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Antiviral activity and mode of action of caffeoylquinic acids from *Schefflera heptaphylla* (L.) Frodin

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Abstract

Schefflera heptaphylla is a popular medicinal plant in southern China. Three caffeoylquinic acid derivatives, namely 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 3-*O*-caffeoylquinic acid, were isolated from this plant and investigated for their antiviral activity against respiratory syncytial virus (RSV). 3,4-Di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid possessed potent anti-RSV activity. The median inhibitory concentrations (IC_{50}) of 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid against RSV were 2.33 μM (1.2 μg/ml) and 1.16 μM (0.6 μg/ml), respectively, in a plaque reduction assay. The dicaffeoylquinic acids exhibited minimal cytotoxicity against HEp-2 cells with median cytotoxic concentration (CC_{50}) higher than 1000 μM. The maximal non-cytotoxic concentration (MNCC) of the two dicaffeoylquinic acids were about 96.7 μM, which suggested their anti-RSV effect was not due to cytotoxicity. The antiviral action of 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid was specific against RSV, as they had no obvious antiviral activity against influenza A (Flu A), Coxsackie B3 (Cox B3), and Herpes simplex type one (HSV-1) viruses. Studies were performed that indicated that the dicaffeoylquinic acids could inhibit RSV directly, extracellularly, but only at much higher concentrations than seen in standard assays. Moreover, they could not inhibit RSV attachment to host cells, and could not protect HEp-2 cells from RSV infection at lower concentrations. The data suggest that the compounds exerted their anti-RSV effects via the inhibition of virus–cell fusion in the early stage, and the inhibition of cell–cell fusion at the end of the RSV replication cycle.

Keywords: *Schefflera heptaphylla*; Dicaffeoylquinic acids; Antiviral activity; RSV

1. Introduction

Human respiratory syncytial virus (RSV), a member of the Paramyxoviradae, is a major cause of lower respiratory tract infections in infants, young children, and even adults. Serological evidence indicates that approximately 95% of children have been exposed to RSV by 2 years of age, and 100% of children have been exposed by the time they reach adulthood (Flasey and Walsh, 2000). Since the immune response to RSV infection is not protective, RSV infections re-occur throughout adulthood (Huntley et al., 2002). The elderly and immunocompromised adults are especially at risk for developing complications or even death associated with RSV infection (Flasey and Walsh, 2000; Douglas et al., 2003).

Serious efforts have been put into finding the effective treatment or prevention of RSV infections (Maggon and Barik, 2004). However, there are no approved vaccines after more than 40 years of research, and the only prophylactic therapies available are RSVIGIV, a polyclonal RSV immunoglobulin, and Synagis (palivizumab), a human monoclonal antibody targeting the RSV fusion protein (Groothuis and Nishida, 2002). Ribavirin is the only antiviral drug approved by the FDA for the treatment of RSV infection, but it is only recommended for use as a small particle aerosol by RSV-infected children who are at high risk of having serious sequelae. The utilization of ribavirin is limited due to its efficacy and toxicity concerns as well as the very long and inconvenient regimen required for its delivery by

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aerosol inhalation (Wyde, 1998; Mills, 1999; Kneyber et al., 2000).

Traditionally medicinal plants are a potential source for search and development of new antiviral drugs, since they can be selected for screening on the basis of their ethnomedicinal use, e.g. against infections (Cos et al., 2003). *Schefflera heptaphylla* (L.) Frodin is a popular medicinal plant in southern China. Its traditional uses mainly are anti-pyretic, anti-inflammatory, and anti-rheumatic (Kitajima et al., 1990; Kong, 2000; Chen et al., 2002). *S. heptaphylla* in Hong Kong is a very common tree with dense foliage, long-stalk, and a smooth brown bark. It is a principal ingredient of a herbal tea widely used for the treatment of common cold in Hong Kong (Kong, 2000). The phytochemical studies of *S. heptaphylla* demonstrated that triterpenoids and their glycosides (saponins) are the major chemical constituents of *S. heptaphylla* (Adam et al., 1982; Lischewski et al., 1984; Schmidt et al., 1984; Kitajima and Tanaka, 1989; Sung et al., 1991a,b,c; Sung and Adam, 1991, 1992; Maeda et al., 1994). Besides, a trisaccharide was isolated from the plant (Sung et al., 1991a). The special components found in *S. heptaphylla* are sulfated triterpenoids and their glycosides (Kitajima et al., 1990; Sung and Adam, 1991). However, little information is available on biological activities of the plant. Our previous investigation indicated that the aqueous extract of *S. heptaphylla* possessed potent anti-RSV activity, and its anti-RSV SI value was found to be higher than that of ribavirin (Li et al., 2004). In this paper, our objectives were to isolate the anti-RSV compounds from *S. heptaphylla*, and then study the mode of action of the active compounds.

2. Materials and methods

2.1. Plant materials

S. heptaphylla (L.) Frodin was collected from the campus of The Chinese University of Hong Kong at Shatin, Hong Kong, China, and was authenticated by Prof. Shiu-Ying Hu of the Department of Biology, The Chinese University of Hong Kong. Voucher specimen was deposited in the herbarium of the Department of Biology and the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong, with accession number of 2433.

