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Antiviral Effect of Flavonol Glycosides Isolated from the Leaf of *Zanthoxylum piperitum* on Influenza Virus

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The ethanol extract of *Zanthoxylum piperitum* (L.) DC. showed *in vitro* antiviral activity against influenza A virus. Three flavonol glycosides were isolated from the EtOAc fraction of *Z. piperitum* leaf by means of activity-guided chromatographic separation. Structures of isolated compounds were identified as quercetin 3-O-β-D-galactopyranoside (1), quercetin 3-O-α-L-rhamnopyranoside (2), kaempferol 3-O-α-L-rhamnopyranoside (3) by comparing their spectral data with literature values. The anti-influenza viral activity of isolates was evaluated using a plaque reduction assay against influenza A/NWS/33 (H1N1) virus. The compounds also were subjected to neuraminidase inhibition assay in influenza A/NWS/33 virus. Compounds 1-3 exhibited antiviral activity against an influenza A virus *in vitro*, and inhibited the neuraminidase activity at relatively high concentrations.

Keywords: *Zanthoxylum piperitum*, Rutaceae, flavonol glycoside, anti-influenza activity, neuraminidase inhibitor

Introduction

Influenza (flu) is an acute respiratory infectious disease caused by influenza viruses. These viruses have a negative single-stranded RNA with eight gene segments, and their subtypes are determined by the antigenicity of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Ge *et al.*, 2010). Influenza neuraminidase (NA) has been established as a primary drug target for the prophylaxis and treatment of influenza infections (Grienke *et al.*, 2012). In general, vaccination is the preferred method of preventing influenza, but antiviral drugs are also considered significant for both the prevention and treatment of influenza, especially in case of unsuccessful vaccination (Schmitz *et al.*, 2007). Although vaccination against influenza virus has been and remains the most attractive way to prevent the infection, its protection is often incomplete due to insufficient vaccine coverage by genetic re-assortment of influenza virus (Lee *et al.*, 2012). The antiviral drugs for influenza have also been limited by the emergence of drug-resistant viral strains and their side-effects (Lee *et al.*, 2012). Thus, the developing novel drugs with high efficacy and low toxicity or alternative control means is still needed to prevent and cure influenza.

Natural products and their derivatives have been historically valuable as a source of therapeutic agents. Natural products provide an abundant chemical diversity that represents a rich source for lead structures in drug discovery. Since the past century, natural products and various compounds isolated from natural sources have been extensively studied for their anti-influenza activity (Wang *et al.*, 2006; Hudson, 2009; Ge *et al.*, 2010; Grienke *et al.*, 2012; Kwak *et al.*, 2013; Lee *et al.*, 2013).

Zanthoxylum piperitum (L.) DC., a deciduous shrub in the family Rutaceae, is mostly distributed in Korea and Japan. The dried pericarp of ripe fruit of *Z. piperitum* is a well-known traditional oriental medicine used as gastrointestinal agents, anthelminthics, detoxicant and diuretic (Mitsuhashi, 1988; Lee, 1996; Jeong and Shim, 2004). Fresh leaves and dried fruits of *Z. piperitum* are also used as a spice to impart a fresh flavor or to suppress any unpleasant fishy and meaty odor in Asian cuisine (Jiang and Kubota, 2001).

Previous phytochemical studies on *Z. piperitum* revealed the presence of two serotonin derivatives (Yanase *et al.*, 2010), several aliphatic acid amides (Hatano *et al.*, 2004; Sugai *et al.*, 2005; Jang *et al.*, 2008), favonoids and polyphenolic compounds (Cho *et al.*, 2003; Kusuda *et al.*, 2006), a glycoprotein (Park *et al.*, 2009), nine glycosides (Jiang *et al.*, 2001), and volatile components (Jiang and Kubota, 2001; Jiang and Kubota, 2004). The leaf and fruit extracts of *Z. piperitum*, and their components are known to have various biological activities such as antioxidation (Cho *et al.*, 2003; Yamazaki *et al.*, 2007), anti-inflammation (Lee *et al.*, 2009; Lee and Lim, 2009; Park *et al.*, 2009), cancer chemoprevention (Kim *et al.*, 1998), vasorelaxation (Li *et al.*, 2010), human acyl-CoA:cholesterol acyltransferase inhibition (Park *et al.*, 2007), anti-influenza viral activity (Choi *et al.*, 2008), antibacterial effect (Hatano *et al.*, 2005; Kusuda *et al.*, 2006), modulation of gastrointestinal tract (Hashimoto *et al.*, 2001), tyrosinase inhibition (Jeong and Shim, 2004), neuro-protective effect (Jeong *et al.*, 2011), and hapatoprotective effect (Lee and Lim, 2008).

