A widespread outbreak of avian influenza throughout East Asia and the ability of these avian viruses to transfer to humans and cause severe infection have raised concerns that such infections could lead to another pandemic of the disease [1]. Antiviral drugs that are effective against such infections hold great promise as means to control these viruses. However, their cost and limited supply may limit their widespread use. The broad-spectrum antiviral drug ribavirin was shown to inhibit influenza-A virus (IAV) and B virus infections in animal models [2–4], but some adverse reactions have been observed in clinical trials with this drug [5]. The administration of ribavirin is limited to a relatively narrow therapeutic range because high doses can induce hemolytic anemia, and excessive doses may exert teratogenic effects [5,6].

In recent years, many researchers have focused on the discovery and development of innovative antiviral
drugs to treat influenza. Since Traditional Chinese Medicine (TCM) has been used to treat diseases in China for thousands of years, many research institutes have focused on TCM because of their efficacy and low toxicity [7,8]. Today, the use of TCM as complementary or alternative medicines is burgeoning globally, including developed countries. Meanwhile, over 80% of the population in developing countries mainly depend on traditional healing modalities, including herbal remedies, for maintaining good health and to treat disease [9,10].

The classical formula Yinqiaosan (YQS) was first documented in the book “Wen Bing Tiao Bian,” which records the theoretical research on warm-hot disease in TCM and its clinical application in the Chinese history. YQS consists of nine herbal components, namely: *Flos lonicerae* (Jinyinhua), *Fructus forsythiae* (Lianqiao), *Fructus arctii* (Niubangzi), *Herba schizonepetae* (Jingjie), *Herba menthae* (Bohe), *Semen sojae praeparatum* (Dandochi), *Herba lophatheri* (Zhuye), *Rhizoma phragmitis* (Lugen), and *Radix glycyrrhizae* (Gan cao). This formula and its modified variations have a lengthy history for the treatment of the common cold, fever, coughing, and other respiratory diseases [11].

A formula comprised of 11 herbs may sound fairly complicated, and thus a simplified formula containing fewer components may be more convenient and more popular in clinical use. Previous studies have demonstrated that YQS inhibits the replication of IAV when administered simultaneously with exposure to IAV, or when YQS is administered during the advanced stage of disease, to treat pneumonia induced in mice by IAV. YQS reduced the lung index and death rate in mice, thus improving their life expectancy [12,13]. The extracts of *Flos lonicerae*, *Fructus forsythiae*, and *Fructus arctii* could down-regulate the expression of Fas, Fasl, tumor necrosis factor-α and tumor necrosis factor receptor-I mRNA in mice with pneumonia induced by influenza virus, and inhibited apoptosis of pulmonary tissue cells [14]. It is well known that the therapeutic effects of herbal medicines are based on the synergistic actions of their main constituents [15,16]. Therefore, in this study, we focused on caffeoylquinic acid, arctium lappa glycoside, aurantiamarin, and forsythin, being the main active components of *Flos lonicerae*, *Fructus arctii*, *Fructus forsythiae*, and *Herba schizonepetae*, respectively, to evaluate the quality and standardization of the four herbs and their preparations [17,18]. Multiple compound determination has been developed to study the quality of such formulae by simultaneously evaluating many active compounds from different herbs in prescriptions [19].

The fingerprinting of TCM product extracts reflects the chemical composition of each herb in the formula, or the distribution of the chemical composition and its content after herbs re-constituting in quantities and dosage. Furthermore, detailed information about whether the components are complete or not can be determined. At present, using information such as the chromatogram peak location, area size and distribution, studies of the fingerprint of TCM can reveal their chemical compositions and pharmacodynamic properties. Presently, the research on Chinese herbal formula fingerprints was in the primary stage, especially the study on the combination of Yinqiaosan fingerprints and pharmacodynamics was scarcely rare [20–22]. Therefore, we sought to establish the fingerprint of YQS and how this was related to its anti-IAV effects.

To achieve this, we evaluated the relative abundance of four key components of YQS in five solvent extracts (water, 30% ethanol, 50% ethanol, 70% ethanol, and 95% ethanol) and compared the IAV-inhibitory effects of these extracts with that of ribavirin *in vitro*.

