

Antiviral Activity of the Plant Extracts from *Thuja orientalis*, *Aster spathulifolius*, and *Pinus thunbergii* Against Influenza Virus A/PR/8/34

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Influenza viruses cause significant morbidity and mortality in humans through epidemics or pandemics. Currently, two classes of anti-influenza virus drugs, M2 ion-channel inhibitors (amantadin and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir), have been used for the treatment of the influenza virus infection. Since the resistance to these drugs has been reported, the development of a new antiviral agent is necessary. In this study, we examined the antiviral efficacy of the plant extracts against the influenza A/PR/8/34 infection. *In vitro*, the antiviral activities of the plant extracts were investigated using the cell-based screening. Three plant extracts, *Thuja orientalis*, *Aster spathulifolius*, and *Pinus thunbergii*, were shown to induce a high cell viability rate after the infection with the influenza A/PR/8/34 virus. The antiviral activity of the plant extracts also increased as a function of the concentration of the extracts and these extracts significantly reduced the visible cytopathic effect caused by virus infections. Furthermore, the treatment with *T. orientalis* was shown to have a stronger inhibitory effect than that with *A. spathulifolius* or *P. thunbergii*. These results may suggest that *T. orientalis* has anti-influenza A/PR/8/34 activity.

Key words: Plant extract, Influenza A virus, antiviral activity, cytopathic effect

Influenza viruses are negative single-stranded RNA viruses of the Orthomyxoviridae family and include influenza A, B, and C types [16]. Influenza A viruses infect birds and mammals while causing significant morbidity and mortality in humans through epidemics or pandemics [11]. It is an important human pathogen that has an impact on

the global health. Influenza epidemics cause between 250,000 and 500,000 deaths every year worldwide [23]. Currently, vaccines and antiviral drugs are crucial for controlling the outbreak of the influenza [7]. Prophylactic vaccines are the most economic and effective strategy for the inhibition of the influenza virus infection. However, commercial vaccines based on the viral surface proteins (hemagglutinin and neuramidase) do not provide effective protection against the new influenza strains owing to the antigenic drift and the antigenic shift [2, 12, 15]. The seasonal vaccine was reported to provide little or no protection against the 2009 pandemic influenza H1N1 virus infection [3, 17, 25]. Because of the time required for manufacture of new strain-matched vaccines, the therapeutic use of the antiviral drugs plays an important role in combating the influenza virus at the initial stage of virus outbreak.

To date, four antiviral drugs have been approved by the FDA for the treatment of influenza virus infection: viral M2 ion-channel inhibitors (amantadine and rimantadine) and viral neuraminidase inhibitors (oseltamivir and zanamivir) [8]. Because amantadine and rimantadine were frequently used for the treatment of influenza, the variants resistant to the M2 inhibitors have been rapidly emerging. The influenza A virus strains that are currently circulating in humans, such as the pandemic 2009 A/H1N1 and the seasonal H3N2, are resistant to both amantadine and rimantadine [1, 10]. Furthermore, these drugs are effective only against the influenza A viruses, making them unsuitable for universal influenza treatment [19].

The neuraminidase inhibitors (oseltamivir and zanamivir) have been shown to be clinically effective against the influenza virus. However, recent studies showed that the influenza A strain (H1N1) isolated from humans is resistant to oseltamivir and zanamivir [24]. These findings emphasize the need for the development of a new antiviral

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agent that has a different viral target strategy to treat the influenza infection. T-705 (favipiravir) that targets the influenza viral polymerase has shown the antiviral effect against influenza *in vitro* and *in vivo* [9, 18]. Since we cannot predict which influenza virus strain will cause the next epidemic and pandemic, it is important to develop new antiviral agents for the effective control of the influenza virus.