During our search for anti-influenza viral compounds from

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natural products, we found that the 95% ethanol extract of *Z. piperitum* leaf possess antiviral activity against influenza A virus. The extract was subjected to bioassay-guided fractionation and isolation. Three anti-influenza viral compounds were isolated from the ethyl acetate fraction of *Z. piperitum*. This paper deals with the isolation, structure determination, *in vitro* anti-influenza viral activity, and neuraminidase inhibitory effect of isolated compounds from the leaf of *Z. piperitum*.

Materials and Methods

General procedure

Melting points were measured on a Gallenkamp melting point apparatus (uncorr.). NMR experiments were performed on Varian Unity INOVA 500 spectrometer with the usual pulse sequences. ESI-MS spectra were obtained on Agilent 1100LC/MSD trap classic. UV spectra were measured on Agilent 8453 spectrophotometer. Column chromatography was carried out on Si gel 60 (230–400 mesh; Merck, Germany), LiChroprep RP-18 (40–63 μm, Merck) and Sephadex LH-20 (25–100 μ, Sigma-Aldrich, USA). TLC was performed on pre-coated silica gel 60 F_{254} plates (Merck) and RP-18 F_{254S} plates (Merck).

Plant material

Leaves of *Zanthoxylum piperitum* (L.) DC. were collected at Jeju, Korea, in August 2011. A voucher specimen is deposited in the School of Pharmacy, Sungkyunkwan University (SKKU-Ph-11-033).

Extraction and isolation

The dried and powdered leaves of *Z. piperitum* (500 g) were extracted with 95% aqueous ethanol (EtOH), three times at room temperature. The combined extracts were evaporated to dryness under reduced pressure, and the residue was suspended in H_2O. The resulting solution was consecutively partitioned with organic solvents to give hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and water fractions. The EtOAc fraction (9.8 g), which showed antiviral activity against influenza A virus, was subjected to column chromatographic separation. The EtOAc soluble was fractionated on a Sephadex LH-20 column with MeOH as eluent to give six fractions (E-1 to E-6). Subfraction E-4 was rechromatographed over a silica gel column using a stepwise elution with EtOAc-MeOH-H_2O (100:5:3, 100:10:7, and 100:13:10) to afford four further subfractions (E-4A to E-4D). Compounds **1** (23.7 mg), **2** (215.1 mg), and **3** (9.8 mg) were obtained by RP-C_{18} column chromatography (55% MeOH-H_2O) from subfraction E-4B. Furthermore, compounds **1** and **3** were afforded from subfractions E-4C and E-4A, respectively.

Quercetin 3-O-β-D-galactopyranoside (**1**): Yellow amorphous powder; mp: 234–235°C; ESI-MS (positive) *m/z* : 487 $[M+Na]^+$; ESI-MS (negative) *m/z* : 463 $[M-H]^-$; UV λ_{max} (MeOH) nm: 257, 267sh, 303sh, 358; ^{1}H-NMR (CD_3OD, 500 MHz) *δ*: 3.45-3.90 (6H, m, Gal-H), 5.16 (1H, d, *J*=8.0 Hz, H-1"), 6.21 (1H, d, *J*=2.5 Hz, H-6), 6.40 (1H, d, *J*=2.5 Hz, H-8), 6.86 (1H, d, *J*=8.5 Hz, H-5'), 7,59 (1H, dd, *J*=8.5, 2.5 Hz, H-6'), 7.83 (1H, d, *J*=2.5 Hz, H-2'); ^{13}C-NMR (CD_3OD, 125 MHz) *δ*: 60.8 (C-6"), 68.9 (C-4"), 72.0 (C-2"), 73.9 (C-3"), 76.0 (C-5"), 93.5 (C-8), 98.7 (C-6), 104.2 (C-1"), 104.4 (C-10), 114.9 (C-2'), 116.6 (C-5'), 121.7 (C-1'), 121.8 (C-6'), 134.6 (C-3), 144.6 (C-3'), 148.8 (C-4'), 157.3 (C-2), 157.6 (C-9), 161.9 (C-5), 165.0 (C-7), 178.4 (C-4).

