Characterization of Neuraminidase Inhibitors in Korean *Papaver rhoeas* Bee Pollen Contributing to Anti-Influenza Activities *In Vitro*

### Authors

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### Key words

- *Papaver rhoeas*
- Papaveraceae
- bee pollen
- *Apis mellifera*
- neuraminidase inhibitors
- anti-influenza activity
- flavonoid glycosides

### Abstract

The active constituents of Korean *Papaver rhoeas* bee pollen conferring neuraminidase inhibitory activities (H1N1, H3N2, and H5N1) were investigated. Six flavonoids and one alkaloid were isolated and characterized by nuclear magnetic resonance and mass spectrometry data. These included kaempferol-3-sophoroside (1), kaempferol-3-neohesperidoside (2), kaempferol-3-sambubioside (3), kaempferol-3-glucoside (4), quercetin-3-sophoroside (5), luteolin (6), and chelianthinfoline (7). All compounds showed neuraminidase inhibitory activities with IC₅₀ values ranging from 10.7 to 151.1 µM. The most potent neuraminidase inhibitor was luteolin, which was the dominant content in the ethyl acetate fraction. All tested compounds displayed noncompetitive inhibition of H3N2 neuraminidase. Furthermore, compounds 1–7 all reduced the severity of virally induced cytopathic effects as determined by the Madin-Darby canine kidney cell-based assay showing antiviral activity with IC₅₀ values ranging from 10.7 to 33.4 µM (zanamivir: 58.3 µM). The active compounds were quantified by high-performance liquid chromatography, and the total amount of compounds 1–7 made up about 0.592 g/100 g bee pollen, contributing a rich resource of a natural antiviral product.

### Abbreviations

- CPE: cytopathic effect
- EDTA: ethylenediaminetetraacetic acid
- HPLC: high-performance liquid chromatography
- HA: hemagglutinin
- MDCK: Madin-Darby canine kidney
- MS: mass
- NA: neuraminidase
- NAI: neuraminidase inhibitors
- NMR: nuclear magnetic resonance
- ODS: octadecyl silica
- SRB: Sulforhodamine B
- TFA: trifluoroacetic acid
- TI: therapeutic index

### Introduction

Bee pollen is a granule-type material of agglutinated flower pollen made by worker honey bees with nectar and bee secretions. The resulting pollen is higher in nutritional value than untreated pollen, and is used as the primary source of protein for the bee hive. Bee pollen has been used in folk medicine from ancient times in many regions of the world for its medical properties to alleviate or cure conditions such as colds, flu, ulcers, and anemia [1]. Recently, biological effects such as antioxi-

- In-Kyoung Lee and Byung Soon Hwang contributed equally to this work.

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Results and Discussion

Influenza NA has been established as a primary drug target for the treatment of influenza infections. Cell culture-based assays need to be performed to evaluate the cytotoxicity and to confirm the anti-influenza activity of potential NAIs. Therefore, we first need to be performed to evaluate the cytotoxicity and to confirm the treatment of influenza infections. Cell culture-based assays.

Results and Discussion

Influenza viruses contain two surface antigens, NA and HA. NA and HA both recognize carbohydrate structures and bind to terminal sialic acid units on the surface of the host cell. When the influenza virus replicates, the binding of HA to its receptor initiates viral entry into the host cell, where NA catalyzes the cleavage of sialic acid. Since NA plays an important role in replication, spread, and pathogenesis during infection, blocking the function of NA is considered to be one of the most promising areas of targeting for the treatment of influenza. In the past decade, many reports have investigated substances related to NA inhibition. It was previously reported that flavonoids and oligo stilbenes are the most prominent natural products with NA inhibitory activity [10]. Therefore, pollen is considered one of the natural sources of NA inhibitors due to its high levels of polyphenols.

