ORIGINAL ARTICLE

Water extract of *Pueraria lobata* Ohwi has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines

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Abstract   Human respiratory syncytial virus (HRSV) infects all age groups and causes bronchiolitis, pneumonia, and acute respiratory distress syndrome with a significant mortality rate. To date, only ribavirin has been used to manage HRSV infection. However, ribavirin is expensive with an only modest effect. Furthermore, ribavirin has several side effects, which means it has limited clinical benefit. *Pueraria lobata* Ohwi (*P. lobata*) is a common ingredient of Ge-Gen-Tang (Kakkon-to) and Sheng-Ma-Ge-Gen-Tang (Shoma-kakkon-to), which are prescriptions of Chinese traditional medicine proven to have antiviral activity against HRSV. Therefore, it was hypothesized that *P. lobata* might be effective against HRSV. To find a cost-effective therapeutic modality, both human upper (HEp-2) and lower (A549) respiratory tract cell lines were used to test the hypothesis that *P. lobata* could inhibit HRSV-induced plaque formation. Results showed that the water extract of *P. lobata* was effective ($p < 0.0001$) against HRSV-induced

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plaque formation. *P. lobata* was more effective when given prior to viral inoculation ($p < 0.0001$) by inhibiting viral attachment ($p < 0.0001$) and penetration ($p < 0.0001$). However, supplementation with *P. lobata* could not stimulate interferon secretion after HRSV infection. In conclusion, *P. lobata* has antiviral activity against HRSV-induced plaque formation in airway mucosa mainly by inhibiting viral attachment and internalization. Further identification of effective constituents could contribute to the prevention of HRSV infection.

Introduction

Human respiratory syncytial virus (HRSV) is a common airway virus that infects patients of all age groups. HRSV is the leading cause of severe viral bronchiolitis [1] and pneumonia [2] in infants and children. The annual mortality rate is 160,000–600,000 deaths worldwide [3]. HRSV is also a threat to adults older than 65 years and to immunocompromised patients by causing pneumonia and exacerbations of pulmonary diseases [4]. HRSV infection may cause acute respiratory distress syndrome in healthy adults with a mortality rate ranging from 40% to 60% [5]. To date, the only Food and Drug Administration-approved agent to manage HRSV infection is ribavirin, which has a limited effect and a marginal clinical benefit [3]. Furthermore, ribavirin needs to be aerosolized for administration to manage HRSV infection. However, it is expensive [6] and has several side effects, such as causing teratogenesis to healthcare workers [7]. Therefore, safe and cost-effective anti-HRSV chemoprevention and/or chemotherapies are urgently needed.

Pueraria lobata Ohwi (*P. lobata*) has been used for thousands of years in China to manage fever, diarrhea, and diabetes [8]. There are several pharmacological activities to support its modern clinical applications in cerebrovascular diseases, cardiovascular diseases, and hyperlipidemia [8]. Furthermore, *P. lobata* has been used as a hangover and/or alcoholism remedy to treat alcohol-related problems [9,10]. *P. lobata* is also a common ingredient in Ge-Gen-Tang (GGT; Kakkon-to) and Sheng-Ma-Ge-Gen-Tang (SMGGT; Shoma-kakkon-to), which are prescriptions of Chinese traditional medicine for airway symptoms. GGT and SMGGT have been proven to have antiviral activity against HRSV [11,12]. Therefore, we hypothesized that *P. lobata* was one of the active constituents of GGT and SMGGT against HRSV. However, the only antiviral activity of *P. lobata* reported is against EV71 [13]. Therefore, we used both human upper (human larynx epidermoid carcinoma cell, HEp-2) and lower (human lung carcinoma cell, A549) respiratory tract cell lines to test the hypothesis that *P. lobata* is effective on HRSV-induced plaque formation.

