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Antiviral activity of *Poncirus trifoliata* seed extract against oseltamivir-resistant influenza virus

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The emergence of oseltamivir-resistant variants of influenza virus has highlighted the necessity for the development of more effective novel antiviral drugs. To date, numerous researchers have focused on developing antiviral drugs using natural resources, such as traditional herbal medicines. *Poncirus trifoliata* is widely used in oriental medicine as a remedy for gastritis, dysentery, inflammation and digestive ulcers. In this study, we investigated the potential antiviral effect of the *Poncirus trifoliata* orange seed extract against influenza virus. An ethanol extract of *Poncirus trifoliata* seeds (PTex) inhibited the activity of influenza viruses, in particular, oseltamivir-resistant strains, in Madin-Darby canine kidney cells. In contrast to oseltamivir, PTex exerted a significant inhibitory effect on the cellular penetration pathway of the virus rather than HA receptor binding. The potent antiviral effect and novel working mechanism of PTex support its further development as an effective natural antiviral drug with a wide spectrum of activity against influenza and oseltamivir-resistant viruses.

***Keywords*:** influenza, *Poncirus trifoliata*, antiviral agent, natural product

Introduction

Influenza virus causes epidemics and pandemics leading to high morbidity and mortality in humans and animals, and thus poses a significant threat to public health (Taubenberger and Morens, 2006). Following the pandemic influenza outbreak in 1918, several subsequent occurrences have been reported in 1957 (H2N2), 1968 (H3N2), 2009 (H1N1), and 2013 (H7N9) (Horimoto and Kawaoka, 2005; Kilbourne, 2006).

Currently, anti-influenza drugs are classified according to their targets, such as M2 ion, neuraminidase (NA) and RNA polymerase (Hossain *et al.*, 2014). Amantadine and rimantadine block release of the virus genome by inhibiting the M2 ion channel (Skehel *et al.*, 1978). Oseltamivir and zanamivir prevent virus spread through inhibiting NA activity (Moscona, 2005). T705 and flutimide block the replication of viral RNA by RNA polymerase (Furuta *et al.*, 2005, 2013). While these drugs can effectively reduce clinical symptoms, their use is limited by side-effects and the emergence of resistant viral strains (Saito *et al.*, 2003; De Jong *et al.*, 2005; Haasbach *et al.*, 2014) Seasonal influenza viruses resistant to the most commonly used antiviral drug, Tamiflu®, have already evolved. Consequently, the development of novel anti-viral drugs to effectively combat emerging Tamiflu-resistant viruses is an urgent medical requirement.

Poncirus trifoliata (PT), also known as trifoliate orange, is closely related to the genus *Poncirus* or *Citrus* and belongs to the Rutaceae family. PT is a tree that grows to over 8 m in height bearing green or yellow fruits resembling small oranges 3–4 cm in size. The trifoliate orange, which originated in China and Korea, is commonly used as traditional medicine for gastritis and allergy (Papa *et al.*, 2014). Various pharmacological activities of PT have been determined, including anti-inflammatory (Shin *et al.*, 2006), anti-bacterial (Kim *et al.*, 1999), anti-anaphylactic (Lee *et al.*, 1996; Park *et al.*, 2005), and anti-cancer effects (Rahman *et al.*, 2015). Although several prenylated flavonoids, coumarins, and triterpenoids from stem bark of PT with antiviral activity against human immunodeficiency virus-1 have been identified (Feng *et al.*, 2010), no studies to date have focused on the potential anti-influenza effect of PT.

In the current study, we investigated the antiviral activity and underlying mechanisms of a dried seed extract of PT against influenza virus. Notably, the PT extract showed antiviral activity against influenza (in particular, oseltamivir-resistant strains) through a novel mechanism distinct from that of oseltamivir.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Cells were maintained in high-glucose Dulbecco's Modified Eagle's medium (DMEM; Gibco BRI) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin at 37°C under 5% CO_2. Influenza virus strain

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A/Puerto Rico/8/34 (H1N1) (PR8) was kindly provided by Professor Song Chang Sun, College of Veterinary Medicine, Konkuk University, Republic of Korea. The oseltamivir-resistant influenza virus with mutated neuramidase (NA-PR8) was kindly provided by the Korean Research Institute of Chemical Technology (KRICT). Viruses were propagated in MDCK cells at 37°C under 5% CO_2. Infectious virus titers were determined using 50% tissue culture infectious dose ($TCID_{50}$) according to Reed and Muench's endpoint method (Reed and Muench, 1938).

