



Fresh ginger (*Zingiber officinale*) has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines

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ABSTRACT

Ethnopharmacological relevance: Ginger, *Zingiber officinale* Roscoe, is a common spice and also a widely used medicinal plant in ancient China. Ginger is an ingredient of Ge-Gen-Tang (Kakkon-to; GGT). GGT has been proved to have antiviral activity against human respiratory syncytial virus (HRSV). However, it is unknown whether ginger is effective against HRSV.

Aim of the study: To find a readily available agent to manage HRSV infection, the authors tested the hypothesis that ginger can effectively decrease HRSV-induced plaque formation in respiratory mucosal cell lines.

Materials and methods: Effect of hot water extracts of fresh and dried gingers on HRSV was tested by plaque reduction assay in both human upper (HEp-2) and low (A549) respiratory tract cell lines. Ability of ginger to stimulate anti-viral cytokines was evaluated by enzyme-linked immunosorbent assay (ELISA).

Results: Fresh ginger dose-dependently inhibited HRSV-induced plaque formation in both HEp-2 and A549 cell lines ($p < 0.0001$). In contrast, dried ginger didn't show any dose-dependent inhibition. 300 $\mu\text{g/ml}$ fresh ginger could decrease the plaque counts to 19.7% (A549) and 27.0% (HEp-2) of that of the control group. Fresh ginger was more effective when given before viral inoculation ($p < 0.0001$), particularly on A549 cells. 300 $\mu\text{g/ml}$ fresh ginger could decrease the plaque formation to 12.9% when given before viral inoculation. Fresh ginger dose-dependently inhibited viral attachment ($p < 0.0001$) and internalization ($p < 0.0001$). Fresh ginger of high concentration could stimulate mucosal cells to secrete IFN- β that possibly contributed to counteracting viral infection.

Conclusions: Fresh, but not dried, ginger is effective against HRSV-induced plaque formation on airway epithelium by blocking viral attachment and internalization.

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1. Introduction

Acute viral respiratory illnesses are the most common human infectious disease. Human respiratory syncytial virus (HRSV) is one of the most common airway viruses. HRSV is a negative-sense, non-segmented, enveloped RNA virus of *Paramyxoviridae*. HRSV has a single antigenic type, two distinct subgroups, and

Abbreviations: A549, Human lung carcinoma cell; ATCC, The American Type Culture Collection; CC₅₀, 50% cytotoxic concentration; DMSO, Dimethylsulfoxide; ELISA, Enzyme-linked immunosorbent assay; FCS, Fetal calf serum; HEp-2, Human larynx epidermoid carcinoma cell; IC₅₀, Minimal concentration required to inhibit 50% cytopathic effect; IFN, Interferon; MEM, Minimum essential medium; PBS, Phosphate-buffered saline; pfu, Plaque forming unit; RSV, Respiratory syncytial virus; *Zingiber officinale*, *Zingiber officinale*, ginger

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multiple subtypes within each subgroup (Dolin, 2012). HRSV causes a variety of respiratory diseases worldwide from common cold in immuno-competent adults to bronchiolitis and pneumonia in infants. HRSV infection can be life-threatening and cause even death in high risk patients, such as infants with congenital cardiac disease, the elderly, patients of cardiopulmonary diseases, immunocompromised adults, and patients with solid organ transplantation (Dolin, 2012; Falsey et al., 2005). Currently, there is no cost-effective strategy for prevention, nor established therapeutic strategy against HRSV infection. Effective preventive or therapeutic modalities against HRSV for severely ill patients are still urgently needed.

