

Full Length Research Paper

Effects of the extract of *Forsythia suspensa* on influenza A H1N1 infection *in vitro*

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The present study investigated the effect of the extract of *Forsythia suspensa* (FSE) on the infection of MDCK cells by anti-H1N1 virus. The protective effect of FSE on the infected MDCK cells was evaluated through quantitative measurement of the changes in the cytopathic effects and by the ultraviolet (UV) absorbance at 600 nm. It was found that FSE was toxic to MDCK cells at a higher concentration, while it had a marked inhibitory effect on cell pathological changes at a lower concentration. Our findings demonstrate that FSE has a significant protective effect on MDCK cells infected in a dose-dependent manner, suggesting that FSE can be developed as an anti-virus agent.

Key words: *Forsythia suspensa*, H1N1 virus, the cytopathic effects.

INTRODUCTION

Acute respiratory tract infections are recognized as a leading cause of acute morbidity in individuals of all ages especially children worldwide (2004; Monto, 2002). The key solution for treating acute viral respiratory tract infections relies on discovering effective antiviral agents. The present study focused on candidates with special characteristics for antiviral from the point of view of traditional Chinese medicine.

Forsythia suspensa is a climbing plant, which is widely distributed throughout China, Korea and Japan (Liu et al., 2010). Having been officially listed in the Chinese Pharmacopoeia, the fruit of *F. suspensa*, named "Lianqiao" in Chinese, has been a well-known traditional Chinese medicine for centuries. According to the maturity level of its fruits, the commercial drugs could be classified into "Qingqiao" (immature) and "Laoqiao" (mature), both of which are official sources of this traditional Chinese medicine.

Lianqiao has been widely used as an antipyretic detoxicant for treatment of various diseases (Piao et al., 2008a; Chang et al., 2008). It has also been shown that *F. suspensa* extract (FSE) is able to suppress vomiting, resist hepatic injury and inflammation for the treatment of infections (Guo Qiang et al., 2009). More than 40 Chinese

medicinal preparations containing Lianqiao or FSE are listed in Chinese Pharmacopoeia, such as "Shuanghuanglian oral solution", "Yinqiao Jiedu tablet", "Qinlian tablet", etc.

FSE contains abundant amount of phenylethanoid glycosides, lignans, flavonoids, terpenes and volatile oils (Zhang et al., 2000). Among these, the phenylethanoid glycosides, lignans, flavonoids compounds were found to be responsible for the various biological effects (Piao et al., 2008a). Previous studies found that FSE exhibited antibacterial, antiviral activities without any adverse effects (Tian et al., 2004). Therefore, the objective of the current study was to investigate the antiviral effect of FSE on acute respiratory infections with H1N1 virus, which may prove useful for further antiviral research on acute respiratory tract infections.

MATERIALS AND METHODS

Reagents

The *F. suspensa* herb was collected from the Department of Agent, Wuhan No.1 Hospital, Wuhan, China (Herbarium no. 221-155-07). Madin-Darby Canine Kidney epithelial cell line MDCK cells were provided by Tongji Medical College, Huazhong University of Science and Technology. The strain of pandemic 2009 H1N1 influenza virus was provided by Institute of medical science viruses, Medical College, Wuhan University Medicine. High glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen, Carlsbad, CA, USA. Fetal calf serum was purchased

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BRL. Ribavirin (purity, 99.3%) obtained from Xinxiang Pharmaceutical Company (Xinxiang, P. R. China).

Extract preparation from *Forsythia suspensa*

FSE was obtained as described in Yue and Piao (Yue Hao et al., 2010). The dried mature fruits (Laoqiao) of *F. suspensa* (500 g) were ground and extracted under reflux conditions with 2500 mL of 80% ethanol. The combined 80% ethanol extracts were sonicated for 1 h three times.

