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Cimicifuga foetida L. Inhibited Human Respiratory Syncytial Virus in HEp-2 and A549 Cell Lines

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Abstract: Human respiratory syncytial virus (HRSV) causes serious pediatric infection of the lower respiratory tract without effective therapeutic modality. Sheng-Ma-Ge-Gen-Tang (SMGGT; Shoma-kakkon-to) has been proven to be effective at inhibiting HRSV-induced plaque formation, and *Cimicifuga foetida* is the major constituent of SMGGT. We tested the hypothesis that *C. foetida* effectively inhibited the cytopathic effects of HRSV by a plaque reduction assay in both human upper (HEp2) and lower (A549) respiratory tract cell lines. Its ability to stimulate anti-viral cytokines was evaluated by an enzyme-linked immunosorbent assay (ELISA). *C. foetida* dose-dependently inhibited HRSV-induced plaque formation ($p < 0.0001$) before and after viral inoculation, especially in A549 cells ($p < 0.0001$). *C. foetida* dose-dependently inhibited viral attachment ($p < 0.0001$) and could increase heparins effect on viral attachment. In addition, *C. foetida* time-dependently and dose-dependently ($p < 0.0001$) inhibited HRSV internalization. *C. foetida* could stimulate epithelial cells to secrete IFN-β to counteract viral infection. However, *C. foetida* did not stimulate TNF-α secretion. Therefore, *C. foetida* could be useful in managing HRSV infection. This is the first evidence to support that *C. foetida* possesses antiviral activity.

Keywords: *Cimicifuga foetida*; Plaque Reduction; Anti-Viral Activity; Respiratory Syncytial Virus.

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Introduction

Human respiratory syncytial virus (HRSV) is a major cause of lower respiratory tract infections in infants, young children, and adults (Falsey and Walsh, 2000). HRSV is the most major viral pathogen of the respiratory tract in infants younger than one year old (Collins and Crowe, 2007; Collins and Graham, 2008). Infection and re-infection with HRSV are most frequent during the first few years of life (Collins and Crowe, 2007). Therefore, of all children that are infected by 24 months, half experienced two infections (Collins and Crowe, 2007). Effective therapeutic modalities are highly needed. However, only supportive care is given to manage HRSV-induced severe lower respiratory tract infection (Collins and Crowe, 2007). Ribavirin is a guanosine analogue that is an inhibitor of inosine monophosphate (IMP) dehydrogenase. It interferes with early events in viral transcription and inhibits ribonucleoprotein synthesis (Wray *et al.*, 1985). Although it was effective in experimentally infected animals, ribavirin has shown little effect on treating HRSV (Collins and Crowe, 2007; Empey *et al.*, 2010; Welliver, 2010). Palivizumab (Synagis) is effective at preventing HRSV infection (Collins and Crowe, 2007). However, it is very expensive and is not effective at the therapy of an established infection (Collins and Crowe, 2007). Therefore, effective chemotherapeutic agents are still urgently needed.

Sheng-Ma-Ge-Gen-Tang (SMGGT; Shoma-kakkon-to) has been used to manage pediatric viral infection. It has been proven to be effective at inhibiting HRSV-induced plaque formation *in vitro* (Wang *et al.*, 2011). *Cimicifuga foetida* L. is a major constituent of SMGGT. *C. foetida* has been used as a medical plant for anti-pyretic and detoxificative purposes in ancient China for thousands of years. *C. foetida* has anti-bacterial, anti-inflammatory, and anti-neoplastic activities (Zhao and Xiao, 2006). Several constituents of *C. foetida* have been proven to have anti-cancer (Sun *et al.*, 2007; Tian *et al.*, 2007), collagenolytic (Kusano *et al.*, 2001), and anti-complement activities (Qiu *et al.*, 2006). However, its anti-viral activity has not been examined. We hypothesized that *C. foetida*, a major constituent of SMGGT, might have activities against HRSV. We used both human upper (HEp2) and low (A549) respiratory tract cell lines to prove that *C. foetida* was effective on cytopathic effects induced by HRSV.

