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In vitro anti-influenza virus effect of total flavonoid from *Trollius ledebouri* Reichb

Yongping Liu[1], Jiming Tong[1], Ying Tong[2], Ping Li[1], Xiaolan Cui[3] and Hongbao Cao[4]

Abstract

Objective: To investigate the *in vitro* antivirus effect of total flavonoid from *Trollius ledebouri* Reichb (TFTLR).

Methods: Madin-Darby canine kidney (MDCK) and Human epithelial type 2 (HEp-2) cell lines were used to test the antivirus effect of TFTLR on nine virus subtypes: four H1N1, one H3N2, and four other subtypes prevalent in North China. Tamiflu, Ribavirin and Lianhua Qingwen were used as active comparators. Comprehensive molecular pathway analyses of TFTLR-H1N1 and TFTLR-H3N2 relationships were also conducted.

Results: TFTLR inhibited MDCK cell lesions induced by H1N1 subtypes (A/FM1/1/47, A/Puerto Rico/8/1934 H1N1, A1/Tianjin Jinnan/15/2009, and A/Brisbane/59/2007) and by the H3N2 Brisbane/10/2009 strain. TFTLR inhibitory concentration $(IC)_{50}$ values against these viruses were 0.13, 0.07, 0.06, 0.14, and 0.07 mg/ml, respectively; and therapeutic index (TI) values were 8.62, 16.0, 18.67, 8.0, and 16.0, respectively. TFTLR showed no effect on parainfluenza virus type 1, herpes simplex virus type 1, respiratory syncytial virus, and coxsackie group B virus type 4. Pathway analysis revealed possible functional therapeutic mechanisms for TFTLR against H1N1 and H3N2 infections.

Conclusion: TFTLR may represent a potential therapeutic agent against influenza A subtypes H1N1 and H3N2 that are prevalent in North China, and should be investigated further.

[1]Laboratory of Pharmacology and Toxicology of Key Laboratory for Research and Development of Chinese Materia Medica in Hebei Province, China; Department of Pharmacology and Toxicology, Institute of Chinese Materia Medica, Chengde Medical University, China
[2]Radiology Department, Affiliated Hospital of Chengde Medical University, Chengde, China
[3]Department of Pharmacology, Institute of Chinese Materia Medica, Chinese Academy of Chinese Medicine sciences, Beijing, China
[4]Department of Genomics Research, R&D Solutions, Elsevier Inc., Rockville, MD, USA

Corresponding author:
Jiming Tong, Institute of Chinese Materia Medica. Chengde Medical University, 129B Research Building, 1 Anyuan Road, Chende, 067000, China.
Email: j.tong@gousinfo.com

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Keywords
Total flavonoid, T. ledebouri Reichb, influenza A virus, respiratory virus, pathway analysis

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Introduction

More than 90% percent of acute respiratory tract infections are induced by respirovirus.[1] Respirovirus invades the respiratory tract to cause local lesions, or takes the respiratory tract as an invasion path to cause ailments in other tissues or organs.[2] Types of virus that invade via the respiratory route include influenza virus, parainfluenza virus, respiratory syncytial virus, measles virus, mumps virus and adenovirus.[2] Respiratory virus antigens can mutate rapidly by antigenic drifting or antigenic switching,[2] and drug-resistant virus strains are continuously detected, demonstrated by reduced or total loss of treatment effect with existing drugs. For instance, Tamiflu is a well-recognized antiviral medication used to treat influenza A,[3] however, over time, Tamiflu-resistant subtypes of influenza A have been detected.[4,5] Thus, there is ongoing demand for the development of novel drugs to treat respiratory virus infections.