2.2. Antiviral-guided isolation of leafstalk extract of S. heptaphylla

Dried and cut leafstalks of *S. heptaphylla* (1.5 kg) were refluxed with 60% ethanol for three times, each time for 1 h. The solution was filtered and evaporated under reduced pressure to obtain a dark brown paste. The paste was suspended in distilled water and then partitioned sequentially with hexane, ethyl acetate, and *n*-butanol to afford hexane fraction, ethyl acetate fraction, and *n*-butanol fraction, respectively. The *n*-butanol fraction (96 g), the most active anti-RSV fraction, was re-suspended in distilled water, and a gray white precipitate then settled down. After removing the precipitate, the supernatant of *n*-butanol fraction was dried under reduced pressure to obtain about 40 g of the residue. The residue (40 g) was subjected to a macroporous resin (D101, Tianjin, China) column eluted with distilled water and 95% ethanol, respectively, to produce an ethanol elution fraction (34 g). The ethanol elution fraction was chromatographed on silica gel 60 PR-18 (40–63 μm, Merck, Germany) column eluted in succession with 35, 40, 50, 70, and 100% methanol. The effluents were combined into eight subfractions (B-1–B-8) according to their TLC patterns as well as their anti-RSV activity. The high polar subfractions from B-1 to B-3 were found to possess potent anti-RSV activity, and were then purified with Sephadex LH-20 (25–100 μm, Fluka, Switzerland) column eluted with 80% methanol to afford three pure compounds that were designated as P-1 (20 mg), P-2 (80 mg), and P-3 (100 mg). The chemical structures of P-1, P-2, and P-3 were, respectively, elucidated as 3-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, and 3,5-di-*O*-caffeoylquinic acid by spectroscopic analyses and comparison with the published chemical data. It is noteworthy that caffeoylquinic acid derivatives were isolated from *Schefflera* plant for the first time. The isolation procedures and chemical structures of three caffeoylquinic acids are displayed in Figs. 1 and 2.

2.3. Viruses and cells

Respiratory syncytial virus (Long strain) and influenza A virus (Flu A/PR/8/34, H1N1 strain) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Herpes simplex virus type 1 (HSV-1, 15577 strain) was kindly provided by Prof. Spencer H.S. Lee of Department of Microbiology and Immunology, Dalhousie University, Halifax, Canada. Coxsackie B3 (Cox B3) was kindly provided by Prof. Jiuxiang Li of Guangzhou Biomedicine Research & Development Center, Jinan University, Guangzhou, China. All virus stocks were stored at −70 °C until use.

RSV was cultured in human larynx epidermoid carcinoma cell line (HEp-2 cells) to contain approximately 5×10^6 pfu/ml. Cultures were aliquoted and kept frozen at −70 °C until use. Cox B3 was grown in HEp-2 cells, HSV-1 in Vero cells (African green monkey kidney cell line), and Flu A in Madin–Darby canine kidney (MDCK) cells. All the cell lines were obtained from the ATCC.

All cell lines were grown in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 25 μg/ml gentamicin (Sigma), and 200 mM L-glutamine (Sigma), which is the growth medium (10% GM). HSV-1-infected cells (Vero cells), RSV-infected cells (HEp-2 cells), and Cox B3 infected cells (HEp-2 cells) were maintained in MEM with 1% FBS, 25 μg/ml gentamicin, and 200 mM L-glutamine, which is the maintenance medium (1% MM). Flu A infected cells (MDCK cells) were maintained in 1% MM supplemented with 1% of