Materials and Methods

Preparation of Hot Water Extracts of Cimicifuga foetida L.

Water extract of air-dried *C. foetida* L. Rhizoma was prepared as reported previously (Yen *et al.*, 1991). The authenticity of *C. foetida* was confirmed by Professor M.H. Yen at the Graduate Institute of Natural Products of Kaohsiung Medical University. Briefly, 100 g of *C. foetida* was shade-dried and decocted for 1 h with 1 L of boiling reverse-osmotic water three times. The decoctions were mixed, filtered, concentrated and lyophilized. The w/w yield of *C. foetida* was 10.7%. The extract of *C. foetida* was then dissolved in minimum essential medium (MEM, Gibco BRL, Grand Island, NY, USA)

and supplemented with 2 or 10% fetal calf serum (FCS) into the final concentrations (10, 30, 100, 300 μg/ml for bioactivity assay and up to 3000 μg/ml for cytotoxicity test) before experiments.

Cells and Virus

Human larynx epidermoid carcinoma cells [HEp-2; ATCC (the American Type Culture Collection) CCL 23] and human lung carcinoma cells (A549 cells; ATCC CCL-185) were used to culture human respiratory syncytial virus (RSV Long strain: ATCC VR-26). Reagents and medium for cell culture were purchased from Gibco BRL. Cells were propagated at 37°C under 5% CO_2 in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B. Virus was propagated on 90% confluent cell monolayer in MEM with 2% FCS and antibiotics as described above. Viral titer was determined by plaque assays and expressed as plaque forming units per ml (pfu/ml). Virus was stored at −70°C until use.

Cytotoxicity Assay

Cytotoxicity of *C. foetida* on proliferating cells was assayed by XTT-based method (Chiang *et al.*, 2002). Briefly, cells (1×10^4 cells/well) were seeded into 96-well culture plates (Falcon; BD Biosciences, USA) and incubated overnight at 37°C under 5% CO_2. Then, the medium was removed and different concentrations (30, 100, 300, 1000, 3000 μg/ml) of *C. foetida* were applied in triplicate. After three days of incubation, the cytotoxicity of *C. foetida* was determined by XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid) kits (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The 50% cytotoxic concentration (CC_{50}) of *C. foetida* was calculated by regression analysis of the dose-response curve generated from the data.

Antiviral Effectiveness Assay by Plaque Reduction Assay

Antiviral activity of *C. foetida* was examined by a plaque reduction assay modified from procedures previously described (Graham *et al.*, 1988; Chen *et al.*, 2010). Briefly, cells (1×10^5/well) were plated in 12-well culture plates for 24 h and were inoculated with a mixture of 200 pfu/well HRSV and various concentrations of *C. foetida* for 1 h. Ribavirin (Sigma, St. Louis, USA) was used as a positive control. After supplement of overlay medium (MEM plus 2% FCS in 1% methylcellulose), they were cultured at 37°C under 5% CO_2 for three days. The monolayers were fixed with 10% formalin, stained with 1% crystal violet, and the plaques were counted. The minimal concentration required to inhibit 50% cytopathic effect (IC_{50}) of *C. foetida* was calculated by regression analysis of the dose-response curve generated from the data.

Time Course Assay

Antiviral activity of *C. foetida* was examined before and after viral inoculation by plaque reduction assay modified from procedures mentioned above (Graham *et al.*, 1988; Chang *et al.*, 2008; Chen *et al.*, 2010). Briefly, cells were seeded and incubated for 24 h as previously described. *C. foetida* of various concentrations was supplemented at −2 h (2 h before viral inoculation), −1 h (1 h before viral inoculation), or 1 h or 2 h (1 h or 2 h after viral inoculation). Supernatant was removed before supplement of overlay medium. They were incubated for a further 72 h as mentioned above. After fixation, crystal violet was supplemented and the plaques were counted.

