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Note

In vitro Anti-Influenza Virus Activity of *Agaricus brasiliensis* KA21

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***Agaricus* is known to have immunostimulatory and anti-tumor effects. However, the antiviral effects of *Agaricus* have not yet been examined. In the present study, the antiviral effects of an extract of *Agaricus brasiliensis* KA21 (AE) on the H1N1 influenza virus (PR8 strain) were investigated.**

The anti-influenza virus effects of AE were examined by using the plaque formation inhibition test. AE inhibited the plaque formation of PR8 in a dose-dependent manner: 98 and 50% (IC_{50}) inhibition at 2.5 and 0.99 mg/mL, respectively. To elucidate the mechanisms of AE, the direct actions and adsorption and invasion inhibition of AE were examined, and were found to have no inhibitory effect on PR8 infection. Thus, *in vitro* antiviral effects may somehow inhibit PR8 after the viral invasion of cells. These results demonstrated that it is expected that AE can effectively prevent the spread of the influenza virus.

Key words：Anti-influenza virus / *Agaricus brasiliensis*.

Influenza A viruses are classified as *Orthomyxoviridae*, single-chain (-) RNA viruses with a spherical particle structure, a diameter of 80-120 nm, an envelope, and a genome with 8 segments. Influenza A viruses have 17 hemagglutinins (HA) and 9 neuraminidase (NA) antigens, the combinations of which cause antigenic mutations through genetic reassortment. Type A influenza virus is highly infectious. Influenza epidemics occur every year. Influenza virus infection generally causes respiratory symptoms and fever, and is a serious public health concern in infants and the elderly because it may cause deaths due to severe cases of pneumonia and encephalitis (Knipe and Howley, 2013). Thus, influenza infection causes social and economic damage.

As a prophylaxis of seasonal influenza virus, the allantoic fluid obtained by egg inoculation with influenza virus is centrifuged and purified, followed by inactivation with an ether treatment to achieve an HA fraction as an inactivated vaccine (Couch, 2008). Currently used seasonal influenza virus vaccines include three or four kinds of virus antigens from influenza virus type A (H1N1 and H1N3), and influenza virus type B. However, they are found to less effectively prevent virus infections of different subtypes due to their diversity (Hampson and Mackenzie, 2006). Anti-neuraminidase agents (e.g., oseltamivir and zanamivir) may relieve the symptoms of influenza infection when used early after its onset, but may result in resistant influenza viruses (Carr et al., 2002; Kiso et al., 2004). Thus, novel antiviral agents

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need to be developed.

In recent years, polysaccharides contained in mushrooms have attracted attention because of their pharmacological effects, for example, their anti-tumor, blood pressure-lowerings, antioxidant, and anti-thrombotic effects (Suga, 2004; Wu et al., 2012; Yamada et al., 2002). In the present study, we examined *Agaricus brasiliensis*, which grows naturally mainly in the Piedade Mountains in southeastern Sao Paulo, Brazil and in the coastal grasslands in Florida and South Carolina, the United States. The pilei of *Agaricus* are 6-11 cm in size and semi-spherical in shape, and their surface is covered with pale gray-brown, fibrous scales. *Agaricus* has been shown to have various physiological effects. These include such as body fat-, visceral fat-, and body weight reducing effects, blood glucose lowering, obesity decreasing, and NK cell activity enhancing effects (Liu et al., 2008). However, the anti-influenza virus activity of *Agaricus* has not yet been examined in detail. Therefore, we herein investigated the effects of field-grown Brazilian *Agaricus brasiliensis* KA21 extract (AE) on type A influenza virus.

Agaricus brasiliensis Strain KA21 was cultivated outdoors in Brazil, and its fruit bodies were washed and dried using hot air at 60℃ or lower. AE was extracted by grinding the fruit bodies of *Agaricus brasiliensis* with D. W. (4℃). The extract was then heat-treated in boiling water for 10 min. Low-molecular-weight substances were removed from AE through a dialysis membrane (14 kDa). The stock solution of AE contained 20 mg lyophilized material/mL.

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM) (Nissui Co. Ltd, Japan) supplemented with 5% fetal bovine serum (FBS), 29.2 μg/mL glutamine, 2.95 μg/mL tryptose phosphate broth (TPB), 75 μg/mL $NaHCO_3$, 20 unit/mL penicillin (Meiji seika Co. Ltd, Japan), and 100 μg/mL streptomycin (Meiji seika Co. Ltd, Japan) in a humidified 5% (v/v) CO_2 incubator at 37℃. A strain of Influenza A virus (strain A/Puerto Rico/8/1934 H1N1: PR8) was used in the present study.