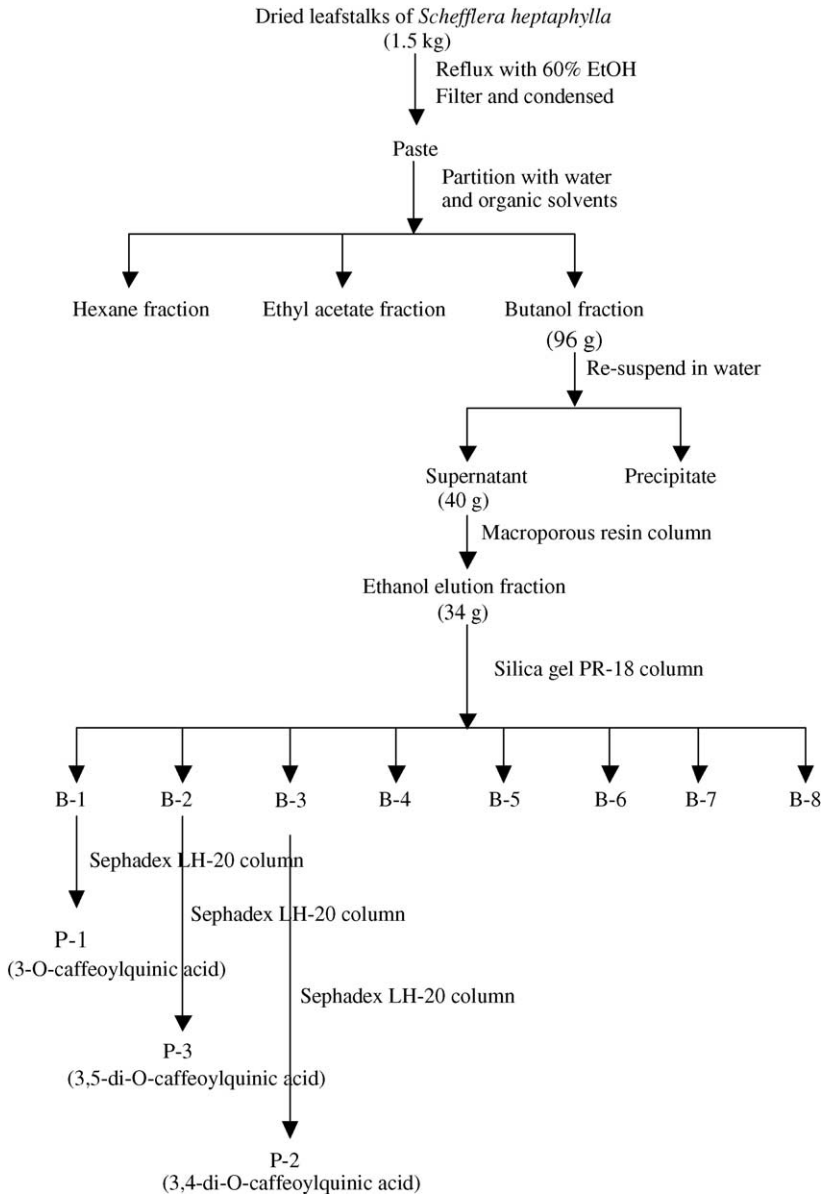
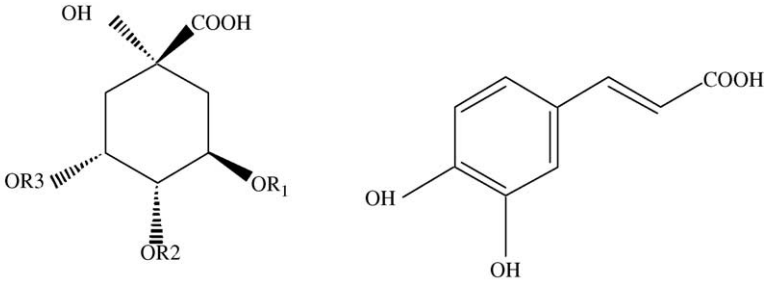


Fig. 1. Isolation and fractionation procedures of the anti-RSV constituents from the leafstalk of *Schefflera heptaphylla*.



3-O-caffeoylquinic acid: R_1 = caffeoyl group, R_2 = H, R_3 = H
3,4-di-O-caffeoylquinic acid: R_1 = caffeoyl group, R_2 = caffeoyl group, R_3 = H
3,5-di-O-caffeoylquinic acid: R_1 = caffeoyl group, R_2 = H, R_3 = caffeoyl group

Fig. 2. Chemical structures of the caffeoylquinic acid derivatives.

L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated typsin (Sigma). All the cells were cultured at 37 °C in a humidified atmosphere supplied with 5% CO_2.

2.4. Cytopathic effects (CPE) reduction assay

CPE reduction assay (Kujumgiev et al., 1999) was adopted to monitor the isolation and purification process of the pure compounds of the medicinal plant *S. heptaphylla* in the present study. The assay was also used to investigate the antiviral activities of pure compounds against Flu A, Cox B3, and HSV-1 viruses. In brief, to confluent cell monolayers in 96-well plate, 0.1 ml suspension containing 100 median tissue culture infectious doses ($TCID_{50}$) of virus and serial two-fold dilutions of the test samples were added simultaneously. To act as the virus control and cell control, the virus suspension and maintenance medium without samples were added, respectively. The plate was incubated at 37 °C under a humidified CO_2 atmosphere for 3–6 days. The virus-induced CPE of the tests was scored under light microscopy in comparison with the parallel virus control and cell control. The concentration that reduced 50% of CPE in respect to that of virus control was estimated from the plots of the data and was defined as 50% inhibitory concentration (IC_{50}).

2.5. MTT reduction assay

Cellular viability was assayed with MTT method (Hussain et al., 1993; Miyamaki et al., 2001). In the assay, different concentrations of samples (100 μl) were applied to the wells of 96-well plate containing confluent cell monolayer in triplicate, while the dilution medium without the sample was used as the control. After 3 days of incubation, 12 μl of the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml in phosphate buffered saline) was added to each well. The plate was further incubated for 3 h to allow MTT formazan formation. After removing the medium, 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. After 15 min, the content in the wells was homogenized on a microplate shake. The optical densities (OD) were then read at a microplate spectrophotometer at double wavelengths of 540 and 690 nm. The median cytotoxic concentration (CC_{50}) was calculated as the concentration of the sample that decreased the number of viable cells to 50% of the cell control through the OD values of viable cells in comparison with non-viable cells. The maximal non-cytotoxic concentration (MNCC) was defined as the maximal concentration of the sample that did not exert cytotoxic effect evaluated from the OD values of non-viable cells.