Materials and methods

Preparation of hot water extracts of *P. lobata*

P. lobata was purchased from herbal shops in Southern Taiwan. The authenticity had been confirmed at least twice through morphological and anatomical identifications by Dr Ming Hong Yen, Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, and by Professor Den En Shieh, Department of Food Science and Technology, Tajen University of Technology, Ping-Tung, Taiwan. A voucher specimen was prepared and deposited at Kaohsiung Medical University Herbarium. A hot water extract of *P. lobata* was prepared as reported previously [13,14]. Briefly, 100 g of *P. lobata* were shade-dried and decocted for 1 hour with 1 L of boiling reverse-osmosis-treated water three times. The decoctions were mixed, filtered, concentrated, and lyophilized. The w/w yield of *P. lobata* was 9.4%. The extract of *P. lobata* was dissolved in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) to the final concentrations of 10 μg/mL, 30 μg/mL, 100 μg/mL, and 300 μg/mL for bioactivity and up to 3000 μg/mL for cytotoxicity tests prior to the experiments.

Human cell lines and HRSV

Human upper and lower respiratory tract cell lines, larynx epidermoid (HEp-2, American Type Culture Collection, ATCC CCL-23), and lung carcinoma cell lines (A549, ATCC CCL-185), were used to culture HRSV (Long strain: ATCC VR-26). Cells were propagated at 37°C under 5% CO_2 in MEM supplemented with 10% FCS and antimicrobials. The virus was propagated on 90% confluent cell monolayer in MEM with 2% FCS and antimicrobials. Viral titer was determined and expressed as plaque forming units (pfu)/mL [12]. The virus was stored at −80°C until use.

Cytotoxicity assay

Cytotoxicity of *P. lobata* on proliferating cells was assayed by XTT {sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid}-based method according to the manufacturer's instructions (Roche, Mannheim, Germany) [12]. Briefly, 10^4 cells/well were seeded into 96-well culture plates and incubated overnight at 37°C under 5% CO_2. The medium was removed and different concentrations (10 μg/mL, 30 μg/mL, 100 μg/mL, 300 μg/mL, 1000 μg/mL, and 3000 μg/mL) of *P. lobata* were applied in triplicate. After 3 days of incubation, the cytotoxicity was determined by XTT kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The 50% cytotoxic concentration (CC_{50}) of *P. lobata* was calculated by the regression analysis of the dose–response curve generated from the data.

Antiviral effect assay

Antiviral activity of *P. lobata* was examined by plaque reduction assay [12]. Briefly, 10^5 cells/well were plated in 12-well culture plates at 37°C under 5% CO_2 for 24 hours and inoculated with a mixture of 200 pfu/well viruses and various concentrations of extract of *P. lobata* at room temperature for 1 hour. Ribavirin (Sigma-Aldrich, St Louis, MO, USA) was the 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 3 days. The monolayer was then fixed with 10% formalin, stained with 1% crystal violet, and plaques were counted. All experiments were done three times in triplicate. The minimal concentration required to inhibit 50% cytopathic effect (IC_{50}) of *P. lobata* was calculated by the regression analysis of the dose–response curve generated from the data.

Time of addition assay

Antiviral activity of *P. lobata* was examined at different time points prior to and after viral inoculation by plaque reduction assay [12]. Briefly, cells were seeded and incubated for 24 hours as previously described. Various concentrations of *P. lobata* were supplemented at 2 hours or 1 hour prior to viral inoculation, or 1 hour or 2 hours after viral inoculation. Supernatant was removed prior to the supplement of overlay medium. Cells were incubated for a further 72 hours and examined by plaque assay. All experiments were done three times in triplicate.

Attachment assay

Plaque reduction assay was used to evaluate the effect of *P. lobata* on viral attachment [12]. Briefly, cells were seeded and incubated for 48 hours. Cells were prechilled at 4°C for 1 hour and the medium was removed. A mixture of 200 pfu/well HRSV was supplemented with various concentrations of *P. lobata*. After incubation at 4°C for another 3 hours, the free virus was removed. The cell monolayer was washed with ice-cold phosphate-buffered saline (PBS) three times, covered with overlay medium, incubated for further 72 hours at 37°C under 5% CO_2, and examined by plaque assay. All experiments were done twice in triplicate.