In the present study, the antivirus effect of total flavonoid acquired from *Trollius ledebouri* Reichb (TFTLR) was tested on nine virus subtypes using *in vitro* cell lines. The viruses investigated comprised four influenza A H1N1 subtypes (A/FM1/1/47, A/Puerto Rico/8/1934 H1N1, A1/Tianjin Jinnan/15/2009, and A/Brisbane/59/2007), one H3N2 subtype (Brisbane/10/2009), and four other viruses: parainfluenza virus type 1 (PIV-1), herpes simplex virus type 1 (HSV-1), respiratory syncytial virus (RSV), and coxsackie group B virus type 4 (CoxB4); all of which are prevalent in North China. Like many other types of herbal medicine, TFTLR, being the main component of Chinese globeflower, is very complex in nature.[6] Detected components include orientoside, vitexin, meletin, cirsimaritin, and orientin-2-O-β-galactoside,[6] however, these are yet to be quantitatively characterised. Despite the lack of characterisation, Chinese globeflower extract has been shown to be effective as a low-cost drug, native of North China, in the treatment of acute upper respiratory tract infection diseases, including tonsillitis, pharyngitis, acute tympanitis and acute lymphangitis.[7] Chinese globeflower appears to exert it's treatment effects on influenza virus infections by inhibiting viral replication, improving the immune function of the body, and improving blood circulation.[8] In addition, previous studies suggest that flavonoids are effective in treating influenza A (H1N1)[9] and related symptoms, including inflammation, cough and fever.[10–13] Therefore, TFTLR acquired from Chinese globeflower may possess anti-respiratory virus effects that are worthy of further study.

The aims of the present study were to investigate: (A) the possible antiviral effect of TFTLR as a whole, on *in vitro* cell lines subjected to infection with nine viral subtypes; and (B) to conduct comprehensive multiple-level pathway analyses using Pathway Studio software, to study the functional associations between TFTLR and two influenza A subtypes, H1N1 and H3N2.

Materials and methods

Experimental materials

Preparation of TFTLR. The herbal medicinal material was collected from Weichang

Bashang grassland, Chengde city, China, and was identified as dry flowers of *T. ledebouri* Reichb by the Institute of Chinese Materia Medica, Chengde Medical College, China. A sample specimen of the material was deposited in the Key Laboratory of Herbal Medicine Research and Development of Hebei Province, China.

The test content (TFTLR) was determined to account for 57% of the total herbal medicinal materials (calibrated using Orientin). TFTLR solution was prepared by the Institute of Chinese Materia Medica of Chengde Medical College (product Batch No. 20090414). A 50 mg/ml stock solution of TFTLR was prepared using high purity water, which was then filter sterilized and stored at 4 °C prior to use. Stock solution was diluted as required for the experiments.

Preparation of active comparator drugs. Tamiflu (Batch No. B1162; Hoffmann-La Roche, Switzerland), Ribavirin granules (Batch No. 090112; China Medicine University Pharmaceutical Company, China), and Lianhua Qingwen capsules (Batch No. 090546; Yiling Pharmaceutical, Shijiazhuang, China) were used as active comparator positive controls. The following stock solutions of each active comparator drug were prepared with high purity water under sterile conditions: a 7.5 mg/ml stock solution of Tamiflu; a 10 mg/ml stock solution of Ribavirin; and a 35 mg/ml stock solution of Lianhua Qingwen. Stock solutions were stored at 4 °C and diluted as required prior to each experiment.

Cell lines, viruses and culture medium. Madin-Darby canine kidney (MDCK) cells were used to test the antivirus effect of the different drugs on influenza A subtypes (four H1N1 subtypes and one H3N2 subtype), and Human epithelial type 2 (HEp-2) cells were used to test the different drugs on PIV-1, HSV-1, RSV and CoxB4 viruses. These viral subtypes were tested as they are the most common disease-causing viruses prevalent in North China (Hebei Province). MDCK and HEp-2 cell lines were purchased from the Cell Centre of Basic Research Institute, Peking Union Medical University, Beijing, China, and the cells were cultured in the ABSL-2 Biological Safety Laboratory of the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, Beijing, China.

Influenza A subtypes, H1N1 (A/FM1/1/47, A/Puerto Rico/8/1934 H1N1, A1/Tianjin Jinnan/15/2009, and A/Brisbane/59/2007 strain), and H3N2 (Brisbane/10/2009 strain) were purchased from the Influenza Research Laboratory of Prevention and Control Institute for Viral Disease, China Disease Control Centre, Beijing, China, where they had been cultured using chick embryos. The 50% tissue culture infectious dose ($TCID_{50}$) values of the five viruses stated above were $10^{-4.5}$, 10^{-4}, 10^{-3}, $10^{-3.5}$, and $10^{-1.5}$, respectively. The PIV-1, HSV-1, RSV and CoxB4 viruses were purchased from the Prevention and Control Institute for Viral Disease, China Disease Control Centre, Beijing, China. The viruses were cultured in this laboratory and their $TCID_{50}$ values were 10^{-5}, $10^{-4.5}$, $10^{-6.5}$, and 10^{-6}, respectively. All viruses were stored at –80 °C prior to use.