2.6. Plaque reduction assay

The inhibitory effects of the caffeoylquinic acids on the replication of RSV in HEp-2 cells were studied by the plaque reduction assay, which was performed according to the method described previously with some modifications (Zhu et al., 2004). Briefly, monolayer of HEp-2 cells was grown on 12-well plate. Approximate 80 plaque-forming units (pfu) of RSV was added to the cells, immediately followed by the addition of various concentrations of the sample. The plate was incubated in 5% CO_2 at 37 °C for 2 h with intermittent rocking at 15-min intervals, and then overlaid with agarose overlay medium containing various concentrations of the test sample. After incubation for 5 days at 37 °C, the infected cells were fixed with 10% formalin, stained with 1% crystal violet, and the number of plaques was counted. The wells overlaid with agarose medium without test sample were used as the control. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control) − (mean number of plaques in sample)] × 100/(mean number of plaques in control). The values of IC_{50}, which was the concentration of test sample required to inhibit up to 50% of virus growth as compared with the virus control group, were estimated from the graphic plots of the data. Ribavirin (Sigma, St. Louis) was used as a positive control drug in the anti-RSV study.

2.7. Direct virucidal assay

The direct effects of the dicaffeoylquinic acids on RSV were performed according to the previously described methods (Barnard et al., 1992; Zhu et al., 2004). The dose-dependent inactivation of RSV by the test sample was examined at different temperatures. At first, RSV was cultured in HEp-2 cells for 5 days. The culture supernatants were aspirated and centrifuged for removing the cellular debris to afford approximately 10^6 pfu/ml of RSV suspension. Then, the test sample was diluted into 80, 40, 20, 10, and 5 μg/ml with 1% MM, and mixed, respectively, with an equal volume of the RSV suspension for 2 h at 37 °C, room temperature (about 25 °C), and 4 °C. The mixture without test sample was used as the control. After 2 h incubation, the mixtures were diluted and their residual infectivity was, respectively, determined with plaque reduction method.

2.8. Pre-treatment assay

To evaluate the effects of dicaffeoylquinic acids on prophylaxis of cell infection, the HEp-2 cells were grown in 12-well plate together with test samples. Firstly, the test sample was diluted as 100, 50, 25, 12.5, and 6.25 μg/ml, respectively, with 10% GM. One milliliter of HEp-2 cell suspension was added to the 12-well plate and mixed, respectively, with 1 ml of different concentrations of the test sample. The mixtures were incubated at 37 °C under 5% CO_2 for 2 days. When confluent cell monolayers formed, the 10% GM containing test sample was removed from the wells. After washing with 1% MM, the cell monolayers were infected with about 80 pfu of RSV to allow plaque formation. The cell monolayers grown in 10% GM without test sample were used as the control in this experiment.

2.9. Inhibition of virus attachment assay

The attachment assay as described by Kimura et al. (2000) was used in this study with minor modification. HEp-2 cell monolayers in 12-well plate were pre-chilled at 4 °C for 1 h. The cell monolayers were then infected with 80 pfu of RSV in the absence or presence of serially diluted samples at 4 °C. After further incubating the infected cell monolayers at 4 °C for another 2 h, the medium was aspirated from the well to remove unabsorbed virus. The cell monolayers were then washed with 1% MM and overlaid with the agarose overlay medium. The plate was incubated at 37 °C under 5% CO_2 for 5 days followed by fixation and staining. The inhibition percentage of test sample on RSV attachment to HEp-2 cells was calculated by the following formula: [(mean number of plaques in control) − (mean number of plaques in sample)] × 100/(mean number of plaques in control).

2.10. Time of addition assay

Time of addition assay was performed according to the method described previously with some modifications (Huntley et al., 2002; Andries et al., 2003). HEp-2 cell monolayers in 12-well plate were infected with RSV (MOI = 1) at 4 °C for 2 h for viral absorption. The unbound RSV was washed out from the cell monolayers with 1% MM. The RSV-bound cells were overlaid with the fresh medium and shifted to 37 °C for further incubation. The test sample (final concentration, 20 μg/ml) was added to the infected cells at 0, 3, 5, 7, 9, 12, and 24 h post-incubation, respectively. The infected cells maintained in 1% MM without adding test sample were used as the control in this experiment. The experiment was terminated when the syncytium of RSV-infected cells was formed (about 48 h post-infection). The culture supernatants were aspirated to determine the extracellular titer of virus with plaque reduction assay. The inhibition percentage of test sample on extracellular virus yield was calculated by the following formula: [(pfu/ml in control) − (pfu/ml in sample)] × 100/(pfu/ml in control).

2.11. Inhibition of virus syncytium formation assay

HEp-2 cell monolayer grown in 96-well plate was infected with MOI = 1 of RSV at 37 °C under 5% CO_2 for 2 h for virus adsorption. The unbound RSV was aspirated from the wells and the cell monolayers were overlaid with the fresh medium. The test sample (final concentration, 20 μg/ml) was added to the infected cells at 3, 5, 7, 9, 12, and 24 h post-infection, respectively. The cell monolayer was observed under light microscope (Olypus Microscope IX71S8F-2) for syncytium formation. The assay was ended when the positive control without test compounds showed syncytium formation of 4+ CPE (about 48 h post-infection) under microscope. The CPE changes in the other wells were graded on a scale of 1+ to 4+, while 4+ grade represented a scenario in which the entire (100%) monolayer in a well showed viral syncytium formation (Sudo et al., 2001; Huntley et al., 2002).