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is a common spice and a widely used medicinal plant, particularly as a broad spectrum anti-emetic agent. Ginger (*Zingiber officinale*) has been proved to have several pharmacologic activities, such as improvement of insulin sensitivity to reduce hyperglycemia and

hyperlipemia, anti-thrombotic and anti-inflammatory activities by inhibiting synthesis of prostaglandins and/or cytokines/chemokines, direct and indirect anti-hypertensive effect, gastrointestinal protective effects against ulceration and emesis, antioxidant and radio-protective effects (Ali et al., 2008; Chrubasik et al., 2005). Besides, ginger has anti-microbial activities against various bacteria, fungi, and nematodes (Ali et al., 2008). Ginger has been proved to be effective on various viruses (Chrubasik et al., 2005; Denyer et al., 1994; Koch et al., 2008; Schnitzler et al., 2007; Sookkongwaree et al., 2006). However, no antiviral activity against HRSV has been reported. Ginger is a common ingredient of Chinese traditional prescriptions for airway infections, such as Ge-Gen-Tang (Kakkon-to; GGT) and Sheng-Ma-Ge-Gen-Tang (SMGGT; Shoma-kakkon-to). GGT (Chang et al., 2012) and SMGGT (Wang et al., 2011) are two different Chinese traditional prescriptions that have been proved to have antiviral activity against HRSV. Therefore, we hypothesized that ginger could be one of the active constituents of GGT and SMGGT against HRSV. We used both human upper (HEp-2) and low (A549) respiratory tract cell lines to test that ginger, fresh or dried, could effectively inhibit plaque formation induced by HRSV infection.

2. Materials and methods

2.1. Preparation of hot water extracts of ginger (*Zingiber officinale* Roscoe)

Clean fresh root-like stems (rhizomes) of ginger were collected from markets in South Taiwan. The medicinal plant sample was authenticated by Dr. Ming Hong Yen, Graduate Institute of Natural Products, KMU, Kaohsiung, Taiwan. Whole mature rhizome at 8 months of age was cleaned, roughly scraped, and completely dried by sunlight to get the dried ginger. Dried ginger was stored in an airtight container in a dry cool place. Rhizomes of fresh and dried gingers were separately sliced into pieces to prepare hot water extracts of gingers as reported before (Chiang et al., 2002). In brief, 100 g of each rhizome was decocted for 1 h with 1000 ml of distilled water repeatedly for three times. The decoctions were collected, mixed, filtered by gauze, concentrated under reduced pressure, and lyophilized to dry. The w/w yields of fresh and dried gingers were 8.5% and 7.5%, respectively. The extracts of gingers were dissolved in minimum essential medium (MEM) (Gibco BRL, NY) 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.

2.2. Cells and virus

Human larynx epidermoid carcinoma cells (HEp-2; ATCC CCL 23) and human lung carcinoma cells (A549 cells; ATCC CCL-185) were used to culture human respiratory syncytial virus (HRSV Long strain: ATCC VR-26). Cells were propagated at 37 °C under 5% CO₂ in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The 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 –80 °C until use.

2.3. Cytotoxicity assay

Cytotoxicities of fresh and dried *Zingiber officinale* on proliferating cells were assayed by XTT-based method (Wang et al., 2011). Briefly, cells (1×10^4 cells/well) were seeded into 96-well culture plates (Falcon; BD Biosciences, USA) and were incubated

overnight at 37 °C under 5% CO₂. Then, the medium was removed and different concentrations (30, 100, 300, 1000, 3000 µg/ml) of *Zingiber officinale* were applied in triplicate. After 3 days of incubation, the cytotoxicities of *Zingiber officinale* were determined by XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid) kits (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The 50% cytotoxic concentrations (CC₅₀) of *Zingiber officinale* were calculated by regression analysis of the dose–response curve generated from the data.

2.4. Antiviral effect assay by plaque reduction assay

Antiviral activities of fresh and dried *Zingiber officinale* were examined by plaque reduction assay (Wang et al., 2011). Briefly, cells (1×10^5 /well) were plated in 12-well culture plates for 24 h and were inoculated with a mixture of virus and various concentrations of extract of *Zingiber officinale* for 1 h. HRSV titer was 200 pfu/well. Ribavirin (Sigma, MO) was used as the positive control. After supplement of overlay medium, MEM plus 2% FCS in 1% methylcellulose, they were cultured at 37 °C under 5% CO₂ for 3 days. Then, the monolayer was fixed with 10% formalin, stained with 1% crystal violet, and plaques were counted. The minimal concentrations required to inhibit 50% cytopathic effect (IC₅₀) of *Zingiber officinale* were calculated by regression analysis of the dose–response curve generated from the data.