**Materials and Methods**

**Reagents and plant material**

High-performance liquid chromatography (HPLC)-grade methanol (Tedia, Fairfield, OH, USA) and other analytical-grade reagents were purchased from KaiXin Chemical Industry Company (Tianjin, China). Milli-Q water (Millipore, Billerica, MA, USA) was used to prepare all the solutions. The reference compounds caffeoylquinic acid, arctium lappa glycoside, aurantiamarin, and forsythin were purchased from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of each standard was over 98% and was suitable for HPLC determination.

The medicinal plants *Flos lonicerae*, *Fructus forsythiae*, *Fructus arctii*, and *Herba schizonepetae* were purchased from the dispensary of Liaoning University of TCM (Shenyang, China) and identified by Dr Xixiang Ying. Voucher specimens are maintained at The Affiliated Hospital of Liaoning University of TCM (Shenyang, China).
Compounds and viruses
Ribavirin was provided by WuZhong Pharmaceuticals Inc. (Batch no. 080615, Jiangsu, China) and 9-day-old embryonated chicken eggs were purchased from YiKang Biological Pharmaceuticals Inc. (Liaoyang, China).

Influenza A/FM1/7 (H1N1) was obtained from The Center for Disease Control and Prevention (Beijing, China). The virus used in the experiments was propagated in the chorioallantoic cavities of 9-day old embryonated chicken eggs for 3 days at 35°C. The infected allantoic fluids were then centrifuged at 1,000 g for 20 minutes and stored as the virus stock solution at −80°C. Samples of the frozen virus was thawed rapidly when needed and diluted with serum-free E-MEM at dilutions ranging from 10−1 to 10−12. Flat-bottomed, 96-well microplates were used for in vitro antiviral experiments and virus titrations.

Apparatus and chromatographic conditions
Analyses were performed on an Agilent Series 1100 HPLC with an ultraviolet detector. All separations were carried out on a Phenomsil ODS C18 column [250 × 4.6 mm; inner diameter (i.d.), 5 μm] (Tianjing, China). The mobile phase consisted of methanol (M) and 4% phosphoric acid solution (P) in a gradient elution mode, which was carried out as follows: 0–5 minutes, linear gradient from M:P [2:98 (v/v)] to M:P (4:96, v/v); 5–10 minutes, mobile phase from M:P (4:96, v/v) to M:P (17:83, v/v); 10–20 minutes, linear gradient to M:P (30:70, v/v); 20–30 minutes, linear gradient to M:P (40:60, v/v); 30–45 minutes, linear gradient to M:P (44:56, v/v); 45–55 minutes, linear gradient to M:P (55:45, v/v); 55–60 minutes, linear gradient to M:P (60:40, v/v). Each run was followed by an equilibration time of 15 minutes. Ultraviolet spectra were monitored at 275 nm, and the flow rate was 1.0 mL/min. The data were collected and analyzed using Chemstation software (Agilent Technologies Co. Ltd., USA).

Preparation of sample solutions
Flos lonicerae, Fructus forsythiae, Fructus arctii, and Herba schizonepetae were ground and mixed. The powder mixture (0.5 g) was weighed and extracted with 20 mL methanol in an ultrasonic water bath (Shanghai Kodos Ultrasonic Instrument Co., Shanghai, China) for 40 minutes. The supernatant was filtered through a 0.45-μm membrane filter and used directly for HPLC analysis.

Method validation
Linearity, precision, repeatability, stability, and recovery were carried out to validate the HPLC method.

Determination of the 50% egg infectious dose (EID50)
The IAV stock solution at dilutions ranging from 10−1 to 10−12 was inoculated into the chorioallantoic cavities of 9 day-old embryonated chicken eggs. Each concentration was inoculated into four embryonated chicken eggs with isotonic NaCl as a control, and incubated at 33–35°C for 48 hours, followed by 4°C for 12 hours. The chorioallantoic cavities were then collected and placed in the flat-bottomed microplates with chicken red blood cells, mixed gently, and then left to stand for 40 minutes. The EID50 was then calculated using the Kärbe formula and was used to determine the starting concentration of virus solution. In this study, we used the 100EID50 [23].