Internalization assay

Effect of *P. lobata* on viral internalization was evaluated at 20-minute intervals following viral adsorption also by plaque reduction assay [12]. Briefly, the cell monolayer was grown in 12-well culture plates and prechilled at 4°C for 1 hour. Cells were infected with 200 pfu/well HRSV and were incubated at 4°C for 3 hours to allow virus binding without internalization. The virus-containing medium was replaced with fresh medium containing various concentrations of *P. lobata*. Cells with various concentrations of *P. lobata* were shifted to 37°C. At 20-minute, 40-minute, or 60-minute intervals following the 37°C shift, acidic PBS (pH 3) was supplemented for 1 minute to inactivate non-internalized virus and followed by alkaline PBS (pH 11) for

neutralization. Then, PBS was replaced by fresh overlay medium. After incubation at 37°C for a further 72 hours, the cell monolayer was examined by plaque assay. All experiments were done three times in triplicate.

Interferon-β assay

After the antiviral effect assay experiment mentioned above, the culture medium was collected and assayed by the interferon (IFN)-β enzyme-linked immunosorbent assay (ELISA) kit (PBL Biomedical Laboratories, Piscataway Township, NJ, USA) according to the manufacturer's instructions. All experiments were done three times in triplicate. The absorbance at $A_{450 \ nm}$ was determined with an ELISA reader (Multiskan EX; Labsystems, Beverley, MA, USA).

Statistical analysis

Results are expressed as mean ± standard deviation [15]. Percentage of control (infection rate; %) was calculated from the plaque counts of *P. lobata* groups divided by that of viral control. Data were analyzed with analysis of variance by JMP 9.0.0 software (SAS, Cary, NC, USA). The Tukey honestly significant difference test was used to compare all pairs of groups in the analysis of variance test. A p value < 0.05 was considered statistically significant.