The combined extracts were concentrated under reduced pressure below 45°C on a rotary evaporator, and freeze dried to give a powdered mass (58.6 g). The crude extract was re-dissolved in water, extracted with CH₂Cl₂ three times and n-BuOH four times in succession. The CH₂Cl₂ fractions and the n-BuOH fractions were combined, respectively. The two fractions were dried on a rotary vaporization to give, respectively, 24.3 g solid residue from the CH₂Cl₂ and 20.5 g from the n-BuOH. An aliquot (200 mg) from CH₂Cl₂ was identified as described in Lu (Liu et al., 2010), based on their mass spectral, ¹H NMR, and ¹³C NMR data, as phillygenin (15.5 mg), phillyrin (79.6 mg), forsythalian A (39.8 mg) and forsythoside A (14.6 mg).

Cell culture and virus preparation

MDCK cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS). Subculture was carried out every 2 - 3 day after it had formed a confluent monolayer. Pandemic 2009 H1N1 influenza virus was serially diluted to 10⁻¹⁰ with non-FCS DMEM culture medium. On 40-well plate, 0.025 mL H1N1 virus with variable dilution and 0.025 mL non-FCS DMEM culture medium were added to each well. Finally, 0.05 mL viable MDCK cells (3×10⁵/mL) were added. Each dilution was quadrupled and normal MDCK cells co-cultured only with DMEM containing 10 mL/L FCS were prepared as negative control at the same time. Then the cells were incubated at 37°C with 50 mL/L CO₂ for 72 h. The cytopathic effects (CPE) were observed under light microscope. The titer at which cells appeared 50% CPE was designated 1×TCID₅₀. A 100×TCID₅₀ was used as the infectious titer in the following experiment. TCID₅₀ was calculated as 10⁻⁶ according to Reed-Muench method (Krah et al., 1991).

Toxicity detection of *Forsythia suspensa* extract

MDCK cells were plated in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO₂ in air. Then, 1 mg/mL FSE was diluted with non-FCS DMEM culture medium serially from 1:2 to 1:1024. A total of the 0.025 mL sample with various concentrations and 0.025 mL of the same culture medium were added to each well on a 96-well plate. Finally, 0.05 mL of viable MDCK cells (3 × 10⁵/mL) was added. Each concentration of the sample was quadrupled. Two controls, MDCK cells co-cultured only with DMEM and 1 mg/mL DMSO, were prepared synchronously. Cells were incubated at 37°C with 5% CO₂ for 72 h. CPE was observed under light microscope and the concentration at which cells appeared < 50% CPE was regarded as the lowest toxic concentration. In addition, cells were stained with 5 mg/mL crystal violet and A₆₀₀ was detected using spectrophotometer.

The anti-H1N1 virus effect detection of *Forsythia suspensa* extract

FSE was diluted serially from 1:512 at which it had no toxicity to

mL FSE with variable concentration was then added to each well of the 96-well plate and 0.025 mL H1N1 virus was overlaid. After incubation at 37°C with 5% CO₂ for 1 h, 0.05 mL viable MDCK cells (3 × 10⁵/mL) were added. Each concentration was quadrupled and four controls, normal MDCK cells co-cultured only with DMEM, 100×TCID₅₀ H1N1 virus and FSE (1:512 mg/mL), ribavirin (0.2 mg/mL ribavirin being positive controls) respectively, were prepared synchronously. Cells were grown for 3 days before detection of CPE under light microscope. After that, the cells were stained with 5 mg/mL crystal violet and A₆₀₀ was measured using spectrophotometer.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± SD. One-way ANOVA was used to evaluate statistical significance. A value of P < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Although, many efforts have been made during these years, there are no licensed vaccines available for preventing respiratory viral infections other than influenza. Recently, many countries have been paying more attention to search for anti-virus agents among Chinese medicinal herbs. The *F. suspensa* is a traditional Chinese medicinal herb. Its pharmacological effects such as antipyretic, antidotal and anti-inflammatory effect were well documented (Zhang et al., 2000). In the present study, we demonstrated that FSE was an effective anti-virus agent, which could have marked respiratory H1N1 virus effect *in vitro*.