Determination of maximum (0%) toxic dose (TD0)
Isotonic NaCl containing 10% penicillin and streptomycin (PS) was used to dilute the herb stock solution at serial concentrations (from stock solution to 1/512). Then, 0.2 mL was added to the embryonated chicken eggs. Each concentration of herb solution was inoculated into four embryonated chicken eggs with isotonic NaCl as a control. The eggs were incubated at 37°C for 48 hours and observed for survival at 12-hour intervals. Embryonated chicken eggs that died within 24 hours were not included in the herbal toxicity analysis.

Determination of the maximum non-blood clotting concentration
Isotonic NaCl containing 0.9% PS was used to dilute the herb stock solution at six concentrations [2 × 10−7 to 2 × 10−1 (7.8 mg/mL to 500 mg/mL)] with double dilution and placed on flat-bottomed microplates. Each concentration was titrated into two cribiform foramina with isotonic NaCl as a control. Then, 1% chicken red blood cell solution was placed into each cribiform foramina. The flat-bottomed microplates were then incubated at 33–35°C for 2 hours to determine the hemagglutination titer.
Time-dependent in vitro IAV-inhibitory effects of the five YQS extracts
Isotonic NaCl containing 0.9% PS was used to dilute the herb stock solution to the maximum non-blood clotting concentration, using the appropriate IAV solution with ribavirin and Hank's solution for comparison. Each preparation (four samples per group) was incubated at 33–35°C for 1, 6, 12, 18, or 24 hours, to determine the time-dependent effect of each sample on hemagglutination.

Evaluation of the therapeutic effect of YQS extracts on embryonated chicken eggs infected by IAV
The 100 EID₉₀ viral suspension was inoculated to eight embryonated chicken eggs and incubated for 2 hours at 37°C. Then, the herb stock solution was inoculated at three concentrations with double dilution (stock solution, 2×10⁻² and 2×10⁻¹); isotonic NaCl and ribavirin were used as a control and active comparator. The samples were incubated for 48 hours at 34°C and then for 12 hours at 4°C. The chorioallantoic cavities were collected and placed in flat-bottomed microplates with 0.1 mL of chicken red blood cells, mixed gently and left to stand for 40 minutes to observe the hemagglutination.

Assessment of the hemagglutination titer
Hemagglutination titer was assessed as previously described [23]. Briefly, the titer was rated as ++++, +++, ++, + or −, where: ++++ indicates that the red blood cells were distributed uniformly on the cribiform foramina of the 96-well flat-bottomed microplates; ++ is similar to +++, but the borders of red cell clots were irregular and the area of distribution was smaller; ++ indicates that red cells formed a ring with small clumps at the edge of the cribiform foramina; + indicates that the red cells had formed small clumps, but the borders were not smooth, and small clumps were found at the edge of the cribiform foramina; − indicates that the red blood cells had formed small clumps in the bottom of the cribiform foramina and the borders were smooth and stereoscopic, and tilting the flat-bottomed microplates caused the red blood cells to form tear shapes.

The hemagglutination titer was calculated as follows. + represents one agglutination unit and is the highest concentration of the virus that caused hemagglutination and was used as the end-point of the assays. The reciprocal of this concentration is the hemagglutination titer. If the end-points are ++++ or +++, they were calculated after data arrangement.

Statistical analysis
SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Continuous data are shown as mean± standard deviation and were analyzed by one-factor analysis of variance. Post hoc analyses were performed using the Least Squares Difference method.

