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Anti-Influenza Activity of Betulinic Acid from *Zizyphus jujuba* on Influenza A/PR/8 Virus

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Abstract

Betulinic acid, a pentacyclic triterpene isolated from Jujube tree (*Zizyphus jujuba* Mill), has been known for a wide range of biological and medicinal properties such as antibacterial, antimalarial, anti-inflammatory, antihelmintic, antinociceptive, and anticancer activities. In the study, we investigated the antiviral activity on influenza A/PR/8 virus infected A549 human lung adenocarcinoma epithelial cell line and C57BL/6 mice. Betulinic acid showed the anti-influenza viral activity at a concentration of 50 μM without a significant cytotoxicity in influenza A/PR/8 virus infected A549 cells. Also, betulinic acid significantly attenuated pulmonary pathology including increased necrosis, numbers of inflammatory cells and pulmonary edema induced by influenza A/PR/8 virus infection compared with vehicle- or oseltamivir-treated mice *in vivo* model. The down-regulation of IFN-γ level, which is critical for innate and adaptive immunity in viral infection, after treating of betulinic acid in mouse lung. Based on the obtained results, it is suggested that betulinic acid can be the potential therapeutic agent for virus infection via anti-inflammatory activity.

Key Words: Betulinic acid, *Zizyphus jujuba*, Influenza A/PR/8, A549, Inflammation

INTRODUCTION

Influenza virus is (-)-strand RNA virus containing viral genome consists of eight segments of single-strand RNA, and is a common cause of respiratory infection known as "the flu". Influenza virus is included in Orthomyxoviridae family and known to have 3 different serotypes including A, B, and C. Among them, serotype B and C were known to infect only human, but serotype A shows broad-spectrum of infection in mammals and even in poultry (Slemons *et al*., 1974; Webster *et al*., 1992).

Influenza A virus have two surface proteins, hemagglutinin (HA) and nuraminidase (NA), and sub-classified by the antigenicity of HA and NA. HA was known to help virus to attach cells and NA is glycoside hydrolase enzyme that cleave the glycosidic linkages of neuraminic acids. Recently, several inhibitors targeting NA were introduced as anti-influenza drug, and known to efficiently prevent the spreading of virus, which includes oseltamivir and zanamivir. However, recently the occurrence of resistant virus against NA inhibitors was reported, and which make us to find other antiviral candidates against influenza virus (Ward *et al*., 2005).

Zizyphus jujuba Mill, (Jujube tree) is indigenous to China over 4000 years and is widely distributed in Europe, eastern Asia, and Australia (Huang *et al*., 2008). Dry fruits of *Z. jujuba* have been utilized as poplar food and tea additives or favor for a long time (Li *et al*., 2007). The extract of *Z. jujuba* is traditionally recognized as an outstanding source of anorexia, fatigue, and loose stools (Guo *et al*., 2010b). To date, it has been revealed that *Z. jujuba* contains the wide range of constituents including flavonoids (Pawlowska *et al*., 2009), triterepenic acids (Guo *et al*., 2010a), phenolic acids and amino acids (Choi *et al*., 2011).

Betulinic acid (BeA) is pentacyclic lupane-type triterpene that are widely distributed throughout the higher plant (Rastogi *et al*., 2015). The jujube tree (*Zizyphus* spp.) is known as one of the most extensively stated sources of BeA produced in considerable quantity (Dubey and Goel, 2013). In recent

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years, BeA has been reported to show a wide range of biological and medicinal properties such as of antibacterial, antimalarial, anti-inflammatory, antihelmintic, antinociceptive, and anticancer activities of BeA (Yogeeswari and Sriram, 2005; Rastogi *et al*., 2015). Especially, its derivatives are a promising new therapeutic agent for the treatment of HIV infection (Dang *et al*., 2013). The interest in BeA with antiviral activities on a few viruses led to examine it against influenza A/PR/8 virus infected in A549 cells and C57BL/6 mice. The aim of the present study was compare the anti-influenza virus activity of BeA *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Isolation of BeA from *Z. jujuba*