Results

Cytotoxicity assay

To determine whether *P. lobata* was toxic to cells, a cytotoxicity assay was performed with the XTT assay. *P. lobata* did not show any cytotoxicity against HEp-2 and A549 cells up to a concentration of 3000 μg/mL (Fig. 1). The estimated CC_{50} was more than 3000 μg/mL. High CC_{50} proved its safety.
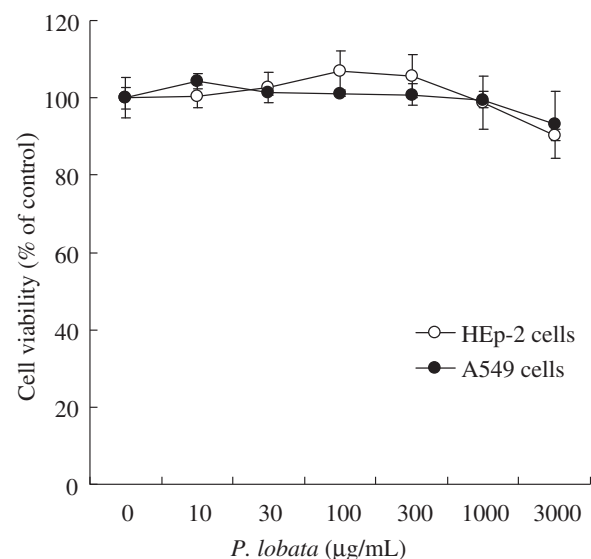
Figure 1. Cytotoxicity assay. *Pueraria lobata* did not show any cytotoxicity against host cells up to the concentration of 3000 μg/mL after 3 days' incubation. Data are presented as mean ± standard deviation of three triplicates.
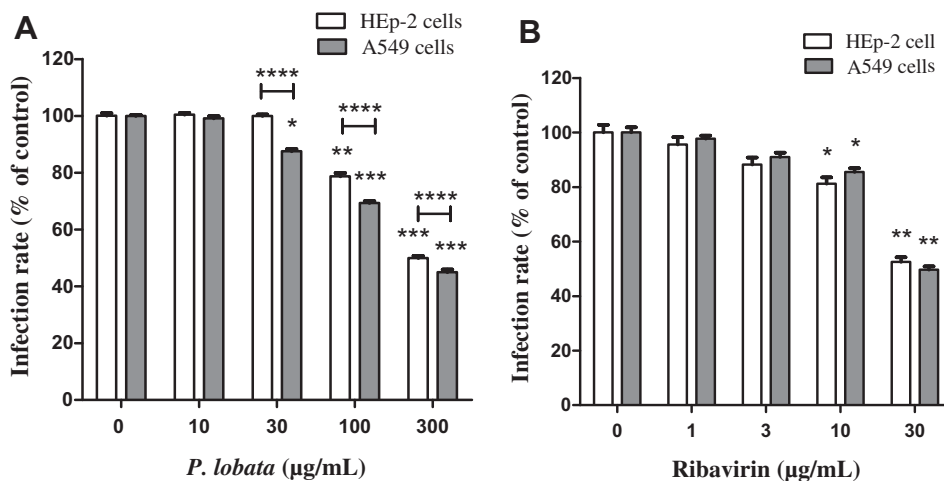
Figure 2. Antiviral effect assay. Both *Pueraria lobata* (A) and ribavirin (B) were dose-dependently ($p < 0.0001$) effective against human respiratory syncytial virus as determined by plaque reduction assay after 3 days' incubation. *P. lobata* was more effective on A549 cells than on HEp-2 cells ($p < 0.0001$). By contrast, ribavirin did not show this difference. Data are presented as mean $\pm$ standard deviation of three triplicates. *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$ were compared to the viral control (0 μg/mL). **** $p < 0.05$ was compared between the different cell types at the same concentrations.

Antiviral effect assay

To determine the anti-HRSV activity, an antiviral effect assay was performed through the plaque reduction method. *P. lobata* and ribavirin were dose-dependently effective against HRSV in HEp-2 and A549 cells (Fig. 2; $p < 0.0001$). *P. lobata* was more effective on A549 than on HEp-2 cells (Fig. 2A; $p < 0.0001$). However, ribavirin did not show this preference on A549 cells (Fig. 2B). The calculated IC_{50} of *P. lobata* was 258.7 μg/mL in A549 cells and 299.8 μg/mL in HEp-2 cells.
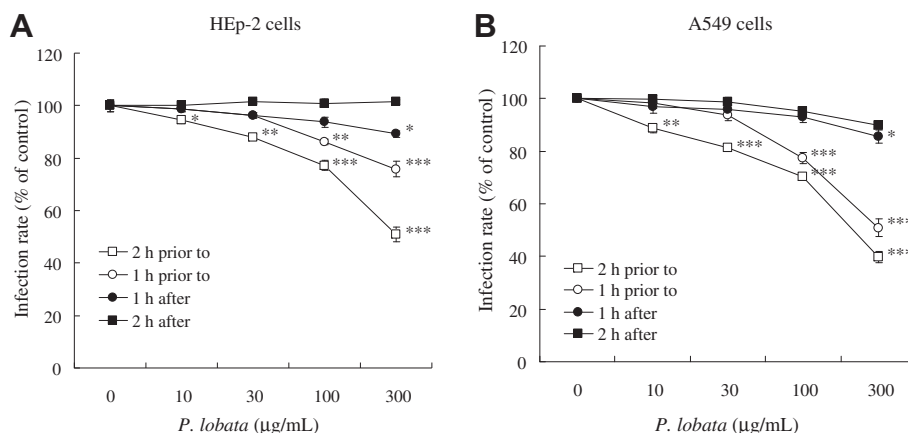
Time of addition assay