Attachment Assay

The effect of *C. foetida* on viral attachment was evaluated by a plaque reduction assay modified from procedures previously described (De Logu *et al.*, 2000; McLellan *et al.*, 2010). Heparin (Sigma, St. Louis, USA) was used as a positive control. Briefly, cells were seeded and incubated for 48 h. The cells were pre-chilled at 4 °C for 1 h and the medium was removed. The cells were infected with a mixture of 200 pfu/well HRSV and various concentrations of *C. foetida*. After incubation at 4 °C for another 3 h, the free virus was removed. The cell monolayer was washed with ice-cold phosphate-buffered saline (PBS) thrice, covered with overlay medium, incubated for further 72 h at 37 °C under 5% CO_2, and examined by plaque assay as described earlier.

Internalization Assay

The effect of *C. foetida* on viral internalization was also evaluated by a plaque reduction assay described earlier (De Logu *et al.*, 2000). Briefly, the cell monolayer was grown in 12-well culture plates and pre-chilled at 4 °C for 1 h. Cells were infected with 200 pfu/well HRSV and incubated at 4 °C for 3 h to allow virus binding without internalization. The virus-containing medium was replaced with fresh medium containing various concentrations of *C. foetida* and cultured at 37 °C. In 20 min intervals, acidic PBS (pH 3) was supplemented for one minute to deactivate un-internalized virus followed by alkaline PBS (pH 11) for neutralization. Then, PBS was replaced by fresh overlay medium. After incubation at 37 °C for further 72 h, the cell monolayer was examined by the plaque assay.

Interferon-β (IFN-β) and Tumor Necrosis Factor-α (TNF-α) Assay

After the experiment of antiviral effectiveness assay mentioned above, the culture medium was collected and assayed by the IFN-β ELISA kit (PBL Biomedical Laboratories, Piscataway, USA) and TNF-α ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instruction. The $A_{450\,nm}$ was determined with ELISA reader (Multiskan EX, Labsystems).

Statistical Analysis

Results were expressed as mean ± standard deviation (S.D.). Percentage of the control (infection rate; %) was calculated from the plaque counts of *C. foetida* groups divided by that of viral control group. Data were analyzed with ANOVA by JMP 7.0.1 software (SAS Institute, Cary, NC, USA). *Tukey* honestly significant difference (HSD) test was used to compare all pairs of groups in the ANOVA test. $p < 0.05$ was considered statistically significant.

Results

Cytotoxicity Assay

C. foetida did not show any cytotoxicity against both HEp-2 and A549 cells at concentrations up to 3000 μg/ml (Fig. 1). Instead, *C. foetida* might slightly increase the proliferation of HEp-2 cells. The estimated CC_{50} was more than 3000 μg/ml. The higher CC_{50} proved its safety.

Antiviral Effectiveness Assay

C. foetida and ribavirin were dose-dependently (Fig. 2; $p < 0.0001$) effective against HRSV in both HEp2 cells and A549 cells. *C. foetida* was more effective in A549 cells (Fig. 2A; $p < 0.0001$). However, the effect of ribavirin was similar in both HEp-2 cells and A549 cells (Fig. 2B). The IC_{50} of *C. foetida* was 67.3 μg/ml in HEp-2 cells and 31.0 μg/ml in A549 cells.

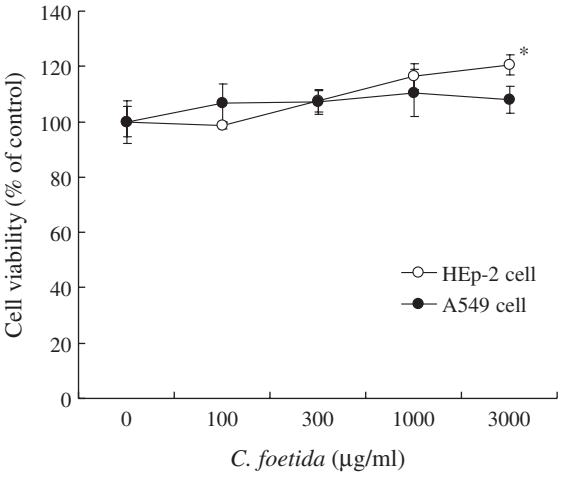


Figure 1. *C. foetida* did not show any cytotoxicity up to 3000 μg/ml. Data were presented as mean ± S.D. of 3 independent experiments.