Papaver rhoeas L. is a species of flowering plant in the poppy family, Papaveraceae. It is sometimes mistaken for Papaver somniferum L., the opium poppy. It was reported that the flavonoids kaempferol, quercetin, luteolin, 8-hydroxyluteolin (hypolaetin), quercetin-3-glucoside (isoquercitrin), kaempferol-3-glucoside (astragalline), and quercetin-3-galactoside (hyperoside) were isolated from the methanol extract of the petals of Papaver rhoeas [11].

Although pollen has traditionally been used to treat the cold and flu, there are no reports of the antiviral activities of P. rhoeas bee pollen. In the present study, we investigated the active agents of Korean P. rhoeas bee pollen for inhibitory activity on NA from influenza virus A H1N1, H3N2, and H5N1, with zanamivir as a positive control. All compounds displayed inhibition of NAs in a dose-dependent manner. The IC50 values of compounds 1–7 were evaluated on the NAs from influenza H1N1, H3N2, and H5N1, with zanamivir as a positive control. All compounds displayed inhibition of NAs in a dose-dependent manner. The IC50 values of compounds 1–7 were evaluated on the NAs from influenza H1N1, H3N2, and H5N1, ranging from 10.7 to 100.5, 25.6 to 143.2, and 12.6 to 151.1 µM, respectively (Table 1). Compound 6, luteolin, exhibited the highest activity against the NAs of all three types. Comparison of the flavonoid glycosides (1–5) with the aglycone (6) indicated that the bulky sugar moiety in compounds 1–5 caused a loss of activity. However, compound 7 (chelianthifoline), an alkaloid, exhibited weak activity.

The manner of inhibition by the compounds was determined to evaluate the relative affinity for NA from the H3N2 influenza virus. To study the mode of inhibition, Dixon plots were used. As shown in Fig. 2, all compounds tested (compounds 1–4, 6, and 7) displayed noncompetitive inhibitory activity, because the Vmax decreased while the Km remained the same with increasing concentrations of inhibitors, which intersected at a non-zero point on the x axis (K). The K values for these compounds were similar to their IC50 values (Table 1). To date, all flavonoids tested were reported to act noncompetitively. Recently, it was reported that essential functional groups of flavonoids to confer good NA inhibitory effects are hydroxyl groups in positions 4′ and 7, an o xo
group in position 4, and a double bond between positions 2 and
3, showing agreement with our results [4].

The antiviral activities of compounds 1–7 against the influenza A
virus were determined using the CPE reduction assay in MDCK
cells to evaluate the cytotoxicity and to confirm the anti-influen-
za activity (Table 1). The IC50 values of these compounds were
10.7–33.4 µM, and the TI values were 2.99–9.35, which was 1.7-
to 5.5-fold higher than that of zanamivir (IC50: 58.3 µM; TI: 1.7).

Luteolin (6) was the most effective with an IC50 value of 10.7 µM
(TI: > 9.35). Interestingly, alkaloid compound 7 demonstrated
more potent activity in the CPE reduction assay than in the
measurement of the neuraminidase inhibition effect. All of the com-
ounds tested were not toxic to MDCK cells, displaying 100% via-
bility at the concentration of 100 µM (Table 1).

After 2 days of infection of MDCK cells with the influenza A virus,
no differences were observed between the mock cells (non-viru-
 lent cells, Fig. 3) and those treated with 10 µM of the com-
ounds (data not shown) in terms of normal morphology, with
typical spread-out shapes. As shown in Fig. 3, severe CPE was
observed in influenza A virus-infected cells that were not treated
with any of the compounds or with zanamivir. Compounds 2, 4, 5,
and 6 potently inhibited the virus-induced CPE in infected MDCK
cells (Fig. 3). Compounds 1 and 7 displayed moderate antiviral
activities, while 3 only weakly reduced the CPE; however, their
activities were comparable to that of zanamivir.

The qualification of the methanol extract and ethyl acetate frac-
tion of bee pollen was performed by HPLC analysis on the basis of
the retention time of the pure compounds and UV spectral data
(Fig. 4). Compounds 1–3 and 6 (peaks 1–3 and 6) were domi-
nant constituents in the ethyl acetate fraction, making up 55%
from the HPLC analysis data. Comparison of the two HPLC pro-
files revealed distinct differences in the peak of compound 6.