Poncirus trifoliata seed extraction

Poncirus trifoliata (PT), cultivated in southern regions of Korea, was the natural source of material used in this study. Dried PT seeds were extracted with ethanol at 65°C for 3 h, and the extract solution filtered and evaporated. The resulting dried PT seed extract (PTex) was dissolved in dimethyl sulfoxide (DMSO; 50 mg/ml stock solution).

Cytotoxicity test

To determine the cytotoxicity of PTex, cell viability was measured via the water-soluble tetrazolium salt (WST) method using an EZ-Cytox kit (Daeil Lab Service) according to the manufacturer's instructions. MDCK cells were seeded on a 96-well plate at a density of 1×10^4 cells/well. After 1 day of culture, cells were treated with serial dilutions of PTex and incubated at 37°C for 2 days. For comparison with the commercial antiviral drug, Tamiflu®, virus-infected cells were treated with various concentrations of oseltamivir phosphate (Sigma) in each experiment. EZ-Cytox solution was added to each well and incubated for 2 h, followed by spectrophotometric measurement of absorbance at 540 nm. The CC_{50} value of samples was defined as the concentration inducing 50% cell death.

Antiviral activity test

To determine the antiviral activity of PTex, MDCK cells were seeded in a 96-well plate and infected with 30 $TCID_{50}$ influenza virus in DMEM growth medium containing 3% BSA, 1 μg/ml trypsin TPCK and 1% penicillin-streptomycin. Next, virus-infected cells were treated with serially diluted PTex or various concentrations of oseltamivir phosphate (Sigma) for comparison. After 48 h, cell viability was determined via the WST method using the EZ-Cytox kit. The EC_{50} of samples was defined as the concentration leading to 50% effective inhibition.

Neuraminidase inhibition (NAI) assay

PTex was mixed with 10 $TCID_{50}$ influenza virus (PR8) for 1 h at room temperature. The mixture was incubated with substrate solution, 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium (4-MU-NANA), while protecting from light at 37°C for 2 h. Optical density was measured with a fluorescence microplate reader (Gemini EM, Molecular Device) at an excitation wavelength of 365 nm and emission wavelength of 450 nm.

RNA isolation and quantitative RT-PCR (qRT-PCR)

MDCK cells in 6-well culture plates were infected with 0.3 MOI influenza virus and treated with 100 μg/ml PTex or 10 μg/ml oseltamivir phosphate, followed by incubation at 37°C for 24 h. Total RNA was isolated from cells using an RNeasy mini kit (Qiagen) and cDNA synthesized from 1 μg RNA using SuperScript II reverse transcriptase (Invitrogen). The PR8 NP-specific primer sequences were 5′-CAGCCT AATCAGACCAAATG-3′ (forward) and 5′-TACCTGCTT CTCAGTTCAAG-3′ (reverse). The primer sequences for GAPDH were 5′-AAGAAGGTGGTGAAGCAGGC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). PCR was performed over 25 cycles under the following conditions: 94°C for 5 min, 20 sec at 94°C, 20 sec at 55°C, 20 sec at 72°C, and 72°C for 10 min. qRT-PCR was performed on real-time PCR system (Applied Biosystems) using the same NP primers. The relative changes normalized against GAPDH were calculated using the ΔCt method.

Immunofluorescence microscopy

MDCK cells seeded onto coverslips in a 4-well plate were incubated overnight at 37°C and subsequently infected with influenza virus in the presence of PTex or oseltamivir phosphate. After 24 h, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 3 min and washed twice with PBS, followed by blocking with 1% BSA for 3 min. Next, cells were treated with murine anti-NP antibodies overnight at 4°C and stained with FITC-conjugated goat anti-mouse IgG antibody at 37°C for 1 h. Nuclei were visualized using mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) and images captured under a fluorescence microscope (Olympus BX51 microscope, Olympus).