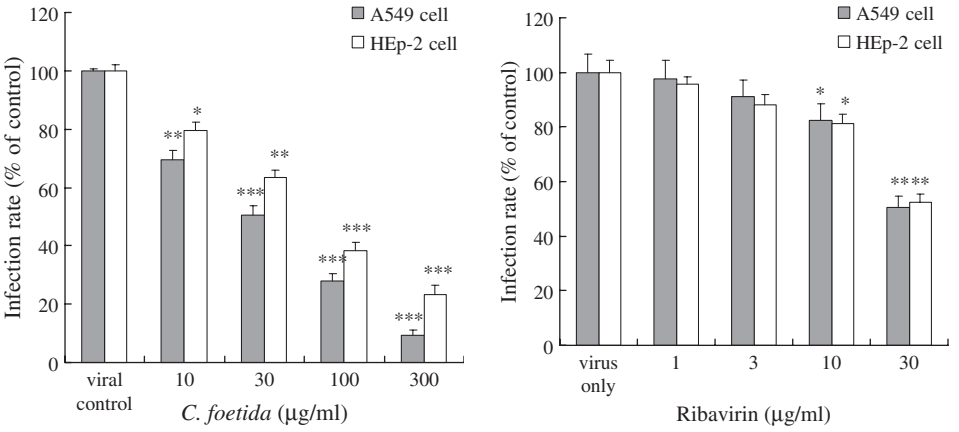


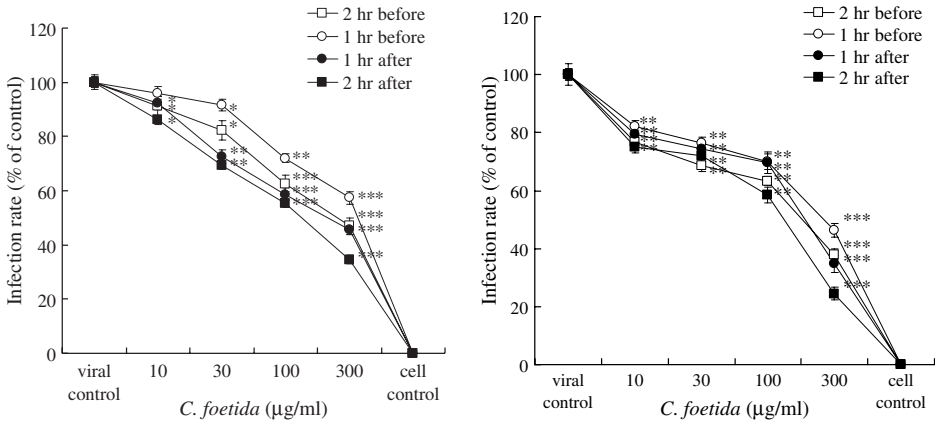
Figure 2. *C. foetida* were effective against HRSV in antiviral effectiveness assay. Both *C. foetida* (A) and ribavirin (B) were dose-dependently ($p < 0.0001$) effective against HRSV determined by the plaque reduction assay. *C. foetida* was more effective in A549 cells ($p < 0.0001$). However, ribavirin did not show the difference. Data were presented as mean ± S.D. of nine tests. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared to the viral control.