The pulverized dried roots (14.5 kg) of *Z. jujuba* were macerated with MeOH (2×60 L) for each one week at room temperature. The MeOH extract was concentrated *in vacuo* to give a crude extract (0.5 kg). The concentrated extract was suspended in H_2O and acidified with 1N HCl to pH 3. The acidic solution was extracted with EtOAc to yield 186.2 g of EtOAc fraction. The aqueous residue was basified with NaOH to pH 9 and extracted with $CHCl_3$ to provide an alkaloid fraction (1.7 g) which was not applied in this study. The EtOAc fraction was subjected to a normal silica gel CC with a mixture of CHCl3 and MeOH (100:1 to 1:1) and give 10 subfractions (EA-1-10). Among them, the EA-3 fraction (50.1 g) was fractioned by a normal silica gel CC with a mixture of $CHCl_3$ and MeOH (100:1 to 5:1), giving seven fractions. Fraction 3 and 4 yielded BeA (betulinic acid, 5.4 g) by the re-crystallization with 100% MeOH.

Betulinic acid (BeA): whitish, amorphous powder, ^{1}H NMR (400 MHz, C_5D_5N) : δ4.95 (1H, s, H-29a), 4.77 (1H, s, H-29b), 3.54 (1H, td, *J*=11.4, 5.0 Hz), 3.46 (1H, t, *J*=7.8 Hz, H-3), 2.74 (1H, td-like, H-13), 2.68 (1H, m, H-11), 2.63 (1H, dt, *J*=12.7, 3.0 Hz. H-16a), 2.26 (1H, m, H-22a), 2.24 (1H, m, H-21a), 1.93 (1H, m, H-12), 1.90 (1H, m, H-15a), 1.87 (1H, m, H-2), 1.79 (3H, s, H-30), 1.76 (1H, t, *J*=11.4, H-18), 1.66 (2H, dt, *J*=13.0 Hz, 3.0, H-1), 1.59 (1H, m, H-22b), 1.56 (2H, m, H-6a and H-16b), 1.52 (1H, m, H-21b), 1.42 (1H, m, H-7a), 1.41 (1H, m, H-6b), 1.40 (1H, m, H-7b), 1.39 (1H, m, H-9), 1.28 (1H, m, H-15b), 1.23 (3H, s, H-23), 1.07 (3H, s, H-27), 1.06 (3H, s, H-24), 1.01 (3H, s, H-26), 0.83 (3H, s, H-25), 0.82 (1H, m, H-5) and ^{13}C NMR (100 MHz, C_5D_5N) : δ178.9 (C-28), 151.3 (C-20), 109.9 (C-29), 72.3 (C-3), 56.6 (C-17), 55.9 (C-5), 51.0 (C-9), 49.8 (C-18), 47.8 (C-19), 42.9 (C-14), 41.1 (C-8), 39.5 (C-1), 39.3 (C-4), 38.6 (C-13), 37.6 (C-22), 37.5 (C-10), 34.8 (C-7), 32.9 (C-16), 31.2 (C-21), 30.3 (C-15), 28.7 (C-23), 28.3 (C-2), 26.1 (C-12), 21.2 (C-11), 19.5 (C-30), 18.8 (C-6), 16.4 (C-24 and C-25), 16.3 (C-26), 14.9 (C-27)

Virus, cells, and reagents

Influenza A/PR/8 virus was obtained from provided by ATCC (American Type Culture Collection, Manassas, VA, USA). A549 cells were purchased from ATCC (Rockville, MD, USA) and maintained in Dulbacco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. Antibiotic-antimycotic solution, FBS, and DMEM were supplied by Gibco BRL (Invitrogen Life Technologies, Karlsruhe, Germany). TPCK-Trypsin was purchased from Pierce (Thermo Fisher Scientific, Rockford, IL, USA). Both sulforhodamane B (SRB) and oseltamivir were purchased from Sigma-Aldrich (St. Louis, MO, USA). The tissue culture plates were purchased from Falcon (BD Biosiences, San Jose, CA, USA). All other chemicals were of reagent grade.

