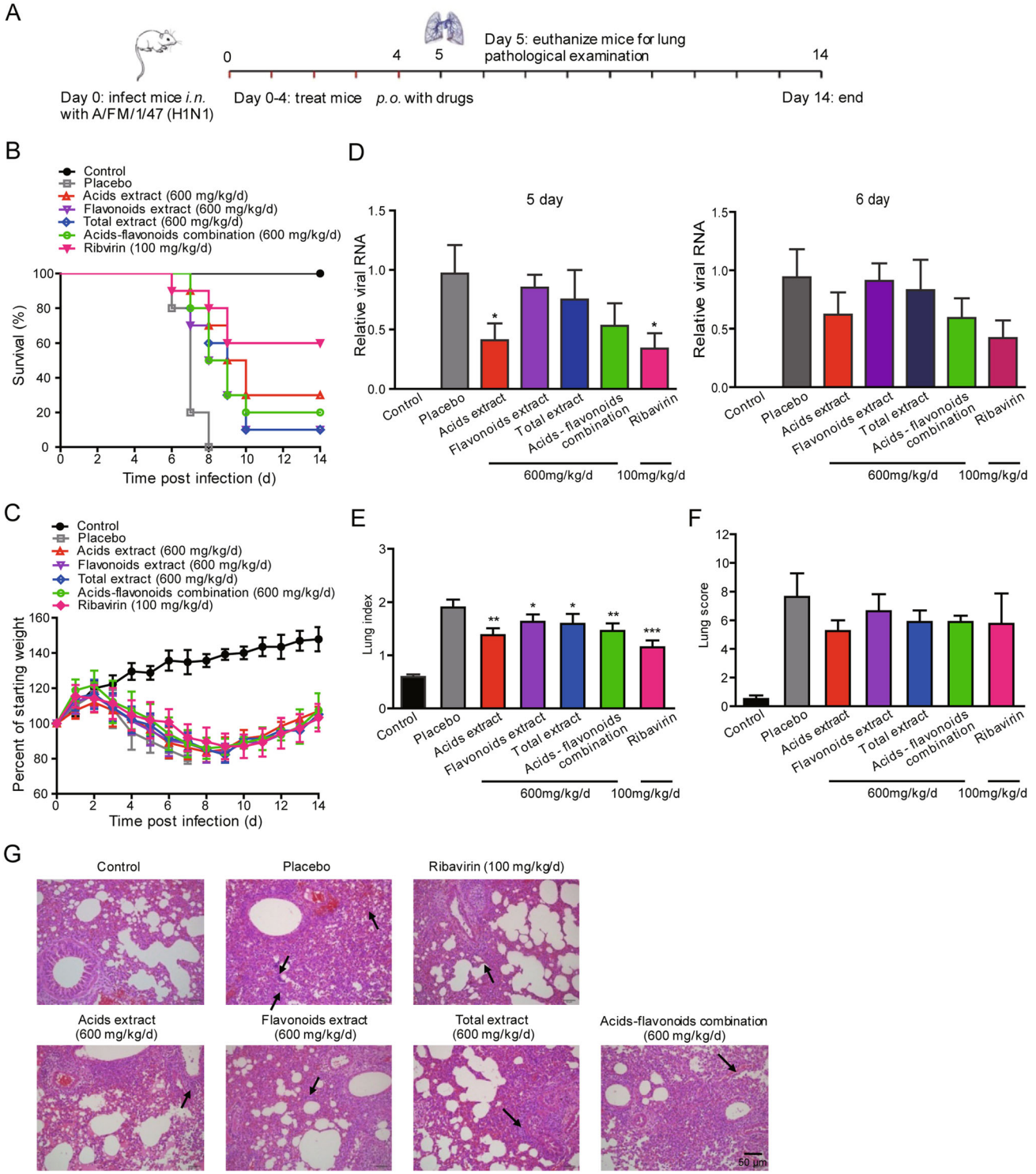

Fig. 3 Therapeutic efficacy of honeysuckle extracts against the influenza virus in mice. **A** Experimental protocol of testing honeysuckle extracts in H1N1-infected mice. Fourteen uninfected ICR mice were as control group. ICR mice were intranasally infected with a mouse-adapted influenza virus H1N1 ($8 \times LD_{50}$). At 2 h post-infection, fourteen mice per group were treated with saline (placebo group), four honeysuckle extracts (600 mg/kg/d), or ribavirin (100 mg/kg/d) twice daily for 5 successive days (days 0–4) by oral gavage, respectively. On day 5 and day 6 post-infection, four mice per group were euthanized for lung pathological examinations. Survival rate (**B**) and body weight (**C**) were monitored for 14 days (n = 10). Viral load (**D**), Lung index (**E**) and Lung score (**F**) of mice infected with influenza virus H1N1 (n = 4). **G** H&E staining of sectioned lungs (n = 4). The histopathological changes are indicated by arrows. Data shown as the mean ± S.D. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the placebo group.