Time Course Assay

C. foetida was effective ($p < 0.0001$) both before and after viral inoculation in both HEp-2 cells and A549 cells in a dose-dependent manner. *C. foetida* had a better effect when given after viral inoculation in HEp-2 cells (Fig. 3A). In HEp-2 cells, the IC_{50} was 261.0 μg/ml (2 h before viral inoculation), 232.8 μg/ml (1 h after viral inoculation), and 151.3 μg/ml (2 h after viral inoculation). Its effect on A549 cells was similar (Fig. 3B); however, with no time-dependent effect. When it was supplemented 2 h after viral inoculation,*C. foetida* could show a better anti-HRSV activity at concentrations higher than 100 μg/ml in A549 cells. Its IC_{50} was 205.4 μg/ml (2 h before viral inoculation), 268.5 μg/ml (1 h before viral inoculation), 212.4 μg/ml (1 h after viral inoculation), and 150.0 μg/ml (2 h after viral inoculation) in A549 cells.

Attachment Assay

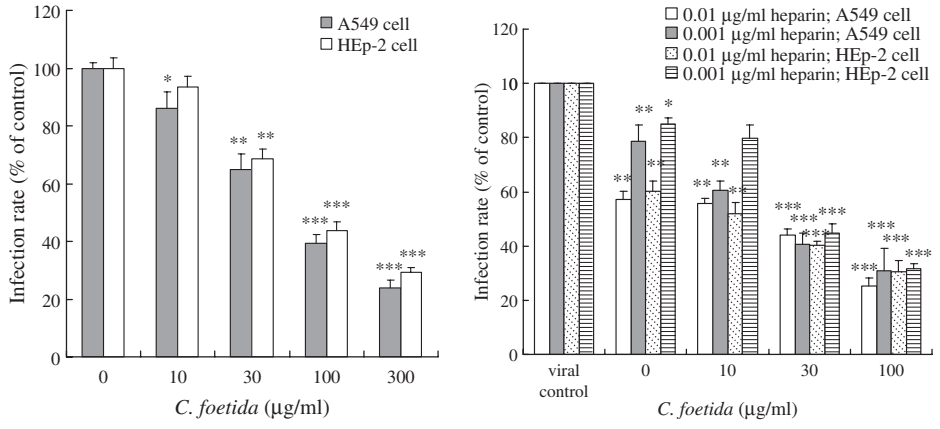
Since *C. foetida* could effectively inhibit HRSV-induced plaque formation when given before HRSV infection, *C. foetida* was hypothesized to be effective on viral attachment and/or internalization. The results of attachment assay confirmed this assumption. *C. foetida* dose-dependently inhibited viral attachment in both HEp-2 cells and A549 cells (Fig. 4A; $p < 0.0001$), with a better effect on A549 cells ($p < 0.0001$). The IC_{50} was 82.1 μg/ml in HEp-2 cells and 70.6 μg/ml in A549 cells. Heparin could dose-dependently prevent RSV attachment (Fig. 4B; p 0.0001). *C. foetida* could further improve the effect of heparin (Fig. 4B; $p < 0.0001$) in both cells. The estimated IC_{50}s of *C. foetida* with



(A) HEp-2 cells (B) A549 cells

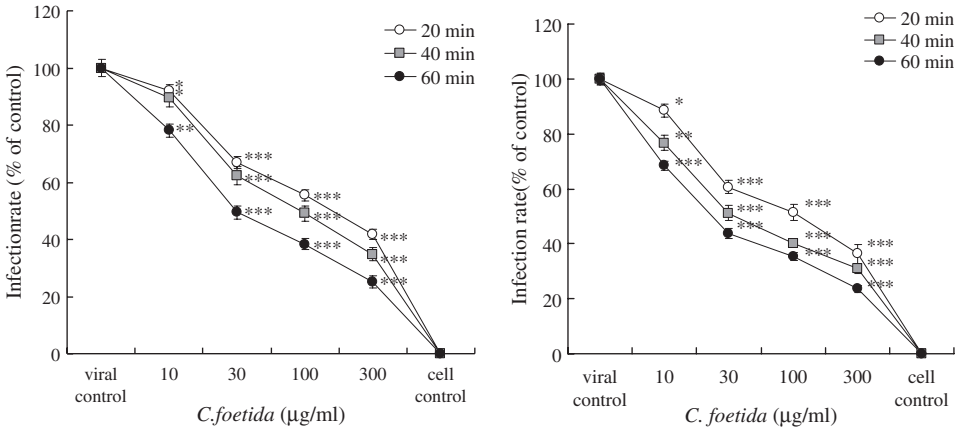
Figure 3. *C. foetida* was effective in both before and after viral inoculation in time course assay. *C. foetida* seemed to be better given after viral inoculation in HEp-2 cells (A). However, this effect was not clear in A549 cells (B). Nevertheless, *C. foetida* was dose-dependently ($p < 0.0001$) effective against HRSV in both HEp-2 (A) and A549 (B) cells. Data were presented as mean ± S.D. of nine tests. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared to the viral control.