Analysis of H1N1 virus titers showed the cells in all quadrupled wells treated with H1N1 virus at titer of 10⁻¹-10⁻⁵ appeared 100% CPE. The cells treated with H1N1 virus at titer of 10⁻⁶, the cells in two wells appeared 100% CPE while the others appeared 50% CPE.

According to the detection of the toxicity of FSE, it was indicated that the cells co-cultured with FSE at concentrations from 1:2 to 1:256 mg/mL appeared CPE of different degrees, while the cells co-cultured with FSE at concentrations from 1:512 to 1:8192 mg/mL displayed no CPE. The 50% toxic concentration was 1:256 mg/mL according to Reed-Muench method. Data presented in Table 1 also indicated that FSE had no toxicity to MDCK cells at concentrations from 1:512 to 1:1024 mg/mL and the cells co-cultured with 1 mg/mL DMSO showed no CPE, indicating that DMSO at this concentration range had no toxicity to MDCK cells. We also evaluated the anti-H1N1 virus effect of FSE. Data presented in Tables 2 - 3 implied that 1:512 to 1:8192 mg/mL FSE showed various protective effects on MDCK cells and the effect was decreased with the increased dilution. There was an obvious correlation between them with the most effective concentrations from 1:526 to 1:2048 mg/mL. The minimal effective inhibitory concentration was 1:8192. The value of

A_{600} obtained using crystal violet staining showed that

Table 1. Cytotoxicity of FSE on MDCK cells shown in the value of A_{600} .

Dilution	Control 1 (Normal MDCK cells)	Control 2 (1 mg/mL MSO)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1 024
Value of A_{600}	1.29 ± 0.012	1.32 ± 0.025	0.15 ± 0.012	0.19 ± 0.013	0.23 ± 0.21	0.26 ± 0.018	0.32 ± 0.023	0.45 ± 0.030	0.61 ± 0.029	0.71 ± 0.041	1.20 ± 0.035	1.25 ± 0.022

The A_{600} value of the cells co-cultured with FSE at concentration from 1:2 to 1:256 mg/mL was lower than that of normal MDCK cells, which showed that FSE had some toxicity to MDCK cells at these concentrations. The A_{600} value became close to that of normal MDCK cells from 1:512 to 1:1024 mg/mL, which indicated that FSE had no toxicity to MDCK cells at these concentrations.

Table 2. Protective effect of FSE on MDCK cells shown in the value of A_{600} .

Dilution	Control 1 (Normal MDCK cells)	^b Control 2 (10^{-4} H1N1 virus)	Control 3 (1:512)	1:1024	1:2048	1:4096	1:8192
Value of A_{600}	1.29 ± 0.023	0.026 ± 0.021	1.11 ± 0.012	1.04 ± 0.025	0.87 ± 0.039	0.91 ± 0.018	0.85 ± 0.026

As shown in the table the value of A_{600} of the cells treated with FSE is higher than that of the cells infected with H1N1 virus while not treated with the FSE (^b $P < 0.01$), indicating the protective effect of FSE on MDCK cells from H1N1 virus infection.

Table 3. Protective effect of FSE on MDCK cells shown in percentage (A_{600} value of cells protected with FSE / A_{600} value of normal MDCK cells).

Dilution	Control 1 (Normal MDCK cells)	^b Control 2 (10^{-4} H1N1 virus)	Control 3 (125 mg/mL ribavirin)	Control 4 (1:512)	1:1024	1:2048	1:4096	1:8192
The ratio of A_{600}	100	15.8 ± 0.023	90.12 ± 0.018	85.11 ± 0.015	80.04 ± 0.006	74.64 ± 0.031	65.91 ± 0.022	62.17 ± 0.015

The percentage of vital cells protected with FSE accounted for compared with normal MDCK cells decreased with the increased FSE dilution, and it was preferable from concentration 1:512 mg/mL to 1:2048 mg/mL. ^b $P < 0.01$ vs group of 10^{-4} H1N1 virus.

starting from 1:512 mg/mL, the value of A_{600} decreased with the increased dilution.