Prior to the experiments, cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM), or Roswell Park Memorial Institute (RPMI) 1640 culture medium (Gibco, a subsidiary of Thermo Fisher Scientific, Rockford, IL, USA) containing 10% of foetal bovine serum (FBS; Tianjin Haoyang Biological Technology Company, China). Cells were cultured in DMEM with 10% FBS for all experimental procedures.

This study was approved by the Hebei Province Key Laboratory of Research and Development of Chinese Materia Medica, Institute of Chinese Materia Medica, Chengde Medical University, Chengde, China. All experiments were

conducted at the ABSL-2 Biosafety Laboratory of the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine.

Experimental methods

Toxicity of TFTLR on MDCK and HEp-2 cells. The TFTLR test solutions were prepared by diluting the stock solution 1:2 to 1:256 with DMEM. A total of 100 µl per well of TFTLR test solutions at different concentrations were added to monolayers of MDCK and HEp-2 cells that had been cultured in 24-well plates. Four wells were tested per TFTLR concentration. Normal control cells were cultured under the same conditions with 100 µl/well of DMEM without TFTLR added. Following the addition of TFTLR or control DMEM, the culture plates were incubated in a 5% CO_2 incubator at 37 °C. Cell lesions (rate of apoptotic/damaged cells) were observed daily under an inverted phase contrast microscope (Olympus, Japan) to determine the minimum concentration that did not induce cell lesions (the maximum non-toxic concentration, TC_0). The median toxic concentration (TC_{50}) was calculated with Reed-Muench assay.[14]

In vitro *antivirus effect of TFTLR.* Cells were cultured to monolayers in 24-well plates, then the culture fluid was aspirated and the cell surface was washed 3 times in DMEM maintenance fluid. MDCK cells were inoculated with a volume of 100 µl/well of each influenza virus strain at the following dilutions of original stocks: $10^{-2.5}$ of A/FM1/1/47, 10^{-2} of A/Puerto Rico/8/1934 H1N1, 10^{-2} of A1/Tianjin Jinnan/15/2009, $10^{-2.5}$ of A/Brisbane/59/2007, and 10^{-2} of Brisbane/10/2009. Hep-2 cells were inoculated with 100 µl/well of PIV-1, RSV, CoxB4, HSV-1 virus fluids at $100 \times$ concentration of the $TCID_{50}$ value. Cells were inoculated in triplicate per viral strain. Following viral inoculation, the culture plates were incubated at 37 °C for 1 h. TFTLR solution of varying concentrations (1, 1/2, 1/4, 1/8, and 1/16 of TC_0 for MDCK and Hep-2 cells) was then added into the MDCK and HEp-2 cell culture plates in volumes of 100 µl/well. Tamiflu (50 mg/ml), Lianhua Qingwen (35 mg/ml) and Ribavirin (10 mg/ml) were used as active comparators. Untreated control cells and virus inoculated positive control cells (without antiviral drug) were cultured simultaneously. All cells were cultured in a 5% CO_2 incubator at 37 °C. Cell lesions were observed daily using an inverted phase contrast microscope. When the level of cell lesions exceeded 75% in the virus positive control, test results for all cells were recorded.

Cell lesions were divided into six levels: (1) Normal growing cells with no observable lesion; (2) The rate of cells with lesions $<10\%$ of the entire cell monolayer; (3) The rate of cells with lesions $<25\%$ of the entire cell monolayer; (4) The rate of cells with lesions $<50\%$ of the entire cell monolayer; 5) The rate of cells with lesions $<75\%$ of the entire cell monolayer; and (6) The rate of cells with lesions $> 75\%$ of the entire cell monolayer. Higher rates of cell lesions equated to less effective drug treatment.

The drug concentration that inhibited the number of cytopathies by 50% (IC_{50}) was calculated using the Reed-Muench method and the therapeutic index (TI) was calculated as follows: $TI = TC_{50}/IC_{50}$. Results were not statistically analysed.