Time-of-addition assay

MDCK cells were infected with 100 $TCID_{50}$ influenza virus at 0 h p.i. and inoculated at 4°C for 1 h. After virus absorption, unbound influenza viruses were washed off twice with PBS. Infected cells were treated with 100 μg/ml PTex or 10 μg/ml oseltamivir for the following time-periods: 0 to 48 h, 1 to 48 h, 2 to 48 h, and 3 to 48 h. After 48 h, cell viability was determined using the WST method.

Attachment inhibition assay

MDCK cells were pre-chilled at 4°C for 1 h. Influenza virus (100 $TCID_{50}$) was pre-absorbed into cells with different concentrations of PTex or oseltamivir phosphate for 1 h at 4°C. Cells were washed twice with ice-cold PBS and replaced with virus growth medium. After 2 days, inhibition of attachment was determined based on cell viability using the WST method.

Hemagglutination inhibition (HI) assay

4 hemagglutination unit (HAU) of influenza virus was incubated with serially diluted PTex for 40 min at room temperature. Virus samples were further treated with two volumes of 1% chicken red blood cells (cRBCs) and incubated for 30 min at room temperature. The extent of hemagglutination was visually observed and the HI concentrations determined.

Table 1. Antiviral activity of PTex against influenza viruses in MDCK cells

Cell line or virus strain	PTex			Oseltamivir phosphate		
	CC_{50}[a] (μg/ml)	EC_{50}[b] (μg/ml)	SI[c]	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Cytotoxic effect						
MDCK	1250 ± 97.33			937.5 ± 86.75		
Influenza viruses						
A/PR8/34 (H1N1)		2.51 ± 0.87	498		3.71 ± 1.35	252.6
A/PR8/34 (H1N1) NA mutant (NA-PR8)		3.91 ± 1.45	319		31.25 ± 4.74	30

[a] CC_{50} mean cytotoxic concentration of 50%, determined with the WST method.
[b] EC_{50} mean effective concentration of 50%, determined with the WST method.
[c] SI value mean selective index, determined based on the CC_{50}:EC_{50} ratio.

Penetration inhibition assay

For the penetration inhibition assay, MDCK cells were pre-chilled at 4°C for 1 h and infected with 100 $TCID_{50}$ influenza virus for 1 h at 4°C. Cells were washed twice with ice-cold PBS and treated with different concentrations of PTex or oseltamivir phosphate for 1 h at 37°C. Subsequently, cells were treated with acid PBS (pH 3) for inactivation of un-penetrated virus and neutralized with PBS (pH 11). After 48 h, cell viability was determined using the WST method.

Statistical analysis

All statistical analyses were performed using GraphPad software version 7.0 (GraphPad Software). For analysis of the significance of differences between treatment and control groups, two-way analysis of variance (ANOVA) or two-tailed student's *t*-test was used. *P* values less than 0.05 were considered statistically significant.

Results

Anti-viral effects of PTex against influenza and oseltamivir-resistant influenza strains

To determine the inhibitory activity of PTex against influenza viruses, PT seed powder was extracted via ethanol solvent extraction or boiling in water. The ethanol extract demonstrated significant antiviral activity. Compared with oseltamivir, PTex showed a 2-fold higher level of SI index against A/PR8/34 (H1N1). The SI value was obtained by dividing the 50% cytotoxic concentration (CC_{50}) by 50% effective concentration (EC_{50}). For groups treated with PTex, the EC_{50} value against normal influenza virus (PR8) was 2.51 μg/ml and SI value was 498 (Table 1). Notably, the antiviral effect of PTex was 7.9-fold higher in Tamiflu-resistant, neuraminidase- mutated influenza virus (NA-PR8). The EC_{50} values of the groups treated with PTex and oseltamivir were 3.91 and 31.25 μg/ml, respectively.