0.01 μg/ml heparin were 19.5 μg/ml and 13.1 μg/ml in HEp-2 and A549 cells, respectively. It is interesting to note that *C. foetida* initially had a higher IC_{50} on viral attachment in HEp-2 cells. However, when it was combined with heparin, *C. foetida* had a lower IC_{50} in A549 cells. Moreover, 0.001 μg/ml heparin unexpectedly showed a synergistic effect with



(A) (B) *C. foetida* and heparin effect

Figure 4. *C. foetida* inhibited viral attachment. (a) SMGGT was dose-dependently effective against viral attachment in both HEp-2 cells and A549 cells ($p < 0.0001$), with a better effect on A549 cells. (b) *C. foetida* could further increase the effect of heparin at concentrations higher than 30 μg/ml. Data were presented as mean ± S.D. of nine tests. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared to the viral control group.



(A) HEp-2 cells (B) A549 cells

Figure 5. *C. foetida* inhibited viral internalization. *C. foetida* was time-dependently and dose-dependently ($p < 0.0001$) inhibited plaque formation caused by HRSV in both HEp-2 (A) and A549 cells (B). The effect was similar in both cell lines. Data were presented as mean ± S.D. of nine tests. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared to the viral control.

30 μg/ml *C. foetida.* The effects of different concentrations of heparin with 30 μg/ml *C. foetida* were similar (Fig. 4B).

Internalization Assay

C. foetida was time-dependently and dose-dependently (Fig. 5; $p < 0.0001$) effective on HRSV internalization in both HEp-2 and A549 cells. The effects were quite similar in both cells. *C. foetida* had a IC_{50} of 118.7 μg/ml (20 min incubation), 37.4 μg/ml (40 min), 25 μg/ml (60 min) in A549 cells, and 180.9 μg/ml (20 min incubation), 94.9 μg/ml (40 min), 29.6 μg/ml (60 min) in HEp-2 cells.

Interferon-β (IFN-β) and Tumor Necrosis Factor-α (TNF-α) Assay

The basal IFN-β and TNF-α secretion in both A549 and HEp-2 cells was similar (Fig. 6). After HRSV infection, IFN-β and TNF-α secretion could be stimulated (Fig. 6; $p < 0.05$). *C. foetida* could stimulate IFN-β secretion in both HEp-2 and A549 cells with or without HRSV infection (Figs. 6A and 6B; $p < 0.0001$). This effect was more prominent on A549 cells ($p < 0.0001$). In contrast, *C. foetida* did not stimulate TNF-α secretion in both HEp-2 and A549 cells with or without HRSV infection (Figs. 6C and 6D).