Quercetin 3-O-α-L-rhamnopyranoside (**2**): Yellow amorphous powder; mp: 184–185°C; ESI-MS (positive) *m/z* : 471 $[M+Na]^+$; ESI-MS (negative) *m/z* : 447 $[M-H]^-$; UV λ_{max} (MeOH) nm: 256, 266sh, 298sh, 346; ^{1}H-NMR (CD_3OD, 500 MHz) *δ*: 0.94 (3H, d, *J*=6. 5 Hz, H-6"), 3.35-4.25 (4H, m, Rha-H), 5.35 (1H, d, *J*=2.0 Hz, H-1"), 6.20 (1H, d, *J*=2.0 Hz, H-6), 6.37 (1H, d, *J*=2.0 Hz, H-8), 6.91 (2H, d, *J*=8.0 Hz, H-5'), 7.30 (2H, d, *J*=8.0 Hz, H-6'), 7.34 (1H, d, *J*=2.0 Hz, H-2'); ^{13}C-NMR (CD_3OD, 125 MHz) *δ*: 16.5 (C-6"), 70.7 (C-5"), 70.9 (C-2"), 71.0 (C-3"), 72.1 (C-4"), 93.5 (C-8), 98.6 (C-6), 102.4 (C-1"), 104.7 (C-10), 115.2 (C-2'), 115.8 (C-5'), 121.7 (C-1'), 121.8 (C-6'), 135.1 (C-3), 145.2 (C-3'), 148.6 (C-4'), 157.4 (C-2), 158.6 (C-9), 162.0 (C-5), 164.7 (C-7), 178.4 (C-4).

Kaempferol 3-O-α-L-rhamnopyranoside (**3**): Yellow amorphous powder; mp: 135–176°C; ESI-MS (positive) *m/z* : 415 $[M+Na]^+$; ESI-MS (negative) *m/z* : 431 $[M-H]^-$; UV λ_{max} (MeOH) nm: 265, 299sh, 339; ^{1}H-NMR (CD_3OD, 500 MHz) *δ*: 0.93 (3H, d, *J*=6.5 Hz, H-6"), 3.45–4.25 (4H, m, Rha-H), 5.38 (1H, d, *J*=1.5 Hz, H-1"), 6.20 (1H, d, *J*=2.0 Hz, H-6), 6.38 (1H, d, *J*=2.0 Hz, H-8), 6.93 (2H, d, *J*=8.5 Hz, H-3', 5'), 7.76 (2H, d, *J*=8.5 Hz, H-2', 6'); ^{13}C-NMR (CD_3OD, 125 MHz) *δ*: 16.5 (C-6"), 70.7 (C-5"), 70.9 (C-2"), 71.0 (C-3"), 72.0 (C-4"), 93.6 (C-8), 98.7 (C-6), 102.3 (C-1"), 104.8 (C-10), 115.4 (C-3', 5'), 121.5 (C-1'), 130.7 (C-2', 6'), 135.1 (C-3), 157.4 (C-2), 158.1 (C-9'), 160.4 (C-4'), 162.1 (C-5), 164.7 (C-7), 178.5 (C-4).

Virus and cell

Human influenza virus A/NWS/33 (H1N1) was propagated in 9- to 11-d-old embryonated chicken eggs for 48 h in an incubator at 37°C. Madin-Darby canine kidney (MDCK) cells were purchased from the American Type Culture Collection (USA) and cultured as monolayers in minimum essential media supplemented with 8% heat-inactivated fetal bovine serum.

Plaque reduction assay

For *in vitro* anti-influenza efficacy of isolated compounds, plaque reduction assay was performed as described previously (Hayden *et al.*, 1980; Hayashi *et al.*, 2010; Shin *et al.*, 2010). Briefly, MDCK cell monolayers in 6-well plates were infected with influenza virus A/NWS/33, and incubated for 40 min. After incubation, the viruses were removed, and infected cells were incubated with 3 ml of medium containing 1% agarose and compounds at different concentrations (250, 125, 62.5, and 31.25 μg/ml) for 48 h at 37°C in 5% CO_2. After 48 h, the monolayers were stained with 1% crystal violet solution, and then plaques were counted. To determine cytotoxicity of compounds in MDCK cells, cell viability assay was performed in 96-well plates with confluent MDCK monolayers. Cells were treated with compounds for 48 h at 37°C in 5% CO_2. After 48 h, each well was stained with 0.034% neutral

red dye for 2 h at 37°C, and then the wells were rinsed, dried, and stored in the dark. Dye was extracted with absolute ethanol-Sorenson citrate buffer (1:1) for 30 min in the dark. The absorbance was measured at 540 nm with a microplate reader (Sunrise; Tecan Trading AG, Austria).

