(+)-pinoresinol-O-β-D-glucopyranoside from *Eucommia ulmoides* Oliver and its anti-inflammatory and antiviral effects against influenza A (H1N1) virus infection

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Abstract. *Eucommia ulmoides* Oliver (Du-Zhong) is an ancient Chinese herbal remedy used for the treatment of various diseases. To date, the effects of its constituent lignans on influenza viruses remain to be elucidated. In the present study, a lignan glycoside was isolated and purified from *Eucommia ulmoides* Oliver. Its structures were identified via extensive spectroscopic analysis, and its antiviral and anti-inflammatory activities, specifically against influenza viruses, were determined via a cytopathic effect (CPE) assay, plaque-reduction assays, a progeny virus yield reduction assay, reverse transcription-quantitative polymerase chain reaction analysis and a Luminex assay. Additionally, western blot analysis was performed to investigate the underlying mechanisms of its effects against influenza viruses. The chemical and spectroscopic methods determined the structure of lignan glycoside to be (+)-pinoresinol-O-β-D-glucopyranoside. The CPE assay showed that (+)-pinoresinol-O-β-D-glucopyranoside exerted inhibitory activities with 50% inhibition concentration values of 408.81±5.24 and 176.24±4.41 μg/ml against the influenza A/PR/8/34 (H1N1) and A/Guangzhou/GIRD07/09 (H1N1) strains, respectively. Its antiviral properties were confirmed by plaque reduction and progeny virus yield reduction assays. Additional mechanistic analyses indicated that the anti-H1N1 virus-induced effects of (+)-pinoresinol-O-β-D-glucopyranoside were likely due to inactivation of the nuclear factor-κB, p38 mitogen-activated protein kinase and AKT signaling pathways. Furthermore, (+)-pinoresinol-O-β-D-glucopyranoside exhibited pronounced inhibitory effects on the expression of influenza H1N1 virus-induced pro-inflammatory mediators, including tumor necrosis factor-α, interleukin (IL)-6, IL-8 and monocyte chemoattractant protein 1. The data obtained suggest that (+)-pinoresinol-O-β-D-glucopyranoside may be a candidate drug for treating influenza H1N1 virus infection.

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Introduction

Influenza A virus (IAV; orthomyxoviridae) infection in humans affects the upper and lower respiratory tracts, which often causes acute respiratory diseases ranging from mild to severe. For example, the symptoms of seasonal influenza virus infection can include fever, headache and chills, although patients recover in days (1), whereas lower respiratory tract infections, including 1918 H1N1 or H51N1, can contribute to alveolitis and diffuse alveolar damage leading to mortality (2,3). Clinically available anti-influenza virus medications include neuraminidase (NA) inhibitors, for example. oseltamivir and zanamivir, and M2 ion channel blockers, including amantadine and rimantadine, which have been shown to be ineffective due to viral genome mutations (4). It was previously

reported that amino acid substitutions in NA (e.g. NA-R292K and NA-Arg292Lys) of H7N9 confer oseltamivir resistance, raising worldwide concerns on preparedness for an influenza pandemic (5).

Interactions between influenza virus hemagglutinin and cell surface sialic acid receptors are important for infection to establish in target cells (6). During viral replication, structural features, including double stranded RNA or 5'-triphosphate RNA, are sensed by the host immune system, leading to elevated pro-inflammatory mediator production and the recruitment of immune cells to the site of infection (7). It is well recognized that the host immune system orchestrates appropriate pro-inflammatory responses to eliminate invading pathogens and clear infected cells. However, it is also becoming clear that viral factors and host immune responses are involved in the pathogenesis of diseases caused by influenza (8). The PB1-F2 protein of H5N1(HK/97) and 1918 H1N1, with an amino acid change at position 66 (N66S), has been found to increase viral virulence (9). Furthermore, exacerbated cytokine production and the dysregulated recruitment of immune cells, including macrophages, following influenza virus infection contribute to the progression of acute lung injury to acute respiratory distress syndrome (ARDS) (10,11). Therefore, data suggests that the most advantageous strategy for the treatment of influenza diseases combines antiviral compounds with immunomodulators.

The activation of host signaling pathways is essential for viral replication and the expression of pro-inflammatory mediators. Activation of the phosphoinositide-3-kinase (PI3K)/AKT, nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK)1/2 and P38 MAPK signaling cascades triggered by influenza virus infection, is significant in viral entry, replication of the viral genome and the nuclear export of viral ribonucleoproteins (vRNPs), but it also elicits an excessive pro-inflammatory response and collateral lung damage (12-15). Therefore, the development of novel compounds that target certain host signaling pathways may be a promising therapeutic direction for diseases caused by influenza.

The herb *Eucommia ulmoides* Oliver (Du-Zhong) has been used in various clinical situations (16,17); traditionally, it was used for strengthening muscles and pulmonary function, reducing blood pressure and preventing miscarriages. Numerous active components have been identified from *Eucommia ulmoides* Oliver, including lignans, polyphenolics, triterpenes and flavonoids (18). Among the active components, the main bioactive components, *Eucommia* lignans, have protective effects against hypertensive renal injury (19). However, their effects on influenza virus infection remain to be fully elucidated. In the present study, the lignan glycoside (+)-pinoresinol-O-β-D-glucopyranoside was isolated from *Eucommia ulmoides* Oliver and subjected to various assays to characterize its inhibitory activity, and the underlying mechanisms, against influenza virus infection.

Materials and methods

General experimental procedures. The nuclear magnetic resonance (NMR) spectra were obtained using Bruker AVANCE-400 NMR spectrometers (Bruker Corporation, Billerica, MA, USA). Analytical high-performance liquid chromatography (HPLC) was performed using the Shimadzu LC-10A instrument (Shimadzu Corporation, Kyoto, Japan) equipped with a DAD detector and a reversed-phase C18 column (5-μm, 4.60x250 mm; Shimadzu Corporation). Preparative HPLC was performed on a Shimadzu LC-8A instrument (Shimadzu Corporation) with a UV SPD-20A detector using a reversed-phase C18 column (5 μm, 20x250 mm). Silica gel (200-300 mesh) and silica gel G plates (both from Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) were used for thin layer chromatography analysis.