To examine the cytotoxicity of AE, the viability of cells treated with AE was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chem. Co., USA) MTT assay. MDCK cells in 96-well culture plates were exposed to serum free MEM (MM) containing serial dilutions of AE. After being treated for 48 h, the cells were incubated with 50 μL of MTT (0.5 mg/mL) at 37℃ for 4 h. After removal of the supernatant, 50 μL of DMSO was added to solubilize the formazan crystals formed, and an optical density at 550 nm was measured using a microplate reader. The 50% cytotoxic concentration (CC_{50}) represented the concentration of AE required to reduce the optical densities of mock-treated wells by 50%. The CC_{50} value of AE was calculated as 10 mg/mL, and the maximum concentration, which showed non-cytotoxicity on MDCK cells by the MTT assay, was 5 mg/mL (data not shown). As a result, this concentration, 5 mg/mL, was used in the antiviral assays.

The antiviral activity of AE against PR8 was evaluated by the plaque reduction assay. Stock virus and AE were diluted by MM. MDCK cell monolayers in 6-well plates were infected with 100 PFU of PR8 and treated with serial dilutions of 5 mg/mL AE for 1 h at 37℃. After removal of the supernatant, the inoculum was replaced with MM containing 0.8% agarose, 0.0005% trypsin, and serial dilutions of 5 mg/mL AE. After incubation for 48 h at 37℃ in 5% (v/v) CO_2 conditions, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The fixed cells were then stained with 0.8% (w/v) crystal violet in 20% ethanol and viral plaques were counted. As a control, the assay was similarly carried out without processing with AE. Inhibition of plaque formation was compared with results using the virus control. The concentration that reduced plaque formation by 50% from the control was estimated from graphic plots and defined as the 50% inhibitory concentration (IC_{50}).

Plaque formation was not inhibited in the control, but was dose-dependently inhibited with AE (29.8, 38.2, 76.4, and 98.1% at 0.31, 0.63, 1.25, and 2.5 mg/mL, respectively) (Fig.1). The plaque formation of PR8 was significantly inhibited by AE. The 50% inhibitory concentration (IC_{50}) of AE on PR8 was approximately 0.99 mg/mL.

In order to determine whether AE had direct virucidal effects on PR8, PR8 suspensions were mixed with equal volumes of 10 mg/mL AE for 1 h at 37℃. The reaction mixture was diluted to 100 PFU/100 μL, and the surviving infectious virus was measured using the plaque assay method. AE (at a final concentration of 5 mg/mL) was allowed to react with PR8 stock solution (6.0×10^6 PFU/mL), followed by the dilution of PR8 to 100 PFU to eliminate the effects of AE on cells. MM was used instead of AE in the control group. Virus titers were determined by the plaque method. No significant differences were observed in mean plaque numbers between the groups (111.0 and 109.0 PFU for the control and AE groups, respectively). Thus, AE had no direct virucidal effects on PR8.

Adsorption assays were performed using published procedures with modifications (De Logu et al., 2000). MDCK monolayers grown in 6-well plates were prechilled at 4℃ for 15 min and then infected with PR8 diluted in MM to 100 PFU for 3 h at 4℃ in the presence or absence of 5 mg/mL AE. MM was then removed and the monolayers were washed twice with PBS. The

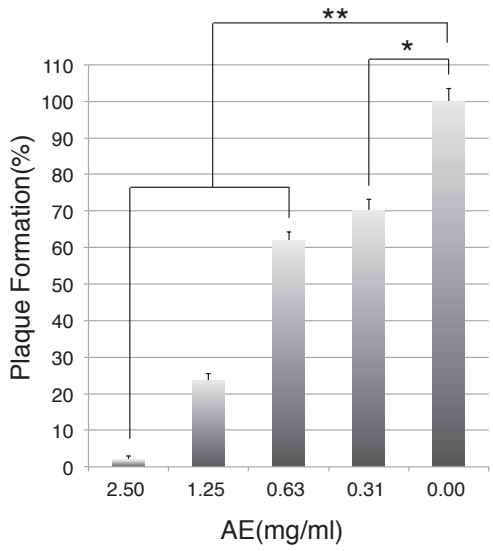

FIG. 1. The anti-PR8 effects of AE by the plaque formation inhibition test (plaque number)
After the inoculation and adsorption of PR8 (100 PFU/0.1 mL) into MDCK cells, an MM-agarose mixture containing serially diluted AE was overlaid to conduct the plaque formation inhibition test. All experiments were performed three times. The results were expressed as the mean ± SE. Each value represents the mean of at least three independent experiments in each group. The significance of the difference between two cell groups was determined using the two-tailed Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$.