In this study, three thousand plant extracts were investigated for their antiviral effects against influenza virus infection using the cell-based screening system. We found three plant extracts that could inhibit the infection by the influenza A/PR/8/34 virus in the cell culture. Furthermore, to examine the effects of the three plant extracts on the replication of A/PR/8/34 virus, we analyzed the quantity of viral RNA by using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

MATERIALS AND METHODS

Preparation of Plant Extracts

The leaves of *T. orientalis* were collected at Jeonbuk, Korea, in March 2004. The whole plant of *A. spathulifolius* was collected at Gyeongsangdo, Korea in July 2008. The stem and the leaves of *P. thunbergii* were collected at Chungnam, Korea in July 2007. All of the voucher specimens including *T. orientalis* (KRIBB 024080), *P. thunbergii* (KRIBB 032007), and *A. spathulifolius* (KRIBB 034098) have been deposited at the Plant Extract Bank in KRIBB, Daejeon, Korea. The plants were extracted with MeOH at 50°C, dried for 24 h, and 20 mg of the resulting solid was dissolved in 100% DMSO solution. This DMSO/plant extract mixture was added to the culture medium such that the effective concentration of DMSO was 0.1% [13, 14]. The culture medium containing the plant extracts in DMSO was used for the antiviral study including the cell viability assay, the cytopathic effect, and the RT-PCR.

Viruses, Cells, and Reagents

Influenza A/Puerto Rico/8/34 (PR/8, H1N1) was kindly provided by Dr. Choi (Chungbuk National University, Korea). The influenza virus was grown for 2 days at 37°C in the allantoic fluid of 10-day-old fertile chicken eggs. The allantoic fluid was then harvested and used for the virus infection. Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDCK cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Oseltamivir carboxylate, the active form of oseltamivir, was provided by Chungnam National University Hospital.

Cell Viability Assay

To determine the effect of the plant extracts on the cell viability, we detected the ATP levels in the cell culture using the CellTiter-Glo Cell Viability Assay (Promega) kit and used them as an indicator of the cell viability [20]. The MDCK cells were seeded in a 96-well

culture plate (2×10^4 cells/well) and incubated at 37°C, 5% CO₂ for 24 h. The cells were then washed twice with PBS and infected with the influenza A/PR/8/34 virus at 10^6 TCID₅₀/ml. After 2 days of infection, the EMEM containing the plant extract (100 µg/ml) or oseltamivir (100 µg/ml) was added to the cell culture. After 3 days of incubation at 37°C, an equal volume of CellTiter-Glo Reagent solution was added to each well. After mixing the contents of each well for 2 min, the plate was incubated at room temperature for 10 min and the luminescence was measured with a VICTOR³ 1420 multilabel counter (Wallac, Perkin-Elmer, Boston, MA, USA).

The percentage of viable cells after the treatment with the plant extracts was calculated as follows: Percentage of viable cells = (Raw signal from the cells treated with the plant extract – signal from the non-treated cells) / (signal from the cells treated with DMSO – signal from the non-treated cells) × 100

Antiviral Activity and Cytotoxicity Test

The antiviral activity of the plant extract against the influenza virus infection was determined using the cell viability and the cytopathic effect (CPE) inhibition assay. The MDCK cells were seeded in 96-well plates. After 1 day of culture at 37°C and 5% CO₂, the cells were infected with the influenza A/PR/8/34 virus (10^6 TCID₅₀/ml). Subsequently, the virus-infected cells were treated with different plant extract concentrations (0.1–100 µg/ml), oseltamivir (0.1–100 µg/ml), or 1% DMSO in PBS. Oseltamivir was used as a positive control and DMSO was used a negative control. After 3 days of incubation, the antiviral activity was determined by the cell viability assay as described above. The plates were then examined by photometric analysis for the cytopathic effect. The cell morphology was observed under the inverted microscope at 10 × 20 magnification.