In vitro antiviral activity assay

Antiviral activity was evaluated by the SRB method using cytophathic effect (CPE) reduction, as previously reported (Song *et al*., 2014). A549 cells were seeded in a 96-well plate at a concentration of 2×10^4 cells per well and were incubated for 24 h. Next day, the diluted virus suspension containing TPCK-trypsin of 1 μg/mL was placed in each well and were added the selected concentration of BeA. Virus-infected non-compound-treated cells were used as viral controls, while non-infected non-compound-treated cells were used as cell controls. After incubation for 2 days, A549 cells were washed with PBS, and ice-cold 70% acetone was added and incubated for 30 min at -20°C. After removing the acetone, 96-well plates were dried in a dry oven for 30 min, after which we added 0.4% (w/v) SRB in 1% acetic acid solution to each well for 30 min at room temperature. SRB was then removed, and the plates were washed with 1% acetic acid before oven-drying. After drying for 1 day, SRB was then solubilized with 10 mM un-buffered Tris-based solution, and the absorbance was then read at 540 nm using a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) with a reference absorbance at 620 nm. The antiviral activity of each test compound in influenza A/PR/8 virus-infected cells was calculated as a percentage of the corresponding untreated control.

Mice and virus infection

C57BL/6 mice between 6 and 7 weeks of age were purchased from SPL laboratory animal company (KOATCH Bio, Pyeongtaek, Korea). Mice were infected intranasally with 5×10^3 pfu/30 μl of influenza A/PR/8 virus. Mice were maintained in animal facility at the Kangwon National University All experiments were approved by the Institutional Animal Care and Use Committees of the Kangwon National University.

Histology and scoring

Lung tissue was washed with PBS containing and fixed in 4% formaldehyde for 1 hour at 4°C. The tissues were dehydrated by gradually soaking them in alcohol and xylene and then embedded in paraffin. The paraffin-embedded specimens were cut into 10 μm sections, stained with hematoxylin and eosin (H&E) and viewed with a digital light microscope (Olympus, Tokyo, Japan). As previously described (Shim *et al*., 2007), we used a scoring system to evaluate the level of lung tissue destruction, epithelial cell layer damage, polymorphonuclear cell infiltration into the site, and alveolitis.

Cytokine analysis

The levels of interferon gamma (IFN-γ), interleukin-1b (IL-1b), and tumor necrosis factor-α (TNF-α) were measured by mouse ELISA Ready-SET-GO kit (eBioscience), according to the manufacturer's instructions.

Statistics

The Kaplan-Meier method was used to determine the statistical significance of differences in survival time. We performed the Log-Rank test (Mantel-Cox), using SPSS 12.0K

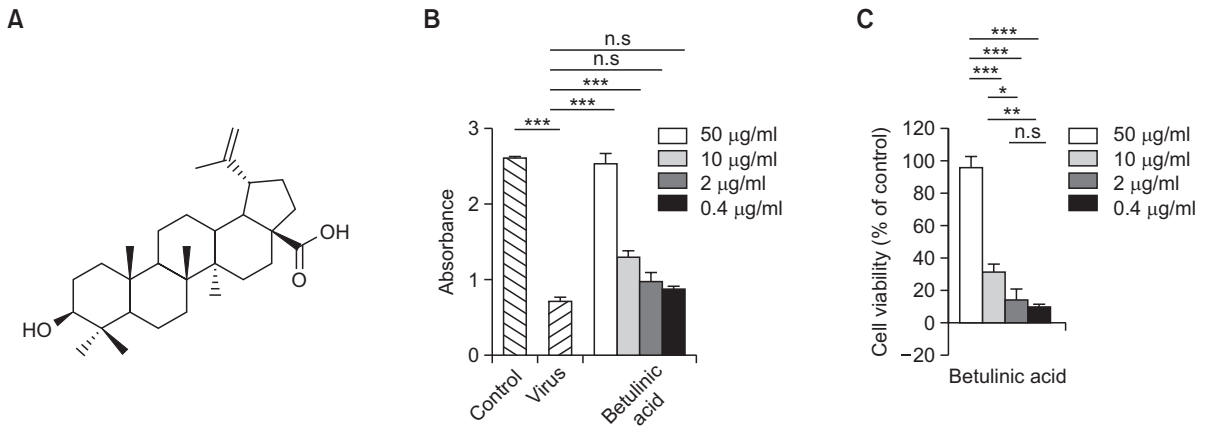

Fig. 1. Antiviral activity BeA against influenza A/PR/8 virus in A549 cells. Antiviral activity of BeA was assessed against influenza A/PR/8 virus in A549 cells. The indicated concentrations of BeA was determined using a concentration series of 0.4, 2, 10, and 50 μM. After 48 h of incubation, the antiviral activity was investigated by CPE reduction assay using SRB. (A) The structure of betulinic acid. (B) Absorbance in each well was measured using microplate reader. ***p<0.0001 and n.s. for not significant. (C) The percent value was calculated by absorbance value as mentioned in Materials and Methods. Results are presented as the mean ± SD of the absorbance and percentage values obtained from three independent experiments carried out in triplicate. *p<0.001; **p<0.03; ***p<0.0001 and n.s. for not significant.