2.5. Time course assay

Antiviral activity of fresh *Zingiber officinale* was examined before and after viral inoculation by plaque reduction assay (Wang et al., 2011). Briefly, cells were seeded and incubated for 24 h. Various concentrations of *Zingiber officinale* were supplemented at –2 h (2 h before viral inoculation), –1 h (1 h before viral inoculation), +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 further 72 h. After fixation, crystal violet was supplemented and plaques were counted.

2.6. Attachment assay

Fresh *Zingiber officinale* was evaluated with its effect on viral attachment by plaque reduction assay (Wang et al., 2011). Briefly, cells were seeded and incubated for 48 h. Cells were pre-chilled at 4 °C for 1 h and the medium was removed. A mixture of 200 pfu/well HRSV and various concentrations of *Zingiber officinale* was supplemented. 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) three times, covered with overlay medium, incubated for further 72 h at 37 °C under 5% CO₂, and examined by plaque assay as described earlier.

2.7. Internalization assay

Effect of fresh *Zingiber officinale* on viral internalization was also evaluated by plaque reduction assay (Wang et al., 2011). 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 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 *Zingiber officinale*. Following 37 °C shift and at 20-min intervals, acidic PBS (pH 3) was supplemented for one minute to inactivate un-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 further 72 h, the cell monolayer was fixed, stained, and examined.

2.8. Interferon- β (IFN- β) and tumor necrosis factor- α (TNF- α) assay

After the experiment of Antiviral effect assay mentioned above, the culture medium was collected and assayed by the IFN- β ELISA kit (PBL Biomedical Laboratories, MD) and TNF- α ELISA kit (R&D Systems, MN) according to the manufacturer's instruction. The $A_{450\text{ nm}}$ was determined with ELISA reader (Mutiskan EX, Labsystems, MA).

2.9. Statistical analysis

Results were expressed as mean \pm standard deviation (S.D.). Percentage of control (infection rate; %) was calculated from the plaque counts of *Zingiber officinale* groups divided by that of viral control. Data were analyzed with ANOVA by JMP 9 software (SAS Institute, NC). 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.

3. Results

3.1. Cytotoxicity assay

Dried *Zingiber officinale* did not show any cytotoxicity against HEp-2 and A549 cells up to the concentration of 3000 $\mu\text{g/ml}$ (Fig. 1). However, fresh *Zingiber officinale* did affect cell viability at concentrations higher than 300 $\mu\text{g/ml}$. The estimated CC_{50} s were 1893.8 $\mu\text{g/ml}$ and more than 3000 $\mu\text{g/ml}$ of fresh and dried *Zingiber officinale*, respectively. High CC_{50} s ensure their safety.

3.2. Antiviral effect assay

Fresh *Zingiber officinale* and ribavirin were dose-dependently effective against HRSV in HEp-2 and A549 cells (Fig. 2a and c; $p < 0.0001$). However, dried *Zingiber officinale* showed a modest activity without clear biological gradient on HEp-2 cells (Fig. 2b). Fresh *Zingiber officinale* was effective at concentration as low as 10 $\mu\text{g/ml}$ with a better effect on A549 cells (Fig. 2a). Nevertheless, ribavirin did not show this difference (Fig. 2c). The calculated IC_{50} of fresh *Zingiber officinale* was 144.9 $\mu\text{g/ml}$ in HEp-2 cells and 73.3 $\mu\text{g/ml}$ in A549 cells.