RT-PCR Analysis

The MDCK cells were seeded into a 96-well culture plate at a concentration of 2.5×10^4 cells per well. After an overnight incubation at 37°C in 5% CO₂, the medium was removed and the cells were washed with PBS. Subsequently, the cells were infected with influenza A/PR/8/34 virus (10^6 TCID₅₀/ml) in the medium containing trypsin-EDTA and treated with 100 µg/ml of the plant extract or oseltamivir. The plate was again incubated at 37°C in 5% CO₂ for 24 or 48 h, and the total RNA was extracted from the cells using a procedure described elsewhere [5]. The amount and the purity of the extracted RNA were determined by spectrophotometry. The presence of the M gene was used as an indicator of a successful replication of the influenza virus. The levels of the gene transcripts were quantified as band density of the target gene to that of the housekeeping gene, GAPDH. The primer sequences were as follows: 5'-AGTGAGCGAGGACTGCAGCGT-3' and 5'-TAGCYT TAGCYGTRGTGCTGGC-3' for the M gene, 5'-CCCATCACCATC TTCCAGGAGC-3' and 5'-CCAGTGAGCTCCCTTCAGC-3' for the GAPDH gene. The cDNA synthesis of each RNA sample and the subsequent DNA amplification were both carried out in a single tube using the gene-specific primers, 100 ng of RNA, and the Transcriptor One-Step RT-PCR Kit (Roche Diagnostics, Mannheim, Germany). The reverse transcriptase reaction was induced by incubating the sample at 50°C for 5 min, and then at 94°C for 5 min. The PCR was performed for 25 cycles at the following times and temperatures: 10 s at 94°C, 30 s at 55°C, 1 min at 68°C, and 5 min at 68°C. The RT-PCR products were visualized using electrophoresis.

Table 1. Antiviral activity of the selected plant extracts.

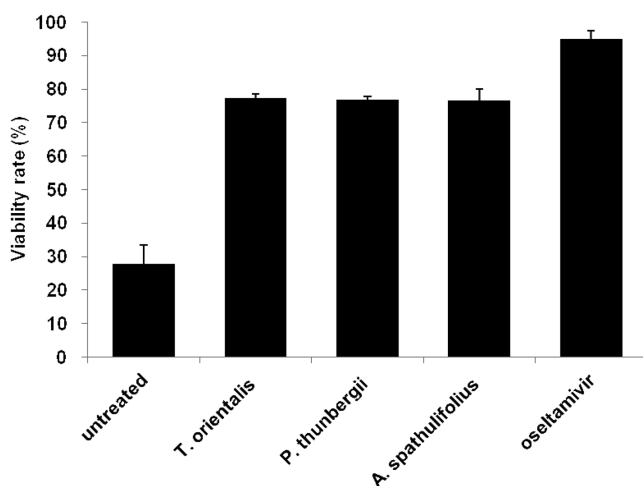
Plant	Part of plant	Antiviral effect of control (%)
		100 µg/ml
<i>Thuja orientalis</i>	Leaves	77.3 ± 3.3
<i>Pinus thunbergii</i>	Stem, leaves	76.8 ± 2.5
<i>Aster spathulifolius</i>	Whole plant	76.7 ± 4.2

^aData are presented values and standard deviation.

RESULTS AND DISCUSSION

Anti-Influenza Effects of Plant Extracts

To find the plant extracts that have antiviral effects, we performed the cell-based screening and measured ATP levels to determine the viability of the cell. MDCK cells were infected with influenza A/PR/8/34 virus with or without 100 µg/ml of plant extract. Oseltamivir was used as a positive control at the concentration of 100 µg/ml and PBS was used as a negative control. We screened 3,000 plant extracts in triplicate, which selected those inducing the cell viability that was more than 70% of the viability shown by the oseltamivir-treated MDCK cells. Among the tested extracts, three plant extracts – *Thuja orientalis*, *Aster spathulifolius*, and *Pinus thunbergii* – had the highest percentages of viable cells at 77.3%, 76.8%, and 76.7%, respectively (Table 1). On the other hand, the oseltamivir-treated cells showed 94.9% viability whereas the cells treated with PBS only had 27.8% viability (Fig. 1). The isolated plant extracts might show the antiviral activity by