3. Results

3.1. Antiviral activity of caffeoylquinic acid derivatives

As shown in Table 1, three caffeoylquinic acid derivatives which were isolated from *S. heptapylla* possessed anti-RSV activity. The antiviral activity of 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid was more potent than that of 3-*O*-caffeoylquinic acid with the IC_{50} values of 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid against RSV being 2.33 μM (1.2 μg/ml) and 1.16 μM (0.6 μg/ml), respectively, in plaque reduction assay. MTT reduction assay indicated that the dicaffeoylquinic acids exhibited minimal cytotoxicity against HEp-2 cells with CC_{50} values higher than 1000 μM. The anti-RSV SI values of the two dicaffeoylquinic acids are much higher than that of ribavirin. Moreover, the MNCC values of the two dicaffeoylquinic acids ensured that their anti-RSV effect was not due to cytotoxicity.

In addition, we also screened the antiviral activities of the dicaffeoylquinic acids against Flu A, Cox B3, and HSV-1 viruses in the present study. The result showed that 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid possessed only a slight anti-HSV-1 activity with IC_{50} values of 81.3 and 62.5 μg/ml, respectively. They could not inhibit Flu A and Cox B3 infections at the concentration up to 100 μg/ml. Thus, the anti-RSV action of the dicaffeoylquinic acids was not only potent but also specific. Further study on the mode of action was carried out to evaluate the stage in which the dicaffeoylquinic acids affect the RSV replication

Table 1
Anti-RSV activity, cytotoxicity, and selective index of two dicaffeoylquinic acids

Compounds	Anti-RSV activity[a] IC_{50} (μM)[b]	Cytotoxicity[c]		Selective index (SI)
		CC_{50} (μM)[d]	MNCC (μM)[e]	
3,4-Di-*O*-caffeoylquinic acid	2.33	1160	96.7	500
3,5-Di-*O*-caffeoylquinic acid	1.16	1300	96.7	1116
Ribavirin	6.97	256	51.2	36.7

[a] The data of anti-RSV activity were measured by plaque reduction assay.
[b] IC_{50}: mean (50%) inhibitory concentration.
[c] The data of cytotoxicity were measured by MTT reduction assay.
[d] CC_{50}: mean (50%) cytototoxic concentration.
[e] MNCC: maximal non-cytotoxic concentration.

cycle. Based on the results of the endpoint titration assay of anti-RSV activity (van den Berghe and Vlietinck, 1991), 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid at 20 μg/ml could completely inhibit high titer of RSV in vitro (data not shown). Thus, the concentration (20 μg/ml) was adopted in the mode of action study of the dicaffeoylquinic acids.

3.2. Extracellular virucidal activity

The effects of dicaffeoylquinic acids on inactivation of RSV infectivity were investigated at 4 °C, room temperature (about 25 °C), and 37 °C, respectively. As shown in Figs. 3 and 4, either 3,4-di-*O*-caffeoylquinic acid or 3,5-di-*O*-caffeoylquinic acid at the concentrations ranging from 5 to 80 μg/ml could directly inactivate RSV to different extent. Pre-incubation of RSV with the dicaffeoylquinic acids resulted in a concentration-dependent reduction of RSV infectivity, and the effect increased with the increase in temperature. 3,5-Di-*O*-caffeoylquinic acid at the concentration of 80 μg/ml could directly inactivate 100% of RSV at 37 °C (Fig. 3B), inactivate more than 90% of RSV at room temperature, and inactivate about 50% of RSV at 4 °C. Similarly, 3,4-di-*O*-caffeoylquinic acid at the same concentration could directly inactivate 60% of RSV at 37 °C (Fig. 3A), inactivate more than 50% of RSV at room temperature, and inactivate 27% of RSV at 4 °C. In this experiment, ribavirin was used as the negative control compound, and any directly virucidal activity was not found at 4 °C, room temperature, and 37 °C.