3.3. Time course assay

Fresh, but not dried, *Zingiber officinale* was effective to inhibit HRSV-induced plaque formation. It was interesting to know whether fresh *Zingiber officinale* was preventive or therapeutic. By the time course assay, fresh *Zingiber officinale* showed time-dependently and dose-dependently effect against HRSV on HEp-2 and A549 cells (Fig. 3a and b; $p < 0.0001$). Fresh *Zingiber officinale* was more effective when given before viral inoculation, particularly on A549 cells (Fig. 3a and b; $p < 0.0001$). Ribavirin showed only dose-dependent, but not time-dependent effect (Fig. 3c and d; $p < 0.0001$). It was obvious that ribavirin, as a small molecule, could readily get into cells. The calculated IC_{50} of fresh *Zingiber officinale* was 212.7 $\mu\text{g/ml}$ (2 h before) in HEp-2 cells, and 26 $\mu\text{g/ml}$ (2 h before), 82.8 $\mu\text{g/ml}$ (1 h before) in A549 cells, respectively.

3.4. Attachment assay

Fresh *Zingiber officinale* showed its better effect when given before viral infection. Therefore, fresh *Zingiber officinale* was supposed to be more effective on viral attachment and/or internalization. The results of attachment assay confirmed this assumption. Fresh *Zingiber officinale* was dose-dependently effective against viral attachment (Fig. 4; $p < 0.0001$), especially in A549 cells ($p < 0.0001$). The calculated IC_{50} was 81.4 $\mu\text{g/ml}$ in HEp-2 cells and 28.2 $\mu\text{g/ml}$ in A549 cells.

3.5. Internalization assay

Fresh *Zingiber officinale* was time-dependently and dose-dependently effective on viral penetration (Fig. 5a and b; $p < 0.0001$), with a better effect on A549 cells. The calculated IC_{50} s were 162.4 $\mu\text{g/ml}$ (40 min), and 88.6 $\mu\text{g/ml}$ (60 min) in HEp-2 cells; 126.1 $\mu\text{g/ml}$ (20 min), 65.9 $\mu\text{g/ml}$ (40 min), and 29.0 $\mu\text{g/ml}$ (60 min) in A549 cells, respectively.

3.6. Cytokines assay

The basal secretions of IFN- β and TNF- α between A549 and HEp-2 cells were not different (Fig. 6). Infection of HRSV modestly increased IFN- β secretion (Fig. 6; $p < 0.05$). Besides, high concentrations of fresh *Zingiber officinale* mildly stimulated HEp-2 and A549 cells to secrete IFN- β (Fig. 6; $p < 0.05$). However, fresh *Zingiber officinale* did not stimulate cells to secrete TNF- α , but rather inhibited its secretion in HEp-2 cells (Fig. S1).

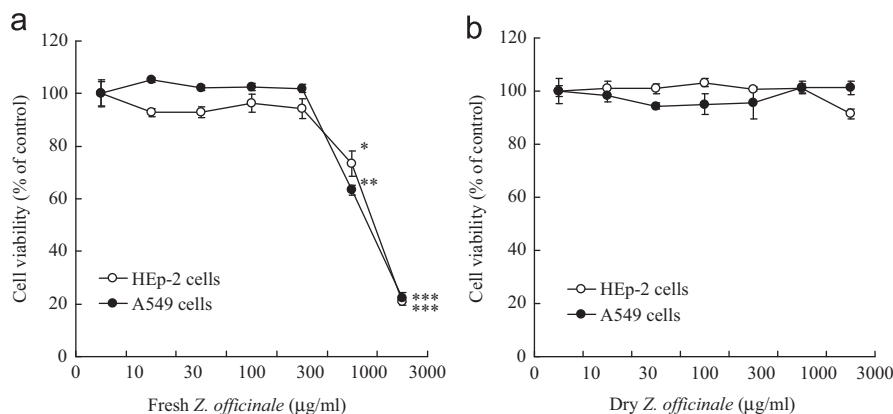


Fig. 1. Cytotoxicity assay. Fresh *Zingiber officinale* (ginger) (a) and dried *Zingiber officinale* (b) did not show any cytotoxicity against host cells up to the concentration of 300 $\mu\text{g/ml}$. However, fresh ginger did affect cell viability at concentrations higher than 300 $\mu\text{g/ml}$. Data are presented as mean \pm S.D. of nine tests.

