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Antiviral activity of *Isatidis Radix* derived glucosinolate isomers and their breakdown products against influenza A *in vitro/ovo* and mechanism of action



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ABSTRACT

Ethnopharmacological relevance: *Isatidis Radix*, the sun-dried roots of *Isatis indigotica* Fortune ex Lindl., is one of the most usually used traditional Chinese medicines. For centuries, the herb has been employed in clinical practice for treatment of virus infection and inflammation. However, its active ingredients remain unclear.

Aim of the study: In the present study, the anti-influenza virus activity of epiprogoitrin, progoitrin, epigoitrin and goitrin, the *Isatidis Radix* derived glucosinolate isomers and their breakdown products, was firstly evaluated *in vitro* and *in ovo* and their mechanism of action was investigated.

Materials and methods: Epiprogoitrin, progoitrin, epigoitrin and goitrin were isolated from *Isatidis Radix* by chiral separation. *In vitro* and *in ovo* evaluations were performed on Madin-Darby canine kidney (MDCK) cells and embryonated eggs respectively, both using protocols including prevention, treatment and virus neutralization. Hemagglutination (HA) and neuraminidase (NA) inhibition assays were performed for further understanding of the antiviral mechanism.

Results: *Isatidis Radix* derived glucosinolate isomers and their breakdown products all exhibited dose-dependent inhibition effect against influenza A virus (H1N1) without toxicity. The antiviral potency of the components was in the order of progoitrin > goitrin > epigoitrin > epiprogoitrin. The attachment of the constituents to the viral envelope conducted to the mechanism of their antiviral action without disturbing viral adsorption or budding.

Conclusion: Taken together, these results are promising for further development of *Isatidis Radix* and may contribute an adjunct to pharmacotherapy for influenza virus infection.

1. Introduction

Influenza viruses are enveloped RNA viruses which can cause morbidity or even mortality in humans by seasonal or pandemic infections. Annually, influenza epidemics lead to 200–500,000 fatal cases globally (Courtin et al., 2017). Currently, therapeutic strategies to lower the mortality rates mainly rely on vaccines and chemical drugs. But usage of vaccines has been often challenged by frequent mutation of the antigen and narrow spectrum (Tao et al., 2017; Zarubaev et al., 2015). On the other side, viral resistance and adverse effects restrict the clinic application of currently available anti-influenza virus drugs including neuraminidase inhibitors, M2 channel blockers and RNA synthesis inhibitors (Hayden and De Jong, 2011; Samson et al., 2013; Correia et al., 2015). Hence, alternative drugs with low toxicity, broad

spectrum and less resistance remain to be developed.

Ethnic medicines are valuable heritage and commercially important resources in the prevention and treatment of viral infectious diseases, a growing number of which are accumulating evidence of experimental and/or clinical efficacy (Li and Peng, 2013; Lia et al., 2019). Among them, *Isatidis Radix* (“Ban-Lan-Gen” in Chinese, the sun-dried root of *Isatis indigotica* Fortune ex Lindl.) is well-known for its antiviral activity. In ethnic medical practice, *Isatidis Radix* has been prescribed for treating fever, pestilence, sore throat, seasonal toxin, papule caused by warm toxin, macula, scarlatina, mumps, swelling abscess, erysipelas and erysipelas facialis (Chinese Pharmacopoeia Commission, 2015). As officially approved medicinal material and products in China, *Isatidis Radix* and its formulations have been used as the major herbal remedies in prevention and treatment against a wide range of viral infections,

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Abbreviations

AAF	amnio-allantoic fluid
CCK8	Cell Counting Kit-8
CPE	cytopathic effect
CRBCs	chicken red blood cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EID ₅₀	50% egg infective dose
FBS	fetal bovine serum
GAP	good agricultural practice
HA	hemagglutination
HAI	hemagglutination inhibition
HPLC	high performance liquid chromatography

IC ₅₀	50% inhibition concentration
MDCK	Madin-Darby canine kidney
MS	mass spectroscopy
NA	neuraminidase
NMR	nuclear magnetic resonance
NPLC	normal phase liquid chromatography
PBS	phosphate buffered saline
RNA	ribonucleic acid
SARS	severe acute respiratory syndrome
SFC	supercritical fluid chromatography
SPF	specific pathogen free
TCID ₅₀	50% tissue culture infective dose
TPCK	N-p-Tosyl-L-phenylalanine chloromethyl ketone

such as seasonal epidemics, severe acute respiratory syndrome (SARS) in 2003 and H1N1 flu outbreaks in 2009 (Lin et al., 2005; Deng et al., 2013). Regardless of the various subtypes of viruses and their rapid mutations, *Isatidis Radix* was found to be effective in clinic against infections caused by influenza viruses with different strains and subtypes (Ke et al., 2012). Notwithstanding the anti-influenza efficacy of *Isatidis Radix* and its preparations is well acknowledged, their active constituents and pharmacological actions have not been elucidated. So far, a few antiviral components have been identified in *Isatidis Radix*, including (3H)-quinazolinone (Li et al., 2013), glycoprotein (Yamada, 1999), epigoitrin, 2,4(1H, 3H)-quinazolinone (Xu et al., 2005),

indirubin (Mak et al., 2004), fructopyrano-(1 → 4)-glucopyranose (Zhang et al., 2013), clemastanin B (Yang et al., 2013), lariciresinol-4-O-β-D-glucopyranoside (Li et al., 2015), polysaccharides (Li et al., 2017), isatithioetherin B and isatithioetherin D (Guo et al., 2018). Among these, the antiviral monomer epigoitrin has received much attention and it was chosen as the marker of antiviral efficacy in *Isatidis Radix* when drafting the 2010 edition of the Chinese Pharmacopoeia.