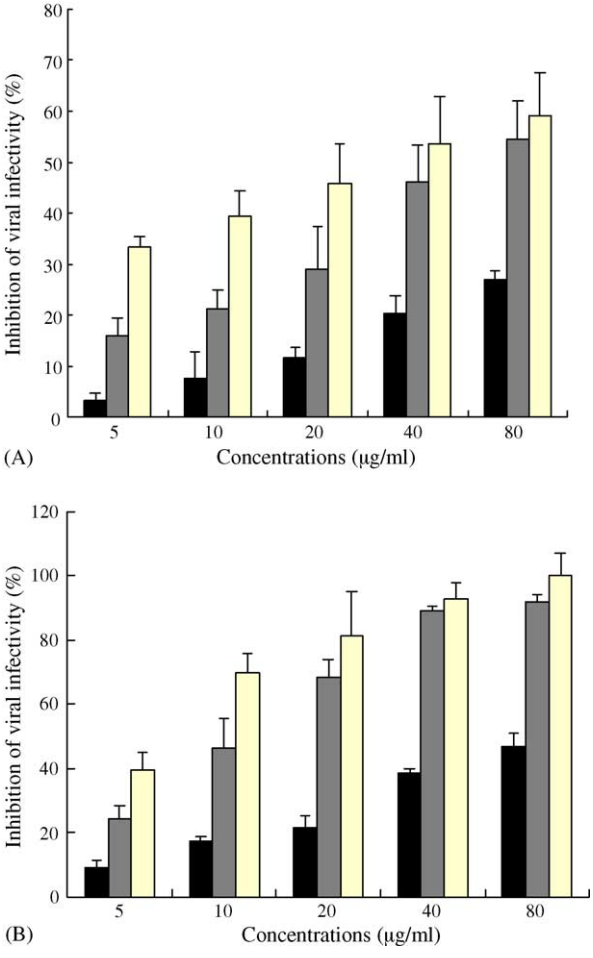


Fig. 3. Direct virucidal effect of: (A) 3,4-di-*O*-caffeoylquinic acid and (B) 3,5-di-*O*-caffeoylquinic acid on RSV at 4 °C [■], room temperature (about 25 °C) [■], and 37 °C [□]. Viral suspensions were pre-incubated with different concentrations of 3,4-di-*O*-caffeoylquinic acid, or 3,5-di-*O*-caffeoylquinic acid, at 4 °C, room temperature, and 37 °C for 2 h, respectively. The titers of the surviving viral mixtures were assayed by plaque reduction method and expressed as a percentage of residual infectivity as compared with the control. Each point represents the average value of two independent experiments.

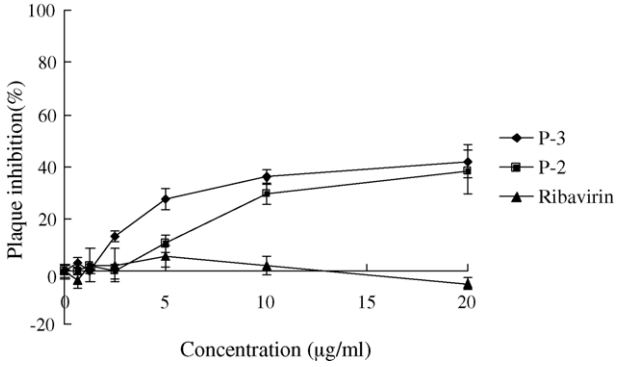


Fig. 4. Effects of two dicaffeoylquinic acids on RSV attachment to host cells. Pre-chilled HEp-2 cell monolayers were incubated with RSV at 4 °C for 2 h in the presence of various concentrations of 3,4-di-*O*-caffeoylquinic acid (P-2), 3,5-di-*O*-caffeoylquinic acid (P-3), or ribavirin, respectively. HEp-2 cell monolayers were then washed with 1% MM and overlaid with the agarose overlay medium for plaque formation assay. Each point represents the average value of two independent experiments.

3.3. Prophylactic activity on RSV infection

In order to evaluate the effects of dicaffeoylquinic acids on prophylaxis of RSV infection, HEp-2 cells were cultured in 12-well plate together with the dicaffeoylquinic acids at the concentrations of 50, 25, 12.5, 6.25, 3.125, and 0 μg/ml of the growth medium (10% GM), respectively. After incubation of the mixtures at 37 °C for 2 days, the growth medium containing the test samples was removed from the wells. The cell monolayers were then infected with about 80 pfu of RSV to allow plaque formation. However, we did not find any obvious reduction of RSV plaques between the treated and untreated (control) cells (data not shown). Thus, neither 3,4-di-*O*-caffeoylquinic acid nor 3,5-di-*O*-caffeoylquinic acid possessed in vitro prophylactic effect on RSV infection.

3.4. Inhibitory activity of dicaffeoylquinic acids against RSV attachment

To examine the inhibitory activity of the dicaffeoylquinic acids on RSV attachment, the monolayer of HEp-2 cells was incubated for 2 h at 4 °C with RSV in the presence of var-

ious concentrations of the test sample. As shown in Fig. 4, the dicaffeoylquinic acids showed weak inhibitory effect on binding of RSV to host cells. Neither 3,4-di-*O*-caffeoylquinic acid nor 3,5-di-*O*-caffeoylquinic acid could inhibit 50% of RSV absorption to HEp-2 cell even at the concentration of 20 μg/ml. In this experiment, ribavirin was not found to possess any inhibitory effect on RSV absorption to HEp-2 cells.

3.5. Time of addition experiment

The experiment was performed to test if there was any effect of the test compounds on RSV-infected cells added at various time intervals, such as 0, 3, 5, 7, 9, 12, and 24 h post-incubation, respectively. The result showed that 3,4-di-*O*-caffeoylquinic acid could completely inhibit RSV replication when it was added before RSV penetration (0 h post-incubation). When 3,4-di-*O*-caffeoylquinic acid was added after RSV penetration (from 3 to 24 h post-incubation), no significant reduction in viral yield was detected (Fig. 5A). Similar result was observed for 3,5-di-*O*-caffeoylquinic acid in the present study (Fig. 5B). In contrast, ribavirin was still effective in suppressing virus replication when it was added at 9 h post-infection. These results indicated that the dicaffeoylquinic acids affected the early stage of RSV replication cycle.

