Screening and evaluation of commonly-used anti-influenza Chinese herbal medicines based on anti-neuraminidase activity

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Available online 20 Oct., 2016

[ABSTRACT] Anti-influenza Chinese herbal medicines (anti-flu CHMs) have advantages in preventing and treating influenza virus infection. Despite various data on antiviral activities of some anti-flu CHMs have been reported, most of them could not be compared using the standard evaluation methods for antiviral activity. This situation poses an obstacle to a wide application of anti-flu CHMs. Thus, it was necessary to develop an evaluation method to estimate antiviral activities of anti-flu CHMs. In the present study, we searched for anti-flu CHMs, based on clinic usage, to select study objects from commonly-used patented anti-flu Chinese medicines. Then, a neuraminidase-based bioassay, optimized and verified by HPLC method by our research group, was adopted to detect antiviral activities of selected 26 anti-flu CHMs. Finally, eight of these herbs, including *Coptidis Rhizoma*, *Isatidis Folium*, *Lonicerae Flos*, *Scutellaria Radix*, *Cyrtomium Rhizome*, *Houttuynia Cordata*, *Gardeniae Fructus*, and *Chrysanthemi Indici Flos*, were shown to have strong antiviral activities with half maximal inhibitory concentration (IC50) values being 2.02 to 6.78 mg·mL⁻¹ (expressed as raw materials). In contrast, the IC50 value of positive control peramivir was 0.38 mg·mL⁻¹. Considering the extract yields of CHMs, the active component in these herbs may have a stronger antiviral activity than peramivir, suggesting that these herbs could be further researched for active compounds. Moreover, the proposed neuraminidase-based bioassay was high-throughput and simple and could be used for evaluation and screening of anti-flu CHMs as well as for their quality control.

[KEY WORDS] Anti-influenza; Chinese herbal medicines; Viral neuraminidase; Bioassay; Screening; Evaluation

[CLC Number] R917

Introduction

Influenza, also known as flu, is an acute infectious respiratory disease caused by infection of influenza viruses such as H1N1 and H5N1 [1]. It could infect human and cause annual influenza epidemics with significant morbidity and mortality [2]. Neuraminidase (NA) is an enzyme that cleaves sialic acid groups from host glycoproteins in order for the influenza virus to be released from the cell, and is also required for influenza virus replication [3]. Currently, blocking the function of neuraminidase is recognized as an effective target to treat influenza presently. Neuraminidase inhibitors (NAIs), such as: oseltamivir, zanamivir, and peramivir, are commonly used to prevent and treat influenza [4]. Resistance of oseltamivir is associated with increased morbidity and poor outcome in severely immunocompromised hosts [5-6]. Peramivir can be offered only as an intravenous formulation because of its low oral bioavailability [7]. Overall, there is a lack of available and effective agents preventing and treating influenza virus infection.

[Received on] 15- Dec.-2015
[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81274026, 81403126, 81330090, 81573676).
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These authors have no conflict of interest to declare.
China has high influenza morbidity. Traditional Chinese medicines have been used to prevent and treat influenza for thousands of years, and have played a significant role in fighting influenza virus pandemic [8]. In recent years, researchers have intensively investigated the antiviral effects of some anti-influenza Chinese herbal medicines (anti-flu CHMs) [8–11]. And many herbs have been proven effective and without drug resistance and serious adverse effects. During the H1N1 epidemic, an evidence-based medical research group conducted a randomized, multicenter trial at 11 medical sites in China and a Chinese herbal compound was demonstrated to be effective against H1N1 influenza, which was similar to oseltamivir [12]. That study has provided important evidences that anti-flu CHMs are effective in treating influenza, and have great potential to prevent and treat influenza.

