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## Original article

## Anti-flu Effect of Compound Yizhihao Granule and Its Effective Components

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## ABSTRACT

**Objective** To research the anti-influenza effect of active ingredients in Compound Yizhihao Granule (CYG). **Methods** The cytotoxicity and cytopathic effects (CPE) were observed under the phase-contrast microscope, besides 50% toxicity concentration ( $TC_{50}$ ) and 50% inhibitory concentration ( $IC_{50}$ ) were also calculated using Reed-Muench method, then the antiviral activity *in vitro* according to Selection Index ( $SI = TC_{50}/IC_{50}$ ) was evaluated. In PR8 virus-infected mice, survival time, death rate, and lung index were observed in order to evaluate the protective effect. Besides, the effective ingredients were determined using HPLC method, and their contents were calculated by external standard method. **Results** CYG could inhibit the influenza virus-induced CPE, with  $IC_{50}$  of 4.6 mg/mL (equal to herbal extracts 262.2  $\mu$ g/mL), and no direct cytotoxic effect at this concentration. PR8-infected mice were ig given CYG, the lung index and mortality were significantly reduced, and survival time was obviously prolonged. HPLC analysis indicated CYG contained many kinds of antivirus active components, including rupestonic acid, epigoitrin, and adenosine. **Conclusion** CYG is an effective natural anti-influenza medicine. Its antiviral effect should be the synergic effect of a variety of antiviral active ingredients.

## Key words

artemisia rupestris; Compound Yizhihao Granule; epigoitrin; influenza virus; *Isatis tinctoria*; rupestonic acid

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## 1. Introduction

Influenza is a contagious illness caused by influenza viruses. When influenza viruses pass from one person to another, because of high mutation risk in the viral genome, the influenza viruses are constantly rearranging their genomes, causing antigenic shift and newly infecting human every year by overcoming protective immunity. Therefore flu pandemics, serious global outbreaks of influenza virus-associated diseases, may happen every few decades or

so, leading to social disruption and economic loss. For example, H1N1 is a type A influenza that caused the first flu pandemic worldwide of the 21st century in 2009 (Al Hajjar and McIntosh, 2010; Taubenberger and Morens, 2010). In children, pregnant women, old people, and immune-compromised people, the infection of H1N1 often led to severe complications, such as pneumonia and death (Kelly et al, 2011). Presently, the main methods for the prevention of influenza is through antiviral drugs and flu vaccination. However, seasonal influenza viruses could constantly mutate

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to evade immune protection or develop drug-resistance (Zhang and Fan, 2011). Until a universal flu vaccine appears, we have to make vaccines for each strain as it evolves. On the other hand, the wide spread use of antiviral drugs likely lead to the spread of drug-resistance. Therefore, it is also necessary to rely on other medications for effective prevention of influenza virus infection. In China, some natural medicines are routinely prescribed to treat flu and common cold. They often showed distinctive advantages, such as multiple effects and serious side effects, and are rarely being observed.

This article would introduce a kind of natural medicine, named Compound Yizhihao Granule (CYG) and discuss its anti-influenza effect and active ingredients.

The medicinal materials of CYG include *Artemisia rupestris* L. and *Isatis tinctoria* L. (woad). *A. rupestris*, known as “Yizihuamanny in Uighur, is a unique species in family Compositae in Xinjiang region (Liu et al, 1999; Song et al, 2005). Woad is a herb in Cruciferae and known as “Wusima” (Liu et al, 1999; Song and Hu, 2005), and its medicinal parts, roots and leaves, were known as *Isatidis Radix* and *Isatidis Folium* (Liu et al, 2000). The two kinds of herbs are Uighur medical conventional medicinal materials. CYG is originally used to treat “naizilai cold” in hospital (Li et al, 2003; Li et al, 1996). In Uighur, “naizilai” means “the invader”, so “naizilai cold” is considered the same as influenza. Thus, after being approved, CYG is widely used in the treatment of influenza and common cold. Though entering clinical practice for many years, relevant study reports are still very limited, especially short of positivism researches about its anti-influenza efficacy. This paper, for the first time, based on empirical study, reported the anti-influenza virus effect of CYG, analyzed its possible antiviral material base, and provided a strong support for its clinical efficacy.