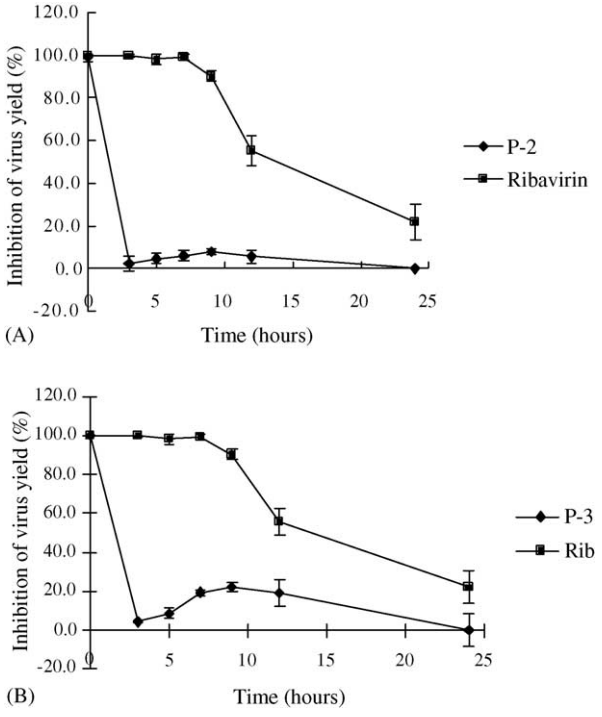


Fig. 5. Effects of time of addition of: (A) 3,4-di-*O*-caffeoylquinic acid and (B) 3,5-di-*O*-caffeoylquinic acid on RSV replication. HEp-2 cells were infected with RSV of MOI = 1. The test compounds, 3,4-di-*O*-caffeoylquinic acid (P-2) and 3,5-di-*O*-caffeoylquinic acid (P-3), were added to make a final concentration of 20 μg/ml at various time intervals of post-incubation (0, 3, 5, 7, 9, and 12 h), respectively. No test compound was added to the control. After 48 h post-infection, their extracellular virus yields were determined and quantitatively analyzed. Data represent the averages of duplicate determinations.

3.6. Inhibitory effects of dicaffeoylquinic acids on RSV syncytium formation

To examine possible time-dependent inhibitory effects of the dicaffeoylquinic acids on syncytium formation induced by RSV, the test compounds were added to the monolayers of RSV-infected cells at 3, 5, 7, 9, 12, and 24 h post-infection, respectively, and observed microscopically for the formation of syncytium. The control group that contained no test compounds showed RSV syncytium formation throughout almost the entire (100%) monolayer of cells in a well (i.e. 4+ CPE) at about 48 h post-infection under microscope. However, the two dicaffeoylquinic acids at the final concentration of 20 μg/ml could completely block syncytium formation of RSV even when they were added at 24 h post-infection (Fig. 6). On the other hand, ribavirin could not inhibit syncytium formation when it was added at 9 h post-infection and afterwards. This result suggested that the two dicaffeoylquinic acids could block syncytium formation induced by RSV at the late stage of RSV replication cycle.

4. Discussion

The present study has demonstrated that the anti-RSV activity of *S. heptaphylla* is mainly attributed to its caffeoylquinic acid constituents. Especially, 3,5-di-*O*-caffeoylquinic acid and 3,4-di-*O*-caffeoylquinic acid possess potent anti-RSV activity with IC_{50} values of 1.16 and 2.33 μM. Caffeoylquinic acid derivatives are isolated from *Schefflera* plant for the first time. *S. heptaphylla* is an Araliaceous plant in which triterpenoids and their glycosides (saponins) are the major constituents. Our research suggests that the minor constituents in medicinal plants may lead to discover more potent lead compounds, and should deserve our attention in future.

The investigation on antiviral spectrum indicated that 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid did not have significant antiviral activities against HSV-1, Flu A, and Cox B3 viruses. The dicaffeoylquinic acids had only slight cytotoxicity. The results suggested that the mode of action of the dicaffeoylquinic acids against RSV maybe different from that of ribavirin (De Clercq, 2002).

In order to study the mode of action of the dicaffeoylquinic acids, we carried out a series of experiments in the present study, including virucidal assay, pre-treatment assay, inhibition of attachment assay, time of addition assay, and inhibition of syncytium formation assay. The result of virucidal assay showed that the dicaffeoylquinic acids could inactivate RSV directly at high concentrations. Besides, the virucidal effects of 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid enhanced with the increase in reaction temperature.