Currently, several in vivo and in vitro methods are used to detect antiviral activity of anti-influenza medicines. Those method mainly include plaque reduction assay, cytopathic effect inhibition assay, MTT test, hemagglutination inhibition test, neuraminidase (NA) activity assay, penetration assay, effect inhibition assay, MTT test, hemagglutination inhibition test, RNA synthesis test, RT-PCR test, and embryo test [13]. Neuraminidase activity assay is a classical, stable, and high-throughput method with a good reproducibility. And it is robust with respect to unspecific matrix interference [14]. To make this method more suitable for evaluation of Chinese medicines, our research group has optimized it previously [15–16]. According to the early work of our group, the antiviral activities of some CHMs determined by the neuraminidase-based bioassay are accurate and objective. Therefore, this neuraminidase-based bioassay was adopted to systemically screen anti-flu CHMs in the present study.

To our best knowledge, there have been no reports on systemic screening of anti-flu CHMs based on neuraminidase-based bioassay and clinic usage. In the present study, a systematic search for anti-flu drugs based on clinic usage was firstly used to select study objects. Then we detected the antiviral activities of the selected 26 herbs using aforementioned neuraminidase-based bioassay and this bioassay was validated by HPLC method which could objectively describe antiviral activities of the CHMs. Finally, ten of these herbs showed strong antiviral activities. These herbs, their extracts, and their anti-flu compounds would be beneficial to defeat the influenza pandemic in the future.

**Materials and Methods**

**Selection of study objects**

A systematic search of the Chinese Patent Medicine Prescription Database in Yaozhi website (http://db.yaozh.com) was conducted to select anti-flu Chinese patent medicines in common use. The database included about 8 000 Chinese patent medicines in the market [17]. The drug reference standards were from Compilation of National Standards of Chinese Patent Medicine [17]. Most of them were from the heat-clearing CHMs in China. For function search, “heat-clearing” was selected as the search term with no other restrictions.

**Materials and reagents**

26 CHMs were purchased from Beijing Lvye Medicine Co., Ltd. (Beijing, China) and identified by Professor XIAO Xiao-He (PLA Institute of Chinese Material Medical, 302 Hospital of People’s Liberation Army, Beijing, China). Neuraminidase Inhibitors Screen Kit was purchased from Beyotime Co., Ltd. (Shanghai, China). Peramivir and sodium chloride injection was obtained from Nanxin Medicine Co., Ltd. (Guangzhou, China). Standard reference of 4-MU was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). The purity was more than 98%. Methanol of HPLC grade was purchased from Fisher Chemicals (Pittsburg, PA, USA). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Glycine was purchased from Amresco Co. (Fountain Parkway Solon, OH, USA). Acetic acid and absolute ethyl alcohol were purchased from Beijing Chemical Factory (Beijing, China). Sodium hydroxide was purchased from Xilong Chemical Co., Ltd. (Chengdu, China). All other chemicals used in the present study were of analytical grade and available locally.

**Sample preparations**

**Sample for preliminary screening** Each powdered herb (about 5.0 g) was weighed precisely and added to 20 mL of 50% (V/V) alcohol. The whole sample was weighed again and then disposed by ultrasonic extraction for 1 h. The loss of weight was compensated by 50% (V/V) alcohol and the sample was filtered. The filtrates were prepared at 250 and 2.5 mg·mL⁻¹ (1 mL of solution contained 250 or 2.5 mg of herb) in 50% alcohol before use. The above solutions were tested after filtrated by 0.22 μm microfiltration membrane.

**Sample for antiviral activity assay** The sample preparation was similar to the aforementioned procedure in the sample for preliminary screening section. The filtrates were prepared at 20, 10, 5, 2.5, and 1.25 mg·mL⁻¹ in 50% alcohol and filtered by 0.22-μm microfiltration membrane before use.

**Sample for detection method validation and positive control** Peramivir and sodium chloride injection with concentration at 3 mg·mL⁻¹ were prepared at 3, 2, 1, 0.5, 0.25 and 0.125 mg·mL⁻¹ in saline and filtered by 0.22-μm microfiltration membrane, which were used as positive controls.