or acids-flavonoids mixture increased survival to 30%, 10%, 10% and 20%, respectively. In addition, administration of the four honeysuckle extracts effectively protected the infected mice from body weight loss (Fig. 3C). Despite a similar trend in body weight loss during the first 9 days post-infection, the mice administered the honeysuckle extracts treatments regained weight starting on day 10, whereas infected mice treated with placebo showed significant body weight loss until their death.

On 'day 5' and 'day 6', we used qPCR to detect the viral load in the lungs of the treated mice. Acids extract and ribavirin treatments significantly inhibited viral RNA expression on day 5 but only slightly suppressed viral RNA expression on day 6 (Fig. 3D). Four mice per group were randomly selected and euthanized for use in lung pathological examinations on day 5. The four honeysuckle extracts treatments effectively suppressed the influenza-induced increases in lung indexes to a different degree (Fig. 3E). At a dosage of 600 mg/kg/d, the lung index of the honeysuckle acids extract-treated mice was 1.4, a statistically significant decrease compared to that of the placebo group (lung index: 1.9). The four honeysuckle extracts treatments administered at a dosage of 600 mg/kg/d protected the lungs of mice from damage caused by influenza virus infection. On day 5, features of extensive lung damage, such as alveolar wall congestion, infiltration of inflammatory cells and bronchial epithelium necrosis, were observed in the placebo group (lung score: 7.63) (Fig. 3F, 3G). In the honeysuckle acids extract-treated mice, the lung damage was notably attenuated (lung score: 5.25).

These data indicated that the honeysuckle acids extract significantly reduced body weight loss, inhibited lung damage and prevented the death of influenza-infected mice. The anti-influenza virus effects of the honeysuckle acids extract was slightly weaker than that of the acids-flavonoids mixture *in vitro*, but *in vivo*, and the pharmaco dynamic study demonstrated the potent therapeutic efficacy of the honeysuckle acids extract against infection with influenza virus H1N1.

Inhibitory Effects of Honeysuckle Extracts against the Neuraminidase Activity of Influenza Viruses

The results of the time-of-drug addition experiment indicated that honeysuckle extracts primarily inhibited the replication and release of the influenza virus after entrance into host cells (Fig. 1E, 1F). Influenza virus neuraminidase can hydrolyze sialic acid residues in progeny virus to promote the release of progeny virus particles from host cells, thus playing an important role in the spread of the influenza virus. Therefore, we speculated that honeysuckle extracts may inhibit the neuraminidase activity of influenza viruses. The inhibitory effects of the honeysuckle extracts treatments on the neuraminidase activity of H1N1, H1N1-H275Y, H3N2 influenza viruses, CHO-expressed H5N1-NA, CHO-expressed H7N9-NA and CHO-expressed H7N9-R294K-NA were examined using MUNANA, a specific fluorescent substrate. Figure 4 shows that the four honeysuckle extracts treatments significantly inhibited the neuraminidase activity of multi-influenza viruses in a dose-dependent manner. Among the four honeysuckle extracts treatments, the flavonoids extract showed the best anti-neuraminidase activity. Moreover, the four honeysuckle extracts treatments had a greater inhibitory effect against recombinant NA from the H7N9 influenza virus than against NA from the other influenza viruses. As shown in Table 2, the IC_{50} value of the honeysuckle flavonoids extract against NA of H7N9 influenza virus was 24.7 μg/mL. These results demonstrated that the honeysuckle extracts treatments had broad-spectrum inhibitory activity against NA in multiple influenza viruses, including the oseltamivir-resistant mutant strains.