In previous study of the authors, the so-called 'epigoitrin' used in former reports (Xu et al., 2005; Ye et al., 2011; Zhang et al., 2013) was found to be mixtures of unequal amounts of epigoitrin ((R)-goitrin, (R)-5-vinyloxazolidine-2-thione) and goitrin ((S)-goitrin, (S)-5-

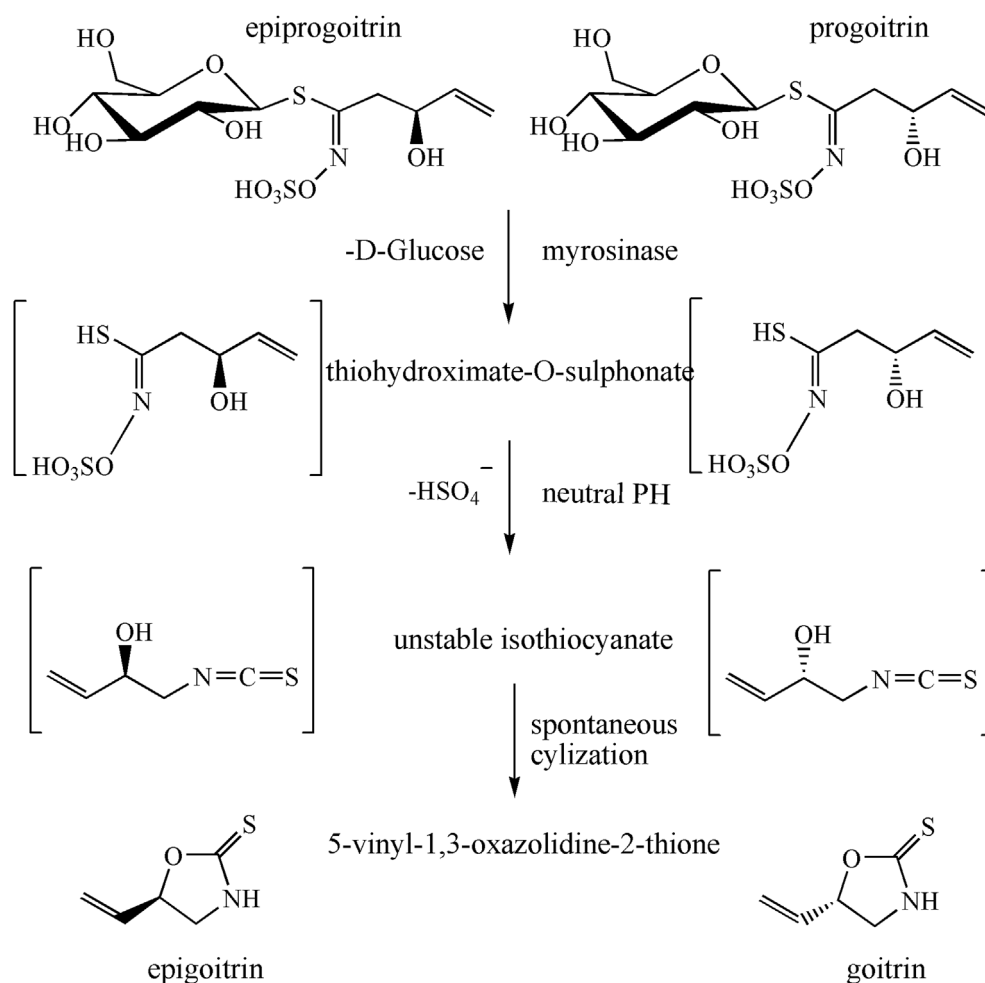


Fig. 1. Breakdown pathway of epiprogoitrin and progoitrin to epigoitrin and goitrin, respectively.

vinylloxazolidine-2-thione) in fact, and the isomers could not be separated by regular C₁₈ column. Consequently, the assay marker for *Isatidis Radix* was changed to (R, S)-goitrin in 2010 and 2015 editions of the Chinese Pharmacopoeia. In addition, (R, S)-goitrin was first resolved by the authors (Nie et al., 2010) using normal phase liquid chromatography (NPLC). Later, novel supercritical fluid chromatography (SFC) and high performance liquid chromatography (HPLC) methods were developed for high-throughput chiral separation and stereospecific assay, respectively (Nie et al., 2016, 2017). On the other hand, epigoitrin and goitrin were verified as breakdown products of their glucosinolate prototypes, epiprogoitrin ((R)-hydroxy-3-butenylglucosinolate) and progoitrin ((S)-hydroxy-3-butenylglucosinolate), on the effect of myrosinase (Fig. 1) (Nie et al., 2011; Xie et al., 2011). As well as being isomers, pure enantiomeric forms of epiprogoitrin and progoitrin were extracted and resolved by the authors from *Isatidis Radix*. In spite of the chemical structures and the biotransformation of the glucosinolates and their breakdown products are illuminated (Xie et al., 2011), the antiviral activity of the monomers and their mode of action remain elusive.