**Detection method validation**

**Detection by microplate reader** 4-Methylumbelliferyl-α-D-N-acetyleneuraminic acid (MUNANA) is a specific substrate of NA and can be converted to 4-methylumbelliforone (4-MU), which can be detected with an emission wavelength of 440 nm and an excitation wavelength of 360 nm. The activity of NA can then be calculated by the fluorescence intensity of 4-MU, and the antiviral effects of medicines can be evaluated through the inhibitory effects in the NA activity.
The experimental groups included enzyme activity control group (E group, NA + MUNANA), control group (C group, MUNANA), test group (T group, NA + sample + MUNANA) and background group (B group, sample + MUNANA). Buffer solution was added into each well of a 96-well plate (70 μL for T group, 80 μL for E and B groups and 90 μL for C group). NA (10 μL) was added into T and E groups, and test solution (10 μL) was added into T and B groups. After mixing for 1 min, the reaction was allowed to run for 30 min at room temperature, and substrate MUNANA was added (10 μL). After incubation at 37 °C for 60 min, the stop buffer [glycine prepared with 25% (V/V) ethanol solution to a final concentration of 0.1 mol·L⁻¹] was added. The fluorescence intensity value was detected by microplate reader (excitation wavelength, 360 nm; emission wavelength: 440 nm; gain adjustment, 70; detection temperature, 37 ºC). Each sample was tested for five times, and the average value was taken. The inhibitory rate of NA was calculated as follows:

\[ I_1 = \frac{(OD_{E} - OD_{C}) - (OD_{T} - OD_{B})}{(OD_{E} - OD_{C})} \times 100\% \]

(\(I_1\): the inhibitory rate of NA detected by microplate reader; \(OD_E\): optical density of enzyme activity control group; \(OD_T\): optical density of test group; and \(OD_B\): optical density of background group.)

**HPLC Analysis**[21]

The assay solutions of the enzyme activity control and test groups were filtered by 0.22-μm microfiltration membrane, and 20 μL of the filtrates was injected onto HPLC to analyze the content of 4-MU. The inhibitory rate of NA was calculated as follows:

\[ I_2 = \frac{M_E - M_T}{M_E} \times 100\% \]

(\(I_2\): the inhibitory rate of NA detected by HPLC; \(M_E\): the content of 4-MU in the enzyme activity control group; and \(M_T\): the content of 4-MU in the test group.)

The analysis of 4-MU was performed using RP-HPLC. An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) was applied. Separation was performed on Agilent Eclipse Plus C18 column (250 mm × 4.6 mm, with 5-μm particle size). The mobile phase consisted of methanol (A) and 0.02% (V/V) ethyllic acid water solution (B). The gradient elution was 10% A from 0 to 14 min, 10%−50% A from 14 to 22 min, and 50% A from 22 to 30 min at a flow rate of 1.0 mL·min⁻¹. The signal was monitored at 322 nm.

4 mg of standard reference of 4-MU was taken into a 10-mL volumetric flask, completely dissolved in methanol to volume, and then diluted with methanol to 100 μg·mL⁻¹. The solution was filtered through a Millipore 0.22-μm membrane filter, and 5 μL filtrate was injected onto the HPLC system for analysis.

**Antiviral activity assay**

According to the NA inhibition of preliminary screening, the herbs having high NA inhibition activity were chosen for further study.

**Results**

**Study objects**

Based on the searching results and usage in the market, 55 anti-flu Chinese patent medicines were chosen. The frequency of each herb used in the 55 formulas was calculated. 26 CHMs with high-frequency (Fig. 1) were selected for testing in the present study. They are shown at numbers later in the paper as their names are complex (Table 1).

**Method validation**

As shown in Fig. 2, the results of HPLC determination were similar to that of assays with microplate reader. Quantitation for 4-MU using HPLC was accurate. So detection by microplate reader was objectively. But the analysis of HPLC was slower than microplate reader, as microplate reader assay only needed seconds to test a sample while HPLC needed about 40 min. So microplate reader was more suitable for the detection of 4-MU than HPLC.