Therefore, these results were consistent with the high neurami-
nidase inhibition shown by the ethyl acetate fraction of bee pollen.
Peaks 8–10 were shown as main constituents (19% from HPLC
analysis of ethyl acetate fraction), but demonstrated no neurami-
nidase inhibitory activity up to 200 µM. The quantitative
amount of compounds 1–7 found in the methanol extract of bee
pollen is shown in Table 2. Calibration curves for compounds
1–7 were linear, as evidenced by the correlation coefficients
(r² = 0.997–0.999). The content of 1 was the highest (about 0.3%

\[\text{Table 1 Inhibitory effect of compounds 1–7 on neuraminidase activities and antiviral activity against influenza A virus in MDCK cells.} \]

<table>
<thead>
<tr>
<th>Compounds</th>
<th>NA inhibition IC50 (µM)</th>
<th>Inhibition type</th>
<th>Cytotoxicity and antiviral activity (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1N1</td>
<td>H5N1</td>
<td>H3N2</td>
</tr>
<tr>
<td>1</td>
<td>85.6 ± 1.4</td>
<td>111.6 ± 1.0</td>
<td>61.0 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>56.2 ± 1.1</td>
<td>80.2 ± 4.8</td>
<td>51.6 ± 1.5</td>
</tr>
<tr>
<td>3</td>
<td>45.3 ± 1.9</td>
<td>51.2 ± 3.1</td>
<td>52.2 ± 2.9</td>
</tr>
<tr>
<td>4</td>
<td>36.3 ± 1.5</td>
<td>61.1 ± 2.1</td>
<td>54.0 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>88.3 ± 3.0</td>
<td>75.1 ± 8.7</td>
<td>112.8 ± 8.2</td>
</tr>
<tr>
<td>6</td>
<td>10.7 ± 0.2</td>
<td>12.6 ± 0.8</td>
<td>25.6 ± 8.3</td>
</tr>
<tr>
<td>7</td>
<td>100.5 ± 9.2</td>
<td>151.1 ± 19.8</td>
<td>143.2 ± 8.9</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>1.5 ± 0.2</td>
<td>3.4 ± 0.1 nM</td>
<td>1.9 ± 0.4 nM</td>
</tr>
</tbody>
</table>

Results were obtained from three independent experiments carried out in triplicate, and are expressed as the mean ± S.D.; a Concentration required to inhibit cell growth by 50%; b concentration required to inhibit virus-induced CPE by 50%; c therapeutic index = CC50/IC50; d the compound used as the positive control; e not determined.

Materials and Methods

**Pollen material**

_P. rhaes_ bee pollen was provided and authenticated by emeritus
professor Kwang-Ryul Choe at Chungnam National University,
Daejeon, Korea, in 2011. P. rhaes bee pollen was collected by
honey bees, _Apis mellifera_ L., at Chungnam province, Korea, in
May–July 2011. A voucher specimen (Y-2011–01) was deposited
at the herbarium of Chonbuk National University, Jeonbuk, Korea.
High-performance liquid chromatography analysis

The qualitative and quantitative analyses of the methanol extract of *P. rhoeas* bee pollen were characterized by the HPLC photo-diode array detection (PDA) system. For qualitative analysis, samples (10 µL) were injected into an analytical ODS column. The mobile phase was composed of 0.04% trifluoro acetic acid in water (A) and methanol (B). The gradient conditions were as follows: 3 min, 20% B; 35 min, 50% B; 40 min, 50% B, with a flow rate of 1 mL/min and the detection at 207 nm. For quantitative analysis, the standard curves of compounds 1–7 were prepared over a concentration range of 31.25–250 µg/mL with four different concentration levels. Samples (15 µL) were injected into an analytical ODS column. The mobile phases and detection conditions were as follows: 20% aqueous methanol (0.04% TFA) for 1, 5, and 7; 25% aqueous methanol (0.04% TFA) for 2 and 3; 30% aqueous methanol (0.04% TFA) for 4; and 40% aqueous methanol (0.04% TFA) for 6. The detection wavelengths were 286 nm for 7, 254 nm for 5, 264 nm for 1–4, and 349 nm for 6. A line for each compound was plotted using linear regression of the peak area vs. concentration. The coefficient of correlation ($r^2$) was used to judge the linearity.

