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Antiviral Constituents from the Bulbs of *Lilium lancifolium*

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A bioassay-guided fractionation of a methanolic extract of the bulbs of *Lilium lancifolium* led to the isolation of seven phenolic compounds (**1-7**) for the first time. Their chemical structures were elucidated on the basis of UV, IR, MS, NMR spectroscopic analyses. All compounds were tested for antiviral activity against respiratory syncytial virus. Compounds **6** and **7** showed significant antiviral activity against respiratory syncytial virus with IC_{50} values of 9.7 and 7.4 µg/mL and selective index values of 14.9 and 24, respectively.

Keywords: *Lilium lancifolium*, Phenolic compouds, Respiratory syncytial virus.

INTRODUCTION

Viruses are the leading cause of respiratory infections in children and adults and are a major cause of morbidity and mortality worldwide. The screening of plants for viral growth inhibitors *in vitro* and the use of the ethnopharmacological approach enhance the probability of identifying new bioactive compounds. Our focus has been on inhibition of respiratory viral infections[1].

Lilium lancifolium is a medicinal plant widely distributed in China. For traditional Chinese medicine, its bulbs have been regularly used as sedatives, antitussive and antiinflammatory agents in China today[2,3]. In our screening program for the potential antiviral agents, seven phenolic compounds were obtained from a methanolic extract of the bulbs of *Lilium lancifolium*. In the present article, we describe the structural elucidation of compound **1-7**, together with the antiviral activities of all these compounds (Fig. 1).

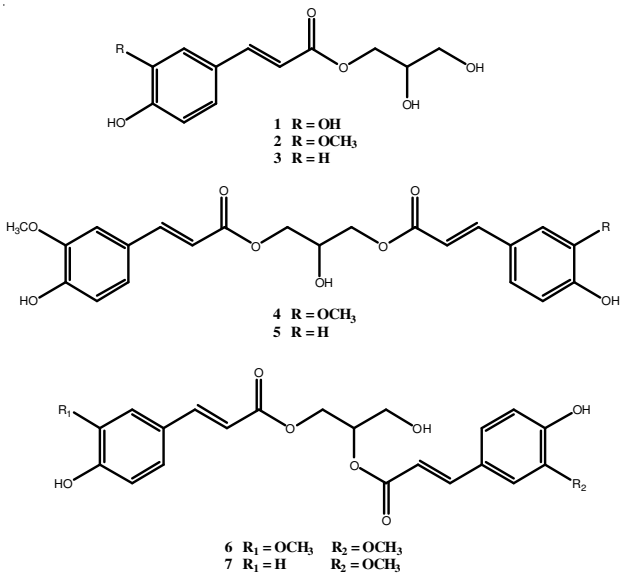

Fig. 1. Chemical structures of compounds **1-7** isolated from the bulbs of Lilium lancifolium

EXPERIMENTAL

Specific rotation measurements were recorded on a Perkin-Elmer 242 MC polarimeter. UV spectra were recorded on a Hewlett-Packard HP-845 UV-visible spectrophotometer. IR spectra were recorded on a Nicolet 470 spectrometer and MS on a Varian MAT-212 mass spectrometer and a Shimadzu GC-MS model QP2010 Plus spectrophotometer, respectively. NMR spectra were recorded on a Bruker AM-400 spectrameter (400 MHz for ^{1}H NMR) or a Bruker DRX-500 (500 MHz for ^{1}H NMR) spectrameter using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. Column chromatography separations were carried out on silica gel (200-300 mesh, Qingdao Haiyang Chemical Co. Ltd, Qingdao, P.R. China), ODS (50 mesh, AA12S50, YMC), Diaion HP-20 (Pharmacia) and Sephadex LH-20 (Pharmacia). All other chemicals used were of biochemical reagent grade.

The bulbs of *Lilium lancifolium* were collected in Shaoyang, Hunan Province of China in September 2011, and a voucher specimen (No. 20110911) was kept at the Chemitry Science and Technology School, Zhanjiang Normal University, China.

Extraction and isolation: Fresh bulbs of *Lilium lancifolium* (9 kg) were cut into pieces and extracted with methanol under reflux. The solvent was removed at reduced pressure to give a viscous residue (734 g). The entire crude extract was suspended in H_2O, and extracted with $CHCl_3$ six times (6 × 3 L) to give a $CHCl_3$ extract (193 g). This extract was then fractionated by silica gel column chromatography (column: 90 × 9 cm) using a mobile phase composed of $CHCl_3$-CH_3OH (30:1-25:1-20:1-10:1-5:1, v/v), and finally with CH_3OH alone to collect six fractions [Fr. 1 (21.7 g), Fr. 2 (18.1 g), Fr. 3 (41.5 g), Fr. 4 (7.2 g), Fr. 5 (13.3 g), and Fr. 6 (61.4 g)]. Fr. 3 (41.5 g) was chromatographed on silica gel (column: 70 × 4 cm) eluting with $CHCl_3$-CH_3OH (40:10) and sephadex LH-20 with $CHCl_3$ and CH_3OH to furnish **4** (12.3 mg), **5** (20.4 mg), **6** (15.3 mg), and **7** (19.8 mg). Fr. 6 (61.4 mg) was passed through a Diaion HP-20 column with a gradient mixture of CH_3OH-H_2O and the CH_3OH eluate fraction was subjected to silica gel column eluting with $CHCl_3$-CH_3OH (20:1; 15:1) and sephadex LH-20 with $CHCl_3$ or CH_3OH to give 1 (13.7 mg), 2 (28.9 mg) and **3** (16.2 mg) as the pure compounds.

