Original article

Anti-influenza virus activity of propolis *in vitro* and its efficacy against influenza infection in mice

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Background: Propolis has been used worldwide as a dietary supplement to maintain and improve human health. We examined whether ethanol extracts of Brazilian propolis exhibit antiviral activity against influenza virus *in vitro* and *in vivo*.
Methods: Among 13 ethanol extracts screened in a plaque reduction assay, four showed anti-influenza virus activity. The anti-influenza efficacy of the four extracts was further examined in a murine influenza virus infection model. The mice were infected intranasally with influenza virus, and the four extracts were orally administered at 10 mg/kg three times daily for seven successive days after infection.
Results: In this infection model, only one extract, AF-08, was significantly effective at 10 mg/kg in reducing the body weight loss of infected mice. The doses of 2 and 10 mg/kg were also effective in prolonging the survival times of infected mice significantly, but 0.4 mg/kg was not. The anti-influenza efficacy of AF-08 at 10 mg/kg was confirmed in a dose-dependent manner in mice. AF-08 at 10 mg/kg significantly reduced virus yields in the bronchoalveolar lavage fluids of lungs in infected mice as compared with the control. The reduction of virus yields by AF-08 at 10 mg/kg significantly corresponded to those induced by oseltamivir at 1 mg/kg twice daily from day 1 to day 4 after infection.
Conclusion: The Brazilian propolis AF-08 was indicated to possess anti-influenza virus activity and to ameliorate influenza symptoms in mice. AF-08 may be a possible candidate for an anti-influenza dietary supplement for humans.

Introduction

Influenza virus infects the respiratory tract in humans and causes a variety of symptoms including fever, nasal secretions, cough, headache, muscle pain and pneumonia. These clinical symptoms often become severe, especially in high-risk groups such as the elderly and infants [1,2]. Amantadine and the neuraminidase (NA) inhibitors, zanamivir and oseltamivir, have been used for the treatment and prevention of influenza virus infection [3–5]. However, amantadine-resistant viruses have begun to appear clinically at a high rate [3,6], and the appearance of resistant viruses has also been reported in treatment with NA inhibitors [7,8]. New anti-influenza virus agents that are effective against resistant viruses and/or are alternative efficacious treatments that supplement the anti-influenza virus activity of the known agents need to be developed.

Propolis has been used worldwide as a folk medicine since ca. 300 BC and as a dietary supplement to maintain or improve human health [9–11]; it is currently used as an alternative medicine in the management of various ailments [12]. Propolis as a crude product is a resinous material collected from various plants by honeybees and contains many kinds of components derived from plant sources. Propolis has been reported to exhibit various biological activities, such as antibacterial, antitumour and immunostimulatory [10,12–17]. It has been also shown to exhibit anti-influenza virus activity *in vitro* [14,18]. However, the anti-influenza virus activity of propolis *in vivo* is unclear.

In the present study, we screened the anti-influenza virus activity of 13 ethanol extracts of Brazilian propolis in a plaque reduction assay. Four ethanol extracts with anti-influenza virus activity *in vitro* were further examined for anti-influenza efficacy in a murine influenza virus infection model. We found that one ethanol extract, AF-08, was significantly effective in

prolonging the survival times of infected mice. AF-08 also significantly reduced virus yields in the bronchoalveolar lavage fluids (BALF) of lungs in infected mice as effectively as oseltamivir. Thus, AF-08, a Brazilian propolis ethanol extract, exhibited anti-influenza virus activity in mice and ameliorated influenza symptoms.

Methods

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 8% and 2% heat-inactivated fetal calf serum (FCS; Equitech-Bio, Inc., Kerrville, TX, USA), respectively [19]. Influenza A/PR/8/34 (H1N1) virus was propagated in MDCK cells. For *in vivo* assays, a mouse-adapted influenza A/PR/8/34 (H1N1) virus was used [20].