The current study aims to go further into the comparative investigation of the anti-influenza activity of the individual glucosinolates and their breakdown products *in vitro* and *in ovo*. Moreover, mechanistic studies were conducted to find their antiviral target and mode of action.

2. Materials and methods

2.1. Plant material and preparation of epiprogoitrin, progoitrin, epigoitrin and goitrin

Isatidis Radix ("Ban-Lan-Gen" in Chinese and "Isatidis root" in English) is the sun-dried root of *Isatis indigotica* Fortune ex Lindl. The scientific name of the plant has been checked with <http://www.theplantlist.org> on 26, August 2019. The plant material, grown in good agricultural practice (GAP) base in Yutian, He-Bei Province, was obtained from Beijing Tongrentang Co., Ltd. The origin was authenticated by Associate Professor Shuai Kang, Curator of the Traditional Chinese Medicine Herbarium of National Institutes for Food and Drug Control, using macroscopic and microscopic analysis according to the Chinese pharmacopoeia (Chinese Pharmacopoeia Commission, 2015). For future reference, its specimen (No. B-1) was deposited at the Traditional Chinese Medicine Herbarium, National Institutes of Food and Drug Control (Beijing, PR China). Epiprogoitrin and progoitrin were extracted and isolated from *Isatidis Radix* based on a similar strategy from a previous report (Xie et al., 2011). First, the pulverized *Isatidis Radix* was extracted with methanol; then the extraction was deproteinized with ammonium acetate and purified with granular activated carbon to obtain the crude extracts of glucosinolates; and, finally, epiprogoitrin and progoitrin were separated from the crude extracts by preparative ion-pair chromatography. Epigoitrin and goitrin were separated from (R, S)-goitrin reference standard (National Institutes for Food and Drug Control, Lot No. 111753–201706) by SFC chiral resolution on a Chiralpak IC column with acetonitrile and carbon dioxide as the mobile phase (Nie et al., 2016). The structures of the purified compound were confirmed by ¹H and ¹³C NMR (Bruker DRX-500), MS (Waters SYNAPT G2) and optical rotation (Rudolph Autopol IV). The purities of the prepared compounds were all determined to be > 98% by HPLC (Xie et al., 2011) and chiral SFC (Nie et al., 2016) methods. Contents of epiprogoitrin, progoitrin, epigoitrin and goitrin in the plant material were determined to be 0.19%, 0.10%, 0.10% and 0.05% respectively by HPLC methods (Nie et al., 2017; Xie et al., 2011). Epiprogoitrin, progoitrin, epigoitrin and goitrin were dissolved in 1 mL of dimethyl sulfoxide (DMSO) and diluted with sodium chloride injection to get test solutions at concentrations. The solutions were kept at 4 °C in a sterilized container.

2.2. Virus, cell and animal

Influenza virus strain A/California/7/2009 (H1N1) NYMC X-179A was from Shanghai Institute of Biological Products Co., Ltd. Continuous Madin-Darby canine kidney (MDCK) cells were maintained in fetal bovine serum (FBS) free Dulbecco's Modified Eagle Medium (DMEM, GIBCO) with TPCK-treated trypsin (8 µg/mL). Ten-day-old specific pathogen free (SPF) embryonated chicken eggs were from Beijing Merial Laboratory Animal Technology Co., Ltd. The clean eggs were fertilized and incubated at 37.2 ± 0.1 °C and 60 ± 5% relative humidity and candled every day to eliminate infertile eggs and dead embryos.

2.3. Reagents

Peramivir (Guangzhou Nanxin Pharmaceutical Co., Ltd.) was used as the reference compound. The reagents used in the study were as follows: sodium chloride injection (Shijiazhuang Siyao Co., Ltd.), phosphate buffered saline (PBS, Hyclone), dimethyl sulfoxide (DMSO, Sigma), Alsever's solution (pH 6.1) an isotonic solution of 2.05% glucose, 0.8% trisodium citrate, 0.42% sodium chloride and 0.055% citric acid in double-distilled water. 0.6% suspension of Leghorn chicken red blood cells (CRBCs) was prepared with equal volume of chicken (male) blood and Alsever's solution (pH 6.1). Then the mixture was washed with PBS for 3 times. The supernatant was discarded and an appropriate amount of blood erythrocytes was diluted with PBS to prepare 0.6% erythrocyte suspension. Cell Counting Kit-8 (CCK8) was from Dojindo. Neuraminidase Inhibitors Screen Kit was from Beyotime Institute of Biotechnology Co., Ltd., which contains 1 mL of neuraminidase, 10 mL of buffer, 1 mL of fluorescent substrate and 1.2 mL of water. Ammonium acetate, granular activated carbon, acetonitrile, glucose, sodium chloride, trisodium citrate and citric acid were from Sinopharm Chemical Reagent Co., Ltd. Carbon dioxide was from Chengweifeng Inc. De-ionized water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.4. Determination of cytotoxicity