Plant material. *Eucommia ulmoides* Oliver was collected from Hubei province (China) and authenticated by Professor Xiping Pan (Guangzhou Medical University, Guangzhou, China).

Extraction and isolation. The air-dried bark (1.5 kg) of *Eucommia ulmoides* Oliver was refluxed with 95% EtOH (v/v, 3x5 l, 1.5 h each). The combined extracts were concentrated *in vacuo* to generate a brown residue (120 g), which was dissolved in H_2O (1.5 l) and subjected to column chromatography (CC) over Diaion HP20 macroporous adsorptive resins, prior to elution with MeOH/H_2O (0:100-95:5). The 95% EtOH (v/v) eluate (13.4 g) was subjected to CC on silica gel and eluted with CH_3Cl/MeOH (95:5-0:100) to generate eight fractions (Fr. 1-9). Fr.6 was separated by preparative HPLC and eluted with MeOH/H_2O (2:8-10:0) to obtain one compound (5.6 mg). The purity of the compound was estimated by HPLC to be >95% and identified as (+)-pinoresinol-O-β-D-glucopyranoside by NMR spectroscopy.

Viruses and cell lines. Influenza A/PR/8/34 (H1N1), A/Hongkong/8/68 (H3N2) and A/Hongkong/Y280/97 (H9N2) were obtained from the American Type Culture Collection (Manassas, VA, USA). Influenza A/Guangzhou/GIRD07/09 (H1N1) and B/Lee/1940 (FluB) were isolated from routine clinical specimens. All viral strains used in the present study were propagated in Madin-Darby canine kidney (MDCK) cells. The viral stocks were stored at -80˚C and titrated in a 50% tissue culture infectious dose ($TCID_{50}$) assay prior to use.

The MDCK and human alveolar A549 cells were obtained from the ATCC and maintained at 37˚C in a humidified atmosphere of 5% CO_2 in DMEM/F12 (1:1) medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The A549 cells were transfected with 20 ng poly (I:C) from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) using 5 μl Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Cytopathic effect (CPE) inhibition assay. The MDCK cell monolayers ($2x10^4$ cells/well) were grown in 96-well plates and inoculated with 100 $TCID_{50}$ of serial influenza virus strains at 37˚C for 2 h. Subsequently, the inoculum was removed and then incubated with 0-1,000 μg/ml of (+)-pinoresinol-O-β-D-glucopyranoside and the positive control oseltamivir carboxylate (TLC PharmaChem., Inc., Canada) at 37˚C, respectively. Following 48 h of incubation, the influenza

virus-infected cells were stained with 0.5% crystal violet solution and observed under a routine light microscope (DM 3000; Leica Microsystems GmbH, Wetzlar, Germany). The 50% inhibition concentration (IC_{50}) of the virus-induced CPE was calculated as previously described (20).

Plaque-reduction assays. The MDCK cell monolayers ($5x10^5$ cells/well) were seeded in 6-well plates and incubated overnight at 37˚C to ensure adherence. The cells were then inoculated with 40 PFU/well of influenza virus, including influenza A/PR/8/34 (H1N1) and influenza A/Guangzhou/GIRD07/09 (H1N1), and incubated at 37˚C with constant agitation. Following 2 h of incubation, the inoculum was removed and replaced with maintenance DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1.5% agarose, 1.5 μg/ml TPCK-trypsin and the indicated concentration of (+)-pinoresinol-O-β-D-glucopyranoside. After 3 days, the cells were fixed in 10% formalin and stained with 1% crystal violet.

Progeny virus yield reduction assay. The A549 cells were grown to 90% confluency in 6-well plates and then infected with influenza (MOI=0.1) with or without the indicated concentration of (+)-pinoresinol-O-β-D-glucopyranoside. After 24 h, the supernatants were harvested, and the confluent monolayers of MDCK cells ($2x10^4$ cells/well) in the 96-well plate were inoculated with 10-fold dilutions of the supernatants at 37˚C for 2 h. Subsequently, the inoculum was removed and replaced with serum-free DMEM containing 1.5 μg/ml TPCK-trypsin. After 48 h, the viral plaques were visualized using trypan blue and observed under a light microscope.

Cell viability assay. The cytotoxic effects induced by (+)-pino resinol-O-β-D-glucopyranoside in A549 cells were evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the A549 cells ($1x10^5$ cells/well) were seeded into 96-well plates and then incubated with (+)-pinoresinol-O-β-D-glucopyranoside at different concentrations (0-1,000 μg/ml) for 48 h. Subsequently, the cells were washed twice with PBS to remove the drug and incubated with 200 μl MTT solution (5 mg/ml) for an additional 4 h. The formazan crystals generated in each well were dissolved with dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). The absorbance was determined at 490 nm using a microplate reader (Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA).

Western blot analysis. The following primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) were used for western blot analysis: NF-κB p65 (cat. no. 8242), phosphorylated (phosphor)-NF-κB p65 (Ser^{536}) (cat. no. 3033), p38 MAPK (cat. no. 8690), phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (cat. no. 4511), AKT (cat. no. 4691), phospho-AKT (Thr^{308}) (cat. no. 13038), ERK1/2 MAPK (cat. no. 4695), phospho-ERK1/2 MAPK (Thr^{202}/Tyr^{204}) (cat. no. 9101), c-Jun N-terminal kinase (JNK) MAPK (cat. no. 9252), phospho-JNK MAPK (Thr^{183}/Tyr^{185}) (cat. no. 4671), cyclooxygenase-2 (COX-2; cat. no. 12282) and GAPDH (cat. no. 5174). The HRP-conjugated secondary antibody (cat. no. BAB1302) was acquired from Multisciences Biotech Co., Ltd. (Hangzhou, China).