**Fig. 1.** Anti-influenza effects of plant extracts.

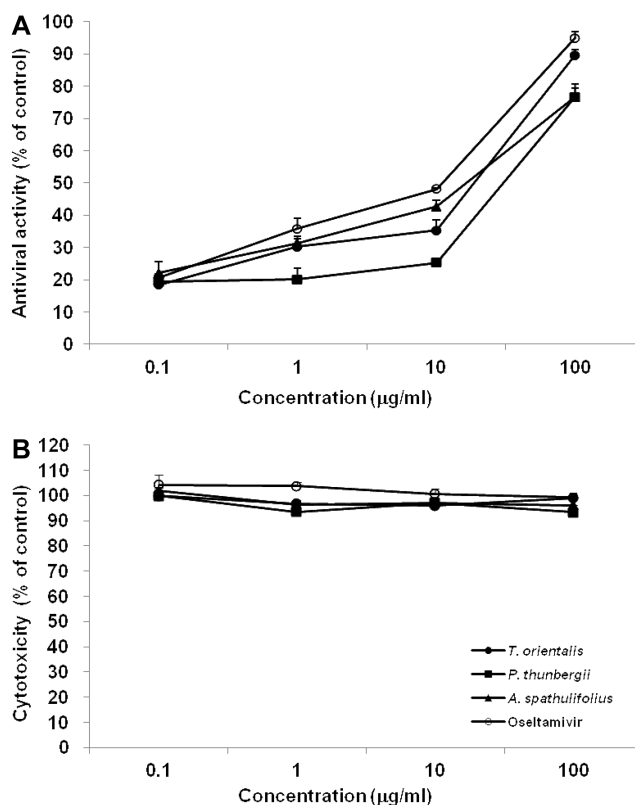
MDCK cells were seeded into 96-well plates and incubated for 24 h prior to the infection with the influenza virus. The MDCK cells were infected with the influenza A/PR/8/34 virus (10^6 TCID₅₀/ml) and then treated with the plant extract (100 µg/ml), oseltamivir (100 µg/ml), or left untreated (negative control). After incubation at 37°C in 5% CO₂ for 2 days, the cell viability was evaluated. The bars represent the means of the triplicate values ± standard deviation.

increasing the viability of the influenza virus-infected cells, but further investigations are required to make a definite conclusion.

Anti-H1N1 Influenza A Virus Activity of the Plant Extracts and Their Cytotoxicity Toward MDCK Cells

Three extracts that led to the highest cell viability were investigated for their antiviral activity by infecting the MDCK cells with the influenza virus in the presence of *Thuja orientalis*, *Aster spathulifolius*, or *Pinus thunbergii*. The antiviral activity of these extracts became higher as their concentration was increased from 0.1 to 100 µg/ml. *A. spathulifolius* and *P. thunbergii* at 100 µg/ml showed moderate antiviral activities of about 60% and 76.8% respectively, whereas *T. orientalis* at the same concentration possessed the higher activity of about 89.5% (Fig. 2A). Oseltamivir that was used as a positive control showed the antiviral activity of about 95% against the influenza A virus at the concentration of 100 µg/ml.

The cytotoxicity of the plant extracts was evaluated using the cell viability assay. Three plant extracts, *Thuja*

**Fig. 2.** Antiviral activity of *T. orientalis*, *A. spathulifolius*, and *P. thunbergii* against the influenza A/PR/8/34 (H1N1) in MDCK cells.

(A) The MDCK cells infected with the influenza and treated with increasing concentrations (0.1–100 µg/ml) of the three plant extracts for 48 h. (B) Viability analysis of the MDCK cells treated with increasing concentrations (0.1–100 µg/ml) of the three plant extracts for 48 h. The curves represent the means of the triplicate values ± standard deviation.

orientalis, *Aster spathulifolius*, and *Pinus thunbergii*, were non-toxic to the MDCK cells as the treatment with the extracts from 0.1 to 100 $\mu\text{g/ml}$ resulted in a high cell viability (about 90–100%). Oseltamivir also had no influence on the cell viability that remained at about 100% for concentrations ranging from 0.1 to 100 $\mu\text{g/ml}$ (Fig. 2B). Since 100 $\mu\text{g/ml}$ of the plants extracts suppressed the virus-induced cell death most effectively with no obvious cytotoxicity, we used this concentration for the following experiments.