**Antiviral activity**

As shown in Fig. 3, the NA inhibition rates of most herbs at 250 mg·mL⁻¹ were similar. Therefore, ten herbs including Coptidis Rhizoma, Isatidis Folium, Lonicerae Flos, Scutellaria Radix, Cyrtomium Rhizome, Houttuynia Cordata, Gardeniae Fructus, Chrysanthemi Indici Flos, Schizonepeta Tenufolia, and Andrographis Herba were screened out based on their NA inhibition rates at 2.5 mg·mL⁻¹ and their usage in clinical treatment for influenza. The NA inhibition rates of these 10 herbs were then determined at concentrations of 20, 10, 5, 2.5, and 1.25 mg·mL⁻¹. According to the dose-effect relationship, the half maximal inhibitory concentration (IC₅₀) value of each herb was calculated by SPSS 19.0.

We next performed statistical analysis of the frequency of herb pairs among the 26 CHMs in 55 anti-flu Chinese patent medicines (Fig. 4). It showed that some high-active herbs had been commonly used with each other in Chinese patent medicines, such as Scutellaria Radix used with Gardeniae Fructus and Scutellaria Radix used with Lonicerae Flos. But some other high-active herbs had not been commonly used with each other, such as Coptidis Rhizoma, Cyrtomium Rhizome and Isatidis Folium. These results indicated that these high-active herbs’ compatibilities are worthy of be reasonably enhanced in therapy for influenza.

**Discussions**

In the present study, a search of anti-flu CHMs based on clinic usage was firstly conducted to select study objects. A neuraminidase-based bioassay was chosen to evaluate antiviral activity. Our results indicated that the neuraminidase-based bioassay could objectively describe...
Table 1  26 high-frequency used CHMs

<table>
<thead>
<tr>
<th>Number</th>
<th>Chinese herbal medicine</th>
<th>Chinese name</th>
<th>Number</th>
<th>Chinese herbal medicine</th>
<th>Chinese name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Artemisiae annuae herba</td>
<td>Qinghao</td>
<td>14</td>
<td>Mori folium</td>
<td>Sangye</td>
</tr>
<tr>
<td>2</td>
<td>Coptidis rhizoma</td>
<td>Huanglian</td>
<td>15</td>
<td>Menthae hapolcalycis herba</td>
<td>Bohe</td>
</tr>
<tr>
<td>3</td>
<td>Rhubarb</td>
<td>Dahuang</td>
<td>16</td>
<td>Artemisiae Annuae Herba</td>
<td>Niubangzi</td>
</tr>
<tr>
<td>4</td>
<td>Houttuynia cordata</td>
<td>Yuxingcao</td>
<td>17</td>
<td>Pogostemonis herba</td>
<td>Guanhuoxiang</td>
</tr>
<tr>
<td>5</td>
<td>Scutellaria radix</td>
<td>Huangqin</td>
<td>18</td>
<td>Andrographis herba</td>
<td>Chuanxilian</td>
</tr>
<tr>
<td>6</td>
<td>Gardeniae fructus</td>
<td>Zhizi</td>
<td>19</td>
<td>Perillae folium</td>
<td>Zisu</td>
</tr>
<tr>
<td>7</td>
<td>Glycyrrhizae radix et rhizoma</td>
<td>Gancao</td>
<td>20</td>
<td>Bupleuri radix</td>
<td>Chaihu</td>
</tr>
<tr>
<td>8</td>
<td>Cyrtomium rhizome</td>
<td>Guanzhong</td>
<td>21</td>
<td>Anemarrhenae rhizoma</td>
<td>Zimu</td>
</tr>
<tr>
<td>9</td>
<td>Chrysanthemi indici flos</td>
<td>Yejuhua</td>
<td>22</td>
<td>Sophorae tonkinensis radix et rhizoma</td>
<td>Shandougen</td>
</tr>
<tr>
<td>10</td>
<td>Forsythiae fructus</td>
<td>Lianqiao</td>
<td>23</td>
<td>Saposhnikoviae Radix</td>
<td>Fangfeng</td>
</tr>
<tr>
<td>11</td>
<td>Lonicerae flos</td>
<td>Jinyinhua</td>
<td>24</td>
<td>Phragmitis rhizoma</td>
<td>Lugeng</td>
</tr>
<tr>
<td>12</td>
<td>Isatidis flium</td>
<td>Daqingye</td>
<td>25</td>
<td>Platycodonis radix</td>
<td>Jiegeng</td>
</tr>
<tr>
<td>13</td>
<td>Schizonepeta tenuofilia</td>
<td>Jingjie</td>
<td>26</td>
<td>Isatidis radix</td>
<td>Banlangen</td>
</tr>
</tbody>
</table>

