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Inactivation of the Influenza Virus by a Supplemental Fermented Plant Product (*Manda Koso*)

Toshihito NOMURA, Reiko YOSHIMOTO, Ryoko KAWABATA, Toshiki MATSUBARA, Seika NARAI, Kosuke ODA, Masaya FUKUSHI, Takashi IRIE, and Takemasa SAKAGUCHI*

Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University,
Hiroshima 734-8551, Japan

ABSTRACT

Manda Koso is a commercial fermented plant product (FPP) made from 53 types of fruits and vegetables that are fermented for more than 3 years. We hypothesized that the FPP can prevent infection by influenza virus and human norovirus. Therefore, we investigated the effects of the FPP on influenza virus and feline calicivirus, a surrogate of human norovirus. We found that 10% FPP inactivated the influenza virus but not the feline calicivirus. Inhibition of the influenza virus was highly concentration-dependent: 1% and 0.3% FPP showed reduced inactivation efficacy. The effects of the FPP on the influenza virus-infected cells were investigated by addition of the FPP to the culture medium after virus infection. No suppressive effect of the FPP on influenza replication in MDCK cells was observed. The results showed that the FPP could inactivate influenza virus by affecting the virus particles.

Key words: anti-viral reagents, cytotoxicity, influenza virus, feline calicivirus

ABBREVIATIONS

DMEM	Dulbecco's modified Eagle's minimum essential medium
FPP	fermented plant product
HAU	haemagglutinating unit
LDH	lactate dehydrogenase
MOI	multiplicity of infection
MTT	3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate-buffered saline
TCID ₅₀	50% tissue culture infectious dose

The influenza virus (IFV), which belongs to the family *Orthomyxoviridae*, is a pathogen that causes respiratory infection. Outbreaks of IFV infection occur every year and sometimes cause fatal diseases such as pneumonia, secondary bacterial pneumonia, and encephalopathy¹². The human norovirus, which belongs to the family *Caliciviridae*, is a causative agent of epidemic gastroenteritis and is the most probable cause of food poisoning in Japan⁶. It is difficult to study the human norovirus because it does not replicate in cultured cells or animals other than humans. Closely related viruses such as the feline calicivirus (FCV) and mouse norovirus are used as surrogate viruses for scientific research.

Manda Koso is a fermented plant product (FPP) made of naturally fermented fruits, plant roots, cereals, marine algae, and *kokuto*, a type of cane sugar. The raw ingredients are crushed and fermented by *Lactobacillus* spp. and yeast generated spontaneously from the raw materials at room temperature for 3 years or more. *Manda Koso* is a well-known natural health food that is consumed in Japan. The FPP is a slightly sweet, black-brown paste composed of 36.9% water, 2.4% proteins and amino acids, 3.7% dietary fibres, 55.2% carbohydrates, and 1.8% ash¹³. Consumption of *Manda Koso* has been reported to reduce fat content without affecting bone weight in ovariectomized rats⁹. The FPP also exhibits free radical scavenging activity². Consumption of *Manda Koso* by fish decreased thiobarbituric acid reactive substances in their tissues³. In addition, intake of the FPP has been recently suggested to improve feed efficiency and intestinal histological status in broilers⁵.

We have reported that plant-derived tannins such as persimmon and green tea tannins efficiently inactivate human and avian IFVs¹¹. IFV particles are aggregated by the tannins and lose infectivity. Resveratrol, a plant-derived polyphenol, and Cinnamomi cortex, a crude drug used in oriental Kampo medicines, have been reported to suppress IFV replication in MDCK cells by affecting the infected cells^{4,8}. Because *Manda Koso* is derived from plants, we expected it to have an effect on IFV or human norovirus. In this study,

* To whom correspondence and reprint request should be addressed.

Address: Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

TEL: +81-82-257-5157 FAX: +81-82-257-5159 E-mail: tsaka@hiroshima-u.ac.jp

we investigated the effects of the FPP on IFV and FCV.

MATERIALS AND METHODS

Reagents

The FPP, *Manda Koso*, was obtained from Manda Fermentation Co., Ltd., Onomichi, Japan. It is a black paste-like material that is rich in nutrients such as carbohydrates, sugars, amino acids, and vitamins, as reported previously¹³. The FPP was dissolved in DMEM and frozen until use.