Because *P. lobata* was effective against HRSV, time of addition assay was done to find out its working point. The effect of *P. lobata* was dose dependent against HRSV on

HEp-2 and A549 cells and was affected by the time of addition (Fig. 3; $p < 0.0001$). *P. lobata* was more effective on A549 cells, particularly when given prior to viral inoculation (Fig. 3; $p < 0.0001$). Therefore, *P. lobata* worked better as a preventive agent. The calculated IC_{50} was 233.5 μg/mL (2 hours earlier) in A549 cells.

Attachment assay and internalization assay

P. lobata showed its better effect when given prior to viral inoculation. Therefore, *P. lobata* was supposed to work on viral attachment and/or internalization. The results of attachment assay confirmed this assumption. *P. lobata* was dose-dependently effective against viral attachment (Fig. 4; $p < 0.0001$), particularly on A549 cells ($p < 0.0001$). The calculated IC_{50} was 157.9 μg/mL in A549 cells and 255.8 μg/mL in HEp-2 cells.

Figure 3. Time of addition assay. The effect of *Pueraria lobata* was dose-dependent ($p < 0.0001$) against human respiratory syncytial virus on HEp-2 (A) and A549 cells (B). This activity was also affected by the time of addition. *P. lobata* was more effective when given prior to viral inoculation ($p < 0.0001$). Data are presented as mean $\pm$ standard deviation of three triplicates. *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$ were compared to the viral control (0 μg/mL).
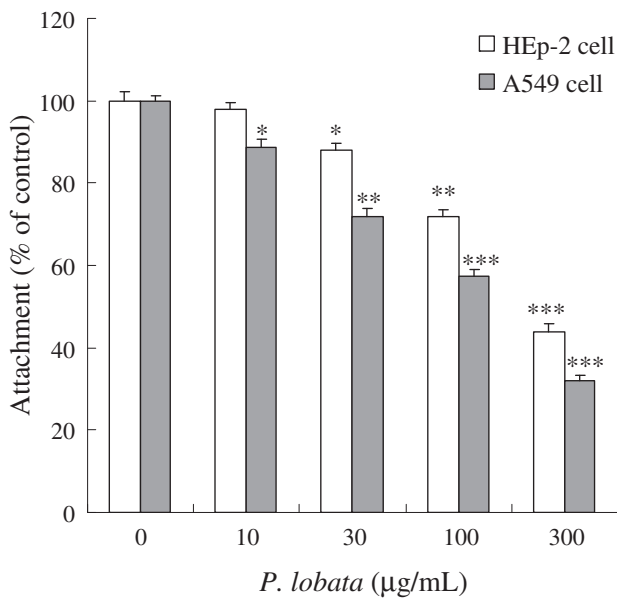
Figure 4. Attachment assay. of *Pueraria lobata* was dose-dependently effective against viral attachment in HEp2 cells and A549 cells ($p < 0.0001$), particularly on A549 cells ($p < 0.0001$). Data are presented as mean ± standard deviation of two triplicates. *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$ were compared to the control group (0 μg/mL).

As for the viral internalization assay, *P. lobata* was time-dependently and dose-dependently effective in inhibiting viral penetration (Fig. 5; $p < 0.0001$), particularly on A549 cells ($p < 0.0001$). The effect was similar to that of attachment assay. The calculated IC_{50} values were 208.2 μg/mL (60 minutes) in A549 cells, and 299.2 μg/mL (60 minutes) in HEp-2 cells, respectively.

IFN-β assay

With regard to the fact that *P. lobata* was effective against HRSV and that IFN could be the main cellular mechanism

against viral infection, it was interesting to ascertain whether the effect of *P. lobata* was mediated by stimulation of IFN secretion. Results showed that *P. lobata* dose-dependently stimulated cells to secrete IFN-β without HRSV infection. Infection of HRSV also induced IFN-β secretion in A549 cells (Fig. 6; $p < 0.05$). However, supplementation with *P. lobata* after viral inoculation had no effect on IFN secretion.