Pathway analyses. Molecular level pathway analyses were conducted to study the potential association between total flavonoid and influenza virus subtypes H1N1/H3N2, using Pathway Studio, version 11.4 (www.pathwaystudio.com). Pathway Studio has been widely used to study modelled relationships between proteins, genes, complexes, cells, tissues and diseases (http://pathwaystudio.gousinfo.com/Mendeley.html).[15] Updated

weekly, the Pathway Studio ResNet database is the largest database among known competitors in the field.[16]

Results

Cell toxicity of TFTLR on MDCK and HEp-2 cells

The TC_0 and TC_{50} of TFTLR and the three positive control drugs (Tamiflu, Ribavirin and Lianhua Qingwen) on MDCK and HEp-2 cells are presented in Table 1. To note, Tamiflu was only tested on MDCK cells and Ribavirin was only tested on HEp-2 cells.

Table 1 Toxicity of different drugs on Madin-Darby canine kidney (MDCK) and Human epithelial type 2 (HEp-2) cell lines

	MDCK cells		Hep-2 cells	
Drugs	TC_0	TC_{50}	TC_0	TC_{50}
TFTLR, mg/ml	0.78	1.12	3.13	4.46
Tamiflu, mg/ml	0.47	0.67	—	—
Ribavirin, mg/ml	—	—	2.5	3.55
Lianhua Qingwen, mg/ml	2.19	3.12	8.75	12.41

TFTLR, total flavonoid from *Trollius ledebouri* Reichb; TC_0, maximum non-toxic concentration; TC_{50}, median toxic concentration.

In vitro *anti-influenza virus effects of TFTLR*

TFTLR showed an inhibition effect on MDCK cell lesions induced by H1N1 subtypes A/FM1/1/47, A/Puerto Rico/8/1934 H1N1 strain, A1/Tianjin Jinnan/15/2009 strain, A/Brisbane/59/2007strain, and by the H3N2 subtype Brisbane/10/2009 strain. The IC_{50} values of TFTLR on these viruses were 0.13,0.07, 0.06, 0.14, and 0.07 mg/ml and TI values were 8.62, 16.0, 18.67, 8.0, and 16.0, respectively (Table 2 and 3).

Tamiflu also showed an inhibition effect on MDCK cell lesions induced by H1N1 subtypes A/FM1/1/47, A/Puerto Rico/8/1934 H1N1 strain, A1/Tianjin Jinnan/15/2009 strain and A/Brisbane/59/2007. The IC_{50} values of Tamiflu were 0.04, 0.04, 0.08, and 0.04 mg/ml, respectively. The TI values were 16.75, 16.75, 8.38, and 16.75, respectively. Tamiflu showed no inhibitory effect on MDCK cell lesions induced by the H3N2 subtype Brisbane/10/2009 strain, with IC_{50} and TI values of 0.12 mg/ml and 5.58, respectively (Table 2 and 3).

Lianhua Qingwen showed minor inhibitory effects on all tested influenza A virus stains, with IC_{50} values of 0.70, 0.78, 1.56, 0.55, and 0.78 mg/ml, respectively. TI values were 4.46, 4.00, 2.00, 5.67 and 4.00, respectively (Table 2 and 3).

Table 2. *In vitro* IC_{50} values of different drugs on Madin-Darby canine kidney (MDCK) cell lesions induced by various influenza viruses

	Drug type					
	TFTLR		Tamiflu		Lianhua Qingwen	
Virus strain	IC_{50} (mg/ml)	TI	IC_{50} (mg/ml)	TI	IC_{50} (mg/ml)	TI
A/FM1/1/47	0.13	8.62	0.04	16.75	0.70	4.46
A/Puerto Rico/8/1934 H1N1	0.07	16.00	0.04	16.75	0.78	4.00
A1/Tianjin Jinnan/15/2009	0.06	18.67	0.08	8.38	1.56	2.00
A/Brisbane/59/2007	0.14	8.00	0.04	16.75	0.55	5.67
Brisbane/10/2009	0.07	16.00	0.12	5.58	0.78	4.00

TFTLR, total flavonoid from *Trollius ledebouri* Reichb; IC_{50}, drug concentration that inhibited the number of cytopathies by 50%; TI, therapeutic index.