The cells were rinsed twice with ice-cold PBS and lysed in RIPA lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and protease inhibitors (Sigma-Aldrich; Merck KGaA). The supernatants from each treatment were collected by centrifugation of the lysates at 13,000 x g for 15 min at 4˚C, and then evaluated to determine the protein concentration using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equivalent quantities of protein (20 μg/lane) were resolved on a 10% polyacrylamide gel and transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were then blocked with 5% non-fat milk (w/v) in 1X TBS/Tween-20 buffer (0.1%, v/v) for 1 h at room temperature prior to incubation with the primary and secondary antibodies. Then, the membranes were incubated overnight at 4˚C with 1:1,000 dilution of primary antibody in 5% BSA (w/v) in TBS/Tween-20 buffer (0.1% v/v). The HRP-conjugated secondary antibody was used to detect the primary antibody at a dilution of 1:500 for 1 h at room temperature. The bands were detected using an enhanced chemiluminescence reaction kit (Amersham; GE Healthcare Life Sciences, Chalfont, UK). The intensity of the phosphorylated bands was quantified using ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD, USA).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR analysis was performed to determine the relative mRNA levels of cytokines and chemokines. Briefly, the influenza A virus-infected cells were treated with the indicated concentrations of (+)-pinoresinol-O-β-D-glucopyranoside. Total cellular RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.); the cDNA was then synthesized from 1 μg total RNA using a PrimeScript™ RT Reagent kit (Takara Bio, Inc., Otsu, Japan), at 37˚C for 15 min followed by 5 sec at 85˚C to inactivate the reaction. The qPCR analysis was performed using a Premix Ex Taq™ Reagent kit (Takara Bio, Inc.), with initial heating to 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing and elongation at 60˚C for 40 sec in an Applied Biosystems 7500 Real-Time PCR system with the primers and probes specified in Table I. GAPDH was used as an internal reference gene. The relative mRNA expression data were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Pro-inflammatory mediator measurements. The inhibitory effects of (+)-pinoresinol-O-β-D-glucopyranoside on the influenza virus-induced production of pro-inflammatory mediators were measured via Luminex assays and ELISAs, respectively. Briefly, the A549 cells in 6-well plates were inoculated with A/PR/8/34 (H1N1) for 2 h, followed by treatment with different concentrations of (+)-pinoresinol-O-β-D-glucopyran oside. Following another 24 h of incubation, the culture supernatants were collected to evaluate the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and prostaglandin E2 (PGE2) using a Luminex kit (Bio-Rad Laboratories, Inc.) and ELISA kits (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols.

Table I. Primers and probe sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Primer and probe	Sequence (5'→3')
TNF-α	Forward	AACATCCAACCTTCCCAAACG
	Reverse	GACCCTAAGCCCCCAATTCTC
	Probe	CCCCCTCCTTCAGACACCCTCA ACC
IL-6	Forward	CGGGAACGAAAGAGAAGCTCTA
	Reverse	CGCTTGTGGAGAAGGAGTTCA
	Probe	TCCCCTCCAGGAGCCCAGCT
IL-8	Forward	TTGGCAGCCTTCCTGATTTC
	Reverse	TATGCACTGACATCTAAGTTCTTT AGCA
	Probe	CCTTGGCAAAACTGCACCTTCAC ACA
MCP-1	Forward	CAAGCAGAAGTGGGTTCAGGAT
	Reverse	AGTGAGTGTTCAAGTCTTCGGA GTT
	Probe	CATGGACCACCTGGACAAGCAA ACC
COX-2	Forward	GAATCATTCACCAGGCAAATTG
	Reverse	TTTCTGTACTGCGGGTGGAAC
	Probe	TTCCTACCACCAGCAACCCTG CCA
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAAGATGGTGATGGGATTTC
	Probe	CAAGCTTCCCGTTCTCAGCC

TNF-α, tumor necrosis factor-α; IL, interleukin; MCP, monocyte chemoattractant protein 1; COX-2, cyclooxygenase-2.

Statistical analyses. All data were analyzed using SPSS v.18.0 statistical software (SPSS, Inc., Chicago, IL, USA) and are presented as the mean ± standard deviation based on at least three independent experiments. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Structural elucidation of (+)-pinoresinol-O-β-D-glucopyranoside. White amorphous powder, 1H-NMR (MeOD,400 MHz):δ7.15 (1H, d, J=8.4 Hz, H-5'), 6.95 (1H, d, J=1.6 Hz, H-2'), 6.92 (1H, dd, J=8.4, 1.6 Hz, H-6'), 6.82 (1H, br, J=8.4 Hz, H-6), 6.80(1H, d, J=1.6 Hz, H-2), 6.77 (1H, d, J=8.4 Hz, H-5), 4.88 (1H, d, J=6.0 Hz, Glc-1), 4.76 (1H, d, J=4.0 Hz, H-7'), 4.71 (1H, d, J=4.0 Hz, H-7), 3.88 (3H, s, 3'-OMe), 3.87 (3H, s, 3-OMe). 13C-NMR (MeOD, 100 MHz): 148.0 (C-3'), 146.17 (C-3), 144.53 (C-4'), 144.36 (C-4), 134.49 (C-1'), 130.78 (C-1), 117.10 (C-6), 116.84 (C-6), 115.02 (C-5'), 113.12 (C-5), 108.64 (C-2'), 108.0 (C-2), 99.87 (Glc-1), 84.54 (C-7), 84.15 (C-7'), 75.24 (Glc-3), 74.87 (Glc-5), 71.94 (Glc-2), 69.76 (C-9'), 69.72 (C-9), 68.36 (Glc-4), 59.5 (Glc-6), 53.8 (3-OMe), 53.45 (3'-OMe), 52.58 (C-8'), 52.39 (C-8). The data were in accordance with the literature regarding (+)-pinoresinol-O-β-D-glucopyranoside (Fig. 1) (22).