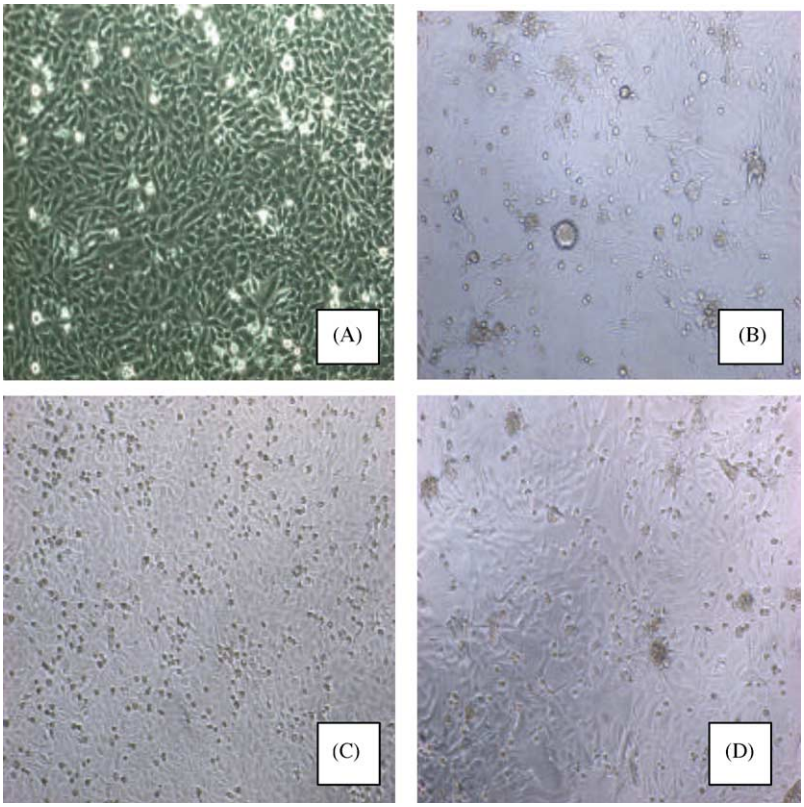


Fig. 6. Light micrographs showing the inhibition of 3,5-di-*O*-caffeoylquinic acid on RSV syncytium formation. The test compound (at final concentration, 20 μg/ml) and ribavirin were added to the RSV infected-cells at 24 h post-infection, respectively. The cell monolayers were photographed at about 48 h post-infection. (A) HEp-2 cell monolayer; (B) 24 h post-infection, untreated RSV infected-HEp-2 cells; (C) 24 h post-infection, 3,5-di-*O*-caffeoylquinic acid treated RSV infected-HEp-2 cells; (D) 24 h post-infection, ribavirin treated RSV infected-HEp-2 cells (magnification of micrographs: ×400).

However, the two compounds did not significantly reduce the RSV infectivity at their IC_{50} concentration (0.6–1.2 μg/ml) and at the concentration (20 μg/ml) used in the mode of action study. Therefore, the anti-RSV activity of dicaffeoylquinic acids was apparently not related to their virucidal abilities. A similar result was found in the attachment inhibition assay. The IC_{50} values of inhibition of viral attachment of two dicaffeoylquinic acids were found to be higher than 20 μg/ml. The result of pre-treatment assay exhibited that two dicaffeoylquinic acids could not protect cultured HEp-2 cells from RSV infection even at high concentrations.

Time of addition study demonstrated that the dicaffeoylquinic acids affected the early stage of RSV replication cycle. When added after RSV penetration into host cells (e.g. 3–24 h post-incubation), the dicaffeoylquinic acids could not inhibit RSV replication. In contrast, ribavirin could not inhibit RSV penetration into host cells, but could inhibit RSV replication when added until 9 h post-incubation. Accordingly, the anti-RSV mode between the dicaffeoylquinic acid and ribavirin appears to be very different.

Inhibition of syncytium formation assay showed that the dicaffeoylquinic acids could block syncytium formation of RSV when they were added from 3 to 24 h post-infection. It is interesting that two time-dependent assays obtained different results. This suggests that dicaffeoylquinic acid exerts its anti-RSV effect via inhibition of two RSV F protein-mediated fusion events: inhibition of virus–cell fusion in the early stage of the replication cycle and inhibition of cell–cell fusion at the end of the replication cycle. This dual mode of action was firstly proposed by Andries et al. (2003).

In addition, some dicaffeoylquinic acids derived from another plant have been reported to possess anti-HIV activity (van den Berghe and Vlietinck, 1991; Cos et al., 2003). It was found that HIV strains, which were made resistant to the dicaffeic acid derivatives, contained several mutations in its envelope glycoprotein gp 120 (Pluymers et al., 2000). Consequently, the primary anti-HIV target of dicaffeoylquinic acids was suggested to be the envelope glycoprotein gp 120. The gp41/120 glycoprotein of HIV structurally resembles the RSV F protein (Andries et al., 2003). Therefore, it is reasonable that the dicaffeoylquinic acids can target RSV F-protein. In recent years, several new classes of fusion inhibitors appear to have significant anti-RSV effect and have distinct mode of antiviral action against RSV, which is different from that of ribavirin. These structurally distinct small molecules are found to inhibit RSV fusion by specific interactions with RSV F-protein (Huntley et al., 2002; Razinkov et al., 2002; Andries et al., 2003; Douglas et al., 2003).

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