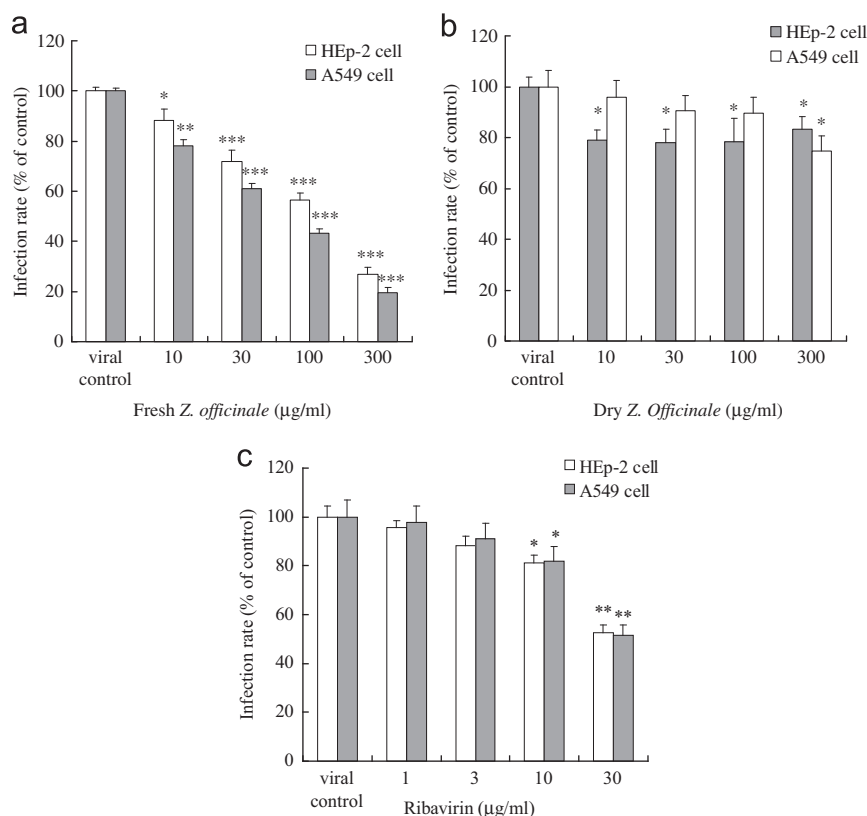


Fig. 2. Antiviral effect assay. Both fresh *Zingiber officinale* (a) and ribavirin (c) were dose-dependently ($p < 0.0001$) effective against HRSV as determined by plaque reduction assay. However, dried *Zingiber officinale* (b) showed some activity without biological gradient. Fresh *Zingiber officinale* was more effective in A549 cells than HEp-2 cells ($p < 0.0001$). Nevertheless, ribavirin did not show this difference. Data are presented as mean \pm S.D. of nine tests. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ were compared to the viral control.

4. Discussion

This study clearly demonstrated that fresh ginger had antiviral activity against HRSV both on HEp2 and A549 cells. 300 $\mu\text{g/ml}$ fresh ginger decreased more than 70% HRSV infection in both HEp-2 and A549 cells, in contrast to dried ginger protecting about 20% from formation of viral plaque in only HEp-2 cells. Dried ginger did not have anti-HRSV activity as fresh ginger had. This might explain why dried ginger is commonly used for metabolic applications, but not for airway viral infections in ancient China. In contrast, fresh ginger had clear antiviral effect against HRSV. This anti-HRSV activity, together with its anti-rhinoviral effect (Denyer et al., 1994), supported its usefulness against airway viral infections. That is why fresh ginger, but not dried ginger, is a common ingredient in prescriptions of Chinese traditional medicine for airway infections, such as Ge-Gen-Tang. Fresh ginger had a much better effect when given before viral inoculation. Fresh ginger inhibited viral attachment and internalization. These effects provided basic mechanisms for its usefulness to prevent HRSV infection. That is why Chinese always have fresh ginger soup to prevent catching cold when get wet in a raining day. Fresh ginger had quite different effect from dried ginger against HRSV. It became interesting why they were so different? One possible explanation is that fresh ginger has quite different active constituents from dried ginger (Jolad et al., 2004, 2005). Some of these changes are probably formed by thermal degradation during isolation which mimics cooking (Jolad et al., 2005). Fresh ginger contains [6]-, [8]- and [10]-gingerols as the major pungent principles and [4]- and [5]-gingerols of trace quantities (Jolad et al., 2004, 2005). Among these, [6]-gingerol is the dominant ginger constituent (Jolad et al., 2004, 2005). During preparation of