Inhibitory effects of PTex on viral RNA and protein synthesis

PTex exerted antiviral effects via a different mechanism to oseltamivir. To determine the underlying mechanism, we initially examined PTex-mediated inhibition of neuraminidase activity. Oseltamivir, a known neuraminidase inhibitor, exerted a significant suppressive effect, even at a low concentration of 19 μg/ml. In contrast, PTex did not suppress viral neuraminidase at a concentration of 478.5 μg/ml (Fig. 1).

We further examined the changes in viral RNA and protein levels following treatment with PTex. Our results showed reduced viral RNA synthesis in the presence of PTex (Fig. 2A). Consistently, relative qRT-PCR analysis showed lower levels of viral RNA after PTex treatment (Fig. 2B). At a concentration of 100 μg/ml PTex, reduction in viral RNA synthesis was markedly greater than that with an equivalent dose of oseltamivir. A 10-fold decrease in viral RNA was observed at a concentration of 10 μg/ml PTex, compared with 100 μg/ml oseltamivir. In addition to suppression of viral RNA, PTex reduced viral protein synthesis, as evident from immunofluorescence analysis (Fig. 3). Our findings suggest that PTex inhibits infection through suppression of viral RNA and protein synthesis.

Anti-influenza activity of PTex at different stages

To investigate whether PTex affects the viral replication cycle, PTex treatment was performed at different stages after virus inoculation. The experimental schedule is depicted in Fig. 4A.

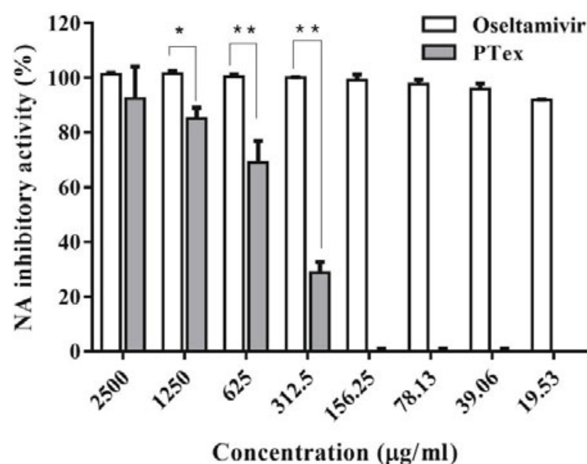

Fig. 1. ***Poncirus trifoliata*** **extract exerted antiviral effects via a different mechanism to oseltamivir.** To determine the underlying mechanism, we initially examined PTex-mediated inhibition of neuraminidase activity. PTex was mixed with influenza virus (PR8) and incubated at room temperature for 30 min. 4-MU-NANA was added to the mixture and incubated at 37°C for 2 h, and the supernatant evaluated using a fluorescence plate reader at an excitation wavelength of 365 nm and emission wavelength of 450 nm. Data are presented as means ± SD for three different samples (*P = 0.001, **P < 0.001 for comparisons between the two groups).