Cytotoxic effect of epiprogoitrin, progoitrin, epigoitrin and goitrin was examined by Cell Counting Kit-8 (CCK8) method. MDCK cells were cultured in a 96-well plate (3 × 10³ cells/well) for 24 h. The culture supernatant was replaced with solutions containing each of the four components at various concentrations (10⁻³–10⁻⁷ mol/L). Six duplicates were used for each concentration. After incubation for 72 h, the culture supernatant was discarded, and 190 µL of DMEM and 10 µL of CCK8 were added to each well. The plates were incubated at 37 °C for 3 h. The fluorescence intensity was recorded by a microplate spectrophotometer (Gemini EM, Molecular Device, USA) at 450 nm. The cell survival rate was calculated, representing the cell viability.

2.5. Cytopathic effect (CPE) inhibition assay

MDCK cells were inoculated with influenza virus A/California/7/2009 (H1N1). The 50% tissue culture infective dose (TCID₅₀) determined according to the Reed-Muench method (Reed, 1938) was 10⁻⁵. Assay of antiviral activity of epiprogoitrin, progoitrin, epigoitrin and goitrin *in vitro* were performed using the CPE and CCK8 methods. MDCK cells were grown in a 96-well culture plate (1 × 10⁴ cells/well) for 24 h. To identify the probably affected viral life cycle, cells were treated with 100 µL of virus at 100 TCID₅₀ and 100 µL of sample solutions at various concentrations (10⁻³ mol/L–10⁻⁷ mol/L) using three different protocols including prevention, treatment and virus neutralization. For protocol 1 (prevention), cells were pre-incubated at 37 °C with sample solutions for 2 h before viral adsorption. Then the cells were washed with PBS and inoculated with virus for 2 h without the compounds and further grown for 72 h. For protocol 2 (treatment),

cells were first inoculated at 37 °C with the virus for 2 h, then washed with PBS and cultured with the compounds for 72 h. For protocol 3 (virus neutralization), sample solutions were mixed with the virus and incubated at room temperature for 30 min, and the mixtures were added to cells and cultured for 72 h. After 3 days' incubation, the solution was discarded and the cells were washed with PBS, then 190 µL of DMEM and 10 µL of CCK8 were added to each well. The plates were incubated in the dark for 3 h at 37 °C. The absorbance was read at 450/630 nm by a microplate spectrophotometer (Gemini EM, Molecular Device, USA). Six duplicates were used for each dilution ratio. Cell controls with/without the sample solutions and virus controls were included. The inhibition ratio was calculated using the following formula: Inhibition activity (%) = (OD_{sample} – OD_{virus})/(OD_{cellular control} – OD_{virus}) × 100%, where OD_{sample} is the optical density of the tested sample at a certain concentration, OD_{virus} is the optical density of the influenza virus control, and OD_{cellular control} is the optical density of normal cells. The 50% inhibition concentration (IC₅₀) was determined by linear extrapolation of the results from serious doses tested.

2.6. Assessment of antiviral potency in embryonated eggs

2.6.1. Virus titration

The 10 day embryonated eggs were inoculated with influenza virus strain A/California/7/2009 (H1N1). The 50% egg infective dose (EID₅₀) calculated using the Reed-Muench method (Reed, 1938) was 10⁻⁹. Likewise, the *in ovo* evaluation was performed using three different protocols. First, the eggs were pre-treated with 0.2 mL of sample solution and incubated at 35 °C for 2 h before administration of 0.1 mL of virus (50 EID₅₀). Second, the eggs were inoculated with 0.1 mL of virus (50 EID₅₀) at 35 °C for 2 h before administration of 0.2 mL of sample solution. Third, 0.1 mL of virus (50 EID₅₀) and 0.2 mL of sample solution were incubated for 30 min at room temperature. Then the mixture was administered to the eggs and incubated at 35 °C for 2 h. Before harvesting, all the eggs were stored at 35 °C for 50 h to facilitate the harvest procedure. The eggs were decapped and the allantoic membrane was carefully moved to sides to pipette out the upper allantoic fluid. After harvesting, the allantoic fluid was subjected to hemagglutination (HA) test.

2.6.2. Hemagglutination (HA) test

A U-bottom 96 well microtiter plate was labeled horizontally 1:2 to 1:128 dilutions. Using a multi-channel pipette, 50 µL of PBS was added to all the wells. 50 µL of allantoic fluid was added to the first well of each row and only PBS is added to the last row. Two-fold dilutions were made by transferring 50 µL from the first well of each column. This was done until the last column and the remaining 50 µL was discarded. 50 µL of 0.6% Leghorn chicken red blood cells (CRBCs) were added to all the wells and were mixed by manual agitation to the plates thoroughly. The plates were incubated for 60 min at room temperature. If the CRBCs formed a button or a ring at the bottom of the wells, it was recorded as negative using a “-” symbol. If hemagglutination occurred, CRBCs were agglutinated and immobilized, it was considered positive and recorded using a “+” symbol. The highest dilution of virus that caused complete hemagglutination was considered as the end-point in HA titration. The results were recorded according to the HA effect of the well at 1:16 dilution. Eggs that still alive were scored as survival and eggs showing no HA activity were scored as negative.