Compounds

Propolis collected by Africanized *Apis mellifera* bees in southern Brazil was extracted with ethanol, and the ethanol extracts were dried. The dried extracts, AF-05, AF-06, AF-07, AF-08, AF-17, AF-18, AF-19, AF-20, AF-M1, AF-M2, AF-M3, G-1 and G-12, were supplied by AMAZONFOOD Ltd. (Tokyo, Japan). These ethanol extracts were dissolved in an appropriate volume of ethanol and diluted with culture medium to make various final concentrations for *in vitro* assays. The concentration of ethanol in each medium was <0.2%. For *in vivo* assays, the ethanol extracts were dissolved in 1% ethanol and administered orally to mice. Ribavirin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in distilled water for a plaque reduction assay. Oseltamivir phosphate was purchased from Roche Diagnostics K.K. (Tokyo, Japan), dissolved in distilled water and administered to mice.

Animals

Female DBA/2 Cr mice (6 weeks old, 17–19 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mice were housed six or seven per cage with food and water *ad libitum* under a 12 h light/12 h dark diurnal cycle (light at 07:30 h). The temperature in the room was kept at 24 ±2°C. The mice were acclimated for at least 3 days before starting experimental procedures. Animal studies followed the animal experimentation guidelines of Kyusyu University of Health and Welfare and were carried out in an approved biosafety level facility.

Plaque reduction assay

Thirteen ethanol extracts of Brazilian propolis were examined for their anti-influenza virus activity using a plaque reduction assay to estimate the possible anti-influenza virus activity *in vitro*. MDCK cells grown in 60 mm plastic dishes were inoculated with 100 plaque-forming units (PFU)/0.2 ml of influenza virus. After adsorption at room temperature for 1 h, the inoculum was removed and the cells were overlaid with 5 ml of a nutrient agarose (0.8%) medium and EMEM, which contained 0.2% bovine serum albumin (BSA; Sigma-Aldrich Japan, Tokyo, Japan), 0.1% glucose (Nacalai Tesque, Inc., Kyoto, Japan), 0.01% DEAE dextran (Sigma-Aldrich Japan, Tokyo, Japan), 3 μg/ml trypsin (Sigma-Aldrich Japan), 0.8% agar (Ina Food Industry Co., Ltd., Nagano, Japan) and various concentrations of ethanol extracts, and maintained in a humidified atmosphere containing 5% CO_2 for 3–4 days. Ribavirin was used as a positive control. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution (Nacalai Tesque). The number of plaques was counted under a dissecting microscope [21,22]. The effective concentrations for 50% plaque reduction (EC_{50}) were determined from a curve relating the plaque number to the concentrations of ethanol extracts.

Cytotoxicity assay

Cytotoxicity of ethanol extracts was examined by the growth inhibition of MDCK cells as described previously [22]. Briefly, MDCK cells were seeded at a concentration of 2.5×10^4 cells/well in 24-well plates and grown with 8% FCS–MEM at 37°C for 2 days. The culture medium was replaced with fresh medium containing ethanol extracts at various concentrations and the cells were grown for a further 2 days. The cells were treated with trypsin and the number of viable cells was determined by the trypan blue exclusion test. The 50% cytotoxic concentrations of ethanol extracts reducing cell viability (CC_{50}) were determined from a curve relating the percentage of viable cells to the concentrations of ethanol extracts.

Influenza virus infection in mice

Efficacies of four ethanol extracts, AF-08, AF-17, AF-19 and AF-M1, which were selected as anti-influenza virus extracts *in vitro,* were examined in an intranasal influenza virus infection model in mice. DBA/2 Cr mice were intranasally infected or mock-infected with a mouse-adapted influenza A/PR/8/34 virus at 1,600 PFU or phosphate-buffered saline (PBS), respectively, under anaesthesia [20,22]. The ethanol extracts at 10 mg/kg were orally administered to the mice by gavage, once at 4 h prior to and twice after virus infection on day 0, and three times daily from day 1 to day 4 or day 6 after infection. A 1% ethanol solution was used for a control. The dosage of 30 mg/kg/day of ethanol extracts for mice corresponds to about 12-fold of the dosage for humans based on body surface area [23] and 10 mg/kg