## 2. Materials and methods

### 2.1 Virus and cell line

Two H1N1 strains [influenza virus A/Beijing/262/95 and influenza virus A/Puerto Rico /8/34 H1N1(PR8)] and MDCK cells were provided by the National Institute for Viral Disease Control and Prevention, China. The virus strains were maintained through cultivation of chicken embryo method, and kept at  $-80^{\circ}\text{C}$  until use. MDCK cells were grown in Eagle's minimum essential medium containing 10% fetal bovine serum.

### 2.2 Animals

Ha/ICR mice [No. SCXK (Xin) 2011-0004] weighing 18–22 g were purchased from the Animal Experimental Center, Xinjiang Medical University, China. Animals were housed in standard cages, five mice per cage, on 12 h light/dark cycle, and air temperature was maintained at  $(22 \pm 2)^{\circ}\text{C}$ . The study was approved by Xinjiang Institute of Materia

Medical, Ethics Committee of Laboratory Animals, and abided the *Guidelines for Care and Use of Experimental Animals* issued by the Ministry of Science of China in 2006.

### 2.3 Standard substances

Rupestonic acid (20121213) was purchased from Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Science; Epigoitrin (111753-201103) and adenosine (110879-200202) were purchased from National Institutes for Food and Drug Control.

### 2.4 Medicinal plants

*Artemisia rupestris* L. was collected from Yili area of Xinjiang region, China ( $81^{\circ}27'05.76''\text{E}$ ,  $44^{\circ}28'42.21''\text{N}$ ) in June 2009. *Isatis tinctoria* L. (woad) was cultivated and collected in Kuqa county, Xinjiang region, China, in September 2009. The two original plants identification was performed by Prof. Wei-jun Yang, and the original plant specimens (*Artemisia rupestris* No. 20090625001, Woad No.c-061) was collected from Xinjiang Institute of Materia Medica, Urumqi, China.

### 2.5 CYG and Ribavirin (RBV) Tablet

CYG was manufactured by Xinjiang Yinduolan Uighur Medicine Co., Ltd. (Lot. 10020104, Drug Approval No. Z20026711). The product was in line with State Food and Drug Administration Standard of China: WS-11137 (ZD-1137)-2002. The adult daily doses were 15–30 g. The treatment program consists of 5–10 g for an adult dose, taken three times a day for approximately one week.

RBV tablet was a positive control medicine, which was manufactured by Huiren Pharmaceutical Co., Ltd., Jiangxi province, China (Lot. 1011025).

### 2.6 Cytotoxicity test

MDCK cells ( $2 \times 10^4/\text{mL}$ ) were incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 24 h in a 96-well plate. Removing the supernatant, CYG solution was added to the cells. The cytotoxicity of CYG on MDCK cells was measured by monitoring cell growth recording and calculating the maximum non-toxic concentration ( $\text{TC}_0$ ) and  $\text{TC}_{50}$  value to indicate non-toxic dosage and cytotoxicity size.

### 2.7 CPE induced by influenza virus A

After incubated for 24 h at  $37^{\circ}\text{C}$ , MDCK cells ( $2 \times 10^4/\text{mL}$ ) were infected the viral suspension and then incubated for 1 h at  $35^{\circ}\text{C}$ . Next, discarded the supernatant and added different sample solution. The cells were re-incubated at  $35^{\circ}\text{C}$ , observed the virus-induced CPE every day, scored according to the following classifications: 0 (CPE = 0%), 1 (CPE < 25%), 2 (25% < CPE < 50%), 3 (50% < CPE < 75%), and 4 (CPE > 75%). Reed-Muench method was

used to estimate  $IC_{50}$  and  $TC_{50}/IC_{50}$  ratio to calculate SI.

## 2.8 Infection mouse model with H1N1

PR8 virus was dropped by nasally in ICR mice, on the day, the infected mice began to take orally CYG, twice daily for 5 d. On day 6 post-infection, the infected mice were weighed and euthanized, the lung of each mouse was weighed to calculate lung index. Another part of the infected mice were observed continuously for 14 d for survival.