adsorbed virus was measured using the plaque assay method. No significant differences were observed in mean plaque numbers between the groups (143.0 and 144.0 PFU for the control and AE groups, respectively). Thus, AE had no inhibitory effects on PR8 adsorption.

To examine the effects on cell membrane penetration during PR8 entry, penetration assays were performed using published procedures with modifications (De Logu et al., 2000). Briefly, approximately 100 PFU of PR8 was adsorbed for 3 h at 4℃ on MDCK cells grown in 6-well plates. The temperature was then abruptly increased to 37℃ to maximize the penetration of the virus. Penetration proceeded for 1 h in the absence or presence of 5 mg/mL AE. Monolayers were then treated with PBS (pH 3) for 1 min to neutralize any remaining attached virus and, after washing twice with MM, cells were overlaid with MM-0.8% agarose without AE. The amount of penetrating virus was measured using the plaque assay method. No significant differences were observed in mean plaque numbers between the groups (134.0 and 134.7 PFU for the control and AE groups, respectively). Thus, AE had no inhibitory effects on PR8 penetration.

The anti-influenza virus activity of *Agaricus brasiliensis* KA21 was investigated in the present study. *Agaricus brasiliensis* KA21 was water-extracted, heated, and dialyzed to remove minerals, enzymes, and low-molecular substances in order to prepare an AE extract and examine its antiviral effects *in vitro*. AE inhibited the growth of influenza virus. These inhibitory effects were not mediated by direct actions on viruses, as observed for the virucidal effects of disinfectants.

The antiviral effects of plant-derived components have recently attracted attention. Polysaccharides purified from *Gracilaria lemaneiformis* have been shown to inhibit the adsorption and replication of influenza virus (Chen et al., 2010), while those purified from *Antrodia camphorata* exhibited anti-hepatitis B virus activity (Lee et al., 2002). The antiviral effects of *Agaricus* may be mediated by the same mechanisms. These effects may be attributed to the inhibition of viral adsorption into cells. However, AE had no inhibitory effects on viral adsorption into cells. We speculated that AE may inhibit viral invasion into cells, similar to the anti-HIV therapeutic agent, maraviroc, which has recently attracted attention (Yuan et al., 2013), and fucoidan derived from Gagome kelp, which has anti-influenza effects (Negishi et al., 2013). However, AE had no inhibitory effects on viral penetration into cells.

The results of these *in vitro* experiments suggested that AE had no direct effects on the virus, but exerted antiviral effects through cells. The polysaccharides of mushrooms have been shown to activate transcription factors, such as NFκ-B and IRF, which induce IFN and cytokine production (Ohta et al., 2007). Interferon activates proteasome subunits to degrade the proteins of hepatitis B virus, thereby exerting antiviral effects (Wieland et al., 2003). AE also activates cytokines, such as interferons, and may exert antiviral effects.

Agaricus brasiliensis has immunity enhancing and blood pressure-lowering effects (Liu et al., 2008; Tsubone et al., 2014). *Agaricus brasiliensis* KA21 contains various proteins, cell wall component β-glucan, and large amounts of minerals (e.g., magnesium, iron, zinc, copper, and selenium) and vitamin B (Liu et al., 2008). The *in vitro* antiviral effects of AE may have been caused by polysaccharides contained in AE, such as β-glucan. The contents of β-glucan in mushrooms vary according to the growing conditions, such as soil, climate, and season (Suga, 2004). *Agaricus brasiliensis* KA21 grown in open culture is more nutritious than that grown in greenhouses (Yamanaka et al., 2013).

In the present study, an extract of *Agaricus brasiliensis* was found to exhibit antiviral effects on influenza virus *in vitro*. Substances that serve as active centers of *Agaricus brasiliensis* need to be identified in future studies.

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