Neuraminidase inhibition assay

A neuraminidase inhibition assay was applied to test the effect of isolated compounds on the neuraminidase activity of influenza virus A/NWS/33, as described previously (Shin *et al.*, 2010; Lee *et al.*, 2012). Briefly, 2-fold dilutions (25 µl) of compounds ranging from 7.8125 to 1,000 µg/ml in reaction buffer (150 mM sodium acetate buffer, pH 7 and 1 mM calcium chloride) were mixed with equal volumes (25 µl) of influenza A/NWS/33 virus solution. After 1 h incubation at room temperature, equal volumes (50 µl) of the substrate solution [4-MU-NANA; 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium; Sigma] were added at a final concentration of 80 µM, and the mixtures were further incubated for 2 h at 37°C in the dark. The fluorescence density was then measured by fluorescence plate reader (Gemini EM; Molecular Devices, USA) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Standard curves were made by plotting the percentage of fluorescence inhibition relative to the activity of virus controls against the logs of compound concentrations. The 50% inhibitory concentration (IC_{50}) was calculated by regression analysis with r2 ≥ 0.9, representing the mean values of 3 independent experiments.

Results

Isolation and structure determination of compounds

The 95% ethanol extract of leaves of *Zanthoxylum piperitum* (L.) DC. was consecutively partitioned with hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. The ethyl acetate fraction, which showed anti-influenza activity, was fractionated by a Sephadex LH-20 column chromatography. Selected fractions were rechromatographed on silica gel, RP-C_{18} columns using different solvent combinations to give compounds **1**-**3**. Compounds **1**-**3** were obtained as yellow amorphous powder. The UV and NMR spectra exhibited absorption maxima and signals, characteristic for flavonol glycoside. The structures of isolated compounds were identified as quercetin 3-O-β-D-galactopyranoside (**1**), quercetin 3-O-α-L-rhamnopyranoside (**2**), kaempferol 3-O-α-L-rhamnopyranoside (**3**) by comparing their spectral data with literature values (Bennini *et al.*, 1992; Mok and Lee, 2013) (Fig. 1).

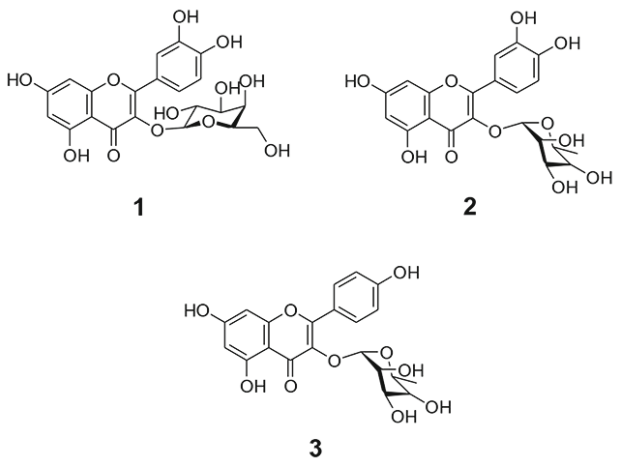

Fig. 1. Structure of compounds 1-3 from the leaf of *Z. piperitum*.

Table 1. *In vitro* antiviral activity of compounds 1-3 against influenza A/NWS/33 (H1N1) virus by using plaque reduction assay

Compound	PFU[a] /ml			
	250 µg/ml	125 µg/ml	62.5 µg/ml	31.25 µg/ml
1	1.25×10^7	2.05×10^7	3.25×10^7	4.00×10^7
2	1.55×10^7	3.15×10^7	4.65×10^7	4.75×10^7
3	N.T.[b]	5.00×10^6	1.25×10^7	3.00×10^7
Control		4.75×10^7		

[a] PFU is a plaque-forming unit
[b] N.T. is not tested, because of its cytotoxicity.
1, quercetin 3-O-β-D-galactopyranoside; **2**, quercetin 3-O-α-L-rhamnopyranoside; **3**, kaempferol 3-O-α-L-rhamnopyranoside; control: tested without compounds

Antiviral activity of isolated compounds on influenza virus *in vitro*

The anti-influenza viral activity of the isolated compounds was evaluated using a plaque reduction assay against influenza A/NWS/33 (H1N1) virus. Compounds were subjected to plaque reduction assay at different concentrations (250, 125, 62.5, and 31.25 µg/ml). Maximum concentration of compounds for plaque reduction assay was determined by their cytotoxicity in MDCK cells. As shown in Table 1, three compounds exhibited antiviral effect, in a dose-dependent