Discussion

In this study, *P. lobata* has for the first time been proven to inhibit HRSV-induced plaque formation in both HEp-2 cells and A549 cells. HEp-2 and A549 cells represent upper and lower respiratory tract mucosal cells, respectively. HRSV mainly targets the low respiratory tract to result in morbidity and mortality [16,17]. Therefore, an effective anti-HRSV agent has to show a better effect on the low respiratory tract mucosa. Our results fulfilled this character that *P. lobata* was effective to protect whole airway mucosa with a better effect on A549 cells (Fig. 2A) to possibly mitigate the disease severity. An effective antiviral agent needs to block the set-up of an infection and/or to block the replication cycle of viruses. Our time of addition assay showed that *P. lobata* was more effective when given prior to viral inoculation by inhibiting viral attachment (Fig. 4) and penetration (Fig. 5). These results indicate that *P. lobata* interferes with the early stages of HRSV infection. Therefore, *P. lobata* potentially prevents the set-up of an infection, rather than blocking the replication cycle of HRSV. All these results were consistent, thus confirming their validity.

Ribavirin has been used to treat HRSV infection of the low respiratory tract in infants and young children [18], in adult females with HRSV pneumonia-induced acute respiratory distress syndrome [5], and in patients after lung [19], liver [20], or allogeneic hematopoietic stem cell transplantation [21]. Nevertheless, the efficacy of ribavirin against HRSV pneumonia in older children and adults has not been established. Therefore, its effect has been questioned [22]. It has been shown that 1 μg/mL ribavirin could
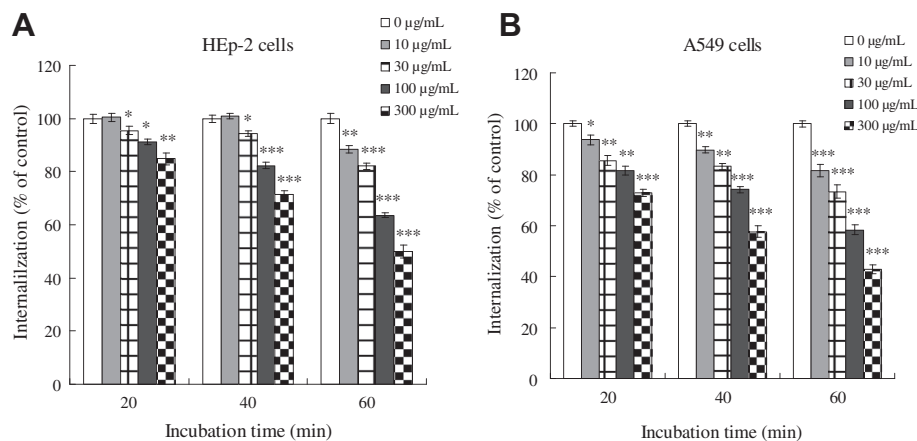
Figure 5. Internalization assay. *Pueraria lobata* was time-dependently ($p < 0.0001$) and dose-dependently ($p < 0.0001$) effective against viral penetration in HEp-2 cells (A) and A549 cells (B). Data are presented as mean ± standard deviation of three triplicates. *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$ were compared to the viral control (0 μg/mL).
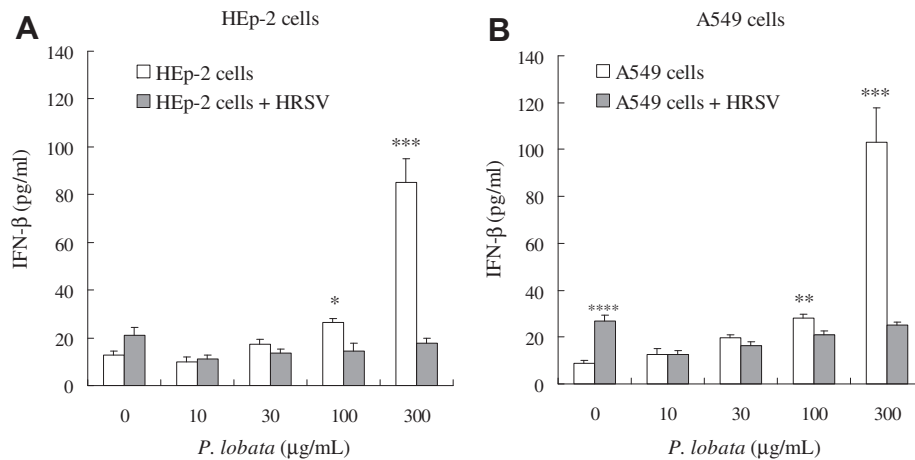
Figure 6. Interferon (IFN)-β assay. Human respiratory syncytial virus infection increased IFN-β secretion in HEp-2 (A) and A549 (B) cells. *P. lobata* stimulated HEp-2 (A) and A549 (B) cells to secrete IFN-β only at high concentrations without human respiratory syncytial virus infection. Data are presented as mean ± standard deviation of three triplicates. *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$ were compared to the group (0 μg/mL). **** $p < 0.05$ was compared to the cell control (0 μg/mL without viral inoculation).