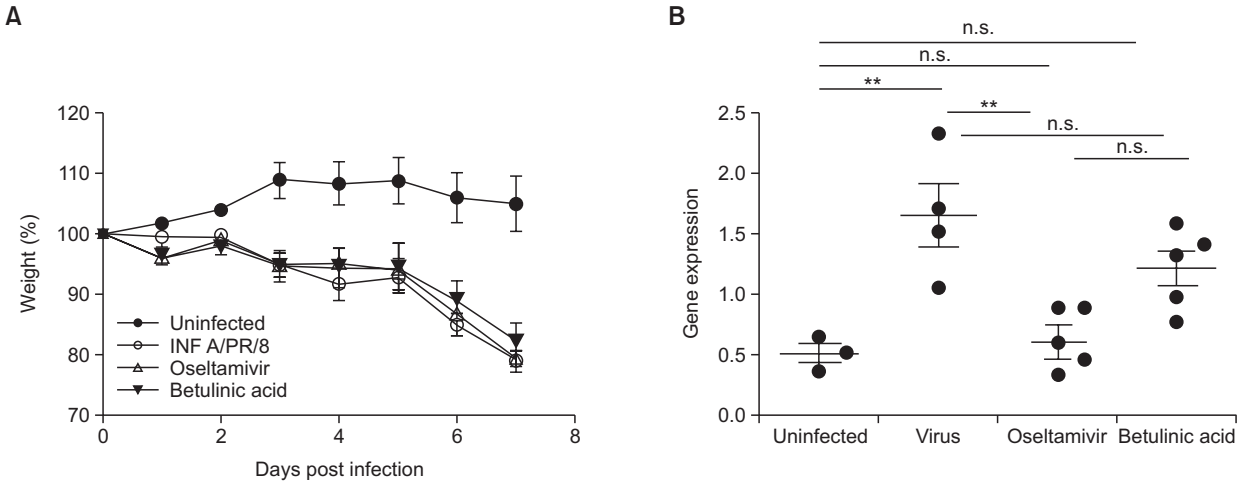

Fig. 2. Active concentration of BeA was determined. (A) 5×10^3 pfu/30 μl of influenza A/PR/8 virus was inoculated. The indicated treatment was started after infection. BeA and oseltamivir were orally administerd daily for 7 days and the survival rate of mice were measured. The body weights of mice were measured. (B) Influenza A/PR/8 virus gene expressions in the lung tissue were detected by real-time PCR 7 days after influenza A/PR/8 virus infection. **p<0.05 and n.s. for not significant.

for Windows. To compare the differences between two groups, Student's *t*-test was used. To compare multiple groups, we carried out one-way ANOVA, followed by the Tukey-HSD post hoc test.

RESULTS

Isolation and Determination of BeA from *Z. jujuba*

BeA were isolated from the methanolic extract of *Z. jujuba* using a series of column chromatography followed by the recrystallization. The structure of BeA was determined on the basis of NMR spectroscopic data (Fig. 1A). In ^{13}C NMR spectrum, the pattern of 30 carbon signals including the characteristic peaks at δ_C 151.3 (C-20) and 109.9 (C-29) exhibited the presence of the lupane-type triterpene having an olefinic bond. Additionally, the signals at δ_C178.9 (C-28) and 78.1 (C-3) showed that this compound bear a carboxylic and hydroxyl moieties, respectively. After confirming the location of characteristic groups by HMBC correlation, it was determined as betulinic acid (BeA) (Yili *et al*., 2009).

Antiviral activity of BeA against influenza A/PR/8/34 virus

The antiviral activities of BeA against influenza A/PR/8/34 were assessed using the SRB method, which monitors the alteration of CPE induced by virus infection. The antiviral assays demonstrated that BeA possessed strong antiviral activity of about 98% against influenza A/PR/8/34 virus at the concentration of 50 μM and antiviral activity of about 30% at the same virus at the concentration 10 μM (Fig. 1B, 1C). BeA was not

toxic to A549 cells with cell viability of about 100% at the concentration of 50 μM (Data not shown).