Cells and virus: Respiratory syncytical virus (RSV) (long strain) and Hep-2 cells were provided by American Type Culture Collection. Hep-2 cell was grown in Dulbecco's modified Eagle's medium (DMEM) containing Eagle's balanced salt solution supplemented with 10 % fetal bovine serum (FBS), 100 U of penicillin per mL, 25 µg of gentamicin per mL, and 2 mM L-glutamine (growth medium). Respiratory syncytical virus-infected cells were maintained in DMEM supplemented with 1 % FBS (maintenance medium).

Cytotoxicity assay: Hep-2 cell cultures were prepared in 96-well plastic plates. After two days of incubations at 37 °C in a CO_2 incubator, when the cell cultures were confluent, culture medium was removed from each well and replenished with 0.1 mL volume of maintenance medium. To test for cytotoxicity, 0.1 mL volume of maintenance medium containing serial 2-fold dilutions of the tested compounds was added to the wells. For the cell control, 0.1 mL maintenance medium without the compound was added. All cultures were incubated at 37 °C, and for 2-5 days of incubation. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, *e.g.*, loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolization in the cytoplasm. The cytopathogenic effect (CPE) was scored (scores, 0 = 0 % CPE; 1 = 0-25 % CPE; 2 = 25-50 % CPE; 3 = 50-75 % CPE; 4 = 75-100 % CPE). The 50 % cytotoxic concentration (CC_{50}), the concentration required to cause visible changes in 50 % of intact cells, was estimated from graphic plots. The maximal non-cytotoxic concentration (MNCC) was determined as the maximal concentration of the natural products that did not exert toxic effect detected by microscopic monitoring[4,5].

Antiviral activity assay: The antiviral activity of **1-7** against viruses was measured by the cytopathogenic effect inhibition assay[4,5]. Twofold serial dilutions of **1-7** were seeded into cell monolayers cultivated in 96-well culture plates, using the MNCC as the higher concentration. An infection control was made in the absence of test compounds. An equal volume of virus suspension was added to the cell monolayers. The plates were incubated at 37 °C in a humidified CO_2 atmosphere (5 % CO_2) for 2-5 days. After that, cytopathogenic effect was observed. The virus-induced cytopathogenic effect was scored as described above in the cytotoxicity assay. The reduction of virus multiplication was calculated as percent of virus control (% virus control = $CPE_{exp}/CPE_{virus\ control} \times 100$). The concentration reducing CPE by 50 % with respect to virus control was estimated from graphic plots and was defined as 50 % inhibited concentration (IC_{50}) expressed in µg/mL. The selective index (SI) was calculated from the ratio CC_{50}/IC_{50}.

RESULTS AND DISCUSSION

The phytochemical study of methanolic extract obtained from the bulbs of *Lilium lancifolium* afforded 7 compounds. Seven phenolic compounds, including 1-*O*-caffeoylglycerol (**1**), 1-*O*-feruloylglycerol (**2**), 1-*O*-*p*-coumaroylglycerol (**3**), 1,3-*O*-diferuloylglycerol (**4**), 1-*O*-feruloyl-3-*O*-*p*-coumaroylglycerol (**5**), 1, 2-*O*-diferuloylglycerol (**6**), 1-*O*-*p*-coumaroyl-2-*O*-feruloylglycerol (**7**) were characterized by comparison of their physical and spectroscopic data with those reported in the literature[6,7]. The structures of all isolates were determined as follows.