Discussion

Honeysuckle has been used for the effective treatment of influenza virus infection for thousands of years in China (Zhou *et al.* 2015). It efficiently inhibits influenza virus replication *in vitro* and *in vivo*. However, the effective components and functional mechanisms had not been previously identified. Here, we performed a systematic evaluation of the activity of the four honeysuckle extracts treatments against the influenza viruses and found that the honeysuckle acids-flavonoids mixture had the strongest inhibitory effect *in vitro*, as indicated by the higher selectivity index ($SI > 23$). To verify whether honeysuckle extracts have broad spectrum activity against influenza virus, we evaluated the effects of four honeysuckle extracts treatments against different influenza virus strains, such as A/FM/1/47 (H1N1) and A/Beijing/32/92 (H3N2). As expected, acids-flavonoids mixture dramatically inhibited both strains.

Influenza virus is an important pathogens of human acute respiratory infection and poses a long-term danger to human health. Additional drug-resistant strains are gradually emerging in addition to oseltamivir resistance of the H1N1 influenza virus, which has persisted since 2007 (Collins *et al.* 2008). The common neuraminidase mutation at amino acid 274 or 275 (H274Y or H275Y) in the influenza virus does not affect its adaptability or replication, which has led to the outbreaks of drug-resistant influenza and epidemics (Hurt 2014; Tarbet *et al.* 2014). Importantly, the honeysuckle acids-flavonoids mixture at a concentration of 20 μg/mL displayed a sufficient inhibitory