was used as a conventional dose of propolis. We also used 0.4 mg/kg and 2 mg/kg ethanol extracts to evaluate the dose-dependent efficacy of ethanol extracts. In order to compare the efficacies of ethanol extracts and oseltamivir, oseltamivir at 1 mg/kg was orally administered to infected mice, once at 4 h prior to and once after infection on day 0, and twice daily from day 1 to day 4. An oseltamivir dosage of >1 mg/kg/day for mice has been shown to be significantly effective against influenza virus infection in a murine infection model [24,25]. We used the dose of 1 mg/kg for mice as a clinical dose. The dose of 0.1 mg/kg was used as a subclinical dose. Six mice from each group were weighed daily from day 0 to day 4 after infection, and the changes were calculated based on the body weight of each mouse on day 0. The number of surviving mice in a group (n=7) was observed daily from day 0 to day 10.

Determination of virus yields in BALF

Virus yields in the BALF of influenza-virus-infected mice have been widely used to evaluate the anti-influenza virus activity of compounds in intranasal influenza virus infection models in mice [22,26–28]. We prepared BALF from four to six infected mice in each group from day 1 to day 4 after infection as previously described by Kurokawa *et al*. [26,27]. The prepared BALF samples were centrifuged (12,000 rpm for 10 min) and the supernatant was stored at -80°C. Virus yields in the supernatant were determined by a plaque assay as described previously [27]. Briefly, confluent monolayers of MDCK cells were incubated with the BALF supernatant serially diluted in PBS containing 1% BSA for 1 h at room temperature. The cells were then overlaid with the nutrient agar (0.8%) medium, maintained for 3–4 days and stained as described above. In this assay, detectable virus titres were >100 PFU/ml. Visualized plaques were counted and the virus titre was expressed as $\log_{10}$ PFU/ml.

Statistical analysis

Statistically significant differences between the EC_{50} and CC_{50} values were evaluated using Student's t-test. Interactions between treated and untreated groups in the changes of net body weights of infected mice and virus yields in the BALF were analysed using the repeated measures two-way ANOVA from day 0 to day 4 after infection. The Kaplan–Meier method followed by a long-rank test was used to evaluate the statistical differences in mortality [22,29]. A P-value <0.05 was considered to be statistically significant.

Results

Screening of propolis for anti-influenza efficacy

Ethanol extracts contain various compounds, some of which may have anti-influenza virus activity or may be cytotoxic. Thus, we compared the EC_{50} and CC_{50} values of each ethanol extract as a way for screening possible anti-influenza virus ethanol extracts *in vitro*. Thirteen ethanol extracts of Brazilian propolis were examined for their anti-influenza virus activity and cytotoxicity to evaluate their possible antiviral activity in a plaque reduction assay as shown in Table 1. In this assay, EC_{50} value of ribavirin as a positive control was 20.2 ±11.7 μg/ml and similar to the result reported by Furuta *et al*. [30]. The EC_{50} values of AF-08, AF-17, AF-19 and AF-M1 were 22.6 ±2.0, 19.5 ±0.8, 34.6 ±4.0, and 60.3 ±8.1 μg/ml, respectively. These EC_{50} values were significantly lower than their CC_{50} values (P<0.05 by Student's t-test), but those of other ethanol extracts were not. Thus, AF-08, AF-17, AF-19 and AF-M1 exhibited possible anti-influenza virus activity *in vitro*. We selected the four ethanol extracts as candidates for *in vivo* screening.