Discussion

HRSV can infect upper respiratory mucosa and replicate initially in the nasopharynx (Collins and Crowe, 2007). HRSV can spread rapidly to the lower respiratory tract possibly

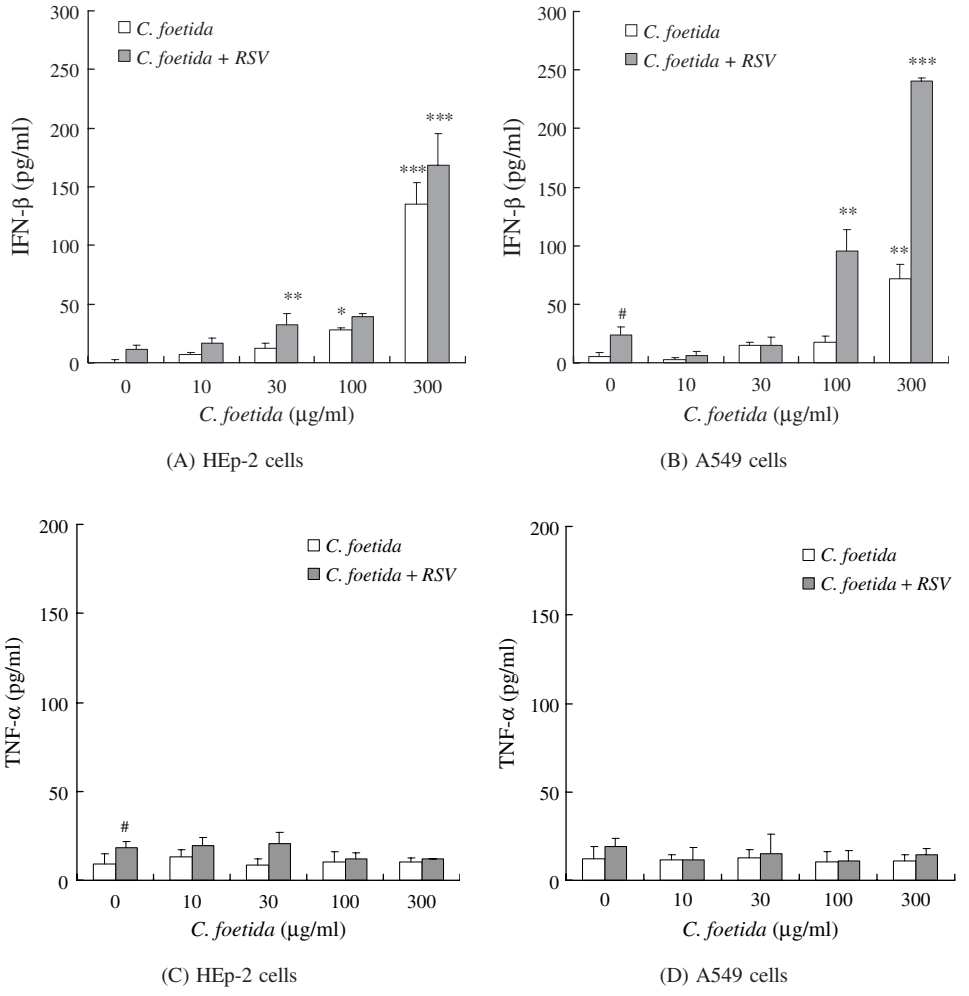


Figure 6. The effect of *C. foetida* on the secretions of interferon (IFN) and tumor necrosis factor (TNF) in HEp-2 cells (A) and (C) and A549 cells (B) and (D). RSV infection might increase IFN-β/TNF-α secretion. *C. foetida* dose-dependently $(p < 0.0001)$ stimulated both cell lines (A) and (B) to secrete IFN-β with or without HRSV infection. In contrast, *C. foetida* did not induce TNF-α secretion with or without HRSV infection in both cells (C) and (D). Data were presented as mean ± S.D. of 12 tests. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared to the control group. $^{\#}p < 0.05$ compared to the cell control.