RESULTS
Method validation
The calibration curves for caffeoylquinic acid, aurantiamarin, arctium lappa glycoside and forsythin were prepared for the concentration ranges of 1.89–189 μg/mL, 3.04–30.4 μg/mL, 5.05–151.5 μg/mL and 0.93–22.3 μg/mL, respectively. The regression equations (regression coefficients) were: y = 15.772x + 45.951 (r = 0.9986), y = 25.231x + 31.844 (r = 0.9992), y = 5.4413x + 28.258 (r = 0.9983), and y = 11.062x – 2.7868 (r = 0.9984), respectively. All calibration curves were plotted based on linear regression analysis of the integrated peak area (y) versus concentration (x, μg/mL) of the four reference standards (caffeoylquinic acid, aurantiamarin, arctium lappa glycoside and forsythin) in the standard solution at six different concentrations. The intra-day precision was evaluated by determining the activity of the standard solutions of the four compounds under the optimized conditions five times within 1 day, with relative standard deviations (RSDs) at 1.1–2.1%. The repeatability was also good, with RSDs ranging from 1.9% to 2.9%, and the stock solutions of the four compounds were stable at room temperature for 0–24 hours with RSDs ranging from 1.9% to 3.0%. The recovery of the four compounds was assessed in six replicates and showed good accuracy with RSD values of <1.72% for the range of 90.0–102.6%.

Determination of the content of four active components in YQS extracts
The content of caffeoylquinic acid, arctium lappa glycoside, aurantiamarin, and forsythin differed according to the extraction methods. The content of caffeoylquinic acid was greatest in the 30% ethanol extract, while content of aurantiamarin was greatest in the 95% ethanol...
X. Wang, O. Hao, W. Wang, et al

The content of arctium lappa glycoside was greatest in water, while the content of forsythin was greatest in the 70% ethanol extract (Table 1).

<table>
<thead>
<tr>
<th>Extract solvent</th>
<th>CAA</th>
<th>AUR</th>
<th>ALG</th>
<th>FOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2.900</td>
<td>0.045</td>
<td>34.347</td>
<td>0.200</td>
</tr>
<tr>
<td>30% ethanol</td>
<td>4.472</td>
<td>0.088</td>
<td>18.748</td>
<td>0.282</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>3.481</td>
<td>0.073</td>
<td>30.597</td>
<td>0.260</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>2.639</td>
<td>0.061</td>
<td>27.832</td>
<td>0.412</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>2.541</td>
<td>0.982</td>
<td>21.020</td>
<td>0.229</td>
</tr>
</tbody>
</table>

*Data presented as %. CAA = Caffeoylquinic acid; AUR = auran-tiamarin; ALG = arctium lappa glycoside; FOR = forsythin.

The EID$_{50}$ of IAV was $10^{-7.25}$ according to the Kärbe formula. All embryonated chicken eggs were alive when incubated with the stock solution of YQS, which means the maximum TD$_0$ of YQS was 500 mg/mL. For comparison, the maximum TD$_0$ of ribavirin was 100 mg/mL.

**EID$_{50}$ and TD$_0$**

The EID$_{50}$ of IAV was $10^{-7.25}$ according to the Kärbe formula. All embryonated chicken eggs were alive when incubated with the stock solution of YQS, which means the maximum TD$_0$ of YQS was 500 mg/mL. For comparison, the maximum TD$_0$ of ribavirin was 100 mg/mL.

**Maximum non-blood clotting concentration**

The maximum non-blood clotting concentration of the five YQS extracts is shown in Table 2. The concentration used in the later studies was the lower one, which would not cause hemagglutination. Therefore, the initial concentration used for this test was $2 \times 10^{-4}$ (31.3 mg/mL).

<table>
<thead>
<tr>
<th>Solvent extract</th>
<th>Dilution ratio</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>$2 \times 10^{-3}$</td>
<td>62.5</td>
</tr>
<tr>
<td>30% ethanol</td>
<td>$2 \times 10^{-3}$</td>
<td>62.5</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>$2 \times 10^{-4}$</td>
<td>31.3</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>$2 \times 10^{-3}$</td>
<td>62.5</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>$2 \times 10^{-4}$</td>
<td>31.3</td>
</tr>
</tbody>
</table>

IAV = Influenza-A virus; YQS = Yinqiaosan.

The maximum non-blood clotting concentration (31.3 mg/mL) inhibited IAV proliferation at each time to different degrees, and the inhibitory effects greatest between 6–12 hours, and then tapered after 18 hours. The IAV-inhibitory effect of the 50% ethanol extract was very significant at each time, particularly at 12 hours (the virus titers in virus group was 1/320 compared with the 50% YQS extract group was 1/10), with the influenza virus-inhibitory effect to 32 multiples.