2.6.3. Experiment design

Epiprogoitrin, progoitrin, epigoitrin and goitrin were assessed at various concentrations at four dose levels (0.625, 1.25, 2.5 and 5 mg/mL) in different experiments with virus concentration of 50 EID₅₀. 3 mg/mL of peramivir was used as the positive control and sodium chloride injection was used as the negative control. Equal dose of virus and DMSO were set as virus control and dilute control, respectively.

Sham-operated group was performed as well. 8 eggs were inoculated in each group.

2.7. Hemagglutination inhibition (HAI) assay

To elucidate the mode of their antiviral action, the inhibition effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on two glycoproteins of the virus were evaluated. First, HAI assay was conducted to investigate the inhibition effect of the components on hemagglutinin.

For hemagglutination (HA) titration, six embryonated chicken eggs were inoculated with 0.1 mL of influenza virus strain A/California/7/2009 (H1N1) (50 EID₅₀) and incubated at 35 °C for 4 days. The amniotic fluid (AAF) was harvested from the live embryos and diluted with PBS from 2⁰ to 2¹¹ for virus replication by the HA assay. Each diluted viral solution was mixed with an equal volume (25 µL) of 0.6% suspension of Leghorn chicken red blood cells (CRBCs) and incubated at 4 °C 25 µL of undiluted virus and PBS were used as the positive and the negative control, respectively. After 60 min, hemagglutination titer was determined. The highest dilution to cause complete agglutination in the HA assay was 128, which was labeled as the hemagglutination titer of the virus.

For the hemagglutination inhibition (HAI) assay, 4 times of the hemagglutination titer (1:32) was used as the standardized hemagglutinin viral solution. 1:1 to 1:128 dilutions of the 5 mg/mL stock solutions of epiprogoitrin, progoitrin, epigoitrin and goitrin were prepared with PBS, respectively. Equal volumes (25 µL) of the standardized hemagglutinin viral solution and the sample solutions were mixed gently and incubated at room temperature for 30 min. 25 µL of the standardized hemagglutinin viral solution and PBS were used as the positive and the negative control, respectively. Then, 25 µL of 0.6% suspension of CRBCs was added to the mixtures and incubated at 4 °C for 60 min. Finally, hemagglutination inhibition effect of the components was observed. The experiment was performed in duplicate for each dilution ratio.

2.8. Neuraminidase inhibition (NA) assay

To evaluate the inhibition effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on neuraminidase, the NA inhibition assay was performed in a 96-well microplate reader using the Neuraminidase Inhibitors Screen Kit. For each well, 70 µL of the reaction buffer, 10 µL of NA and 10 µL of sample solution were added. The reaction mixture was vibrated for 1 min and incubated at 37 °C for 2 min for full interaction. Then 10 µL of the fluorescent substrate was added and the entire mixture was vibrated for 1 min and incubated at 37 °C for 1 h. Epiprogoitrin, progoitrin, epigoitrin and goitrin were diluted with water to prepare sample solution at different concentrations (0.625, 1.25, 2.5 and 5 mg/mL). Peramivir at different concentrations (0.375, 0.75, 1.5 and 3 mg/mL) were used as the positive control and water was used as the negative control. Triplicates were used for each dilution ratio. The fluorescence was recorded by a microplate spectrophotometer (Gemini EM, Molecular Device, USA) and the excitation wavelength and the emission wavelength were set at 322 nm and 450 nm, respectively. The inhibition rate (%) was calculated as follows: NA inhibitory activity (%) = [1 – (F_s – F₀)/(F_m – F₀)] × 100%, where F_s is the fluorescence intensity of the sample, F₀ is the absorbance of the background, and F_m is the absorbance of the negative control.

2.9. Statistical analysis

All experiments were carried out in triplicate and the data are presented as means ± standard deviations. The values of TCID₅₀, EID₅₀ and IC₅₀ were calculated using SPSS 16.0 software.

3. Results

3.1. Cellular toxicity of epiprogoitrin, progoitrin, epigoitrin and goitrin

The results of the cytotoxicity test indicated that epiprogoitrin, progoitrin, epigoitrin and goitrin at all investigated concentrations showed no influence on cell viability. The maximum non-toxic dose of *Isatis Radix* derived glucosinolate isomers and their breakdown products on MDCK cells was 10^{-3} mol/L and the CPE assay was performed at this dose and below.

3.2. Anti-influenza activity of epiprogoitrin, progoitrin, epigoitrin and goitrin in vitro

Epiprogoitrin, progoitrin, epigoitrin and goitrin were evaluated for their antiviral activity against the influenza virus strain A/California/7/2009 (H1N1) on MDCK cells via cytopathic effect (CPE) inhibition assay. The mode of action of the components was studied. When the cells were pre-treated with epiprogoitrin, progoitrin, epigoitrin and goitrin before viral adsorption or the cells were treated with the components after virus incubation, no obvious protective effect was observed. However, when the cells were treated with pre-reacted virus and component, a pronounced CPE inhibition effect was detected. Epiprogoitrin, progoitrin, epigoitrin and goitrin exhibited dose-dependent anti-influenza virus activity with an IC_{50} value of 0.44 ± 0.03 , 0.19 ± 0.01 , 0.36 ± 0.02 and 0.19 ± 0.02 μ M, respectively (Fig. 2).

