Antiviral Activity of Zanthoxylum Species against Influenza Virus

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ABSTRACT: We aimed to investigate the antiviral activity of Zanthoxylum species against influenza virus A/WS/33, A/PR/8 and B/Lee/40 used by sulforhodamine B (SRB) assay and the action of leaves extracts of Zanthoxylum piperitum on life cycle of influenza virus A/WS/33. Among the twelve extracts, only the leaf extract of Z. piperitum exhibited strong antiviral activity at low concentration of less than 10 μg/ml with no cytotoxicity (50 μg/ml) against all of three viruses. In addition, only oseltamivir showed antiviral activity with IC_{50} of 65.3 μg/ml against influenza A/WS/33 among the viruses. Furthermore, the leaf extract of Z. piperitum suppressed infection of influenza virus A/WS/33, when added just prior (−1 hr) or after virus inoculation (0 hr). Leaf extract of Z. piperitum directly affect the infectivity of influenza virus A/WS/33 particles. Therefore, Leaf extract of Z. piperitum exhibited higher antiviral activity against three influenza viruses than that of the oseltamivir, which directly interacts with influenza A/WS/33 particles, affecting the initial stages of infection such as receptor binding and virus entry.

Key Words: Influenza virus, Zanthoxylum piperitum, Osetamivir, Cytotoxicity, Antiviral activity

INTRODUCTION

Highly pathogenic RNA viruses can be defined as those causing contagious or transmissible animal/or human diseases. Such diseases are characterized by the potential for severe and rapid transmission, irrespective of national borders, resulting in serious socio-economic and public health consequences. Influenza viruses infect humans and several animal species, causing respiratory complications with high morbidity and mortality rates (Webster et al., 1992). Two commercially available neuraminidase inhibitors, inhaled zanamivir and oral oseltamivir, have demonstrated clinical benefits in the prevention and treatment of seasonal influenza infections (Abed & Boivin, 2006; Moscona, 2006). However, their utility has been limited by side-effects and the emergence of resistant viral strains (Gubareva et al., 1998; Kiso et al., 2004; Le et al., 2005).

Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses, compounds from that are increasingly being investigated for their ability to combat viral infections, with successful cases being recorded for the treatment of Hepatitis viruses in animals and humans (Thyagarajan et al., 1988; Thyagarajan et al., 1990; Venkateswaran et al., 1987).

As many viruses are not yet curable, the development of safe, effective and inexpensive antiviral drugs are among top global health priorities. This includes the influenza virus. Many research groups in Asia, Europe and America have paid particular attention to the development of antiviral agents from traditional plant medicines (Jassim & Naji, 2003). The Zanthoxylum species has been used for centuries as source of spices in Asian cuisine and traditional Asian medicine (Bryant & Mezine, 1999; Cho et al., 2003;
Shibata et al., 1999). Species within this genus are generally characterized by the presence of compounds such as alkaloids, lignans, and coumarins, and known to have many benefits such as antibiotic and anti-inflammatory effects, antioxidative abilities, and antimalarial activities (Hisatomi et al., 2000; Matu & Staden, 2003; Talapatra et al., 1973; Weenen et al., 1990).

The root bark and stems of one species within this genus, Z. schinifolium, have been reported to exhibit antiviral activity against Hepatitis B Virus (HBV) (Chen et al., 1997). In this study, we investigated the antiviral activity of four plants from the Zanthoxylum species against influenza viruses A/WS/33, A/PR/8 and B/Lee/40 via sulforhodamine B (SRB) assay, and the action of leaf extract of Z. piperitum on the life cycle of influenza virus A/WS/33.

**MATERIALS AND METHODS**

1. Virus, Cell line and Reagents Preparations

Influenza virus A/WS/33, A/PR/8 and B/Lee/40 were kindly provided by ATCC (American Type Culture Collection, Manassas, VA, USA) and were propagated in Madin-Darby canine kidney (MDCK) cells at 37°C. MDCK cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic. Antibiotic-antimycotic, trypsin-EDTA, FBS and MEM were supplied by Gibco BRL (Grand Island, NY, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, NJ, USA). Sulforhodamine B (SRB) was purchased Sigma-Aldrich (St. Louis, MO, USA). Oseltamivir (F. Hoffmann-La Roche Ltd, Switzerland) were purchased from a pharmacy in Korea as prescribed by a medical doctor. All other chemicals were a reagent grade.

