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

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[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 (IC_{50}) values being 2.02 to 6.78 mg·mL^{-1} (expressed as raw materials). In contrast, the IC_{50} 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

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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]. Zanamivir is administered by inhalation and may have adverse reaction for patients with asthma or chronic obstructive airway disease [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

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preventing and treating influenza virus infection.

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, andhave 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, protein synthesis 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-cleaning” 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 [15-16, 18-20] 4-Methylumbelliferyl-*α*-D-*N*-acetylneuraminic acid (MUNANA) is a specific substrate of NA and can be converted to 4-methylumbelliferone (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 con-