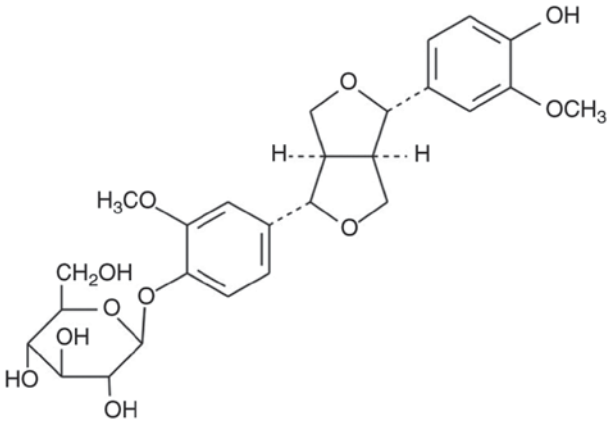

Figure 1. Chemical structure of (+)-pinoresinol-O-β-D-glucopyranoside.

Anti-influenza effects of (+)-pinoresinol-O-β-D-glucopyranoside in vitro. The present study initially evaluated the anti-influenza effects of (+)-pinoresinol-O-β-D-glucopyranoside using a CPE reduction assay. The MDCK cells were inoculated with 100 $TCID_{50}$ of influenza viruses and then incubated with a concentration series of (+)-pinoresinol-O-β-D-glucopyranoside or oseltamivir carboxylate following removal of the inoculum. (+)-pinoresinol-O-β-D-glucopyranoside was found to reduce the CPE induced by two influenza A/H1N1 viral strains (A/PR/8/34 and A/Guangzhou/GIRD07/09), with IC_{50} values of 176.24-408.81 μg/ml and SI values of 1.80-4.17 (Table II). However, (+)-pinoresinol-O-β-D-glucopyranoside did not exhibit antiviral effects against influenza virus A/Hongkong/8/68 (H3N2), A/Hongkong/Y280/97 (H9N2) or B/Lee/1940 (FluB) (Table II). The activity against A/PR/8/34 and A/Guangzhou/GIRD07/09 was confirmed using plaque reduction assays and progeny virus yield reduction assays. As shown in Fig. 2A, (+)-pinoresinol-O-β-D-glucopyranoside treatment significantly reduced plaque formation in the A/PR/8/34 and A/Guangzhou/GIRD07/09 (H1N1) virus-infected cells. Furthermore, the progeny virus titers of the two virus strains were significantly decreased by treatment with (+)-pinoresinol-O-β-D-glucopyranoside at the concentration of 250 or 500 μg/ml (Fig. 2B). Together, these results suggested that (+)-pinoresinol-O-β-D-glucopyranoside inhibits influenza A H1N1 viruses.

Effects of (+)-pinoresinol-O-β-D-glucopyranoside on A549 cell viability. To select appropriate concentrations for further experiments, the A549 cells were incubated with increasing concentrations of (+)-pinoresinol-O-β-D-glucopyranoside for 48 h. Following this, cell viability was assessed with an MTT assay to evaluate the potential cytotoxicity of lignan (+)-pinoresinol-O-β-D-glucopyranoside. The results showed that (+)-pinoresinol-O-β-D-glucopyranoside did not affect the viability of A549 cells up to a concentration of 1,000 μg/ml (Fig. 3). Therefore, the pharmacological effects of (+)-pinoresinol-O-β-D-glucopyranoside on viral infection were investigated using a concentration range of 150-450 μg/ml.

Table II. Anti-influenza virus efficacy of (+)-pinoresinol-O-β-D-glucopyranoside.

Virus type and strain	(+)-pinoresinol-O-β-D-glucopyranoside (μg/ml)			Oseltamivir (μg/ml)		
	TC_{50}	IC_{50}	SI[a]	TC_{50}	IC_{50}	SI[a]
A/PR/8/34 (H1N1)	736.49±34.51	408.81±5.24	1.80±0.11	>1,000	0.041±0.01	>1,000
A/GZ/GIRD07/09 (H1N1)	736.49±34.51	176.24±4.41	4.17±0.30	>1,000	0.022±0.01	>1,000
A/HK/8/68 (H3N2)	736.49±34.51	>737	<1	>1,000	0.098±0.01	>1,000
A/HK/Y280/97 (H9N2)	736.49± 4.51	>737	<1	>1,000	0.756±0.12	>200
B/Lee/1940 (FluB)	736.49±34.51	>737	<1	>1,000	11.51±1.19	>100

[a]SI was calculated as the ratio of TC_{50} to IC_{50}. SI, selectivity index; TC_{50}, 50% toxic concentration; IC_{50}, 50% inhibition concentration.