Antiviral activity of BeA against influenza A/PR/8 virus infected mice

To confirm the anti-influenza activity of BeA *in vivo*, BeA or oseltamivir were administered to mice as follows: BeA (10 mg/kg/dose) or oseltamivir (30 mg/kg/dose), dissolved in PBS, was intraperitoneally administered daily for 7 days after influenza virus infection. The mice were infected intranasally with 30 μL of influenza A/PR/8 virus suspension 5×10^3 plaque forming unit (PFU).

We monitored daily the body weight of influenza A/PR/8 virus-infected mice for all experiments to compare anti-influenza virus effect of BeA with oseltamivir treatment. However, BeA and oseltamivir did not attenuated body weight loss induced by infection of influenza A/PR/8 virus (Fig. 2A). Further evidence of the anti-influenza effects of BeA on influenza A/PR/8 virus-infected mice lung tissue was provided viral gene expression by real-time PCR analysis. However, BeA did not influence on influenza A/PR/8 virus replication (Fig. 2B). Interestingly, however, influenza infected mice treated with BeA significantly attenuated pulmonary pathology including increased necrosis, numbers of inflammatory cells and pulmonary edema induced by influenza A/PR/8 virus infection, assessed at 7 days post infection, as compared with vehicle- or oseltamivir-treated mice (Fig. 3A). In addition, there are significant difference in scoring system to evaluate the level of lung tissue destruction, epithelial cell layer damage, polymorphonuclear cell infiltration into the site, and alveolitis as compared with influenza A/PR/8 virus infected group (Fig. 3B).

Alteration of pulmonary cytokine signature in PR8-infeced mice after the treatment of BeA and oseltamivir

Virus-induced cytokines conduct a major role in recruiting leukocytes to the site of infection and activating innate immune responses to induce inflammation. Despite their protective roles, severe inflammation induced by cytokine storm was known to be associated with influenza-induced pulmonary pathology. To evaluate cytokines production at protein levels, mice were infected and treated by the same scheme as before and 6 hrs after final administration of BeA and oseltamivir, lungs from mice were obtained. The levels of cytokines including IFN-γ, IL-1β and TNF-α in lungs were measured with ELISA. The intranasal infection of influenza A/PR/8 virus increased the levels of IFN-γ, IL-1β and TNF-α at day 7 post infection as compared with PBS-treated control mice. Oseltamivir did not induce significant changes in the IFN-γ, IL-1β and TNF-α levels over vehicle treatment in influenza A/PR/8 virus-infected mice. Interestingly, at day 7 post infection, although BeA did not reduce the level of IL-1β and TNF-α, it significantly decreased the levels of IFN-γ as compared with that of oseltamivir treatment (Fig. 4).