Effects of *T. orientalis*, *A. spathulifolius*, and *P. thunbergii* on the PR8 Virus-Induced CPE

Influenza infection causes a cytopathic effect (CPE) that is signified by the cellular detachment as well as the cell rounding, swelling, and finally death [6, 21]. In order to evaluate the effect of the three selected extracts on the CPE of the infected cells, the CPE reduction assay was performed. The MDCK cells were first infected with the influenza virus and treated with the plant extracts at the concentration of 100 $\mu\text{g/ml}$. Using an inverted microscope,

the morphology of the uninfected cells were observed (Fig. 3A) and compared with that of the influenza virus-infected cells. Unlike the normal cells, the infected cells showed a rounded-up appearance and were detached from the plate (Fig. 3B). As shown in Fig. 3C, the oseltamivir-treated cells did not show any visible CPE formation. Similarly, the *T. orientalis* extract also prevented the virus-induced CPE very effectively (Fig. 3D), but *A. spathulifolius* and *P. thunbergii* only had a weak CPE inhibition effect. (Fig. 3E, 3F). The microscopic evaluation suggested that *T. orientalis*, *A. spathulifolius*, and *P. thunbergii* had protective effects against the PR8 virus infection at 48 hpi. Considering these results, the inhibitory activity of the plant extracts against the virus-induced cell death can be thought to be roughly correlated with the cell viability assay described earlier (Fig. 1).

Effects of *T. orientalis*, *A. spathulifolius*, and *P. thunbergii* on Viral mRNA Synthesis

The amount of the viral mRNA that is synthesized in the initial stage of the virus infection was determined from the

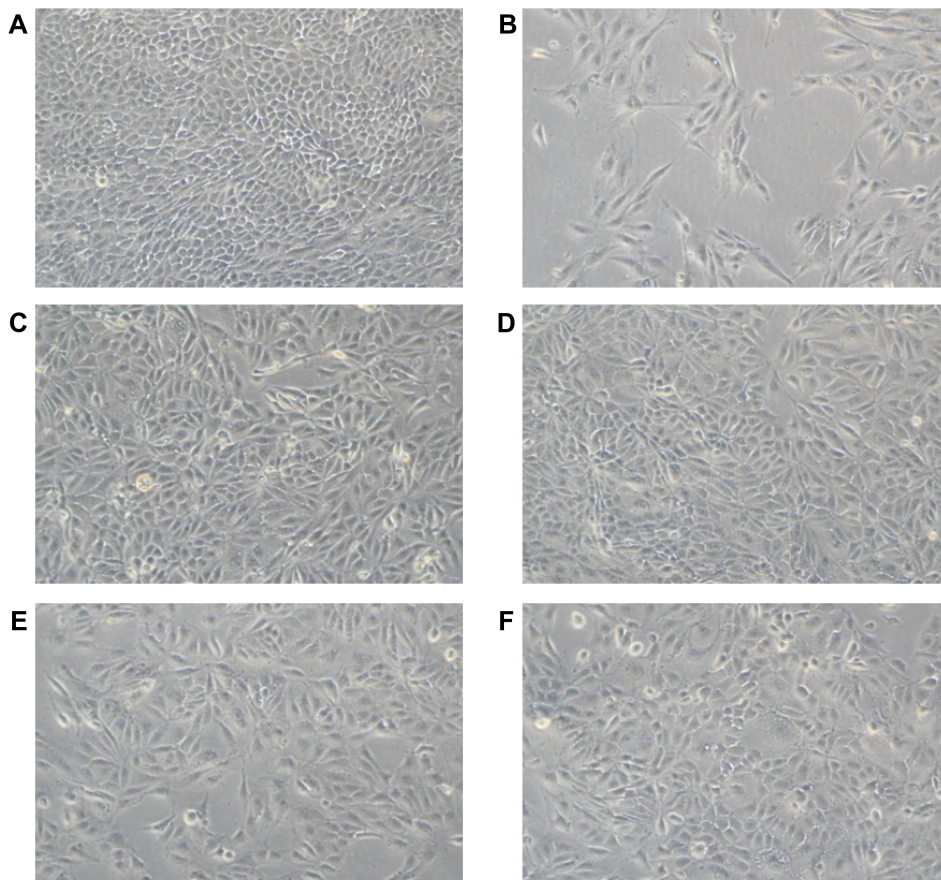


Fig. 3. The inhibition effect of *T. orientalis*, *A. spathulifolius*, and *P. thunbergii* on the influenza virus-induced CPE in the MDCK cells. The virus-infected cells were treated with oseltamivir or the plant extracts at 100 $\mu\text{g/ml}$. After incubation at 37°C in 5% CO_2 for 48 h, the morphology of cells was photographed using a microscope. (A) Uninfected cells; (B) Influenza virus-infected cells treated with PBS; (C) Influenza virus-infected cells treated with oseltamivir; (D) Influenza virus-infected cells with *T. orientalis*; (E) Influenza virus-infected cells with *P. thunbergii*; (F) Influenza virus-infected cells with *A. spathulifolius*.