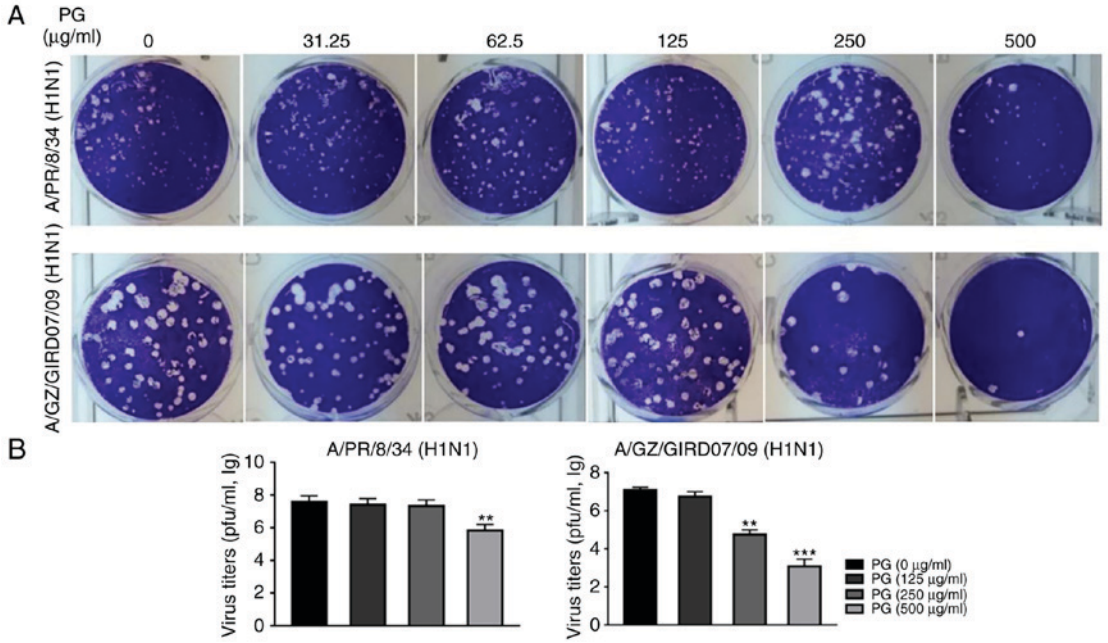

Figure 2. Antiviral effects of PG. (A) MDCK cells were infected with 40 PFU/well of influenza virus A/PR/8/34 (H1N1) and A/Guangzhou/GIRD07/09 (H1N1). Following adsorption of the virus for 2 h, the inoculum was removed, and the cells were overlaid with DMEM containing 1.5% agarose with serial dilutions of PG. After 3 days, the cells were stained and images were captured. (B) A549 cells were infected with influenza virus A/PR/8/34 (H1N1) and A/Guangzhou/GIRD07/09 (H1N1) (MOI=0.1). The indicated concentrations of PG were added to the medium. After 24 h, supernatants were collected, and virus titers were determined using a plaque assay. Data are expressed as the mean ± standard deviation. **P<0.01 and ***P<0.001, vs. untreated infected cells. PG, (+)-pinoresinol-O-β-D-glucopyranoside.

Effect of (+)-pinoresinol-O-β-D-glucopyranoside on influenza virus-induced cellular signaling. Studies have revealed that influenza A virus exploits multiple host cell signaling pathways to facilitate self-replication (23,24). It has been suggested that the pharmacological inhibition of cellular signaling may be a potential strategy for controlling viral infection (25). The results of the study indicated that (+)-pino resinol-O-β-D-glucopyranoside possesses antiviral activity against influenza A H1N1, therefore, whether the anti-H1N1 virus activity was associated with the inhibition of signaling pathways required for influenza virus infection was determined. Treatment with (+)-pinoresinol-O-β-D-glucopyran oside significantly decreased the influenza H1N1-induced activation of multiple cellular signaling pathways, including the NF-κB, p38, MAPK and AKT pathways, but not the JNK or ERK MAPK pathways (Fig. 4A and B). As these pathways may also have been activated by viral products,

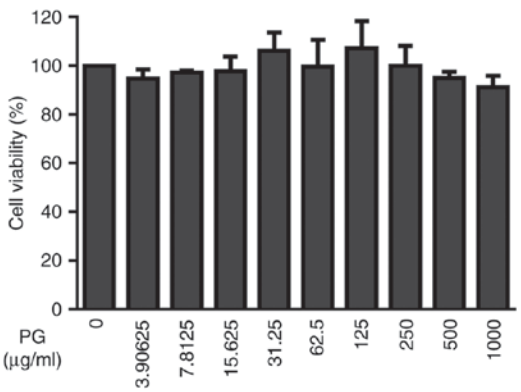

Figure 3. Effect of PG on A549 cell viability. A549 cells were treated with increasing concentrations of PG for 48 h, and cell viability was determined with an MTT assay. The percentage cell viability was calculated as the absorbance of PG-treated cells relative to the untreated cells. Data are expressed as the mean ± standard deviation. PG, (+)-pinoresinol-O-β-D-glucopyranoside.