Efficacies of the four ethanol extracts selected *in vitro* were examined in a murine influenza virus infection model. In this model, the weight changes of infected mice are a useful indicator of the morbidity of mice after a challenge with influenza virus [29,31]. We used 10 mg/kg of ethanol extract as a dose for mice, which corresponded to a human dosage. However, because we did not have sufficient amounts of ethanol extracts for the influenza virus infection experiments using many mice and their long-term administration, efficacies of the four ethanol extracts were screened by the weight changes of infected mice from day 0 to day 4 after infection. As shown in Figure 1, the mean body weights of infected mice administered 1% ethanol decreased markedly from day 0 to day 4 after infection compared with those in mock-infected mice (P<0.05 by the repeated

Table 1. Anti-influenza virus activity and cytotoxicity of propolis

Propolis	EC_{50}, μg/ml	CC_{50}, μg/ml
AF-05	62.0 ±3.4	61.8 ±4.9
AF-06	60.0 ±3.7	58.3 ±0.6
AF-07	59.1 ±3.9	55.0 ±1.1
AF-08	22.6 ±2.0*	49.9 ±2.7
AF-17	19.5 ±0.8*	50.2 ±0.1
AF-18	45.2 ±7.8	149.2 ±6.6
AF-19	34.6 ±4.0†	101.9 ±0.5
AF-20	<10	10
AF-M1	60.3 ±8.1†	132.3 ±1.6
AF-M2	101.2 ±2.0	ND
AF-M3	111.6 ±25.1	ND
AF-G1	54.3 ±3.5	40
AF-G12	36.8 ±16.9	ND
Ribavirin	20.2 ±11.7	ND

The effective concentrations for 50% plaque reduction (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) values were determined as described in the text. Each value indicates the mean ±SE of three independent experiments. *P<0.05 and †P<0.01 versus CC_{50} values by Student's t-test. ND, not done.

measure two-way ANOVA from day 0 to day 4). The administration of AF-08 at 10 mg/kg significantly reduced the loss of mean body weight in the infected mice from day 0 to day 4 after infection ($P<0.05$ by the repeated measure two-way ANOVA from day 0 to day 4). AF-17, AF-19 and AF-M1 at 10 mg/kg did not reduce the loss of body weight in infected mice significantly (data not shown). The oral administration of AF-08 at 10 mg/kg for 11 days did not produce significant body weight loss in uninfected mice (data not shown). Thus, among the four ethanol extracts, only AF-08 was found to exhibit a potential anti-influenza efficacy in mice without toxicity.

Confirmation of anti-influenza efficacy of AF-08

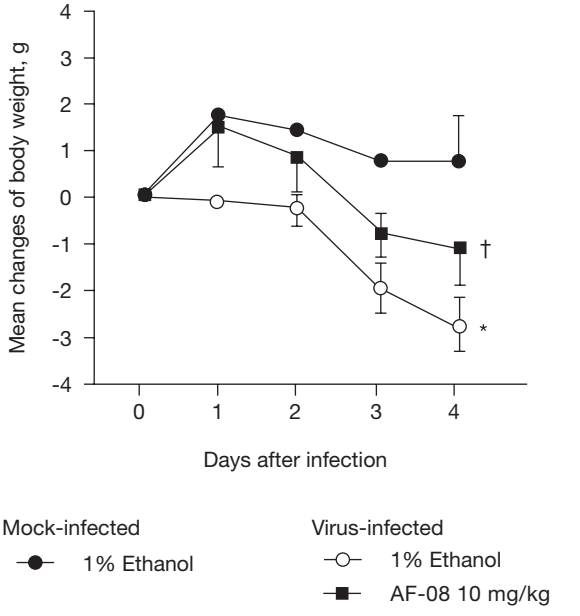
To confirm the potential anti-influenza efficacy of AF-08, we examined the dose-dependent efficacy of AF-08 on the survival rate of infected mice (Figure 2). The infected mice were orally administered AF-08 at 0.4, 2 or 10 mg/kg. All infected mice administered 1% ethanol as a control were dead by day 10 after infection. However, 14.3%, 28.6%, and 71.4% of infected mice administered AF-08 at 0.4, 2 and 10 mg/kg, respectively, were still alive on day 10 after infection. AF-08 at 2 and 10 mg/kg was significantly effective in prolonging the survival times of infected mice as compared with the control (8.3 ±0.7 days, $P<0.05$ and 9.3 ±0.5 days, $P<0.01$, respectively, compared with the control of 6.1 ±0.6 days by the Kaplan–Meier method), but the dose of 0.4 mg/kg was not (7.9 ±0.7 days, $P=0.08$). Thus, AF-08 was effective against influenza virus infection in a dose-dependent manner and its anti-influenza efficacy in mice was confirmed.