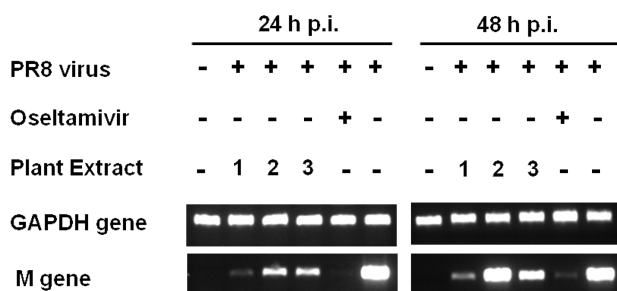


Fig. 4. Effects of *T. orientalis*, *A. spathulifolius*, and *P. thunbergii* on the viral mRNA synthesis using RT-PCR.

Replication of the virus from the MDCK cells before the infection as well as 24 and 48 h after the infection with the influenza A/PR/8/34 virus in the presence of the plant extract (100 µg/ml), oseltamivir (100 µg/ml), or vehicle alone (control, 0.1% DMSO). The gene expression levels were assessed by the standard RT-PCR. GAPDH served as a loading control. Plant extract 1: *T. orientalis*; 2: *P. thunbergii*; 3: *A. spathulifolius*.

infected cells treated with the plant extracts and the result was compared with those from the oseltamivir-treated cells. The RNA extraction was performed at 24 and 48 h after the infection with the influenza A/PR/8/34 virus. The treatment with 100 µg/ml of *T. orientalis* decreased the viral mRNA synthesis at 24 and 48 h after the infection, whereas the cells treated with oseltamivir at the same concentration also exhibited a small amount of the viral mRNA synthesis, resulting in a narrow band (Fig. 4). On the other hand, *P. thunbergii* and *A. spathulifolius* did not decrease the viral mRNA synthesis. As a positive control, GAPDH mRNA signals from the cells treated with the plant extracts had no significant difference from the cells treated with oseltamivir and the untreated cells (Fig. 4). These findings indicate that *T. orientalis* decreases the viral mRNA synthesis as effectively as oseltamivir does.

Influenza viruses are respiratory pathogens that affect humans and are responsible for substantial morbidity and mortality. The recent emergence of the viruses resistant to the NA inhibitors and the M2 ion-channel inhibitors has raised concerns about new pandemic influenza strains. It is thus crucial to develop strategies for the protection and control of the current and the next pandemic influenza. Our studies evaluated the antiviral efficacy of plant extracts selected through cell-based screening. Three extracts were found to possess potent anti-influenza virus activity in the cell cultures. Among the plant extracts, *T. orientalis* at 100 µg/ml was shown to have the highest inhibitory effect against the influenza virus. These results are similar to previous studies in which quercetin 3-rhamnoside exhibited antiviral effects against the influenza virus [4]. The influenza virus replication cycle can be divided into the following stages: entry into the host cell; release of the viral genes into the cytoplasm; replication, transcription, and translation of the viral genome; assembly and budding at the host cell plasma membrane; and release of the progeny

virus [22]. Our findings showed the inhibition of the influenza A/PR/8/34 virus infection by *T. orientalis* and suggest that the infection prevention mechanism of the plant extract might involve the blockage of the virus attachment to the host cells or the inhibition of the virus replication. However, further studies are needed to determine the exact inhibitory mechanism of the plant extract.

This study suggests that if a single component that has anti-influenza activity is identified from the *T. orientalis* extract, it might have a great potential to be used as an effective agent for the treatment of influenza virus infections. We will also conduct more studies to evaluate whether these treatments can result in the antiviral activity in animals.

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