Conclusion

In conclusion, the present study indicated that FSE

may have a significant antiviral effect on acute respiratory tract infections with H1N1 virus infection.

This could be useful for further antiviral research on acute respiratory tract infections. However, further study is needed to clarify its antiviral mechanism on H1N1 virus, and its

anti-viral effects on other viruses.

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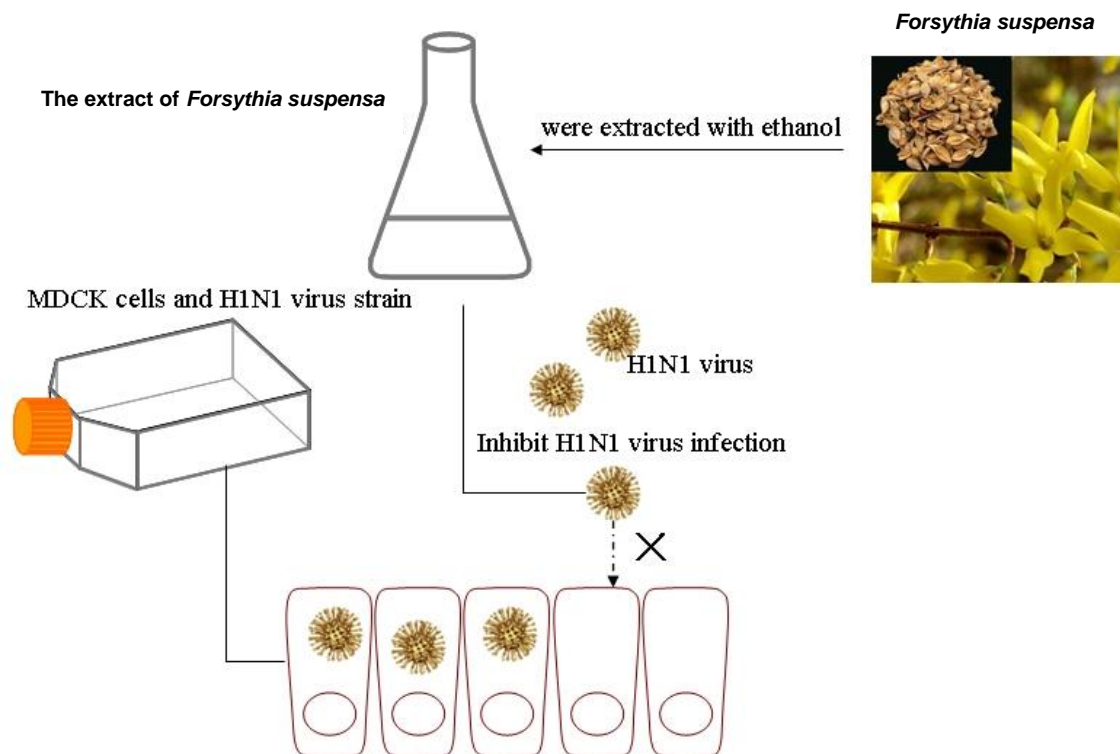


Figure 1. *In vitro* anti-influenza A H1N1 effect of FSE. The extract of *Forsythia suspensa* (FSE) contains abundant amount of phenylethanoid glycosides, lignans, flavonoids compounds and is known have an effect on multiple biological functions, including antiviral activities. Our findings demonstrate that FSE can inhibit H1N1 virus in MDCK cells effectively and can be considered as potential agents for therapy of infectious diseases caused by H1N1 virus infection.

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