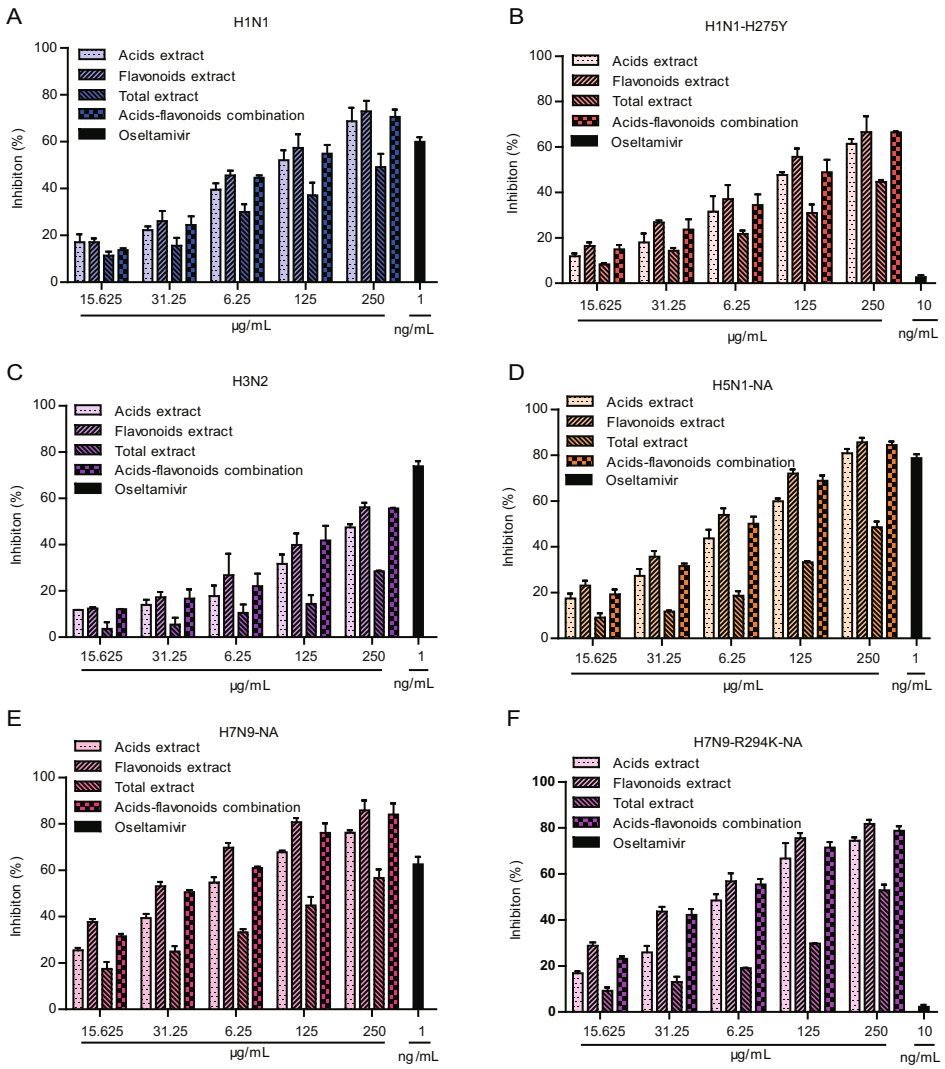

Fig. 4 Inhibition of neuraminidase activity by four honeysuckle extracts. Neuraminidase (NA) inhibition of four honeysuckle extracts (15.625–250 μg/mL) against the influenza viruses: (**A**) H1N1, (**B**) oseltamivir-resistant H1N1-H275Y, (**C**) H3N2, (**D**) CHO-expressed H5N1-NA, (**E**) CHO-expressed H7N9-NA and **F** CHO-expressed H7N9-R294K-NA. Oseltamivir carboxylate (1 nmol/L or 10 nmol/L) was used as a positive control. The results were shown as the mean ± S.D. of three independent experiments.

Table 2 IC_{50} of honeysuckle extracts against neuraminidase activity of influenza A virus.

NA	IC_{50}[a] ± SD (μg/mL)			
	Acids extract	Flavonoids extract	Total extract	Acids-flavonoids
H1N1	112.3 ± 17.7	90.9 ± 8.6	305.1 ± 23.7	100.1 ± 11.4
H3N2	332.6 ± 34.5	196.0 ± 23.4	947.3 ± 39.5	203.8 ± 9.9
H5N1	78.8 ± 5.5	54.7 ± 3.4	283.9 ± 29.0	63.8 ± 0.6
H7N9	55.9 ± 5.1	24.7 ± 2.3	170.0 ± 5.2	35.2 ± 3.1
H1N1-H275Y	150.4 ± 13.6	108.4 ± 17.0	350.1 ± 36.9	125.7 ± 14.7
H7N9-R294K	80.1 ± 6.3	44.1 ± 5.4	291.2 ± 26.2	53.1 ± 6.0

The results were the mean ± S.D. of three independent experiments.

[a]Concentration of drugs required to reduce the activity of neuraminidase by 50%.

effect on the oseltamivir-resistant H1N1-H275Y strain, with an inhibition rate of 47.8%, while the inhibition rate of OC against H1N1-H275Y was only 3.5% (Cheng *et al.* 2016; Jin *et al.* 2018).

Furthermore, the oral administration of the honeysuckle acids extract treatment effectively protected mice infected with H1N1 influenza virus from death. At a dosage of 600 mg/kg/d, they reduced body weight loss, alleviated influenza-induced acute lung injury, improved lung

parameters and conferred a 30% survival rate on the mice. These data are not consistent with the results we observed *in vitro* showing that acids-flavonoids mixture had the best antiviral activity. We believe the potential reason is that the crude extracts have different metabolism and absorption rates *in vitro* and *in vivo*. We will carry out experiment on drug metabolism in the future.

The time-of-drug addition experiment showed that the honeysuckle extract inhibits influenza virus release primarily because of their effective inhibition of NA. In particular, the honeysuckle extracts treatments had inhibitory activity against NA from H1N1, H3N2, H5N1, H7N9 and the oseltamivir-resistant mutant strain. Honeysuckle extracts may interact with the surface proteins of influenza virus, such as NA, which is important for virus release from host cells (Sakai *et al.* 2017). However, further in-depth study is still needed. It remains to be revealed whether honeysuckle extracts have inhibitory effects through other mechanisms, such as the inhibition of RNA polymerase activity, inhibition of influenza virus vRNP outside the nucleus, and/or activation of host cell immune function.

In conclusion, our study found that among the four honeysuckle extracts, including acids extract, flavonoids extract, total extract and acids-flavonoids mixture, the acids extract components of honeysuckle are the key antiviral component. They suppresses IAVs infection mainly by inhibiting viral NAs activity. With its broad-spectrum antiviral activity against IAVs, honeysuckle acids extract shows great potential for the clinical management of influenza virus infections.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The related expression was observed in the animal experiments and was approved by the Science and Technology Department of Jiangsu Province (SYXK 2016-0011) and the Animal Ethics and Experimentation Committee of China Pharmaceutical University. All institutional and national guidelines for the care and use of laboratory animals were followed.

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