Extraction and isolation

The dried pollen (400 g) was extracted with 70% methanol (1 L × 3) for 24 h at room temperature, and the solution was concentrated under reduced pressure to obtain a crude extract (150 g). The extract was partitioned successively with n-hexane, chloroform, ethyl acetate, and butanol. The ethyl acetate fraction exhibited the most potent inhibitory activity against H1N1 neuraminidase. Therefore, the ethyl acetate fraction (3 g) was then subjected to a C18 Sepak cartridge eluting with a gradient of increasing methanol (10–100%) in water to give four fractions (Fr. I–IV) based on the silica TLC pattern and HPLC analysis. Fractions I and II were followed by reversed-phase preparative HPLC with 35% aqueous MeOH (0.04% TFA) and 40% aqueous MeOH (0.04% TFA), respectively, at a flow rate of 6 mL/min. Compounds 1 ($t_R$ 34 min, 33.2 mg), 2 ($t_R$ 44 min, 35.5 mg), 3 ($t_R$ 22 min, 13.7 mg), and 5 ($t_R$ 22 min, 13.7 mg) were isolated from fraction I, while 4 ($t_R$ 79 min, 22.3 mg) was obtained from fraction II. Fraction III was chromatographed on a Sephadex LH-20 column with MeOH, and then purified by reversed-phase preparative HPLC with a 50–80% aqueous MeOH gradient (0.04% TFA) at a flow rate of 8 mL/min to yield 7 ($t_R$ 26 min, 9 mg). Compound 6 (14.2 mg) was obtained by column chromatography of a silica gel eluted
with CHCl₃-MeOH (10:1) and a Sephadex LH-20 eluted with CHCl₃:MeOH (1:1) from fraction IV.

**Neuraminidase inhibitory activity**

Neuraminidase inhibitory activity was estimated using a previously reported fluorometric (FL) method, with some modifications [14]. Recombinant influenza A virus H1N1, H3N2, and H5N1 NAs were purchased from R&D systems. The substrate for the FL assays, 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid (MUNANA), is cleaved by NA to release N-acetyl-α-D-neuraminic acid (NANA) and the fluorescent compound 4-methylumbelliferone (MU). Therefore, NA inhibition by an NA inhibitor causes a reduced fluorescence of the MU. The substrate, 50 µL of 0.2 mM MUNANA, was mixed with 90 µL of 50 mM Tris buffer (containing 200 mM NaCl, 5 mM CaCl₂, pH 7.5) at room temperature. Ten microliters of the sample and 50 µL of H1N1 NA (50 ng/mL) were added to a well in a plate. The mixture was then recorded at the excitation and emission wavelengths of 365 nm and 445 nm, respectively, with a POLAR OPTIMA. In the case of H3N2 and H5N1 NAs, 25 mM MES buffer (containing 500 mM NaCl, 5 mM CaCl₂, pH 6.5) and 50 mM MES buffer (containing 500 mM NaCl, 5 mM CaCl₂, pH 6.5) were used, respectively, and H5N1 required an activation period of 24 h at 37°C before the assay. The other methods were the same as those for H1N1 NA. The 50% inhibitory concentration (IC₅₀) for enzymatic activity of neuraminidase was determined from the dose-response curve using Excel software.

$$\text{Inhibition} \% = \left[1 - \frac{(S - S₀) \times 100}{C₀} \right] \times 100$$

Where C is the fluorescence of the control (enzyme and substrate) after 30 min of incubation, C₀ is the fluorescence of the control (enzyme and substrate) after incubation, and S₀ is the fluorescence of the tested samples at zero time.