by aspiration of secretions (Collins and Crowe, 2007). HRSV primarily causes morbidity and mortality by the pathology of the lower respiratory tract (Collins and Crowe, 2007). Therefore, management of HRSV infection needs an effective strategy to inhibit viral infection of both upper and lower respiratory tracts. This experiment showed that *C. foetida* was effective at inhibiting RSV-induced plaque formation in both human upper (HEp-2) and lower (A549) respiratory tract cells. Therefore, *C. foetida* could inhibit viral replication in the nasopharynx. Furthermore, 300 μg/ml *C. foetida* could inhibit HRSV-induced plaque

formation to less than 10% of the control in lower respiratory tract (A549) cells. Therefore, higher concentrations of *C. foetida* could largely inhibit HRSV-induced morbidity and mortality. When given after viral inoculation, *C. foetida* had a similar effect to that of giving after HRSV inoculation in the time course assay. Its preventive activity was further supported by an attachment assay and an internalization assay. Therefore, *C. foetida* was effective at preventing and managing HRSV infection. Heparin is highly effective at preventing HRSV attachment (McLellan *et al.*, 2010). *C. foetida* could further increase the effect of heparin. Furthermore, *C. foetida* had a synergistic effect with 0.001 μg/ml heparin. Therefore, *C. foetida* could have mechanisms difference from those of heparin. Other than preventing viral attachment and internalization, *C. foetida* could stimulate IFN-β to prevent HRSV infection. HRSV infection will induce cellular production of IFN-β and TNF-α (McCann and Imani, 2007). Both IFN and TNF contribute to innate immunity against viral infection (Bartee *et al.*, 2008; Benedict *et al.*, 2003; McFadden *et al.*, 2009). *C. foetida* could stimulate both HEp-2 and A549 cells to secrete IFN-β with or without HRSV infection. Therefore, along with direct cytoprotection, *C. foetida* could be useful for preventing and managing viral infection by stimulating IFN-β. Although it is a potent antiviral cytokine, TNF-α can activate p38MAPK to induce apoptosis of bronchial epithelia (Gallelli *et al.*, 2010). *C. foetida* could not induce TNF-α secretion in both HEp-2 and A549 cells. Therefore, *C. foetida* could be active against HRSV infection without inducing apoptosis of respiratory mucosa. Most of the therapeutic reagents under development aimed at inhibiting viral entrance (Empey *et al.*, 2010; Welliver, 2010). However, *C. foetida* was also effective after viral inoculation, especially on HEp-2 cells, a human larynx epidermoid carcinoma cells. HRSV replicates initially in the upper respiratory mucosa (Collins and Crowe, 2007). Therefore, *C. foetida* could be a better candidate to manage RSV infection.

In this study, *C. foetida* had low IC_{50}s in antiviral effectiveness assay in which both HRSV and *C. foetida* were concomitantly supplemented. Therefore, the IC_{50}s should show a logical trend between antiviral effectiveness assay and time course assay. However, the IC_{50} was 31.0 μg/ml in A549 cells when *C. foetida* was concomitantly supplemented with HRSV. When *C. foetida* was supplemented 2 h before viral inoculation in A549 cells, the IC_{50}s were 205.4 μg/ml. The IC_{50} did not change much from 268.5 μg/ml (1 h before viral inoculation), 212.4 μg/ml (1 h after viral inoculation), to 150.0 μg/ml (2 h after viral inoculation). The IC_{50} in antiviral effectiveness assay was much lower than that of time course assay. It lacked a logical trend between the time course assay and the antiviral effectiveness assay. This might raise a question about the validity of the experiment. However, our results clearly showed *C. foetida* was also effective when supplemented after viral inoculation. During the time course assay, *C. foetida* was removed before the supplement of overlay medium. Nevertheless, *C. foetida* remained there in the antiviral effectiveness assay to exert its antiviral effect. Therefore, the results of the antiviral effectiveness assay were the summation of all effects in the time course assay, so it was reasonable to have a lower IC_{50} in the antiviral effectiveness assay.

C. foetida could prevent RSV infection by inhibiting viral attachment, internalization, and by stimulating IFN-β secretion. Furthermore, *C. foetida* could be effective at inhibiting plaque formation after HRSV inoculation. *C. foetida* was quite different from therapeutic

reagents under development that aimed at inhibiting viral entrance only (Empey *et al.*, 2010; Welliver, 2010). Therefore, *C. foetida* is worthy to be further evaluated for its activity and mechanisms against HRSV.

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