Table 3. *In vitro* antivirus effects (lesion score) of different drugs on Madin-Darby canine kidney (MDCK) cell lesions induced by influenza viruses

		Virus strain				
Treatment group	Drug concentration, mg/ml	A/FM1/1/47	A/Puerto Rico/8/1934 H1N1	A1/Tianjin Jinnan/15/2009	A/Brisbane/59/2007	Brisbane/10/2009
TFTLR	0.78	1111	1111	1111	1111	1111
	0.39	1222	1111	1111	2121	1111
	0.20	3222	2222	1111	3333	2112
	0.10	4443	3333	2222	4444	2233
	0.05	5555	4444	3444	5555	4444
Tamiflu	0.47	1111	1111	1112	1111	1111
	0.23	1111	2211	3322	1111	2222
	0.12	1111	2222	3333	2222	3344
	0.06	3333	3333	4445	3333	5555
	0.03	4444	5555	6666	4444	6666
Lianhua Qingwen	2.19	1122	2222	3333	1111	22222
	1.10	3333	3333	5555	1221	3333
	0.55	3444	4444	6666	3344	4444
	0.27	4555	6666	6666	4455	6666
	0.14	5566	6666	6666	6666	6666
Normal Cell control	—	1111	1111	1111	1111	1111
Virus control	—	6666	6666	6666	6666	6666

Data presented as lesion scores from four repeated experiments.
Lesion scores were divided into 6 levels: (1) Normal growing cells with no observable lesion; (2) The ratio of cells with lesions <10% of the entire cell monolayer; (3) The ratio of cells with lesions <25% of the entire cell monolayer; (4) The ratio of cells with lesions <50% of the entire cell monolayer; 5) The ratio of cells with lesions <75% of the entire cell monolayer; and (6) The ratio of cells with lesions >75% of the entire cell monolayer. Higher lesion scores equate to less effective drug treatment.
TFTLR, total flavonoid from *Trollius ledebouri* Reichb.

In vitro *antiviral effects of TFTLR using HEp-2 cells*

TFTLR showed no inhibitory effect on HEp-2 cell lesions induced by PIV-1, HSV-1, RSV and CoxB4 viruses. Ribavirin showed a strong inhibitory effect on HEp-2 cell lesions induced by all four virus subtypes, with IC_{50} values of 0.11, 0.22, 0.22, and 0.22 mg/ml, respectively. The TI values for Ribavirin were 32.27, 16.14, 16.14, and 16.14, respectively. Lianhua Qingwen showed minor inhibitory effect on HEp-2 cell lesion induced by RSV, CoxB4, with IC_{50} values of 3.12 and 6.23 mg/ml, and TI values of 3.98 and 1.99, respectively. Lianhua Qingwen showed minor inhibitory effect on HEp-2 cell lesion induced by PIV-1 and HSV-1 (Table 4).

Pathway analyses

Since TFTLR demonstrated strong antivirus effects for both H1N1 and H3N2 subtypes, comprehensive pathway analyses were performed to study the possible association between total flavonoid and both H1N1 and H3N2 at the molecular level. Pathway analyses showed that flavonoid may be linked to both H1N1 and H3N2 through multiple pathways, as shown in Figure 1. Different flavonoids have been

Table 4. *In vitro* antivirus effects (lesion score) of different drugs on Human epithelial type 2 (HEp-2) cell lesion induced by PIV-1, HSV-1, RSV and CoxB4 viruses

Treatment group	Concentration, mg/ml	Virus strain			
		PIV-1	HSV-1	RSV	CoxB4
TFTLR	3.13	4444	5555	5555	6666
	1.56	5555	6666	5555	6666
	0.78	6666	6666	6666	6666
	0.39	6666	6666	6666	6666
	0.20	6666	6666	6666	6666
Ribavirin	2.50	1111	2222	2222	2222
	1.25	1111	2222	2222	2222
	0.63	1111	2222	2222	2222
	0.32	1111	3333	3333	3333
	0.16	1111	4444	4444	4444
Lianhua Qingwen	8.75	6666	6666	2222	3333
	4.38	6666	6666	3333	4444
	2.19	6666	6666	5555	5555
	1.10	6666	6666	6666	6666
	0.55	6666	6666	6666	6666
Normal cell control	—	1111	1111	1111	1111
Virus control	—	6666	6666	6666	6666