dried ginger, the active constituents can be changed (Jolad et al., 2005). For example, the concentrations of gingerols, the major constituents of fresh ginger, were reduced in dried ginger. In contrast, the concentrations of the major gingerol dehydration products, shogaols, increased (Jolad et al., 2005). [6]-, [8]- and [10]-gingerols are turned into shogaols during the commercial drying process (Ali et al., 2008; Jolad et al., 2005). However, [6]-gingerol, as the dominant constituent of both fresh and dried gingers (Jolad et al., 2005), did not have any effect on HRSV (unpublished data). Therefore, the active constituents should be those contained only in fresh ginger which needs to be identified in the future.

The results of time course assay of fresh ginger were somewhat similar to those of GGT (Chang et al., 2012). This similarity could be easily explained if fresh ginger was the main active ingredient of GGT. However, GGT contains only 16% (w/w) of fresh ginger. There could be a possibility that methodologic pitfalls cause a systemic bias so that all time course assays would show similar results. We used ribavirin to prove that our results were valid. Ribavirin is a synthetic nucleotide that freely gets into cells. Ribavirin had completely different results of time course assay that showed only dose-dependent effect without any time difference. Therefore, fresh ginger showing its usefulness to prevent viral infection was valid. Fresh ginger could inhibit HRSV attachment and internalization. HRSV attaches to and penetrates into cells by G protein and the fusion (F) protein, respectively (Dolin, 2012). Therefore, fresh ginger might exert its effect on G and F protein. If this was true, fresh ginger should have a similar effect on HEp-2 and A549 cells. Nevertheless, fresh ginger has a better effect on A549 cells with lower IC_{50} s to inhibit viral attachment and internalization. Therefore, fresh ginger might