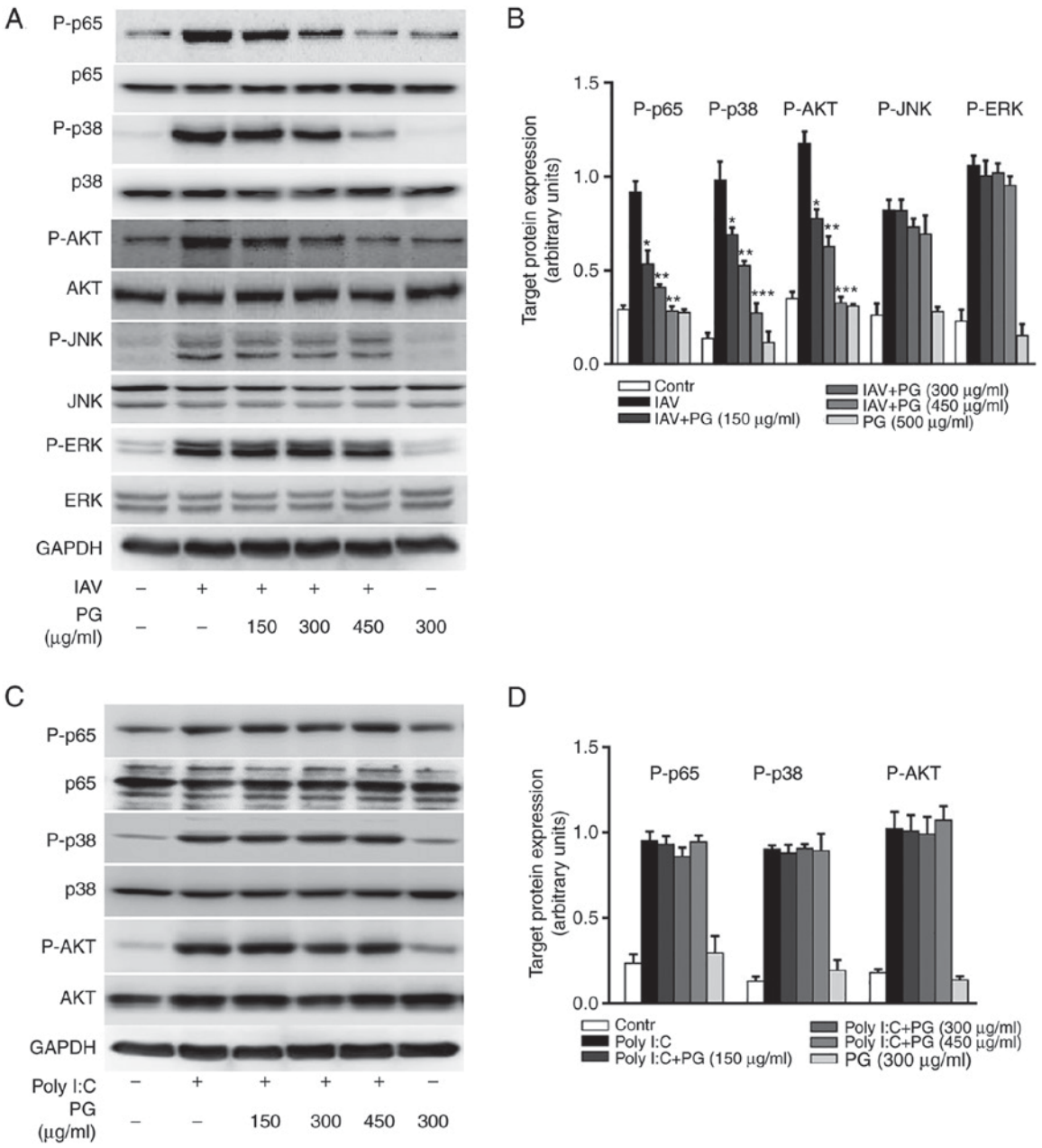

Figure 4. PG treatment inhibits IAV-induced activation of cellular signaling pathways. (A) A549 cells seeded in 6-well plates were either mock-infected or infected with influenza virus A/PR8/34 (H1N1) (MOI=0.1), and then cultured in the presence or absence of PG (150-450 μg/ml). Following cell lysis for 24 h, equal quantities of protein lysates were analyzed via western blot analysis using the indicated antibodies. (B) Quantification of indicated phosphorylated proteins. (C) A549 cells were transfected with 20 ng poly (I:C) using Lipofectamine 2000 in the presence or absence of PG (150-450 μg/ml) for 24 h. The cells were lysed and the lysates were subjected to western blot analysis. (D) Quantification of indicated phosphorylated proteins. Quantification was performed using the ImageJ software (normalized to GAPDH protein levels). Data are expressed as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01 and ***P<0.001, vs. untreated infected cells. PG, (+)-pinoresinol-O-β-D-glucopyranoside; IAV, influenza A virus; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; P-, phosphorylated.

whether (+)-pinoresinol-O-β-D-glucopyranoside affected synthetic mimics of viral RNA poly (I:C)-mediated pathway activation was investigated. As shown in Fig. 4C and D, it was found that (+)-pinoresinol-O-β-D-glucopyranoside did not affect the poly (I:C)-induced activation of NF-κB, p38 MAPK or AKT signaling. Taken together, these results suggested that (+)-pinoresinol-O-β-D-glucopyranoside inactivates multiple cellular signaling pathways triggered by viral infection, therefore exerting antivirus effects against H1N1.

Effects of (+)-pinoresinol-O-β-D-glucopyranoside on the influenza virus-induced expression of pro-inflammatory mediators. The high or low pathogenic influenza virus-induced hyperinduction of pro-inflammatory mediators was mediated though specific host cellular pathways, which are considered to affect the severity of influenza diseases (26,27). To determine whether (+)-pinore sinol-O-β-D-glucopyranoside can affect the H1N1 influenza virus-induced expression of pro-inflammatory mediators though the inhibition of cellular signaling, the present study