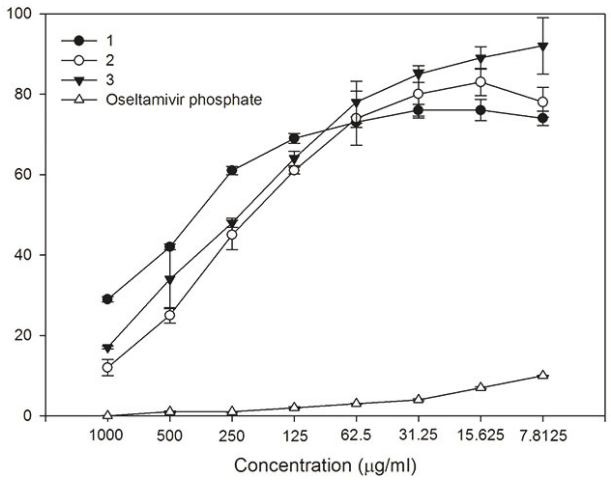

Fig. 2. Inhibitory effect of compounds 1-3 on the viral neuraminidase activity. The solutions of compounds (25 µl) in serial two-fold dilutions were mixed with equal volumes of influenza A/NWS/33 virus solution (25 µl). The substrate solution (4-MU-NANA; 50 µl) was added, and incubated for 2 h at 37°C. Neuraminidase activity was determined by fluorescence density. Each point represents the mean ± SEM for three independent experiments.

manner, on influenza A/NWS/33 virus. Among the isolated compounds, compound **3** revealed the strongest antiviral activity *in vitro*.

Inhibitory effect of isolated compounds on viral neuraminidase activity

A neuraminidase inhibition assay was employed to test the effect of isolated compounds on the neuraminidase activity of influenza A/NWS/33 virus. As shown in Fig. 2, the activity of viral neuraminidase was decreased by the compounds in a dose-dependent manner. The compounds showed neuraminidase inhibitory activity at relatively high concentrations. For compounds **1**-**3**, reduction by half in enzyme activity (IC_{50}) was observed at 434, 211, and 273 μg/ml, respectively, which are exceedingly higher than that of oseltamivir phosphate.

Discussion

Control of influenza virus infection is a significant public health concern. Although chemically synthesized antiviral drugs such as amantadine, oseltamivir, zanamivir, and peramivir have been useful for the prevention and treatment of influenza, their application has been restricted by the emergence of drug-resistant viral strains and their side-effects. Thus, the developing new drugs or alternative control means is still needed to prevent and cure influenza. Many plant extracts and isolated compounds from natural products have been reported to have anti-influenza viral activities (Wang *et al*., 2006; Hudson, 2009; Ge *et al*., 2010; Grienke *et al*., 2012).

Zanthoxylum piperitum have been reported to possess various biological activities and phytochemical constituents. Even though leaf extract of *Z. piperitum* has previously been shown to have anti-influenza acivity against influenza A/WS/33, A/PR/8, and B/Lee/40 viruses, its active constituents have not yet been identified (Choi *et al*., 2008).

In continuation of our search for anti-influenza viral compounds from natural products, we have found that the 95% ethanol extract of *Z. piperitum* leaf, showed antiviral activity against influenza A virus. The extract was subjected to bioassay-guided fractionation and isolation. Three anti-influenza viral compounds were isolated from the ethyl acetate fraction of *Z. piperitum*, and their structures were identified as quercetin 3-O-β-D-galactopyranoside, quercetin 3-O-α-L-rhamnopyranoside, kaempferol 3-O-α-L-rhamnopyranoside by comparing their spectral data with literature values. The anti-influenza viral activity of the isolates was evaluated using a plaque reduction assay against influenza A/NWS/33 (H1N1) virus. Three isolated compounds exhibited antiviral activity against an influenza A virus *in vitro*. The compounds were also subjected to inhibitory activity against neuraminidase, an enzyme perceived to play a key role for the release of newly made virus particle from infected cells by cleavage of sialic acid moieties on target cell receptor. The effect of compounds **1**-**3** was evaluated using neuraminidase inhibition assay in influenza A/NWS/33 virus. The activity of neuraminidase decreased significantly by compounds **1**-**3**; however, half reductions of enzymatic activity were shown at relatively high concentrations. The inhibitory activity data of oseltamivir phosphate was included as a positive control for comparison. The neuraminidase inhibitory activity for isolated compounds is exceedingly weaker than that of oseltamivir phosphate, a well-known neuraminidase-specific inhibitor in clinical use. In conclusion, although isolated compounds were not shown potent anti-influenza activity, this study has suggested that flavonol glycosides from *Z. piperitum* may be considered as neuraminidase inhibitors and antiviral agents against influenza A virus.

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