**Virus and cell culture**

The influenza A virus was provided by ATCC (American Type Culture Collection) and propagated in 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 solution. Antibiotic-antimycotic solution, tryptic-EDTA, FBS, and MEM were purchased from Gibco BRL. Tissue culture plates were purchased from Falcon. SRB and zanamivir (purity of > 98%) were purchased from Sigma-Aldrich.

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**Table 2** Qualitative and quantitative analysis of compounds 1–7 found in the methanol extract of *P. rhoeas* bee pollen.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>tₑ (min)</th>
<th>Dried pollen (mg/100 g)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.8</td>
<td>294.9 ± 4.5</td>
<td>kaempferol-3-sophoroside (1)</td>
</tr>
<tr>
<td>2</td>
<td>18.6</td>
<td>154.8 ± 1.3</td>
<td>kaempferol-3-neohesperidoside (2)</td>
</tr>
<tr>
<td>3</td>
<td>20.1</td>
<td>24.5 ± 1.3</td>
<td>kaempferol-3-sambubioside (3)</td>
</tr>
<tr>
<td>4</td>
<td>24.8</td>
<td>ND (&lt; 1)</td>
<td>kaempferol-3-glucoside (4)</td>
</tr>
<tr>
<td>5</td>
<td>12.9</td>
<td>63.2 ± 1.0</td>
<td>quercetin-3-sophoroside (5)</td>
</tr>
<tr>
<td>6</td>
<td>32.7</td>
<td>24.9 ± 0.5</td>
<td>Luteolin (6)</td>
</tr>
<tr>
<td>7</td>
<td>10.4</td>
<td>30.1 ± 0.9</td>
<td>Chelianthifoline (7)</td>
</tr>
<tr>
<td>8</td>
<td>24.0</td>
<td>NT</td>
<td>Unknown</td>
</tr>
<tr>
<td>9</td>
<td>27.0</td>
<td>NT</td>
<td>Unknown</td>
</tr>
<tr>
<td>10</td>
<td>29.6</td>
<td>NT</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* Values are means (n = 3); b standard deviation; c not detected; d not tested.
Assays of antiviral activity and cytotoxicity

Antiviral activity and cytotoxicity were evaluated by the SRB method, which involves assessing the degree of reduction in the CPE [15]. Briefly, MDCK cells were seeded in 96-well culture plates at a concentration of 2 × 10^4 cells/well. The cells were then incubated for 18–24 h at 37°C, after which the medium was removed and the cells were washed with PBS. After washing, 0.09 mL of diluted virus suspension and 0.01 mL of medium supplemented with trypsin-EDTA and compounds 1–7 or zanamivir, the positive control, were added in 10-fold dilutions ranging from 0.1 to 100 µM. Cells were incubated at 37°C in 5% CO₂ for two days. After washing once with PBS, 100 µL of cold (~20°C) 70% acetone were added to each well, and the cells were fixed for 30 min at ~20°C. The acetone was then removed, and the 96-well plates were dried in an oven for 30 min. After drying, 100 µL of 0.4% (w/v) SRB in 1% acetic acid solution were added to each well, followed by incubation for 30 min at room temperature. Unbound SRB was then removed, and the plates were washed five times with 1% acetic acid. Plates were then placed in a drying oven for 24 h. Bound SRB was solubilized with 100 µL of 10 mM unbuffered Tris-base solution, and the plates were incubated for 30 min. The resulting absorbance at 540 nm was obtained using a VERSAmax microplate reader, with the reference absorbance set to 620 nm. Zanamivir was used as a positive control in the CPE inhibition test.

The morphology was assessed using a light microscope at 20 × 10 magnification. Images were recorded with Leica Application Suite V2.7 software. The 50% inhibitory concentration (IC₅₀) and 50% cytotoxic concentration (CC₅₀) were calculated by regression analysis.

Supporting information

NMR assignments of compounds 1–7 are available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References