2. Plant and Sample Preparations

Four plants, Zanthoxylum piperitum, Zanthoxylum schinifolium, Zanthoxylum coreanum, and Zanthoxylum planispinum were collected from Mt. Gwanggyo (Suwon, Korea), air-dried in room temperature and pulverized. Each 100 g sample of the specimen plants was extracted twice with 600 ml of methanol at room temperature for 3 days and filtered (Whatman No. 2). The combined filtrate was concentrated to dryness by rotary evaporation at 40°C. Each extract was solubilized in 1 ml DMSO and stored at -20°C.

3. Cytotoxicity and Antiviral Assay

MDCK (Madin-Darby canine kidney) cells grown to confluence in 96-well plates were exposed to different concentrations of the antiviral compounds (three wells per compound concentration) in maintenance medium for 2 days at 37°C, in parallel with the virus-infected cell cultures. For each antiviral compound, three wells were used as controls (non-drug-treated cells). After 2 days of incubation, cytotoxicity was evaluated via SRB assay as previously described (Lin et al., 1999). The concentration of antiviral compound that reduced the viability of MDCK cells to 50% of the control was estimated as the 50% cytotoxic concentration (CC₅₀).

To calculate the CC₅₀ values, the results were transformed to percentage of controls and the CC₅₀ values were graphically obtained from the dose-response curves (Microsoft Office, 2003).

Antiviral activity against influenza viruses were determined via SRB assay, as mentioned above. Infectivity of virus stock was determined as infectivity of the virus by SRB ID₅₀ (50% infective dose). MDCK cells in 96-well tissue culture plates were used when confluent. Culture medium was removed and the cells were washed with 1 × phosphate buffered saline (PBS). Following this, 0.09 ml of the diluted virus suspension containing CCID₅₀ (50% cell culture infective dose) of the virus stock were used to produce an appropriate cytopathic effects within 2 days after infection and 0.01 ml of medium supplemented with trypsin-EDTA containing an appropriate concentration of the antiviral compound. The antiviral activity of each test material was determined with four concentrations ranging from 0.1 to 100 µg/ml. A ten-fold dilution scheme for each compound was employed. Three wells were used as virus controls (virus-infected non-drug-treated cells) while three wells were used as cell controls (non-infected non-drug-treated cells). The culture plates were incubated at 37°C in 5% CO₂ for 2 days and observed for appropriate cytopathic effects (CPE). Subsequently, the 96-well plates washed 1 times with 1 × PBS, while 100 µl of cold (-20°C) 70% acetone were added to the top of each well and left for about 30 min at -20°C. After 70% acetone was removed, 96-well plates were left to dry in an oven for 30 min. 100 µl of 0.4% (w/v) SRB in 1% acetic acid solution were added to each well and left at room temperature for 30 min. SRB were removed and the plates washed 5 times with 1% acetic acid before oven drying. 96-well plates were then left to dry in...
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1. Cytotoxicity and Antiviral Activity of Specimen Extracts against Three Influenza Viruses

We used sulforhodamine B (SRB) assay in order to determine the cytotoxicity of test plant extracts. Oseltamivir exhibited no cytotoxicity at concentration of 50 μg/ml (Table 1). Only bark extract of Z. schinifolium and root extract of Z. coreanum exhibited cytotoxicity at concentration of 50 μg/ml (Table 1).