## 2.9 Statistical analysis

Statistical comparisons of data were carried out with ANOVA and *t*-test between groups using the SPSS 17.0 system (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL: SPSS Inc.). A value of  $P < 0.05$  was considered statistically significant.

## 2.10 Analysis of active compositions

HPLC (1260 HPLC Instrument and Chemstation for LC Systems Workstation, Agilent Co., America) was employed to analyze the percentage contents of rupestonic acid, epigoitrin, and adenosine in CYG. The chromatographic separation was performed using a Phenomenex ODS-A(250 mm × 4.6 mm, 5 μm) column with a flow rate of 1.0 mL/min

and the sample injection column volume was 10 μL; The column temperature was maintained at 35 °C. All analytes were monitored at 245 nm. Rupestonic acid was separated using the mobile phase consisted of 0.4% phosphoric acid aqueous solution (A) and acetonitrile (B), and the flowing gradient program was 0–30 min, 10%–40% B; 30–55 min, 40%–10% B; 55–65 min, 10%–10% B. Epigoitrin and adenosine were separated using the mobile phase consisted of water (A) and methanol (B), and the flowing gradient program was 0–20 min, 1%–15% B; 20–40 min, 15%–40% B; 40–50 min, 40%–70% B; 50–60 min, 70%–85% B; 60–75 min, 85%–1% B; 75–85 min, 1% B.

## 3. Results

### 3.1 Antiviral activity in vitro

In cytotoxicity test, both  $TC_0$  and  $TC_{50}$  (18.8 and 111.7 mg/mL) of CYG significantly exceeded those of RBV ( $TC_0$  and  $TC_{50}$  of 617.2 and 4937.6 μg/mL), which suggested that the maximally tolerated dose was much higher than RBV.

The *in vitro* results showed CYG was capable of inhibiting virus-induced CPE at the concentration of 18.8–1.2 mg/mL (Figure 1), the average  $IC_{50}$  value was 4.6 mg/mL, and SI was 24.3 (Table 1). Therefore, CYG may directly inhibit *in vitro* replication of influenza virus and display less toxic than antiviral drug.

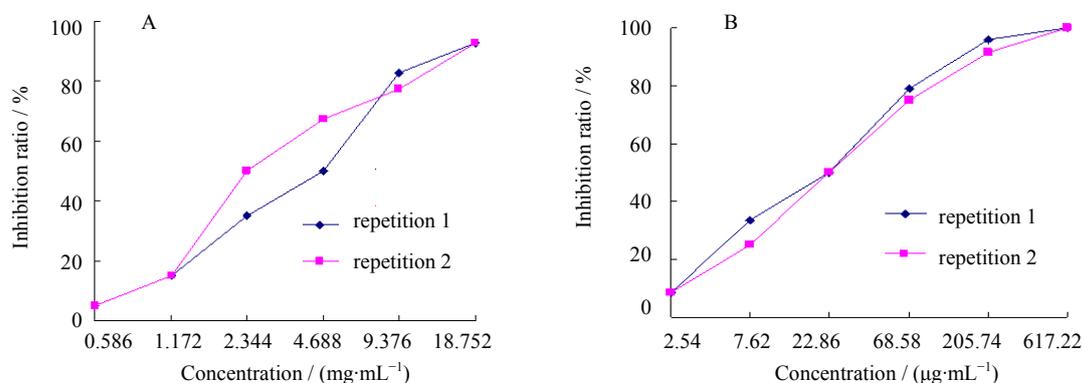


Figure 1 Inhibition ratios of CYG (A) and RBV (B) on CPE at concentration of 18.8–1.2 mg/mL

Table 1 Inhibition of CYG on CPE induced by influenza virus

Virus	RBV		CYG	
	$IC_{50}$ / (μg·mL <sup>-1</sup> )	SI	$IC_{50}$ / (mg·mL <sup>-1</sup> )	SI
A/Beijing/ 262/95	35.6 ± 6.8	138.7	4.6 ± 1.7	24.3