The number will be shown later indicating the name of herb.
antiviral activities through assay validation with the HPLC method (Fig. 2). Then, viral NA inhibition activities of 26 commonly-used anti-influenza CHMs were studied through neuraminidase-based bioassay. Antiviral activities among these herbs could be obviously distinguished. Finally, ten of these herbs were found to exert strong antiviral activities, especially the first eight CHMs, including Coptidis Rhizoma, Isatidis Folium, Lonicerae Flos, Scutellaria Radix, Cyrtomium Rhizome, Houttuynia Cordata, Gardeniae Fructus, and Chrysanthemi Indici Flos.

Outbreaks of H5N1, H1N1, and other influenza warn us that all the people in the world are still under health threat of potentially influenza pandemic. Although chemical drugs do have some effects on influenza, they are still unsatisfactory...
The recombination of 17 HA and 10 NA subtypes will infect more various species and cause great difficulty for treating influenza [1-2]. So it is essential to find more active agents for the prevention and treatment of influenza. The ten herbs screened out from 26 herbs based on the NA inhibition rate and usage in the clinic showed strong antiviral activities (Figs. 3B and 3D). Their viral NA IC₅₀ values for the first eight CHMs were under 5 mg·mL⁻¹. If the concentration was converted to 1 the quantity of herb extract per mL of sample (the extract yield was estimated at 10% due to 50% (V/V) alcohol as solution), most of their viral NA IC₅₀ values may be lower than that of positive control peramivir. Furthermore, the active component in herbs may have a stronger antiviral activity. It indicated that these herbs should be further researched and developed to find potential active compounds and create novel anti-flu agents [18].

However, there were always criticisms on the quality of traditional Chinese medicines. It imposes restrictions on recognition and application of anti-flu Chinese medicines worldwide. The reason for that is the limitation of current quality control methods that are mostly through content control of marker chemicals [22]. We collected marker chemicals in the 26 commonly-used CHMs’ quality control methods based on Chinese Pharmacopoeia [22] and analyzed the relevance between marker chemicals and antiviral activities by literature review [23-28]. We found that marker chemicals for only about 33% anti-flu CHMs had relevance with antiviral activities, and about 15% anti-flu CHMs did not have content control. Meanwhile, the mode of CHMs curing diseases is multicomponent. Single or limited active component for quality control of CHMs cannot reflect bioactivity comprehensively or ensure quality. Therefore, current chemical quality control methods of anti-flu CHMs may not guarantee their quality. However, the neuraminidase-based bioassay could objectively describe antiviral activities of CHMs. And it is simpler and less time-consuming than the current methods for quality control. What’s more, it is high-throughput and could lead to greater efficiency in drug screening. Thus, the neuraminidase-based bioassay is appropriate for quality control of anti-flu CHMs.

In conclusion, the neuraminidase-based bioassay as a fast, efficient and objective method could be adopted to screen and evaluate anti-influenza CHMs. Findings in the present study may offer a reference for establishing major composition of clinical anti-flu medications and for developing more potent anti-flu Chinese medicines. The eight herbs screened out in our research should be further investigated for their applications in treating influenza.

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Cite this article as: HAN Xue, et al. / Chin J Nat Med, 2016, 14(10): 794–800.

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Cite this article as: HAN Xue, et al. / Chin J Nat Med, 2016, 14(10): 794–800.