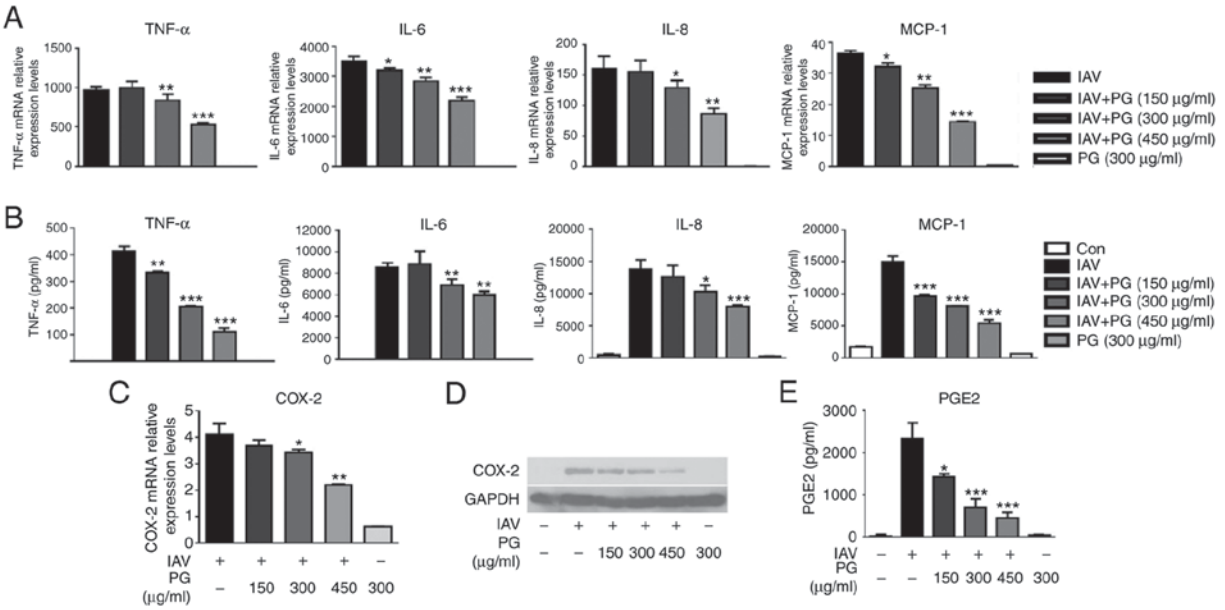

Figure 5. PG treatment reduces IAV-induced expression of pro-inflammatory mediators. (A) A549 cells were either mock-infected or infected with IAV/PR8/34 (H1N1) (MOI=0.1) in the presence or absence of PG (150-450 μg/ml), and then lysed with TRIzol reagent for 24 h. Total RNA was isolated and RT-qPCR analyses were performed to measure the gene expression of TNF-α, IL-6, IL-8 and MCP-1. (B) Culture supernatants of H1N1 virus-infected A549 cells treated with different concentrations of PG were harvested for the evaluation of cytokines and chemokines using a Luminex assay. (C) mRNA and (D) protein levels of COX-2 in H1N1 virus-infected A549 cells with treated with different concentrations of PG were analyzed via RT-qPCR and western blot analyses, respectively. (E) PGE2 levels in the culture supernatants from virus-infected A549 cells with/without PG treatment were evaluated using ELISAs. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001, vs. untreated infected cells. PG, (+)-pinoresinol-O-β-D-glucopyranoside; IAV, influenza A virus; Con, control; TNF-α, tumor necrosis factor-α; IL, interleukin; MCP, monocyte chemoattractant protein 1; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

assessed the expression of pro-inflammatory mediators at the mRNA and protein levels via RT-qPCR and Luminex assays, respectively. As shown in Fig. 5A and B, treatment with (+)-pinoresinol-O-β-D-glucopyranoside decreased the H1N1 influenza virus-induced upregulation of cytokine and chemokine expression, including that of TNF-α, IL-6, IL-8 and MCP-1, in a concentration-dependent manner. Furthermore, it was found that treatment with (+)-pinoresinol -O-β-D-glucopyranoside suppressed the H1N1 virus-induced production of COX-2 (Fig. 5C and D) and that of the derived PGE2 (Fig. 5E). These results indicated that (+)-pinoresin ol-O-β-D-glucopyranoside decreased the H1N1 influenza virus-induced expression of pro-inflammatory mediators via the inhibition of multiple signaling pathways.

Discussion

In previous decades, the increasing incidence of antiviral drug-resistant influenza viruses has highlighted the urgency for novel antiviral drugs. Compounds from Chinese herbal medicines have gained interest in the development of novel antiviral medications as they tend to possess multiple activities and a broad safety window. In the present study, a lignan compound was isolated from *Eucommia ulmoides* Oliver and its structure was subjected to extensive spectroscopic analysis; it was identified as (+)-pinoresinol-O-β-D-glucopyr anoside. Further investigation showed that the antiviral and anti-inflammatory effects of (+)-pinoresinol-O-β-D-glucopyr anoside against influenza virus infection likely occur through the inhibition of AKT, NF-κB, and p38 MAPK signaling.

Our previous study reported on the structure of a novel lignan glycoside [(+)-pinoresinol 4-O-[6'-O-vanilloyl]-β-D-glucopyranoside)] from the latex of *Calotropis gigantean*, comprised of (+)-pinoresinol-O-β-D-glucopyranoside moiety and a vanilloyl group, which possessed antiviral activity though the retention of vRNPs in the nucleus (28). Additionally, it was found that (+)-pinoresinol-O-β-D-gl ucopyranoside did not have any inhibitory effects on influenza A/PR/8/34 (H1N1) virus with an IC_{50} value >348.6 μM and SI value <1.0 (28). In the present study, the antiviral effect of (+)-pinoresinol-O-β-D-glucopyranoside was re-evaluated, and the compound was found to have antiviral activity against the influenza A/PR/8/34 (H1N1) virus with an IC_{50} value of 408.81±5.24 μg/ml (785.37±10.07 μM) (Table II), which was higher than previously reported and for that of (+)-pinoresinol 4-O-[6'-O-vanilloyl]-β-D-glucopyranoside. These results suggested that (+)-pinoresinol-O-β-D-glucopyranoside was not potent enough to exert inhibitory effects on the influenza A/PR/8/34 (H1N1) virus at low doses due to the absence of a vanilloyl moiety. The antiviral properties of (+)-pinores inol-O-β-D-glucopyranoside were confirmed by the result that treatment reduced influenza A/GZ/GIRD07/09 (H1N1) virus-induced CPE in MDCK cells (Table II).