Comparison of anti-influenza virus activity of AF-08 and oseltamivir in mice

The anti-influenza virus activities of AF-08 and oseltamivir against influenza virus were compared in mice. For this comparison, 0.1 and 1 mg/kg of oseltamivir were used as doses corresponding to subclinical and clinical human doses in mice, respectively. Administration of AF-08 at 10 mg/kg was confirmed to reduce the body weight loss in infected mice significantly ($P<0.05$ by the repeated measure two-way ANOVA from day 0 to day 4, Figure 3A). Oseltamivir at 1 mg/kg significantly reduced the body weight loss of infected mice, but 0.1 mg/kg did not. There was no significant difference between the mean body weights of infected mice administered AF-08 at 10 mg/kg and oseltamivir at 1 mg/kg from day 1 to day 4. Thus, the efficacy of AF-08 at 10 mg/kg against influenza infection was comparable to that of oseltamivir at 1 mg/kg in mice.

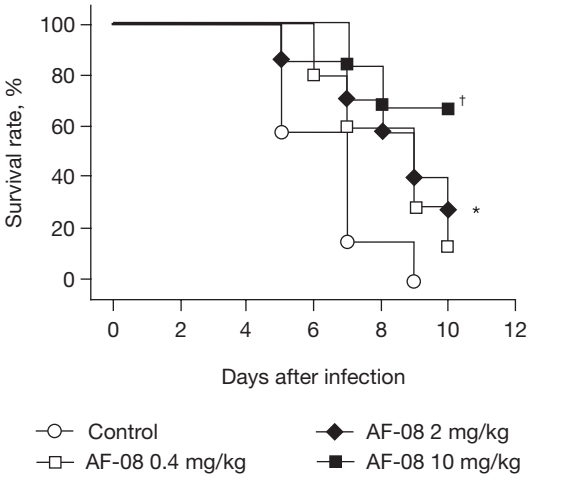
When virus yields in the BALF of infected mice were compared in infected mice administered AF-08

Figure 1. Effect of AF-08 on body weight of influenza-virus-infected mice



Influenza-virus-infected mice were orally administered AF-08 at 10 mg/kg three times daily from day 0 to day 4 after infection and mock-infected mice were orally administered 1% ethanol, as described in the text. The changes of body weight ($n=6$) are expressed as the mean ±SE in each group. *$P<0.05$ compared with mock-infected mice and †$P<0.05$ compared with infected mice administered 1% ethanol by the repeated measure two-way ANOVA from day 0 to day 4 post-infection.

Figure 2. Effect of AF-08 on survival rate of influenza-virus-infected mice



Influenza-virus-infected mice were orally administered 1% ethanol (control) and AF-08 at 0.4, 2 and 10 mg/kg three times daily from day 0 to day 6 after virus infection as described in the text. Seven mice were used in each group. *$P<0.05$ and †$P<0.01$ compared with 1% ethanol-administered infected mice by the Kaplan–Meier method from day 0 to day 10 after infection.

at 10 mg/kg and oseltamivir at 1 mg/kg, both AF-08 at 10 mg/kg and oseltamivir at 1 mg/kg significantly reduced virus titres compared with the control ($P<0.05$ by the repeated measure two-way ANOVA from day 1 to day 4, Figure 3B). However, oseltamivir at 0.1 mg/kg did not significantly reduce virus yields in infected mice. There was no significant difference between the virus yields in infected mice administered AF-08 at 10 mg/kg and oseltamivir at 1 mg/kg ($P=0.06$ by the repeated measure two-way ANOVA from day 1 to day 4). Thus, AF-08 at 10 mg/kg significantly reduced virus yields in the BALF of infected mice as effectively as oseltamivir at 1 mg/kg.