reduce more than 50% of HRSV-induced plaque formation with a noticeable decrease of syncytial size at 3 μg/mL [23]. By contrast, our experiment showed that ribavirin of 3 μg/mL was barely effective and it caused only approximately 15% reduction of plaque formation at 10 μg/mL (Fig. 2B). Ribavirin could easily penetrate into HEp-2 and A549 cells to exert its antiviral effect against HRSV [24]. Compared with previously reported results, our data are more compatible with the clinical effects. Therefore, ribavirin is not a good candidate to manage HRSV infection, particularly for lower respiratory tract infection.

SMGGT and GGT [11,12] are different Chinese traditional prescriptions. *P. lobata* is one of the common ingredients of GGT and SMGGT. *P. lobata* was effective at the concentrations of 30 μg/mL on A549 cells and 100 μg/mL on HEp-2 cells. *P. lobata* inhibited viral attachment and penetration. All these results were consistent, thus confirming their validity. *P. lobata* showed a similar activity against HRSV as GGT and SMGGT. These results supported our hypothesis that *P. lobata* was one of the active ingredients of SMGGT and GGT against HRSV. Nevertheless, there are four common ingredients in GGT and SMGGT: *P. lobata*; *Glycyrrhiza uralensis* [25]; *Paeonia lactiflora* [26], and *Zingiber officinale* [24]. All these common ingredients have potent antiviral activity against HRSV. Therefore, although *P. lobata* is one of the active ingredients, it is not the major component in these traditional prescriptions.

IFN enhances the cellular resistance against viral infection [27,28] and this can be the main cellular mechanism against viral infection [29]. *P. lobata* stimulated HEp-2 and A549 cells to secrete IFN-β without HRSV infection (Fig. 6). However, the stimulatory effect disappeared after viral challenge (Fig. 6). It has been proposed that viral F protein can induce the production of IFN-β [30,31]. However, viral NS1, NS2, and G proteins mediate the evasion of cellular IFN response for viral advantage [32]. Therefore, improvement of IFN secretion can be beneficial against HRSV infection. Nevertheless, *P. lobata* failed to stimulate interferon secretion after HRSV infection.

In conclusion, *P. lobata* could possibly be used to prevent HRSV infection mainly by inhibiting viral attachment and internalization. Further identification of the effective constituents is necessary to develop the cost-effective therapeutic modalities against HRSV.

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