The antiviral drug, oseltamivir was used as a positive control, while extracts were tested for their inhibitory activity against influenza virus type A/WS/33. Oseltamivir showed weak antiviral activity with IC_{50} of 65.3 μg/ml against influenza A/WS/33 (Table 1). Leaf extract of Z. piperitum, bark extract of Z. coreanum, stems, and fruit and leaf extract of Z. planispinum exhibited anti-influenza virus A/WS/33 activity with IC_{50} of 8.89, 93.6, 99.6, 8.88, 8.18 μg/ml, respectively. Most notably, leaf extract of Z. piperitum, and fruit and leaf extract of Z. planispinum exhibited strong antiviral activity at less than 10 μg/ml (Table 1). Also, leaf extract of Z. piperitum, bark extract of Zanthoxylum coreanum, and stem and leaf extract of Z. planispinum exhibited anti-influenza virus A/PR/8 activity with IC_{50} of 9.28, 96.1, 85.52, 9.02 μg/ml, respectively. Oseltamivir did exhibit antiviral activity of less than 50% at concentration of 100 μg/ml against influenza virus A/PR/8 (Table 1). Leaf extract of Z. piperitum and Z. planispinum exhibited strong antiviral activity at concentrations of less than 10 μg/ml (Table 1). Furthermore, oseltamivir did exhibit antiviral activity of less than 50% at concentration of 100 μg/ml against influenza virus B/Lee/40 (Table 1). Leaf extract of Z. piperitum, and fruit extract of Z. planispinum exhibited anti-influenza virus B/Lee/40 activity with IC_{50} of 0.44 and 6.24 μg/ml, respectively. Leaf extract of Z. piperitum and fruit extract of Z. planispinum exhibited strong antiviral activity at concentration of less than 10 μg/ml (Table 1).
Table 1. Antiviral activity of Zanthoxylum species against influenza viruses in MDCK cells.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Tissue sampled</th>
<th>CC₅₀ᵃ</th>
<th>IC₅₀ᵇ</th>
<th>TIᶜ</th>
<th>IC₅₀ᵇ</th>
<th>TIᶜ</th>
<th>CC₅₀ᵃ</th>
<th>IC₅₀ᵇ</th>
<th>TIᶜ</th>
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<tbody>
<tr>
<td>Zanthoxylum piperitum</td>
<td>Roots</td>
<td>718.0</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Hearts</td>
<td>&gt; 100</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
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<tr>
<td></td>
<td>Leaves</td>
<td>88.7</td>
<td>8.89±0.26</td>
<td>9.99</td>
<td>9.28±0.22</td>
<td>9.56</td>
<td>0.44±0.06</td>
<td>201.59</td>
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<td>Zanthoxylum schinifolium</td>
<td>Barks</td>
<td>54.9</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Leaves</td>
<td>73.6</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Zanthoxylum coreanum</td>
<td>Leaves</td>
<td>&gt; 100</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Barks</td>
<td>1391.0</td>
<td>93.6±0.44</td>
<td>14.9</td>
<td>96.1±4.13</td>
<td>14.47</td>
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<td>Roots</td>
<td>41.2</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Zanthoxylum planispinum</td>
<td>Stems</td>
<td>300</td>
<td>99.60±0.44</td>
<td>3.01</td>
<td>85.52±7.49</td>
<td>3.51</td>
<td>N.D.ᵈ</td>
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<td></td>
<td>Barks</td>
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<td>N.D.ᵈ</td>
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<td>−</td>
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<tr>
<td></td>
<td>Fruits</td>
<td>98.6</td>
<td>8.88±0.32</td>
<td>11.1</td>
<td>6.24±0.92</td>
<td>15.80</td>
<td>N.D.ᵈ</td>
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<tr>
<td></td>
<td>Leaves</td>
<td>72.1</td>
<td>8.18±1.47</td>
<td>8.82</td>
<td>9.02±0.18</td>
<td>7.88</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
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<td>Oxeitamivir</td>
<td></td>
<td>178.98</td>
<td>65.3±15.2</td>
<td>2.74</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>N.D.ᵈ</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

ᵃ The 50% cytotoxic concentration for target cells (MDCK cells) in µg/ml.
ᵇ Concentration of compound in µg/ml producing 50% inhibition of virus-induced cytopathic effects.
ᶜ Therapeutic index (TI = CC₅₀/ IC₅₀).
ᵈ IC₅₀ value within concentration of compound to test not calculated because maximum inhibition rate was arrived under 50%.

Fig. 1. The effects Zanthoxylum piperitum leaves extract on the infectivity of influenza virus A/WS particles. Influenza virus A/WS particles were incubated with 20 µg/ml of leaf extract at 4°C for 1 hr. Subsequently Vero cells were incubated with treated or untreated virus extracts at 4°C for 1 hr. Unbound virus was removed by extensive washing and infection was continued by cultivating cells in infection medium without leaf extract at 37°C. Antiviral activity was determined by titration using SRB assays 2 days post-infection.