Data presented as lesion scores from four repeated experiments.
Lesion scores were divided into 6 levels: (1) Normal growing cells with no observable lesion; (2) The ratio of cells with lesions <10% of the entire cell monolayer; (3) The ratio of cells with lesions <25% of the entire cell monolayer; (4) The ratio of cells with lesions <50% of the entire cell monolayer; 5) The ratio of cells with lesions <75% of the entire cell monolayer; and (6) The ratio of cells with lesions >75% of the entire cell monolayer. Higher lesion scores equate to less effective drug treatment.
TFTLR, total flavonoid from *Trollius ledebouri* Reichb; PIV-1, parainfluenza virus type 1; HSV-1, herpes simplex virus type 1; RSV, respiratory syncytial virus; and CoxB4, coxsackie group B virus type 4.

observed to inhibit influenza A virus replication,[17] possibly through the flavonoids inhibiting viral reproduction and stimulating autophagy,[18] which in turn may mediate antiviral immunity.[19] Moreover, flavonoids may attenuate influenza A related symptoms, such as fever, cough, inflammation and immune response, possibly through the inhibition of important regulatory enzymes involved in arachidonic acid metabolism.[20]

Pathway analysis also showed that the anti-influenza A virus effect of flavonoid may involve mechanisms at the genetic level. As shown in Figure 1, flavonoids induce the activation of nuclear factor, erythroid 2 like 2 (NFE2L2),[21] while NFE2L2 activation can reduce influenza A H3N2.[22] In addition, flavonoids can significantly decrease the gene expression of interleukin (*IL*)-6 and tumour necrosis factor (*TNF*)-α,[23] while IL-6 and TNF-α are biomarkers of influenza A H1N1 infections.[24] This may also partly explain why flavonoids can inhibit influenza A-related symptoms, including fever, cough and inflammation, all of which are related to the activity of IL-6 and TNF-α.[11]

Supporting references for each relationship in Figure 1 are available for download via the following weblink: (http://gousinfo.com/database/Data_Genetic/Network_sup

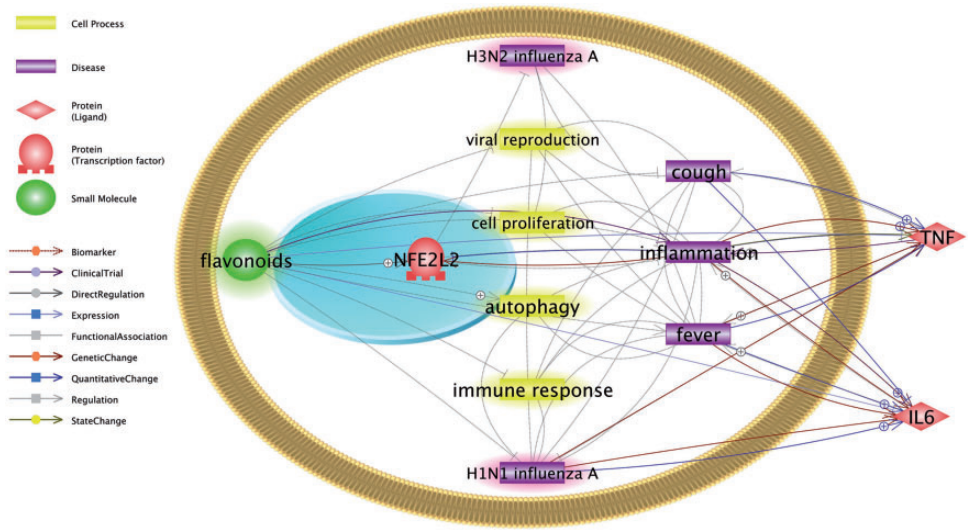

Figure 1 Pathway analyses of the potential associations between flavonoids and influenza A subtypes, H3N2 and H1N1. The pathway was generated using Elsevier Inc. software, Pathway Studio, version 11.4 (http://www.pathwaystudio.com)

porting_info_Flavonoids.xlsx). This weblink includes the relationship type, titles, and related sentences where a relationship has been identified. This information can be used to identify the detailed descriptions regarding how flavonoids may contribute to the treatment of influenza A virus infection.