**Therapeutic effect of YQS extracts on IAV-infected embryonated chicken eggs**

As summarized in Table 4, there were significant differences ($p<0.05$) between each extract (water, 30%, 50%, 70%, and 95% ethanol) and each concentration (500, 250 and 125 mg/mL) versus the IAV group. This means that each solvent extract with different concentration were all effective in IAV-inhibitory on the embryonated chicken eggs. Of interest, the 50% ethanol extract at concentrations of 500 mg/mL and 250 mg/mL was superior to ribavirin ($p<0.05$). Thus, the 50% ethanol extract may be the optimal extract of YQS to inhibit IAV in vitro, and should serve as a baseline for in vivo experiments of YQS for the treatment of viral infection.
Composition and efficacy of Yinqiaosan

DISCUSSION

Optimization of the HPLC conditions
To achieve good separation, we investigated a range of HPLC analytical parameters including the separation column, mobile phase and elution mode. The separation of each chromatographic peak was not optimal using a Phenomsil ODS C18 column (150 × 4.6 mm; i.d., 5 μm). Therefore, we first used gradient elution modes with methanol/water, acetonitrile/water and acetonitrile/methanol/water containing different concentrations of acetic acid for the mobile phase. We found that the four compounds showed markedly different polarities, and they did not separate well using isocratic and gradient elution. We next tested different concentrations of methanol and aqueous phosphoric acid. However, the chromatographic peaks split when the phosphoric acid concentration was lower than 0.4%. Finally, we found that the separation was improved using a Phenomsil ODS C18 column (250 × 4.6 mm; i.d., 5 μm) using a solvent system composed of methanol (M)/water containing 0.4% phosphoric acid solution (P) as the mobile phase in a gradient elution mode. Because all of the compounds could be detected using this approach and with adequate adsorption, the detection wavelength was set at 275 nm. Under these conditions, the chromatograms of the four references compounds and YQS extracted with water, and 30%, 50%, 70%, or 95% ethanol are shown in Figures 1 and 2.

Relationship between the fingerprints of each extract and IAV-inhibitory effects
In this study, we focused on the characterization of the four main active ingredients of YQS in different solvent extracts. Figure 1 shows typical chromatograms for the four reference compounds, and Figure 2 shows the chromatograms for the four components of YQS in the five solvent extracts. The retention times of caffeoylquinic acid, arctium lappa glycoside, aurantiamarin, and forsythin were approximately 24.6 minutes, 35.9 minutes, 40.2 minutes, and 41.7 minutes, respectively, and the total chromatographic run-time was 60 minutes. There was a wide variation in the content of the four components between the five extracts. The content of caffeoylquinic acid was greatest in the 30% ethanol extract, while content of aurantiamarin was greatest in the 95% ethanol extract. The content of arctium lappa glycoside was greatest in the water extract, while the content of forsythin was greatest in the 70% ethanol extract. However, the IAV-inhibitory effect of YQS was greatest with the 50% ethanol extract, which indicates that these affects are achieved through synergistic activity of all of the components rather than an individual component.
This study differs from previous studies that investigated the anti-viral effects of YQS. Reference documentation on the molecular fingerprints of TCM formulae are still relatively scarce, including for YQS. Shiyou et al. focused on the effective parts in each herb against influenza virus and the chemical constituents of YQS, including the isolation of flavonoid and its relative content [17,18,24]. In our study, our evaluation of YQS were based on its use in clinical practice, considering the mechanism and curative effects of the YQS formula rather than individual herbs or specific active components. We also evaluated how the fingerprint of YQS was related to its IAV-inhibitory effects, and it was proposed on the base of effective single component after component identification. Quality control of the formulation is closely related to its pharmacodynamic action, which is very important in terms of maintaining the quality of medicines, to enhance the effects of the formulation, and to modernize TCM.

Some of the chromatographic peaks changed when YQS was extracted using different solvents. For example, a chromatographic peak at the retention time of 13 minutes was prominent in the 95% ethanol extract but was not well defined in the other extracts (Figure 2).

Thus, it seems that some components show solvent specificity. Although this peak was not a focus of the present study, it should be considered in future studies.