### 3.2 Antiviral activity in vivo

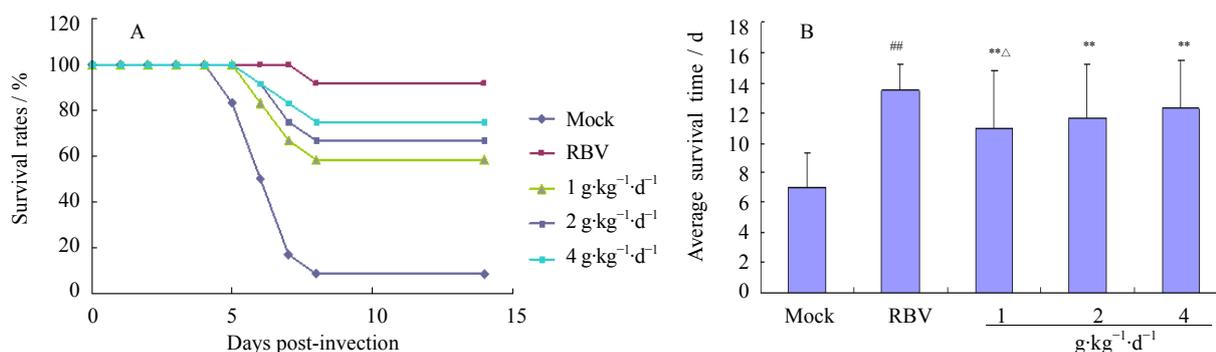
To dynamically observe the interfering effect of CYG on infected mice, we found almost all the virus control group mice died within 6–9 d after infection, with a 91.7% mortality rate. On the contrary, the CYG of mice in varying

clinically relevant doses groups were markedly protected against PR8 virus challenge, with the survival rate more than 80% 9 d post-infection. Till experiment ended, the average survival time of CYG groups extended significantly, the life extension rates of CYG 2 and 4 mg/kg groups reached up to 65.7% and 75.7% (Table 2); Meanwhile the mortality rates were as low as 33.3% and 25% (Figure 2). In addition, after orally taking CYG for 5 d, the lung indexes of infected mice were reduced significantly, which indicated CYG could alleviate inflammation of lungs (Figure 3). It was worth noting that compared with RBV group, CYG 2 or 4 mg/kg groups had no significant difference in mortality and average survival time, so it is with lung index (Table 3). These mean that CYG as a herbal supplement has the powerful effect in combating influenza virus infection.

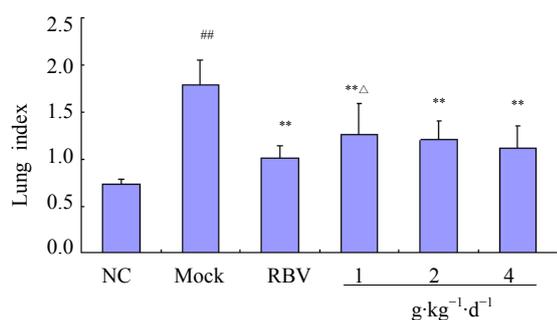
**Table 2** Effect of CYG on mortality

Groups	Single dose / (g·kg <sup>-1</sup> )	Died / Total	Mortality / %	Survival rate / %	Average survival time / d	Life extension rate / %
Mock	–	11/12	91.7	8.3	7.0 ± 2.4	–
RBV	0.06	1/12	8.3**	91.7**	13.5 ± 1.7**	92.8
CYG	1	5/12	41.7**	58.3**	11.0 ± 3.7** <sup>△</sup>	57.1
	2	4/12	33.3**	66.7**	11.6 ± 3.5**	65.7
	4	3/12	25.0**	75.0**	12.3 ± 3.2**	75.7

\*\**P* < 0.01 vs Mock group; <sup>△</sup>*P* < 0.05 vs RBV group; same as below

**Figure 2** Effect of CYG with clinically relevant doses on risk of death (A) and survival time (B) in influenza infected mice