Influenza viruses exploit multiple host cell signaling cascades to facilitate their replication. An increasing number of reports have demonstrated that the suppression of cellular signaling using pharmacological agents can limit the spread of influenza. The inhibition of NF-κB activity by acetylsalicylic acid or Bay 11-7082 inhibited influenza virus propagation via the retention of vRNP in the nucleus, and effectively reduced

viral titers *in vitro* and *in vivo* (13,29). Furthermore, the synthesis of eight segments of the viral RNA (vRNA) genome was reduced by an NF-κB inhibitor (30). Similarly, the inhibition of PI3K/AKT signaling confirmed its importance in viral processes, including viral uptake, vRNA synthesis and vRNP nuclear export (14,31). Phosphorylation of the early-endosomal protein EEA1 and anti-apoptotic factor B-cell lymphoma 2 by P38 MAPK has been reported to enhance the endocytosis of virus particles and the nucleocytoplasmic export of viral NP proteins, and this was eradicated following treatment with a P38 MAPK-specific inhibitor (15,32). Findings indicated that inhibition of the NF-κB, p38 MAPK, and AKT signaling pathways by specific inhibitors exerted antiviral activity. However, certain compounds from Chinese herbal medicines with NF-κB inhibition activity did not exhibit broad antiviral activity and the detailed mechanism was not revealed (28,33). In concordance, although the present found that the virus-induced NF-κB, p38 MAPK, and AKT signaling pathways were inhibited by (+)-pinoresino l-O-β-D-glucopyranoside, (+)-pinoresinol-O-β-D-glucopy ranoside did not exert inhibitory effects on influenza virus A/Hongkong/8/68 (H3N2), A/Hongkong/Y280/97 (H9N2) or B/Lee/1940 (FluB) (Table II). In the present study, the reasons why lignan (+)-pinoresinol-O-β-D-glucopyranoside, with its NF-κB, p38 MAPK, and AKT signaling inhibition properties, did not show broad antiviral activity were not elucidated. The results suggested that the inhibition activity of natural compounds from traditional Chinese medicine on cellular molecules was not potent enough, compared with specific inhibitors. It is anticipated that investigations in the future will elucidate the detailed mechanism. The possible underlying mechanism of (+)-pinoresinol-O-β-D-glucopyranoside against influenza infection may involve inactivation of the NF-κB, P38 MAPK and PI3K/AKT signaling pathways (Fig. 3).

The results of the present study demonstrated that (+)-pino resinol-O-β-D-glucopyranoside decreased the expression of TNF-α, IL-6, IL-8 and MCP-1 (Fig. 5A and B). During influenza virus infection, the abnormal activation of host signaling pathways leads to an excessive inflammatory response, which is considered to result in lung tissue injury and may progress to ARDS (34). In patients infected with seasonal influenza viruses, the levels of cytokines, including IL-6, TNF-α and interferon (IFN)-γ-inducible protein 10 (IP-10), were elevated on day 1 but had declined rapidly by day 5 (35). By contrast, the persistent elevation of cytokines in patients infected with avian H7N9 or H5N1 viruses resulted in poor outcomes and even mortality (27,36). Dysregulation among pro-inflammatory cytokines has served as a hallmark of influenza disease severity (37). The suppression of NF-κB signaling has been shown to decrease the influenza virus-mediated expression of IL-6, IL-8, MCP-1 and RANTES *in vitro* and *in vivo* (13). p50 subunit deficiency in mice attenuated an array of NF-κB-targeted genes induced by influenza A (H5N1) (38). P38-mediated signaling is also involved in the initiation of pro-inflammatory cytokine synthesis. Treatment with a p38 MAPK inhibitor (SB203580) reduced the H5N1 virus-mediated expression of cytokines and chemokines, including TNF-α, IP-10, MCP-1 and RANTES (39). The cytokine levels, including those of IP-10 and MCP-1, in patients with severe influenza A virus infection were positively correlated with the expression of P38 MAPK in $CD4^+$ lymphocytes (40). During viral replication, the viral products, including viral RNA sensed by host pattern recognition receptors can also activate cellular signaling and initiate the expression of pro-inflammatory cytokines. In examining whether that the anti-inflammatory effects of (+)-pinoresino l-O-β-D-glucopyranoside is due to its antiviral property or the inhibition of cellular signaling triggered by viral products, the present study found that treatment with (+)-pinoresinol-O -β-D-glucopyranoside did not affect the poly (I:C)-mediated activation of NF-κB, p38 kinase or AKT signaling (Fig. 4B). These results suggested that the anti-inflammatory effects of (+)-pinoresinol-O-β-D-glucopyranoside were a result of its antiviral effects. Therefore, it was hypothesized that the inhibitory effects of (+)-pinoresinol-O-β-D-glucopyranoside on infection-activated NF-κB and p38 kinase led to a decrease in the influenza virus-induced expression of pro-inflammatory cytokines.

Previous studies have reported that NF-κB and p38 kinase signaling are required for the expression of COX-2, which is involved in the pathogenesis of pneumococcal pneumonia and influenza H5N1 viral disease (41-43). From the data presented in the present study, the inhibitory effects on NF-κB and p38 kinase signaling by (+)-pinoresinol-O-β-D-glucopyranoside treatment were correlated with the decreased expression of COX-2 and PGE2 (Fig. 5C-E). Previous studies have demonstrated that COX-2 deficiency or inhibition significantly reduced virus-induced inflammation and changes in body temperature, and protected against life-threatening influenza challenge (44,45). Notably, the delayed combination of antiviral agents with COX-2 inhibitor treatment significantly prolonged the survival of mice infected with H5N1 (46). Additionally, Coulombe *et al* revealed that PGE2 impaired the type I IFN-mediated antiviral response (47). Therefore, it appears that suppression of the expression of COX-2 and PGE2 by (+)-pinoresinol-O-β-D-glucopyranoside is beneficial to the host during influenza infection.

In conclusion, the present study found that (+)-pinoresin ol-O-β-D-glucopyranoside from *Eucommia ulmoides* Oliver exerts antiviral and anti-inflammatory effects through NF-κB, P38 MAPK and AKT signaling pathway inhibition in influenza virus-infected cells. Therefore, it was hypothesized that the product possesses multiple biological activities and low toxicity, and that it may be a promising anti-influenza candidate drug.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding authors on reasonable request.

Author contributions

ZY, XP and ZJ conceived and designed the experiments; JL, XL, BZ, XC, PX and HJ performed the experiments; JL and XL analyzed the data; JL, XL and BZ wrote the manuscript. ZY, XP and ZJ contributed to revisions of the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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