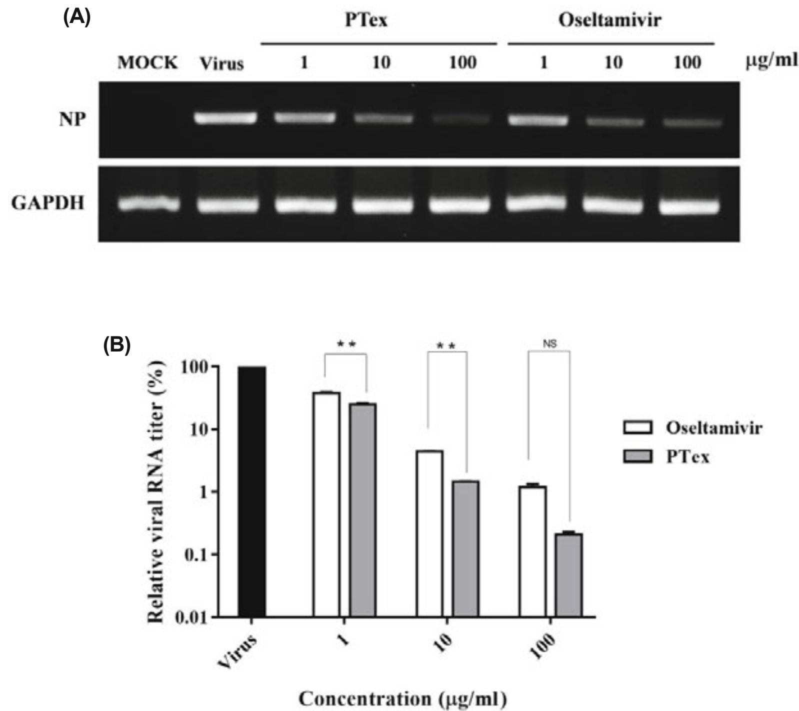

Fig. 2. ***Poncirus trifoliata*** **extract affects influenza virus in RNA synthesis.** MDCK cells were infected with influenza virus (PR8) in the presence of PTex and oseltamivir. After 24 h, total RNA was isolated from cells and cDNA synthesized. (A) The NP RNA level was detected via RT-PCR using specific primers, identified via gel electrophoresis and normalized to that of GAPDH. (B) qRT-PCR analysis of NP RNA expression using specific primers. Values are presented as means ± SD relative to the RNA titer of virus-infected cells (**$P < 0.001$ for comparison between the two groups).

Influenza virus (PR8)-infected cells were treated with PTex or oseltamivir at concentrations of 100 and 10 μg/ml, respectively. PTex showed higher antiviral activity during 0–48 h and 1–48 h, compared with the later stages of infection, with very limited anti-viral effects at later time-points (Fig. 4B). These results suggest that PTex interferes with the early stages of viral replication.

Inhibitory effect of PTex on viral entry

To determine whether influenza virus entry is inhibited by PTex, we conducted an attachment assay. After 1 h of PTex treatment at a concentration of 15.63 μg/ml, cells showed 100% viability. Compared with PTex, oseltamivir exerted a lower inhibitory effect. The hemagglutination inhibition (HI) assay involves suppression of virus-induced red blood cell