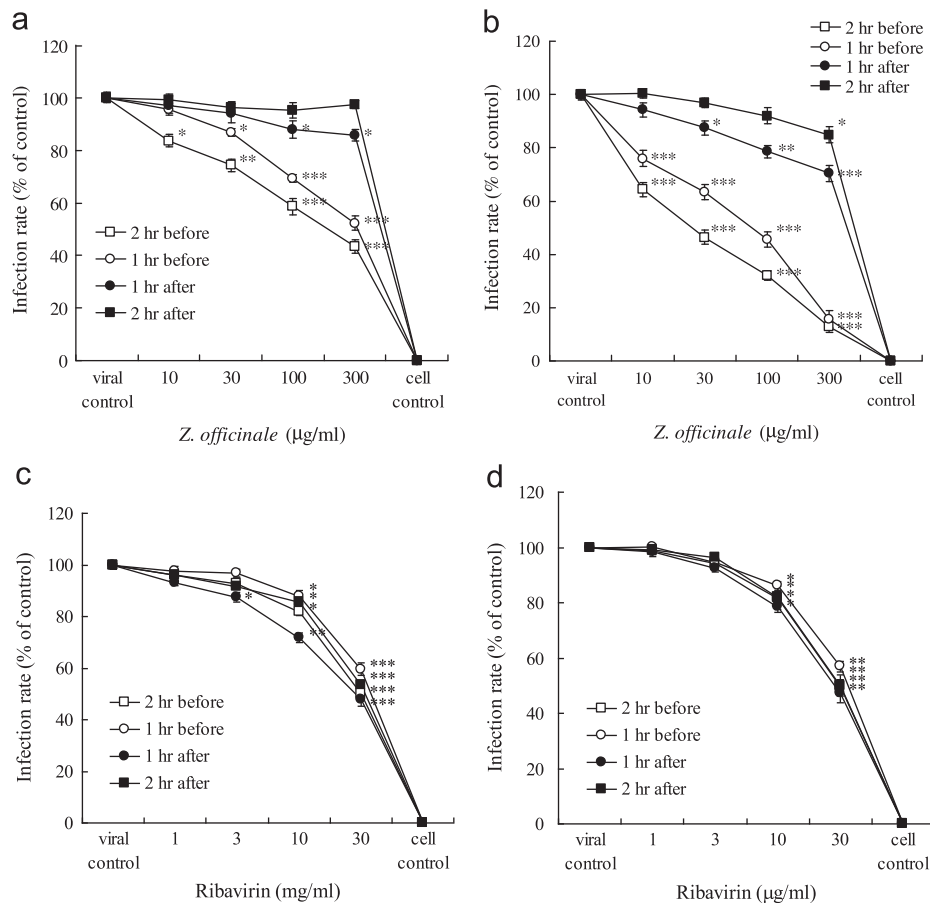


Fig. 3. Time course assay. Fresh *Zingiber officinale* was time-dependently ($p < 0.0001$) and dose-dependently ($p < 0.0001$) effective against HRSV in HEP-2 cells (a) and A549 cells (b). Fresh *Zingiber officinale* was more effective to inhibit plaque formation when given before viral inoculation ($p < 0.0001$), especially in A549 cells. Ribavirin did not show any time-dependent effect on HEP-2 (c) or A549 cells (d). Data are presented as mean \pm S.D. of nine tests. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ were compared to the viral control.

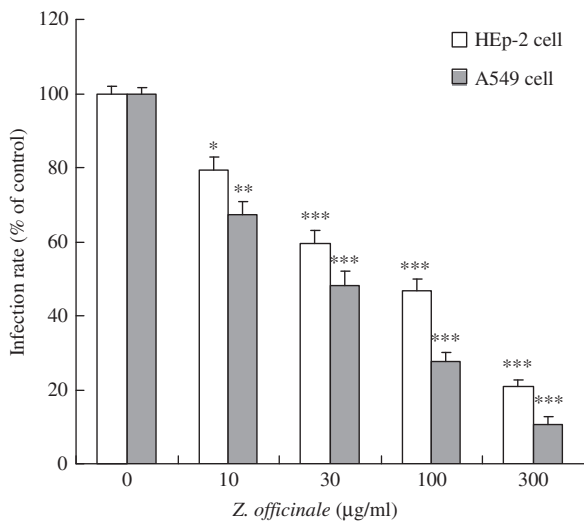


Fig. 4. Attachment assay. Fresh *Zingiber officinale* was dose-dependently effective against viral attachment in HEP2 cells and A549 cells ($p < 0.0001$), with a better effect on A549 cells ($p < 0.0001$). Data are presented as mean \pm S.D. of six tests. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ were compared to the control group.

local inflammation and viral replication in low respiratory tract are essential for disease control. Fresh ginger could inhibit viral spreading by inhibiting viral attachment and penetration to minimize viral production. These effects were better on low respiratory tract mucosal (A549) cells. Besides, fresh ginger could stimulate epithelial cells to secrete IFN- β that contributed to the inhibition of viral replication. However, only at high concentration could fresh ginger stimulate HEP-2 and A549 cells to secrete IFN- β . This effect was not impressive comparing to its effective inhibition of plaque formation at low concentrations. Therefore, stimulation of IFN- β might not be the major anti-viral mechanism of fresh ginger. Ginger has anti-inflammatory activity through its inhibition on productions of prostaglandins and inflammatory cytokines (Ali et al., 2008; Chrubasik et al., 2005). Therefore, fresh ginger might be beneficial to inhibit the pathology of low respiratory tract during HRSV infection. The immunity develops after HRSV infection is not long-lasting so that patients can be repeatedly infected (Dolin, 2012). A safe and cost-effective strategy to manage repeated infection is of importance during endemic periods. Palivizumab (synagis) is effective for prevention but is very expensive. Fresh ginger is very cheap and generally considered to be safe with only some minor adverse effects (Ali et al., 2008). Fresh ginger could be a safe, readily available, and cost-effective strategy to prevent HRSV infection in high risk patients.