Fig. 2. The time course of Zanthoxylum piperitum leaves extract addition on influenza virus A/WS replication in Vero cells. Ten µg/ml of each compound was added at either before (−1 hr), during (0 hr), or after (1, 2, 4, 6, 8, 12 and 24 hrs) virus infection. After 2 days, inhibition was evaluated by SRB method and expressed as the inhibition rate. Each value is the result of mean ± S.D. of three independent experiments.

2. Analysis of influenza virus A/WS/33 infectivity with Z. piperitum Leaves Extract

Analysis was performed to ascertain whether leaf extract of Z. piperitum interact with the viral particle. Antiviral activity of pre-incubation with leaf extract of Z. piperitum resulted in 94%. Similarly, continuous presence of the extract during infection led to 93% antiviral activity (Fig. 1).

3. Study of Time Course

To study the inhibitory effect of Z. piperitum leaf extract, which possess a broad spectrum of antiviral activities on the stages of virus infection, the compounds were added at different periods (before, during, and after) of influenza virus A/WS/33 infection. Results showed that the leaf extract suppressed influenza virus A/WS/33 infection, when
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added just prior (−1 hr) or after the virus inoculation (0 hr). The inhibitory rate was higher than 60% (Fig. 2). However, the inhibitory rates declined to 15% or less when added at post (1, 2, 4, 6, 8, 12 and 24 hrs) infection (Fig. 2).

**DISCUSSION**

Influenza viruses are classified as types A, B, and C. The gradual antigenic drift of influenza virus types A and B, coupled with occasional antigenic shifts makes the control of infections problematic (Shaw et al., 1992). Only two classes of anti-influenza virus drugs are currently accessible. These are viral neuraminidase inhibitors (oseltamivir and zanamivir) and viral M₂ ion channel protein inhibitors (amantadine, rimantadine) (De Clercq, 2004). The viral M₂ ion channel protein inhibitors and neuraminidase inhibitors are not fully effective due to the frequent emergence of drug-resistant viral variants and their lack of activity against the influenza B virus (Betakova et al., 1996; Gubareva et al., 2001; Thompson et al., 2004; Silver, 2003). As a result, there is a urgent need for broad-spectrum antiviral drugs that exert inhibitory effects against currently active human viruses.

The objective of this study was to determine whether methanol extracts of different plant tissues would function as an antiviral agent against influenza A or B virus in vitro. Subsequently, we also attempted to elucidate the ability of *Z. piperitum* leaf extract on influenza virus multiplication. Oseltamivir, used for treatment of flu, exhibited weak antiviral activity against influenza virus A/WS/33 and no activity against influenza virus A/PR/8 and B/Lee/40. Among the specimen plant extract, only leaf extract of *Z. piperitum* exhibited antiviral activity against influenza viruses A/WS/33, A/PR/8 and B/Lee/40, higher than that of oseltamivir. Our results showed that leaf extract of *Z. piperitum* inhibit the growth of influenza strains A/WS/33, A/PR/8 and B/Lee-40 with differences in inhibitory efficacy among the strains.

To elucidate the ability of *Z. piperitum* leaf extract on influenza virus A/WS multiplication, we closely examined actions during the infection cycle. We demonstrated that leaf extract affected the early stages of viral infection cycle, such as receptor binding, virus entry, and internalization. Based on these result, it can be concluded that compounds in the leaf extract of *Z. piperitum* do interact with the particle of influenza virus A/WS/33, as pre-exposure did alter the infectivity of the influenza virus (Fig. 1). Based on the results of the time-of-addition, pre-incubation of the MDCK cells with *Z. piperitum* leaf extract followed by washing did protect the cells from A/WS/33 infection (Fig. 2). Furthermore, the extract can only inhibit A/WS infection when added on, pretreatment (−1 hr), and during virus inoculation (0 hr), but not after 1 hr or later (Fig. 2). This suggests that the mode of action is derive from inhibiting the absorption of virus (Fig. 1 and Fig. 2).

**CONCLUSION**

We aimed to investigate the antiviral activity of *Zanthoxylum* species against influenza virus A/WS/33, A/PR/8 and B/Lee/40 used by SRB assay and only the leaf extract of *Z. piperitum* exhibited higher antiviral activity against three influenza viruses than that of the oseltamivir, which directly interacts with influenza A/WS/33 particles, affecting the initial stages of infection such as receptor binding and virus entry.

**LITERATURE CITED**


Gubareva LV, Webster RG, Hayden FG (2001) Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase