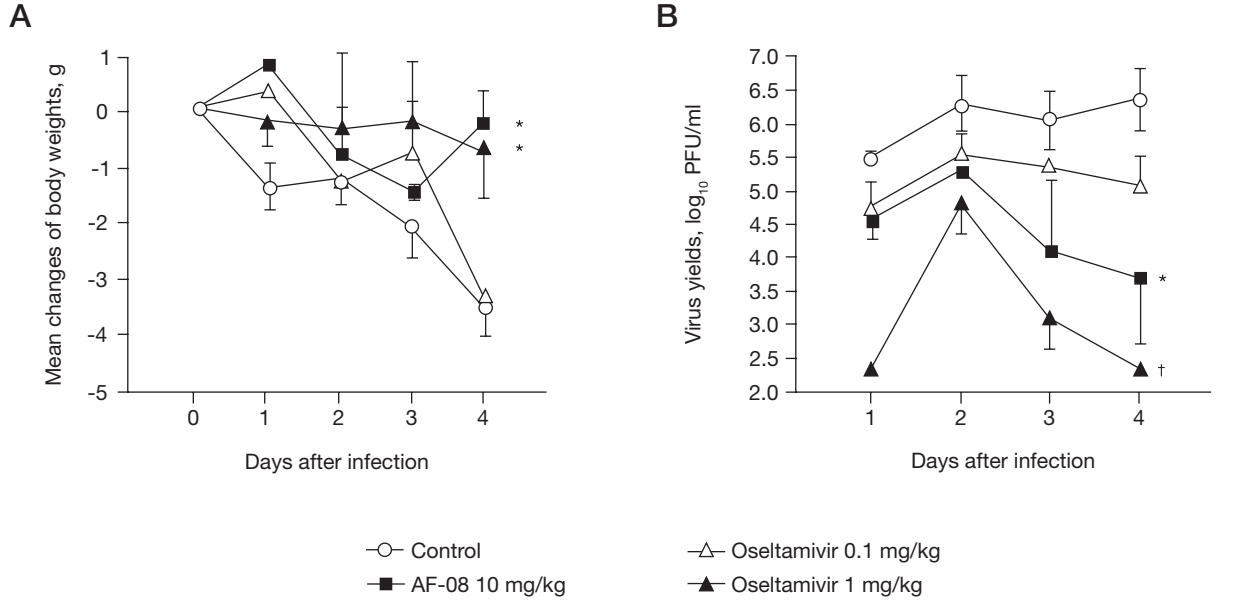
Discussion

Propolis is a natural product that is known worldwide as a folk medicine and used as a dietary supplement because of its wide range of biological activities [9–17]. However, the efficacy of propolis against influenza has been unknown *in vivo*. In this study, we found that an ethanol extract of Brazilian propolis, AF-08, exhibited anti-influenza virus activity *in vitro* and *in vivo*. This is the first study demonstrating the *in vivo* anti-influenza virus activity of propolis as a crude product. Brazilian propolis has been reported to have a chemical composition and pharmacological activity that is different from others produced in temperate zones [10,15–17,32]. AF-08 may be characterized as a propolis having possible anti-influenza components *in vivo*.

Because most propolis has been used practically in the form of ethanol extracts in humans, we examined the anti-influenza virus activity of ethanol extracts. The ethanol extracts contain many kinds of compounds, some of which may be active, whereas others may be cytotoxic. We screened the anti-influenza virus activity of 13 ethanol extracts in plaque reduction assays and selected four ethanol extracts with significantly lower EC_{50} values than CC_{50} values (Table 1). As the first screening, a plaque reduction assay is suitable to select ethanol extracts with potent anti-influenza virus activity. However, this assay is not suitable to demonstrate the anti-influenza virus activity of ethanol extracts containing compounds that are activated metabolically *in vivo* and those that act antivirally though biological response *in vivo*. Thus, the four extracts were suggested to contain some components with potent anti-influenza virus activity.