Discussion

Trollius ledebouri Reichb, of the Ranunculaceae family, is one type of Chinese globeflower used for the treatment of acute upper respiratory tract infections, tonsillitis, pharyngitis, acute tympanitis, and acute lymphangitis.[7] Total flavonoid is the natural product and the main effect fraction of *T. ledebouri* Reichb, and its components include orientoside, vitexin, meletin, cirsimaritin, and orientin-2-O-β-galactoside.[6] Previous studies have shown that TFTL and TFTLR have significant antibacterial, anti-inflammatory, antipyretic and antiviral effects.[12,13] Due to the complexity of quantitatively measuring the sub-components of TFTLR, the current study focused on testing the antiviral effect of TFTLR as a whole.

To better evaluate the antiviral effect of TFTLR, the present study tested the *in vitro* antivirus effect of TFTLR with 1, 1/2, 1/4, 1/8 and 1/16 times the TC_0 value of TFTLR on MDCK and HEp-2 cell lines. Tamiflu, Ribavirin and Lianhua Qingwen were used as active comparator positive control drugs. TFTLR was shown to inhibit influenza A subtypes, including A/FM1/1/47, A/Puerto Rico/8/1934 H1N1, A1/Tianjin Jinnan/15/2009, A/Brisbane/59/2007 and Brisbane/10/2009. The IC_{50} values were 0.13, 0.07, 0.06, 0.14 and 0.07 mg/ml, respectively. With concentrations of 1 or 1/2 times the TC_0 value, the anti-influenza virus effects of TFTLR were shown to be similar to that of Tamiflu. No inhibitory effect by TFTLR was observed on HEp-2 cell lesions induced by the PIV-1, HSV-1, RSV and CoxB4 viruses.

Molecular pathway analyses supported the present experimental results and showed that there were multiple functional

pathways that connect flavonoid and the influenza A subtypes H1N1 and H3N2 (Figure 1). For example, a flavonoids–NFE2L2–H3N2 pathway was revealed, in which flavonoids induce the activity of NFE2L2,[25] and NFE2L2 activation can reduce the activity of H3N2.[22] A flavonoids–IL-6–H1N1 pathway was also revealed, in which flavonoids inhibit the production of IL-6 (pro-inflammatory cytokines),[26] while IL-6 plays a role in the development of H1N1 infection.[27] These may partially explain the anti-H1N1 effect of flavonoids.[10]

Plant flavonoids have also been reported to attenuate the immune response and interfere with viral reproduction,[17] which adds background evidence to the findings that flavonoids can inhibit influenza A related symptoms including fever, cough and inflammation.[11,28]

The results of the present study are limited by several factors. First, the results regarding the therapeutic effects of TFTLR were not statistically analysed. Although TFTLR appeared to demonstrate effectiveness on H1N1 subtypes and the H3N2 subtype in MDCK cells, the results of the study need to be investigated further to elucidate the antiviral properties of flavonoids using drugs tests, or via complementary treatments using TFTLR, in humans and human cell types *in vitro*. Further, the present study employed cell line experimental data and literature-based pathway analysis to study the anti-influenza virus effect of TFTLR. Another effective approach for further investigation would be to use, e.g., gene expression data, to analyse the TFTLR treatment effect on anti-influenza virus. Finally, the present study tested the treatment effect of TFTLR as a whole extract, however, TFTLR comprises multiple chemical components. Thus, the antivirus function of the subcomponents of TFTLR should be studied to determine which are the active components in TFTLR, in order to facilitate the understanding of its treatment mechanisms.

In conclusion, both *in vitro* cell-line experiments and pathway analysis supported the effectiveness of TFTLR in treating influenza A subtypes H1N1 and H3N2 infections that are prevalent in North China. With relatively low cost, the results of the present study suggest that TFTLR from *T. ledebouri* Reichb may possess therapeutic potential for the treatment of influenza A versus infections. The results of the present study also suggest that TFTLR may not be an effective drug candidate for treating infections caused by PIV-1, HSV-1, RSV and CoxB4 viruses. To the best of the present authors' knowledge, this is the first study to test the antivirus effect of TFTLR extracted from Chinese globeflower, which is native to Northeast China. Considering the component complexity of TFTLR, the authors suggest that further investigations are required to evaluate the antivirus effect of TFTLR on respiratory viruses that are prevalent in North China.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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