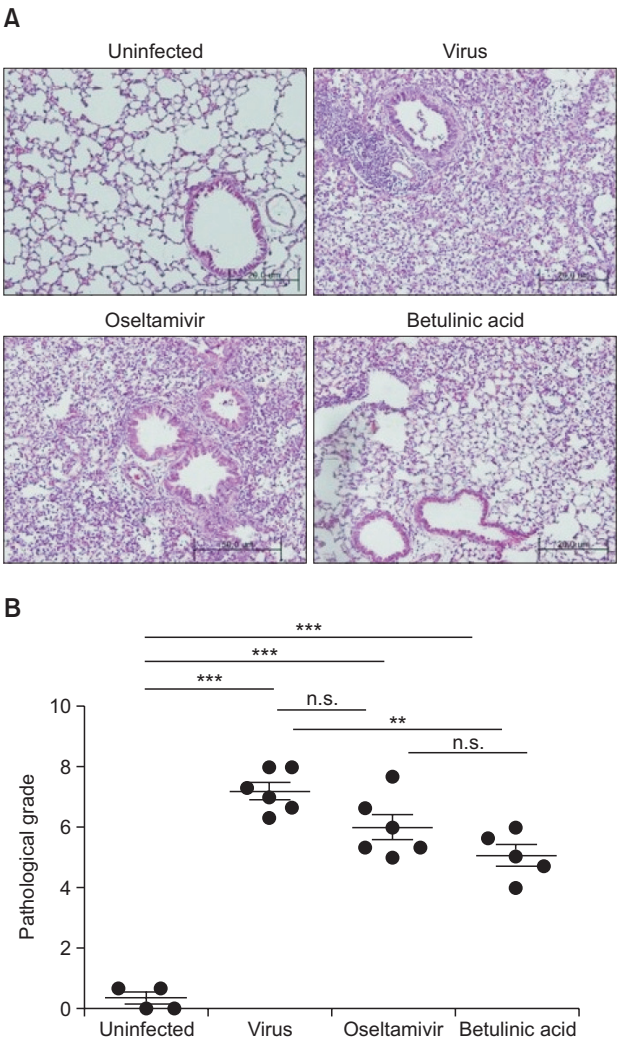

Fig. 3. Anti-influenza virus activity of BeA and oseltamivir in the mice. Mice were infected with 5×10^3 pfu/30 μl of influenza A/PR/8 virus, orally administered with BeA and oseltamivir for 7 days after virus infection. Mice were sacrificed at 7 days post infection, and lung sections were prepared as described in Materials and Methods. (A) Representative H&E stained samples of lung section from uninfected or influenza A/PR/8 virus-infected mice were shown at 200× magnification. Infected mice were treated with BeA or oseltamivir. (B) Pathological grade of mice which were administrated orally for 7 days after infection. ***p*<0.01; ****p*<0.001, and n.s. for not significant.

DISCUSSION

The present study demonstrated that BeA inhibits the proliferation of influenza A/PR/8 virus with a dose dependent manner (0.4-50 μM) in A549 cells without significant cytotoxicity. After confirming antiviral activity of BeA *in vitro* model, we further studied whether BeA exerts sufficient therapeutic efficacy in influenza virus infected mouse model. Although BeA did not restore the body weight loss after the infection of influenza A/PR/8 virus similar to oseltamivir used as the positive control, the histochemical staining result showed BeA significantly reduced the inflammation and pulmonary edema induced by influenza virus.

Also, the change of IFN-γ level after treating of BeA in mouse lung supported that BeA can be the potential therapeutic agent for the inflammation by virus infection. IFN-γ has been known to have critical for innate and adaptive immunity in some viral, bacterial and protozoal infections. Collectively, these results suggested that BeA decrease inflammatory cytokine levels, especially IFN-γ, and help influenza A/PR/8 virus-

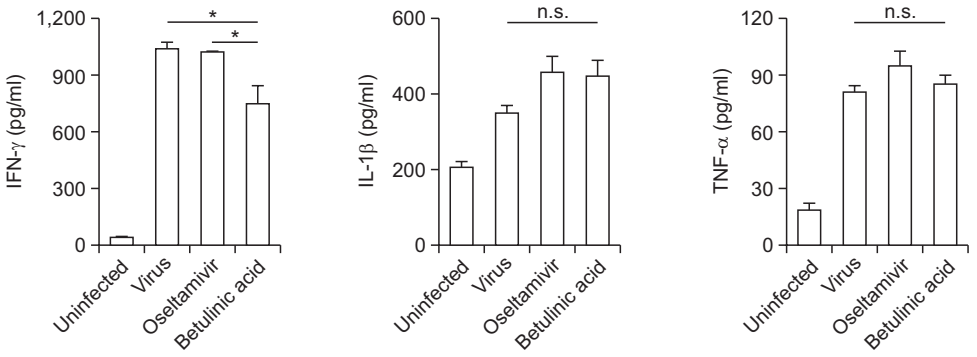

Fig. 4. Cytokine quantitation changed from BeA or oseltamivir in mice. Lung infl ammation cytokine levels were evaluated according to ELISA. C57BL/6 mice were intranasally infected with 5×10^3 pfu/30 μl of influenza A/PR/8 virus, and orally administered with BeA (10 mg/kg) and oseltamivir (5 mg/kg). Orally administration daily for 7 days and sacrificed in mice after 6 hrs. Proinflammatory cytokines and chemokine were measured in lung by ELISA. *p<0.02 and n.s. for not significant.

infected mice to rapidly recover from severe pulmonary inflammation. Although further studies are necessary to clarify the detailed anti-influenza mechanisms, it is suggested that BeA can be the potential therapeutic agent for treating of influenza viral infection via anti-inflammation.

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