3.3. Anti-influenza activity of epiprogoitrin, progoitrin, epigoitrin and goitrin in ovo

In agreement with results of *in vitro* test, epiprogoitrin, progoitrin, epigoitrin and goitrin failed to exhibit prevention or treatment effect against influenza virus A (H1N1) *in ovo*. However, when the chicken embryos were treated with the pre-mixed solutions of the samples at a concentration of 5 mg/mL and the virus, their allantoic fluid showed no HA effect on CRBCs. As shown in Table 1, *Isatis Radix* derived glucosinolate isomers and their breakdown products were all found to neutralize influenza virus A (H1N1) in a dose dependent manner. The minimum concentrations for epiprogoitrin, progoitrin, epigoitrin and

Table 1

Neutralization effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on influenza virus A (H1N1) in embryonated eggs ($n = 3$).

Group	Compound concentration, mg/mL	Survival	Negative	Inhibition rate, %
Sham-operated group	N/A	8	8	N/A
Virus control group	N/A	8	0	N/A
Dilute control group	N/A	8	8	N/A
Negative control group	N/A	8	8	N/A
Positive control group	3.0	8	8	100
Epiprogoitrin	0.625	8	0	0
	1.25	8	2 \pm 1	25
	2.5	8	4 \pm 1	50
	5.0	8	8	100
Progoitrin	0.625	8	8	100
	1.25	8	8	100
	2.5	8	8	100
	5.0	8	8	100
Epigoitrin	0.625	8	2 \pm 1	25
	1.25	8	8	100
	2.5	8	8	100
	5.0	8	8	100
Goitrin	0.625	8	4 \pm 1	50
	1.25	8	8	100
	2.5	8	8	100
	5.0	8	8	100

goitrin exhibiting significant inhibition (100% inhibition rate) were 5.0, 0.625, 1.25 and 1.25 mg/mL, respectively. At the minimum investigated concentration of 0.625 mg/mL, the inhibition rate for epiprogoitrin, progoitrin, epigoitrin and goitrin were 0, 100%, 25% and 50%, respectively. At all the concentrations, the components were found to be safe.

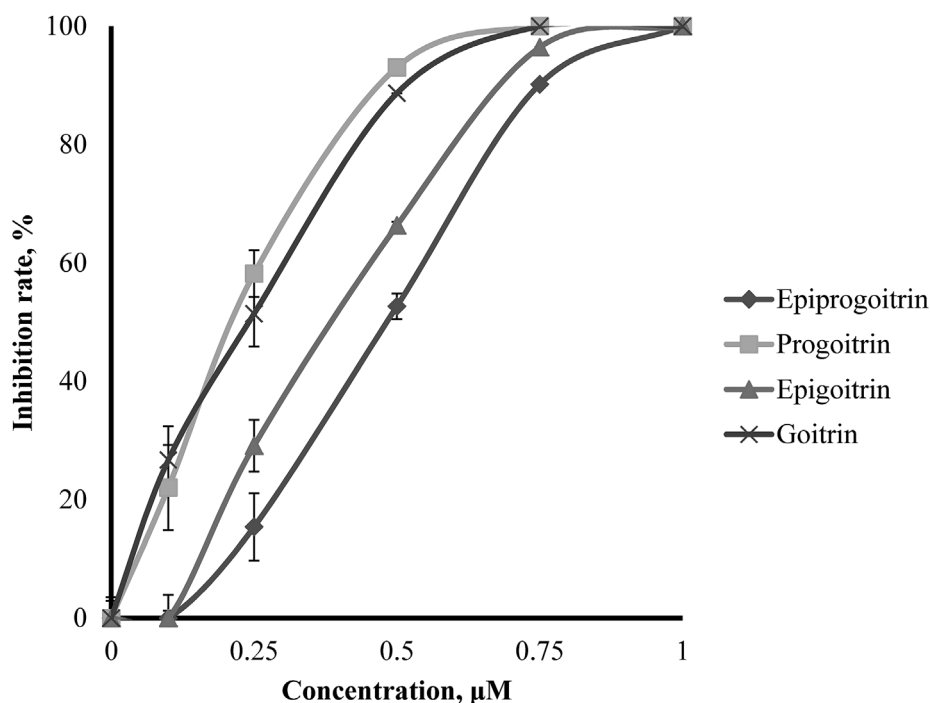


Fig. 2. Inhibitory effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on influenza virus A (H1N1) in Madin-Darby canine kidney (MDCK) cells ($n = 3$). 100 μ L of sample solutions at different concentrations were mixed with 100 μ L of virus at 100 TCID₅₀. After incubation at room temperature for 30 min, the mixtures were added to the cells. After incubation at 37 °C in 5% CO₂ for 72 h, the antiviral activity was investigated by cytopathic effect (CPE) inhibition assay and Cell Counting Kit-8 (CCK8) method.