**Rational for determining the maximum non-blood clotting concentration**

In this experiment, some YQS extracts above a certain concentration caused agglutination of chicken blood cells, which aroused our attention. Was this phenomenon caused by a physical or a chemical reason? If it was a chemical effect, YQS may contain a component that could cause chicken red blood cell agglutination. For this reason, we determined the maximum non-blood clotting concentration of YQS. Based on a literature review, it seemed likely that this component was a lectin. Indeed, there are almost 1,000 types of plant lectins, which are widely distributed in groups such as Leguminosae, Solanaceae, Euphorbiaceae, Gramineae, Lilaceous, and Amaryllidaceae. Legume lectin is the most abundant, with more than 600 varieties. Several recent studies have shown that several plants used in TCM, including Radix isatidis, Ramulus visci, and Aloe, contain lectins. It has been reported that lectins can promote lymphocyte proliferation in mice and suppress the

**Figure 2.** High-performance liquid chromatography chromatograms of the four components for Yinqiaosan extracted in (A) water, (B) 30% ethanol, (C) 50% ethanol, (D) 70% ethanol, and (E) 95% ethanol. CAA = Caffeoylquinic acid; AUR = auranthanarin; ALG = arctium lappa glycoside; FOR = forsythin.
propagation of tumor cells [25–28]. In pilot studies, we commonly found chicken red blood cell agglutination, which prompted our suspicion that the YQS extracts also contain lectin components, although whether this is derived from a single or several herbs is unclear. Therefore, we used the maximum non-blood clotting concentration to ensure that the extract itself did not cause agglutination of the chicken blood cell and ensure the findings only reflected the direct effects of YQS extracts on IAV inhibition.

**Assessment of IAV inhibition**
The hemagglutination titer was determined to reflect the effect of YQS extracts on IAV activity. Microscopic and other quantitative method could also be applied in future studies to evaluate the relationship between the fingerprints of the YQS extracts and IAV-inhibitory effects. Of interest, the therapeutic effect of the 50% ethanol extract of YQS at concentrations of 500 mg/mL and 250 mg/mL was superior to that of ribavirin. However, the toxicity of YQS formulations has not been reported and needs further research.

In conclusion, we used a simple quantitative method based on HPLC to analysis the relative content of the four main active components of YQS. The method offered good linearity, precision, repeatability, stability, and recovery. Using this method, we successfully established the HPLC fingerprints of the four components in YQS in five solvent extracts. The study also indicated the possibility that the five solvent extracts of YQS can inhibit IAV in embryonated eggs in vitro. YQS extracted in 50% ethanol yielded the greatest effect and was superior to ribavirin. Taken together, the results of this study provide valuable evidence supporting the clinical use of YQS.

**ACKNOWLEDGMENTS**
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X. Wang, O. Hao, W. Wang, et al


不同提取溶剂下銀翹散中四種成分指纹图谱的建立及其外抗甲型流感病毒效果

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3中國醫科大學 臨床藥理學院

採用高效液相色譜法同時測定不同提取溶劑（水提、30% 醇提、50% 醇提、70% 醇提、95% 醇提）下銀翹散中綠原酸、橙皮苷、牛蒡苷和連翹苷四種有效成分的含量。結果顯示上述四種有效成分在 0.93–151.5 mg/L 範圍內呈線性關係（R² > 0.9983）；平均回收率為 91.3 – 100.5%，RSD = 1.89%。並較好的建立了不同溶劑提取下的銀翹散中四種活性成分的指纹圖譜。本次研究還採用體外直接抑制流感病毒和雞胚抗病毒實驗技術觀察不同提取工藝銀翹散抗流感病毒療效。結果顯示 50% 醇提法的銀翹散提取物在 500 mg/mL 和 250 mg/mL 濃度時，其抗流感病毒作用最強，治療效果優於利巴韋林對照組（p < 0.05）。因此 50% 醇提法可能為銀翹散抗 IV 最佳提取工藝。該實驗結果表明銀翹散提取物顯示出了較好的體外抗流感病毒作用，為銀翹散以後用於流感治療的可能性作出了評價。

關鍵詞：指纹圖譜，A 型流感病毒，銀翹散
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