Mock: virus control \*\**P* < 0.01 vs Mock group

**Figure 3** Effect of CYG with clinically relevant doses on symptoms of pneumonia mice

NC: normal control; Mock: virus control

<sup>##</sup>*P* < 0.01 vs NC group; \*\**P* < 0.01 vs Mock group; same as below

**Table 3** Decreased lung indexes by CYG ( $\bar{x} \pm s$ , *n* = 10)

Groups	Single dose / (g·kg <sup>-1</sup> )	Lung index	Inhibition ratio / %
NC	–	0.74 ± 0.04	–
Mock	–	1.79 ± 0.27 <sup>##</sup>	–
RBV	0.06	1.01 ± 0.14**	43.6
CYG	1	1.26 ± 0.34 <sup>△</sup>	29.6
	2	1.20 ± 0.19**	33.0
	4	1.12 ± 0.23**	37.4

### 3.3 Contents of effective components

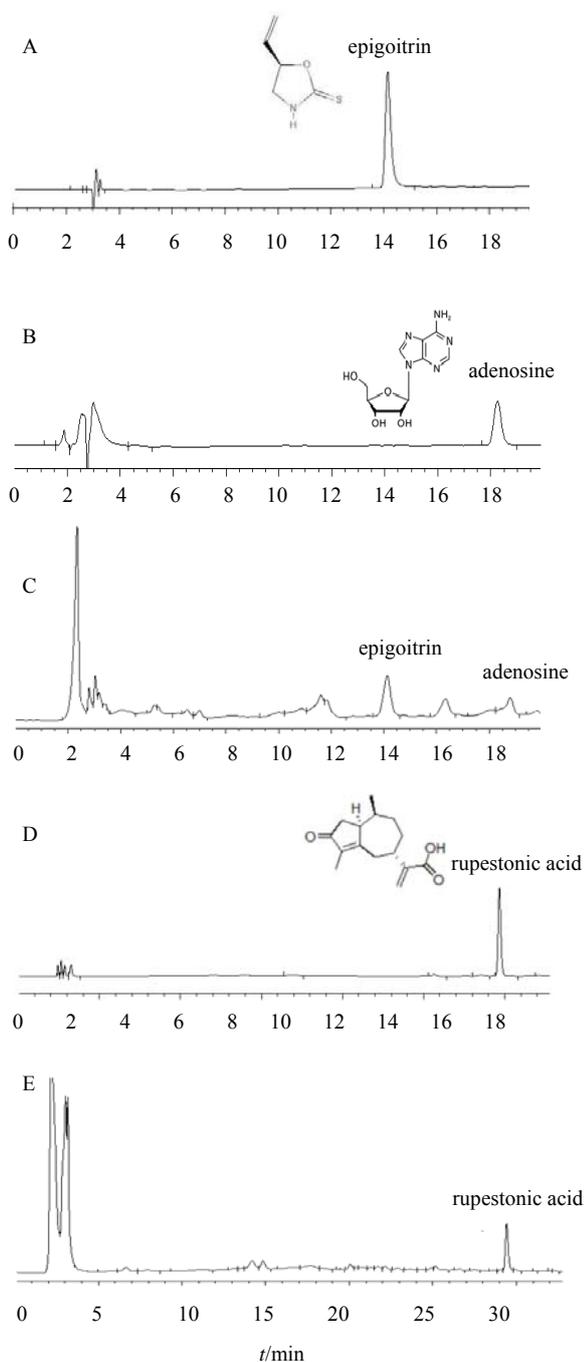
Several kinds of antiviral ingredients were detected through HPLC analysis (Figure 4), according to the peak area to calculate by external standard method, CYG contains rupestonic acid (0.5469 µg/mg), epigoitrin (0.0119 µg/mg), and adenosine (0.0687 µg/mg).

## 4. Conclusion and discussion

Uyghur medicine is a small branch of traditional medicine, and similarly, to use natural drugs is one of its main features. In recent years, it attracted more and more attention, because of not only its unique academic thinking, but also many Uyghur medicine natural products, including CYG, which achieves desired clinical effects.