3.4. Effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on hemagglutinin

The inhibitory effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on hemagglutinin was examined to characterize the mode of their antiviral action. As shown in Fig. 3, hemagglutinin was not inhibited in the reaction solution used as positive control, nor in the presence of epiprogoitrin, progoitrin, epigoitrin or goitrin, even at the highest concentration (5 mg/mL) which exhibited antiviral activity.

3.5. Effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on neuraminidase

NA has been regarded as one of the most important target of the anti-influenza virus activity of the drugs. As shown in Table 2, peramivir presented dose-dependent NA inhibitory activity with IC_{50} value of 0.69 ± 0.05 mg/mL. But no apparent inhibition effect of epiprogoitrin, progoitrin, epigoitrin or goitrin on NA was observed, even at the highest concentration (5 mg/mL).

4. Discussion

As a highly contagious disease, influenza causes high morbidity and mortality in epidemics each year. Due to adverse effect and viral resistance, development of novel natural anti-influenza drugs becomes necessary and urgent. *Isatis Radix* is used for ages to cure symptoms similar to influenza and has been reported to show satisfactory efficacy on respiratory viruses, but the mechanism remains unilluminated till now (Xu et al., 2019). Different types of compounds have been isolated from this traditional medicine including alkaloids, lignans and flavonoids, etc. For the first time, antiviral effect and mode of action of glucosinolates and their biotransformed products from *Isatis Radix* were discussed in this study.

Two pairs of enantiomers, namely epiprogoitrin, progoitrin, epigoitrin and goitrin, were prepared from *Isatis Radix* by chiral separation. First, CPE inhibition assays were designed in three different ways to examine their inhibitory effect against influenza virus strain A/California/7/2009 (H1N1) *in vitro*, and to determine at which stage they act in the viral replication cycle. The results indicated that the

Table 2

Inhibitory effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on neuraminidase ($n = 3$).

Group	Compound concentration, mg/mL	Inhibition rate, %
Positive control	0.375	35.80 ± 1.25
	0.75	50.25 ± 2.64
	1.5	71.53 ± 4.72
	3.0	79.43 ± 3.81
Epiprogoitrin	0.625	0
	1.25	0
	2.5	0
	5.0	0
Progoitrin	0.625	5.95 ± 0.68
	1.25	0.12 ± 0.04
	2.5	0
	5.0	0
Epigoitrin	0.625	0
	1.25	0
	2.5	0
	5.0	0
Goitrin	0.625	9.67 ± 1.77
	1.25	3.85 ± 0.70
	2.5	2.13 ± 0.56
	5.0	0.50 ± 0.07

antiviral activity of epiprogoitrin, progoitrin, epigoitrin and goitrin was mainly attributed to their virucidal effect, but not action on host cell protection or post-virus-adsorption interruption. Virus, rather than cell, was more probably to be the action target of the components. Hence, further insight into the antiviral activity *in ovo* was performed by hemagglutination test on the allantoic fluid of embryonated eggs. In agreement with previous observations, the four components were all found to significantly reduce viral titer in chicken embryos when mixed with virus before infection. Taken together, it was concluded that epiprogoitrin, progoitrin, epigoitrin and goitrin showed obvious virucidal effect on influenza virus A (H1N1) *in vitro* and *in ovo*.

For a deeper understanding of the mechanism of the antiviral action, the effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on hemagglutinin and neuraminidase was evaluated by hemagglutination inhibition (HAI) assay and neuraminidase inhibition (NA) assay. Influenza virus is an enveloped RNA virus with hemagglutinin (HA) and