Among the four ethanol extracts selected *in vitro*, only AF-08 administered orally at 10 mg/kg significantly reduced the body weight loss of infected mice (Figure 1) and prolonged the survival times (Figure 2). The dose of 2 mg/kg of AF-08 was also effective in prolonging the survival times of infected mice, but 0.4 mg/kg was

Figure 3. Effects of AF-08 and oseltamivir on body weight loss and virus yield in BALF of influenza-virus-infected mice



Influenza-virus-infected mice were orally administered 1% ethanol (control), AF-08 at 10 mg/kg or oseltamivir at 0.1 and 1 mg/kg from day 0 to day 4 after infection, as described in the text. **(A)** Changes of body weight ($n=6$) are expressed as the mean ±SE in each group. *$P<0.05$ compared with 1% ethanol-administered infected mice by the repeated measures two-way ANOVA from day 1 to day 4 post-infection. **(B)** The virus yields in bronchoalveolar lavage fluids (BALF; $n=4–6$) were expressed as the mean ±SE in each group represented. *$P<0.005$ and †$P<0.001$ compared with 1% ethanol-administered infected mice by the repeated measures two-way ANOVA from day 1 to day 4 post-infection. PFU, plaque-forming units.

not (Figure 2), indicating that the efficacy of AF-08 to influenza was dose-dependent. It is possible that some components of AF-08 possess anti-influenza efficacy and that the efficacy of the components is dependent on the amount administered to mice. In the *in vitro* screening, AF-08 showed cytotoxicity. However, no body weight loss was observed in mice administered AF-08 at 10 mg/kg. Thus, some anti-influenza components in AF-08 might be selectively absorbed from the alimentary tract in mice and exhibit anti-influenza efficacy that was not associated with toxicity in mice.

AF-08 at 10 mg/kg significantly reduced virus yields in the BALF of infected mice as well as 1 mg/kg oseltamivir did (Figure 3B). In this experiment, the body weight loss was similar in infected mice given AF-08 at 10 mg/kg and oseltamivir at 1 mg/kg (Figure 3A). The suppression of virus growth by AF-08 as a crude mixture *in vitro* was also confirmed in mice (Figure 3B). Thus, it was strongly suggested that AF-08 contains some anti-influenza virus components. Propolis as a crude mixture and its various components have been reported to exhibit immunomodulatory action [11–13]. Although we examined the effect of AF-08 at 10 mg/kg on the levels of interferon (IFN)-α as a factor of natural immunity [33] and IFN-γ and interleukin-12 as Th1 cytokines [27,34] in the BALF of infected mice from day 1 to day 4 after infection, no changes of the cytokine levels due to AF-08 were seen (data not shown). Therefore, the anti-influenza virus activity of some components contained in AF-08 rather than the activation of host immunity by AF-08 probably resulted in the suppression of influenza virus growth in mice.

We used the dose of 10 mg/kg of AF-08 as the dose for mice corresponding to the dose used as a human dietary supplement in our infection models. A dose of 1 mg/kg of oseltamivir was also used in mice as a comparable dose for humans used in the same model. Both 10 mg/kg of AF-08 and 1 mg/kg of oseltamivir exhibited similar anti-influenza virus activities against influenza virus infection in mice. Because AF-08 as a propolis is already used as a dietary supplement by humans, it may be reasonable to expect it to be used also for the treatment of influenza virus infection in humans. For the practical use of AF-08 as an anti-influenza dietary supplement, we are now evaluating the mechanism of antiviral action of the active components.

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Disclosure statement

The authors declare no competing interests.

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