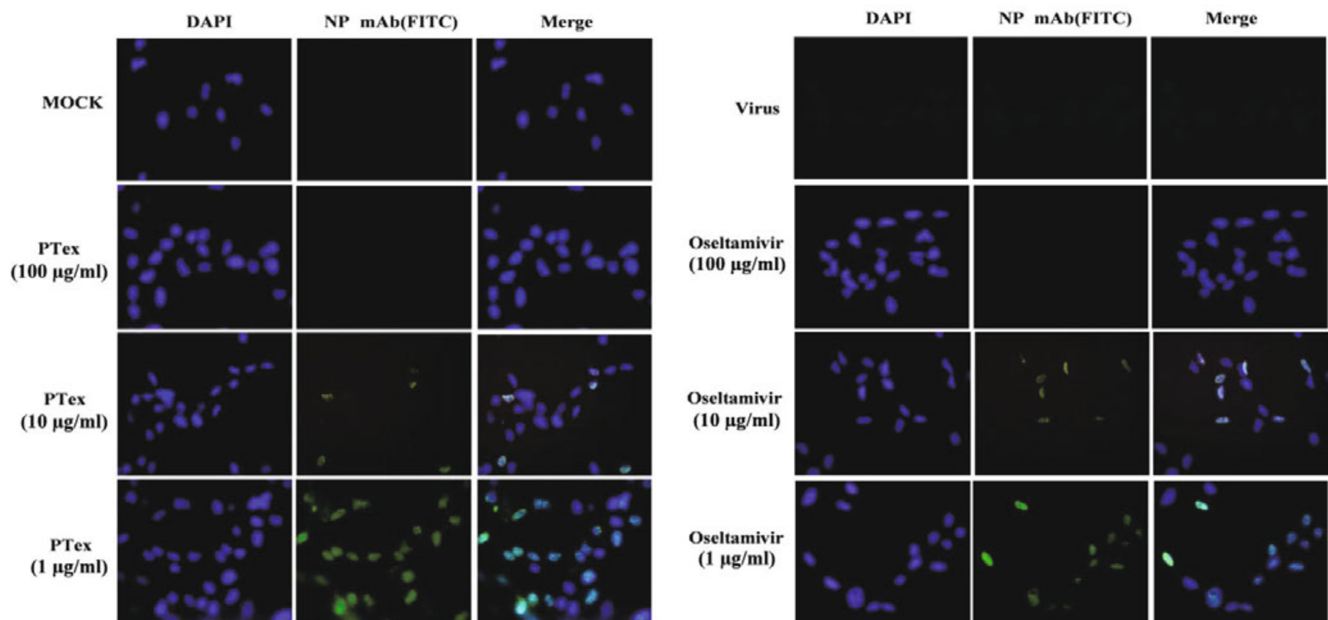

Fig. 3. ***Poncirus trifoliata*** **extract affects influenza virus in protein synthesis.** MDCK cells were infected with influenza virus (PR8) in the presence of PTex and oseltamivir for 24 h. Viral NP protein was detected via fluorescence microscopy using specific monoclonal and FITC-conjugated goat anti-mouse IgG antibodies (green). Nuclei were visualized with DAPI (blue) (original magnification, 200×).

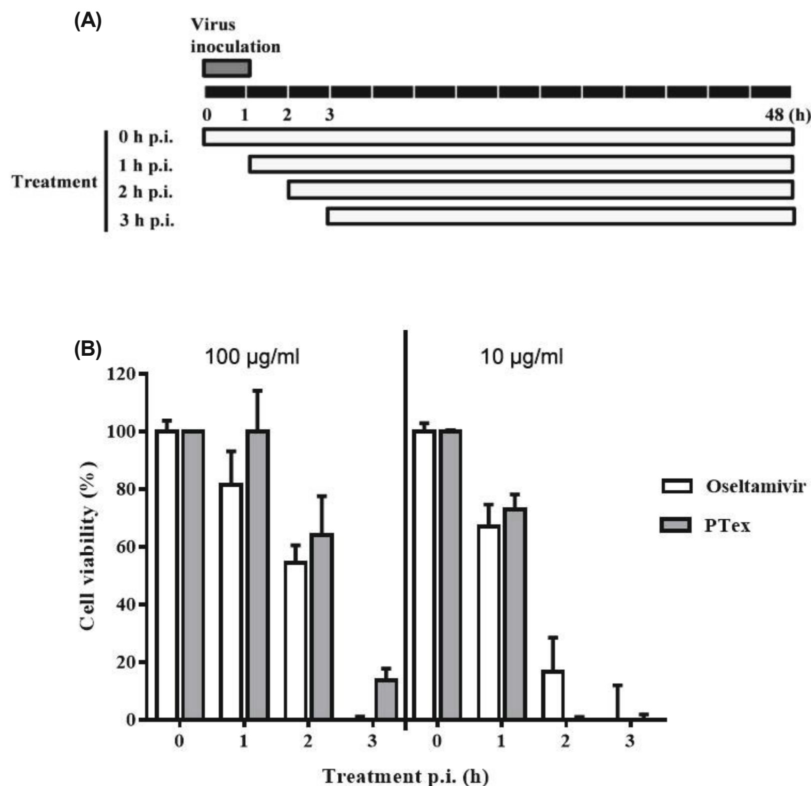

Fig. 4. ***Poncirus trifoliata*** **extract inhibits the early stages of viral replication.** (A) Schematic diagram of the time-of-addition assay. MDCK cells were infected with 100 $TCID_{50}$ influenza virus (PR8) for 1 h. Cells were treated with the two concentrations of PTex and oseltamivir over different times. (B) Calculation of cell viability with the WST method. Values are presented as means ± SD based on four different samples.

(RBC) agglutination through inhibitory effects of candidate agents on HA1. Accordingly, the HI assay was conducted to determine whether PTex inhibits adsorption of influenza virus by HA1. We observed influenza virus-induced RBC agglutination and no inhibitory effects of PTex on agglutination within a concentration range of 3.12 to 100 μg/ml (Fig. 5B), implying that PTex does not affect the HA1 region of influenza virus.