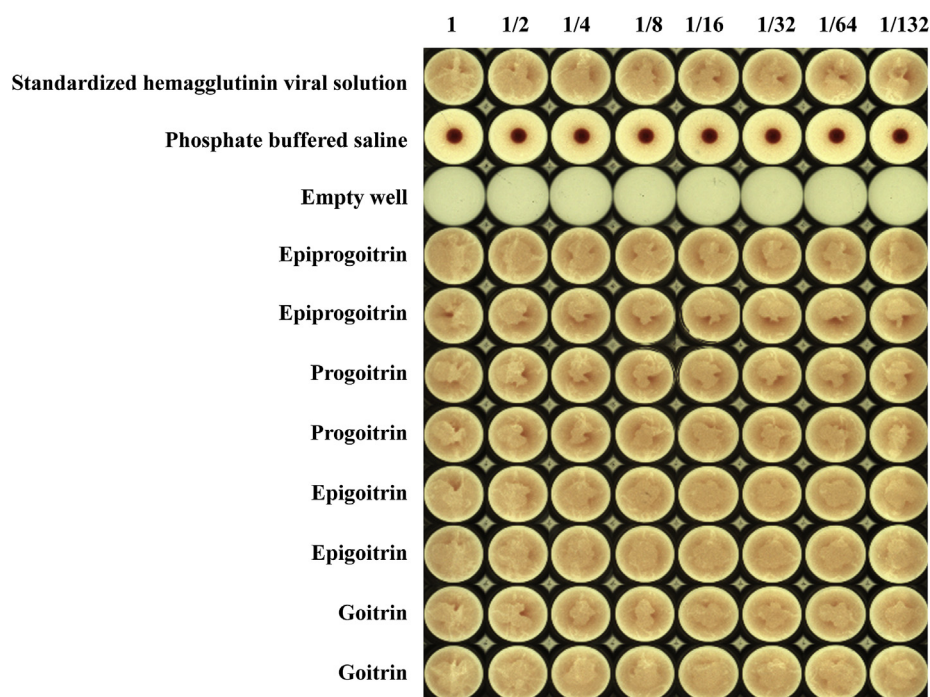


Fig. 3. Effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on hemagglutinin. Effect of epiprogoitrin, progoitrin, epigoitrin and goitrin on hemagglutinin was evaluated by hemagglutination inhibition (HAI) assay. Epiprogoitrin, progoitrin, epigoitrin and goitrin at a concentration of 5 mg/mL was serially diluted 2-fold and added to the 96-well plate. The standardized hemagglutinin viral solution was added to the 96-well plate. Then, 0.6% chicken red blood cells suspension was added to the mixtures as described in the Material and Methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

neuraminidase (NA) embedded in the lipid envelope. Viral adsorption to cells is the initial step of influenza infection, which is performed via the binding between sialic acid on the cell surface and hemagglutinin of the virus (Skehel and Wiley, 2000). Results of HAI assay showed that exposure to epiprogoitrin, progoitrin, epigoitrin or goitrin did not affect the hemagglutinin activity of H1N1 virus. The components were indicated not to interfere with the binding of sialic acid and hemagglutinin. On the other hand, for the release of the influenza virus from the cell, NA functions as an enzyme cleaving sialic acid groups from the host glycoproteins (Huang et al., 2008). Epiprogoitrin, progoitrin, epigoitrin and goitrin were found to exert low inhibition rates on neuraminidase by a NA inhibition assay. The components were indicated not able to prevent budding of progeny virus from the cell membrane. Therefore, it is implied that epiprogoitrin, progoitrin, epigoitrin and goitrin play their antiviral activity by acting as virus neutralizers, which associate with some component on the viral envelope without interfering with viral adsorption or budding.

Based on the IC₅₀ values obtained from CPE inhibition assay, the activity of the compounds *in vitro* was in the order of progoitrin = goitrin > epigoitrin > epiprogoitrin. Comprehensive comparison of the minimum concentrations exhibiting 100% inhibition and the inhibition rate at a minimum investigated concentration of 0.625 mg/mL obtained from HA assay on embryonated eggs, the activity of the compounds *in ovo* was in the similar order of progoitrin > goitrin > epigoitrin > epiprogoitrin. When comparing the potency of the antiviral activity between the monomers, no remarkable difference was found between the precursor and the myrosinase-derived product (epiprogoitrin vs. epigoitrin and progoitrin vs. goitrin). On the contrary, considerable discrepancy was observed between the enantiomers (epiprogoitrin vs. progoitrin and epigoitrin vs. goitrin). The gap between the antiviral potency of progoitrin and epiprogoitrin was larger than that between goitrin and epigoitrin. S-enantiomers (progoitrin and goitrin) claimed higher activity than R-enantiomers (epiprogoitrin and epigoitrin). It is implied that the heteroaromatic chain/ring is the main functional group of the *Isatis Radix* derived glucosinolates isomers and their breakdown products. And cyclization of the heteroaromatic chain to the oxazoline ring has no obvious influence on the function. The stereogenic center (chiral carbon atom) in the heteroaromatic chain of epiprogoitrin and progoitrin and in the oxazoline ring of epigoitrin and goitrin is an important structural feature from both chemical and biological points of view. Lucky enough, all the components were found to be safe both to the cells and the embryos.

5. Conclusions

In conclusion, experimental results indicated that epiprogoitrin, progoitrin, epigoitrin and goitrin, the *Isatis Radix* derived glucosinolates isomers and their breakdown products, could neutralize the influenza virus strain A/California/7/2009 (H1N1), thus exhibiting antiviral efficacy *in vitro* and *in ovo*. The finding enriched the pharmacology and the mechanism for research and development of *Isatis Radix*, the most common traditional medicine used for treating influenza. Further mechanistic studies implied that the above components had no inhibitory effect on hemagglutinin and neuraminidase. Thus, the attachment to the viral envelope without disturbing viral adsorption or budding to host cells was suggested to contribute critically to the mode of antiviral action of the components. Potential activity on other influenza virus targets like endonuclease, viral polymerase, nucleoprotein or M2 protein needs future exploration. With accurate anti-viral efficacy and low toxicity, epiprogoitrin, progoitrin, epigoitrin and goitrin from *Isatis Radix* are expected to be used as promising antiviral drug resources. Consequently, further validation including *in vivo* evaluation and clinical trials are required before introducing them from the research into practical application. However, whether the glucosinolates and their enzymatic hydrolysates could inhibit other influenza viruses or not is unknown and the molecular mechanism still needs to be

clarified by further investigation.

Author contributions

Lixing Nie and Yanlin Wu designed and carried out the experiments, analyzed the data, and wrote the manuscript. Zhong Dai checked the data. Shuangcheng Ma edited the article. All authors approved and reviewed the manuscript.

Declaration of competing interest

None.

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