Compound **1**, which had the molecular formula $C_{12}H_{14}O_6$, deduced from the EI-MS (*m/z* 253.0716 $[M-H]^-$) and ^{13}C NMR spectrum. The ^1H and ^{13}C NMR spectrum of **1** showed the presence of a caffeoyl group [δ (H) 7.05 (1H, d, *J* = 2.0 Hz, H-2'), 6.76 (1H, d, *J* = 8.0 Hz, H-5'), 6.96 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 6.27 (1H, d, *J* = 16 Hz, H-8'), and 7.56 (1H, d, *J* = 16 Hz, H-7'); δ (C) 127.1 (C-1'), 115.0 (C-2'), 146.7 (C-3'), 149.7 (C-4'), 116.3 (C-5'), 123.2 (C-6'), 147.9 (C-7'), 114.8 (C-8'), and 168.2 (C-9')] and a glycerol group [δ (H) 4.24 (2H, m, H-1), 3.82 (1H, m, H-2), 3.63 (2H, d, *J* = 5.5 Hz, H-3); δ (C) 66.7 (C-1), 71.4 (C-2), 64.2 (C-3)]. All the information mentioned above supports compound **1** be a phenolic monoglyceride. Comparison of the ^{13}C NMR and ^1H NMR data of **1** with 1-*O*-caffeoylglycerol showed that the signals were coincident[6]. Thus, **1** was assigned as 1-*O*-caffeoylglycerol. In the same way, compounds **2** and **3** were identified as 1-*O*-feruloylglycerol[6] and 1-*O*-*p*-coumaroylglycero[l6], respectively.

The molecular formula of compound **4** was determined to be $C_{23}H_{24}O_9$ by the negative ion at *m/z* 443.4237 $[M-H]^-$ in the HREI-MS. The ^1H NMR and ^{13}C NMR of **4** indicated the presence of two feruloyl moieties [δ (H) 7.64, 6.31 (each 2H, d, *J* = 16.0 Hz, trans alkene protons), 7.21 (2H, d, *J* = 2.0 Hz, H-2' and H-2"), 6.83 (2H, d, *J* = 8.0 Hz, H-5' and H-5"), 7.10 (2H, d, *J* = 8.0, 2.0 Hz, H-6' and H-6"), 3.89 (6H, s, 2 × OMe); δ (C) 127.7 (C-1' and 1"), 111.8 (C-2' and 2"), 149.1 (C-3' and 3"), 149.5 (C-4' and 4"), 116.7 (C-5' and 5"), 124.1 (C-6' and 6"), 147.9 (C-7' and 7"), 115.1 (C-8' and 8"), 168.0 (C-9' and 9"), and 56.7 (C-OMe)] and a glycerol group [δ (H) 4.41 (4H, m, H-1 and H-3), 4.17 (2H, m, H-2); δ (C) 67.9 (C-1 and C-3), 68.1 (C-2)]. Comparison of the ^{13}C NMR and ^1H NMR data of **4** with 1, 3-*O*-diferuloylglycerol showed that the signals were coincident[7]. Therefore, compound **4** was elucidated as 1, 3-*O*-diferuloylglycerol. In the same way, compounds **5**, **6** and **7** were identified as 1-*O*-feruloyl-3-*O*-*p*-coumaroylglycerol[6], 1, 2-*O*-diferuloylglycerol[6], and 1-*O*-*p*-coumaroyl-2-*O*-feruloylglycerol[6], respectively.

Compounds **1-7** were tested for their antiviral activity against respiratory syncytical virus (Table-1). Among these compounds, **1**, 2-*O*-diferuloylglycerol (**6**) and 1-O-*p*-coumaroyl-2-*O*-feruloy-lglycerol (**7**) showed potent antiviral activity against respiratory syncytical virus with IC_{50} values of 9.7 and 7.4 µg/mL and selective index values of 14.9 and 24, respectively, comparable to that of Ribavirin, an approved drug for the treatment of respiratory syncytical virus infections in humans. The other compounds demonstrated moderate or weak antiviral activity against respiratory syncytical virus.

TABLE-1
ANTIVIRAL ACTIVITY OF COMPOUNDS
ISOLATED FROM THE BULBS OF *Lilium lancifolium*

Compounds	IC_{50} (µg/mL)[a]	CC_{50} (µg/mL)[b]	Selective index[c]
1	41.3	248.7	6.0
2	41.3	149.4	3.6
3	37.9	248.7	6.6
4	22.6	110.5	4.9
5	30.7	110.5	3.6
6	9.7	98.3	10.1
7	7.4	110.5	14.9
Ribavirin	2.6	62.5	24.0

[a]IC_{50} is the concentration of the sample required to inhibit virus-induced CPE 50 %, [b]CC_{50} is the concentration of the 50 % cytotoxic effect, [c]SI, CC_{50}/IC_{50}

Conclusion

Seven phenolic compounds (**1-7**) were isolated from the bulbs of *Lilium lancifolium* for the first time. Their chemical structures were elucidated on the basis of UV, IR, MS, NMR spectroscopic analyses. All compounds were tested for antiviral activity against respiratory syncytial virus. Compounds **6** and **7** showed significant antiviral activity against respiratory syncytical virus with IC_{50} values of 9.7 and 7.4 µg/mL and selective index (SI) values of 14.9 and 24, respectively. Thus, this research suggested that the phenolic compounds from the methanolic extract may be primary antiviral constituents.

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