Inhibitory activity of PTex on penetration of influenza virus

The above experiments established that PTex inhibits the attachment of influenza virus to cells but not binding via HA1. Next, we investigated the effects of PTex on cell penetration by the virus. In the penetration assay, influenza virus bound the cell surface at 4°C, followed by endocytosis at 37°C in the presence of PTex. Notably, PTex exerted a significant inhibitory effect on viral penetration with an EC_{50} of ~1 μg/ml,

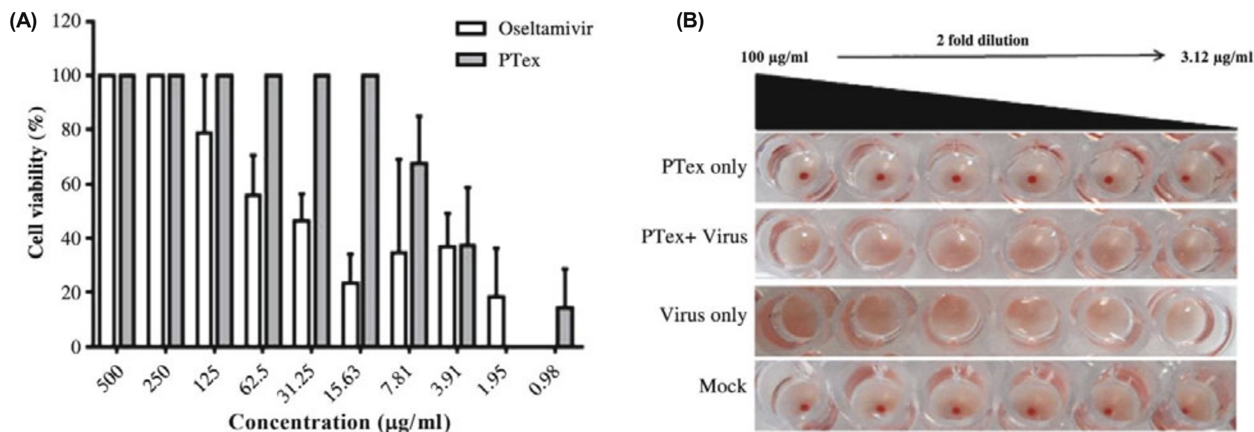

Fig. 5. ***Poncirus trifoliata*** **extract inhibits virus entry but does not suppress receptor binding of influenza virus.** (A) Attachment inhibition assay. Influenza virus (PR8) was pre-absorbed into cells treated with PTex and oseltamivir for 1 h at 4°C. Cells were washed twice and replaced with virus growth medium. After 2 days, cell viability was calculated using the WST method. Values are presented as means ± SD based on four different samples. (B) HI assay. Two-fold diluted PTex was incubated with an equivalent volume (4 HA) of influenza virus (PR8) for 40 min at room temperature. Two volumes of 1% chicken RBC were added to the mixtures and incubated for 30 min at room temperature. Viral HA-mediated RBC agglutination was monitored.

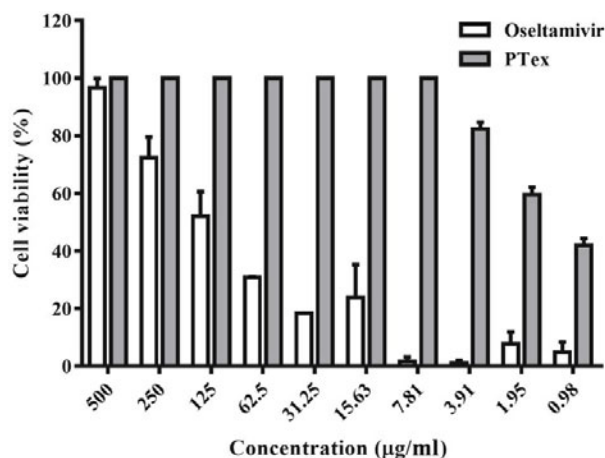

Fig. 6. ***Poncirus trifoliata* extract significantly inhibits viral penetration.** Influenza virus (PR8) was pre-absorbed on MDCK cells for 1 h at 4°C, followed by incubation with serially diluted PTex and oseltamivir at 37°C for 1 h. Unpenetrated viruses were inactivated with PBS (pH 2) for 1 min and neutralized with PBS (pH 11). After 2 days, cell viability was calculated with the WST method. Values are presented as means ± SD based on four different samples.

which was more pronounced than its effects on cell attachment. Compared with PTex, oseltamivir that inhibits the activity of NA crucial for viral release had little effect (Fig. 6). Our results suggest that PTex effectively inhibits viral penetration processes, including viral entry, M2 ion channel-mediated acidification, HA-mediated cell-cell fusion and genome uncoating.