According to Uyghur medicine theory, four substances (water, fire, soil, and air) are associated with the occurrence of illness, and they lead to four kinds of attributes in the human body: hot, cold, damp, and dryness. The four attributes exist similarly in plants, animals, and minerals. Therefore, Uyghur medicines are also divided into four attributes (Hamulati, 2003). In accordance with the therapeutic principle of Uyghur medicine, the attribute of a disease and the treating drugs should be the opposite (Aibai and Dawuti, 1995). “Naizilai cold” is also considered a “hot-type” disease, so the herbs with “cold attribute” can be used to treat it (Yisakejiang, 2005). *A. rupestris* and woad both belong to the herbs with “cold attribute”, and are mainly used to treat “hot-type” cold, as well as hepatitis when they could be used individually (Cheng and Li, 2010). Therefore, using these two herbs of compound preparation to treat “naizilai cold” is reasonable.

Some studies for plant extracts report also to some extent support CYG as a treatment for the flu. For example, the *A. rupestris* extracts can inhibit many kinds of virus, include influenza A<sub>3</sub> virus, parainfluenza virus, and respiratory syncytial virus, etc (Ji et al, 2007; Xiao and Aibai, 2008). The woad roots extracts can inhibit the influenza virus both *in vivo* and *in vitro* (Li et al, 2010; Xu et al, 2005), especially, the extracts of the woad roots and leaves significantly inhibits influenza A virus *in vitro* (Liu et al, 2000).



**Figure 4** Active constituents analysis of epigoitrin reference substance (A), adenosine reference substance (B), epigoitrin and adenosine detected in CYG samples (C), rupestonic acid reference substance (D), and rupestonic acid detected in CYG samples (E)

Our research provided empirical support for the antiviral effects of CYG for the first time. It is worth noting that from the  $IC_{50}$  value of CYG (4.6 mg/mL), we could calculate  $IC_{50}$  value of the herbal extracts to be 262.2  $\mu$ g/mL, and this very close to the recognized herbal extracts efficacy criteria, that generally below 100  $\mu$ g/mL (Cos et al, 2006), indicating that CYG is highly active at inhibiting influenza

virus. On the other hand, the  $TC_{50}$  of CYG is far above that of RBV, suggesting that the toxicity of CYG is well below the antivirus agents in nucleoside. Even more important, CYG clinical equivalent dose was very effective to combat influenza viral infections, for the main observation indexes, we find no significant difference between CYG and RBV groups. These results suggest that CYG plays a role in directly inhibiting influenza virus. However, what material foundation supports it having such an antiviral effect?

It is reported that *A. rupestris* mainly contains sesquiterpenoids, such as rupestonic acid and isorupestonic acid (Liu et al, 1985), and flavonoids (Ji et al, 2007), they are all the antiviral substances (Xiao et al, 2008). Many studies show rupestonic acid and its most derivatives all can inhibit the influenza virus (Yong et al, 2009; 2011; Zhao et al, 2012). Woad contains nucleoside, such as adenosine and uridine (Liu et al, 2003), sulphur compounds, such as epigoitrin (Liu et al, 2002), as well as indole alkaloid (Zhang et al, 2005). They have also been verified to be the antiviral ingredients (Liu et al, 2003; Xu et al, 2006; An et al, 2008), and among them, epigoitrin is considered the important anti-influenza composition in *Isatidis Radix* (Xu et al, 2006; Zhang et al, 2013), adenosine also acts the same role (Liu et al, 2003). Meanwhile, it is an accepted broad-spectrum antiviral substance. There are reports that four active components in woad and their combination have anti-adenovirus activity (Zhang et al, 2005).

In this study, we have found CYG contains many kinds of the known antiviral active ingredients, including rupestonic acid, epigoitrin and adenosine, and that its effective components are likely more complex, because there are some unknown ingredients needing to be further identified. To be sure that the CYG's antiviral effect should be the multicomponent synergy results.

Though the antiviral material basis of CYG still needs more exploring, our studies have already supported its extensive adaptability and excellent efficiency in clinic, that is, not only for the common cold, CYG as an anti-influenza natural drug, also has a good application prospect. As time goes on, more and more people will know and accept its healing properties.

#### Conflict of interest statement

The authors declare no conflict of interest.

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