In conclusion, fresh *Zingiber officinale* is cheap and could prevent HRSV infection largely by inhibiting viral attachment, internalization, and possibly stimulating IFN- β secretion. Fresh *Zingiber officinale* has been safely used for thousand years worldwide to manage various

have different mechanisms, other than inhibiting viral G and F proteins. Further studies will be needed to find out the exact mechanisms. HRSV causes morbidity and mortality through inducing severe bronchiolitis or pneumonia (Dolin, 2012). Inhibition of

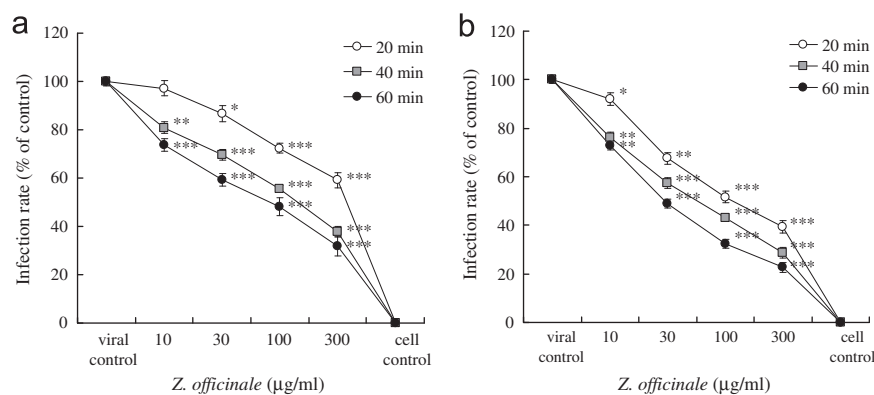


Fig. 5. Internalization assay. Fresh *Zingiber officinale* was time-dependently ($p < 0.0001$) and dose-dependently ($p < 0.0001$) effective against viral penetration in HEP-2 cells (a) and A549 cells (b). Data are presented as mean \pm S.D. of nine tests. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ were compared to the viral control.

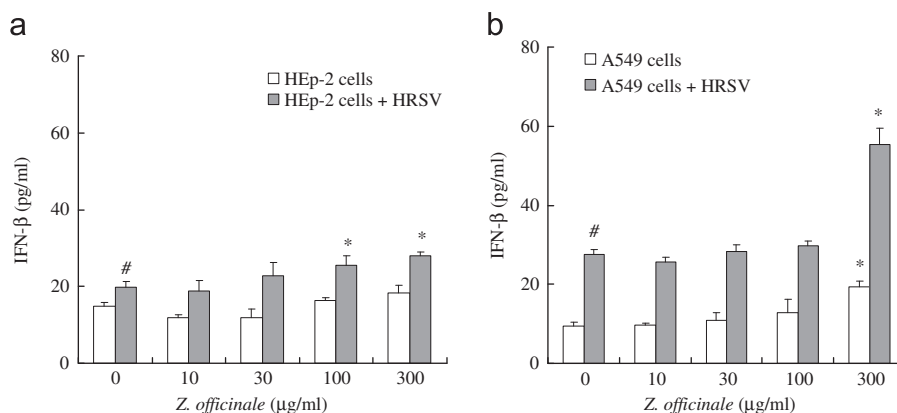


Fig. 6. Interferon- β (IFN- β) assay. HRSV infection increased IFN- β secretion in HEP-2 (a) and A549 (b) cells. Fresh *Zingiber officinale* stimulated HEP-2 (a) and A549 (b) cells to secrete IFN- β only at high concentrations. Data are presented as mean \pm S.D. of nine tests. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ were compared to the control group (0 $\mu\text{g/ml}$). # $p < 0.05$ was compared to the cell control (cell 0 $\mu\text{g/ml}$).

diseases. Our results provide the first evidence to support its possible use to prevent HRSV infection.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2012.10.043>.

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