Discussion

Influenza viruses are highly infectious and a major cause of epidemics and pandemics. Owing to the emergence of viral strains resistant to currently available treatments, the development of novel, effective therapeutic agents remains a critical medical requirement (Hurt *et al.*, 2011; Hsieh *et al.*, 2016).

Data from the current study showed that *Poncirus trifoliata* seed extract can effectively suppress influenza virus replication. In contrast to oseltamivir, PTex has the same antiviral effect in oseltamivir-resistant influenza virus as well as oseltamivir-sensitive influenza virus (Table 1). Both oseltamivir and PTex combination, which act in different stages of viral replication, showed efficacy up to concentrations as low as 1 μg, making it difficult to identify a synergistic effect (data not shown). We think that this should be clarified in future animal experiments.

PTex suppressed viral RNA transcription and protein synthesis (Figs. 2 and 3) at the early stages of the influenza virus cycle (Fig. 4). Studies on influenza virus entry showed that virion-containing endosomes were found within 5 min after adsorption, uncoating occurred between 5 and 90 min, and vRNPs accumulated between 13 and 90 min in the nucleus (Matlin *et al.*, 1981; Yoshimura *et al.*, 1982). Also according to the paper on antiviral drug against influenza virus, a time-of-addition assay has shown that efficacy at 0, 1, 2 h has an effect in the early stages (Yang *et al.*, 2013; Wang *et al.*, 2017). Therefore these results suggesting that the extract at the early stages of influenza virus replication block the virus attachment, acidification, HA-mediated cell-cell fusion, and uncoating.

PTex effectively blocked binding of the influenza virus to cells. At the early stages of virus infection, HA, a major membrane protein, is involved in adsorption to cells. HA1 is reported to play a role in binding of virus to cell surface receptors (Lin *et al.*, 2016). While PTex suppressed entry of influenza virus, as determined from the binding inhibition assay (Fig. 5A), the compound did not affect HA1 (Fig. 5B), clearly indicating that binding of virus is suppressed via a route distinct from HA receptor inhibition. Penetration inhibition assay is used to demonstrate the inhibition of penetration of various viruses such as herpes simplex virus and human immunodeficiency virus as well as influenza viruses (Lin *et al.*, 2011; Hsieh *et al.*, 2012; Liu *et al.*, 2013; Ho *et al.*, 2014). Thus, we demonstrate that PTex exerted a potent inhibitory effect against endocytosis in penetration inhibition assay (Fig. 6). It supports the theory that antiviral activity is mediated through hindering the cell penetration pathway rather than cellular binding.

Influenza viruses attach to the sialic acid receptor on the host cell surface via HA and form early endosomes, followed by acidification by M2. Due to acidification, HA is cleaved into HA1 and HA2, which promotes HA-mediated cell-cell fusion that plays an important role in uncoating. M2 inhibition and HA-mediated cell-cell fusion assays (Skehel and Wiley, 2000; Sriwilaijaroen and Suzuki, 2012; Michalek *et al.*, 2015) revealed low inhibitory effects of PTex on M2 and HA2 (data not shown). Further studies are necessary to determine the precise antiviral mechanisms of PTex.

In conclusion, the anti-influenza mechanism of action of PTex is distinct from that of oseltamivir. While oseltamivir mainly inhibits release of influenza virus by NA, PTex acts on the viral endocytosis pathway. This novel mode of antiviral activity may have crucial clinical significance. Our experiments showed anti-viral activity of PTex against both influenza virus (PR8) and Tamiflu-resistant influenza virus (NA-PR8) with non-neuraminidase inhibitory activity. In view of the emerging oseltamivir-resistant virus strains, we propose that PTex has utility as a potent antiviral agent with a wide spectrum of activity against influenza and oseltamivir-resistant viruses.

Acknowledgements

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