Cells and viruses

The MDCK(+) cells, which are canine kidney-derived cells⁷, were originally purchased from Sumitomo Dainippon Pharma (Osaka, Japan), and CRFK cells, which are feline kidney-derived cells, were purchased from ATCC and were propagated in Dulbecco's modified minimum essential medium (DMEM; Wako Chemicals) supplemented with 10% foetal calf serum, penicillin G (100 units/ml; Meiji Seika Pharma, Tokyo, Japan), and streptomycin (100 µg/ml; Meiji Seika Pharma). Human IFV A/Udorn/72 (H3N2) was propagated in embryonated chicken eggs¹¹. The FCV F9 strain, purchased from ATCC, was propagated in the CRFK cells.

Assay for inactivation of virus particles

The anti-viral assays for IFV and FCV were performed using the standard TCID₅₀ method, as described previously [11]. In brief, 90 µl of the FPP solution and 10 µl of the virus solution were mixed and incubated for 3 min at room temperature. The solution was then 10-fold serially diluted with DMEM. MDCK(+) cells or CRFK cells in a 96-well plate were inoculated with 50 µl of the diluted virus solution in quadruplicate or octuplicate. After 1 hr of adsorption, the inoculum was removed, and the cells were incubated in 100 µl/well of DMEM. When the cytopathic effects had fully developed after several days, the cells were fixed with ethanol and acetate (5:1) and further stained with 0.5% amido black 10B in 45% ethanol and 10% acetate. The 50% endpoint of the viral infection was determined using the Behrens–Karber method, and 50% tissue culture infectious dose (TCID₅₀) was calculated. Water was used instead of the FPP solution, and the infectivity was used as the virus infectivity of a mock-treated control. The anti-viral effects were estimated by comparing the FPP-treated infectivity with the mock-treated infectivity.

Assay of the effects of the FPP on the infected cells

MDCK cells were inoculated with IFV at an input multiplicity of infection (MOI) of 0.2 and maintained for 1 hr in a CO₂ incubator with occasional tilting. After adsorption, the inoculum was removed, and the cells were washed once with phosphate-buffered saline (PBS) and maintained in DMEM containing various concentrations of the

FPP. A part of the culture medium was collected at 1 hr (just after virus adsorption), 12 hr, and 24 hr. The haemagglutinating units (HAUs) in the samples were measured using chicken erythrocytes, and infectious titres (infectivities) were measured using the standard TCID₅₀ method with MDCK(+) cells. In separate experiments, MDCK cells were infected with IFV at an input MOI of 5.

Lactate dehydrogenase assay

MDCK cells were incubated in DMEM containing 10%, 5%, 2.5%, 1.3%, 0.6%, 0.3%, or 0.2% FPP for 24 hr, and the released lactate dehydrogenase (LDH) was measured using a colorimetric method with the Cytotoxicity LDH Assay Kit-WST (Dojin-do Molecular Technologies, Inc, Kumamoto, Japan) and the plate reader TriStar LB941 (Berthold Technologies, Wildbad, Germany) with absorbance at 485 nm. The LDH value was set to 100% after NP-40 treatment, and the relative ratio of each sample was calculated.

MTT assay

The FPP was subjected to cytotoxicity assays by using Cell Proliferation Kit I (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Briefly, confluent MDCK cells in a 96-well plate were exposed to 100 µl/well of DMEM containing various concentrations of the FPP for 24 hr in a CO₂ incubator. Then, 10 µl of 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and the cells were further incubated for 4 hr. The cells were solubilized, and optical absorbance at 570 nm was measured using a microtiter plate reader (Model 680; Bio-Rad Laboratories, Hercules, CA). Cell viability was estimated by comparing the values of the FPP samples with that of DMEM without FPP.

RESULTS

Effects of the FPP on viruses

To investigate the effects of the FPP on virus particles, the FPP was incubated with IFV for 3 min, and infectivity after the treatment was measured. The FPP at 10% and 3% almost completely inactivated IFV, and at 1% and 0.3%, partly inactivated IFV in a dose-dependent fashion (Fig. 1A). Prior filtration through a 0.22 µm filter weakened the inactivation (Fig. 1B), indicating that some type of large aggregates may be involved in the IFV inactivation.

Inactivation of FCV, a surrogate of human norovirus, was also investigated. The FPP had no effect on FCV, even at a high concentration of 10% (Fig. 1C). The FCV stock contained abundant host cell proteins because the stock had been prepared from infected cell lysates. We speculated that such proteins or other ingredients may have weakened the inactivation activity of the FPP.

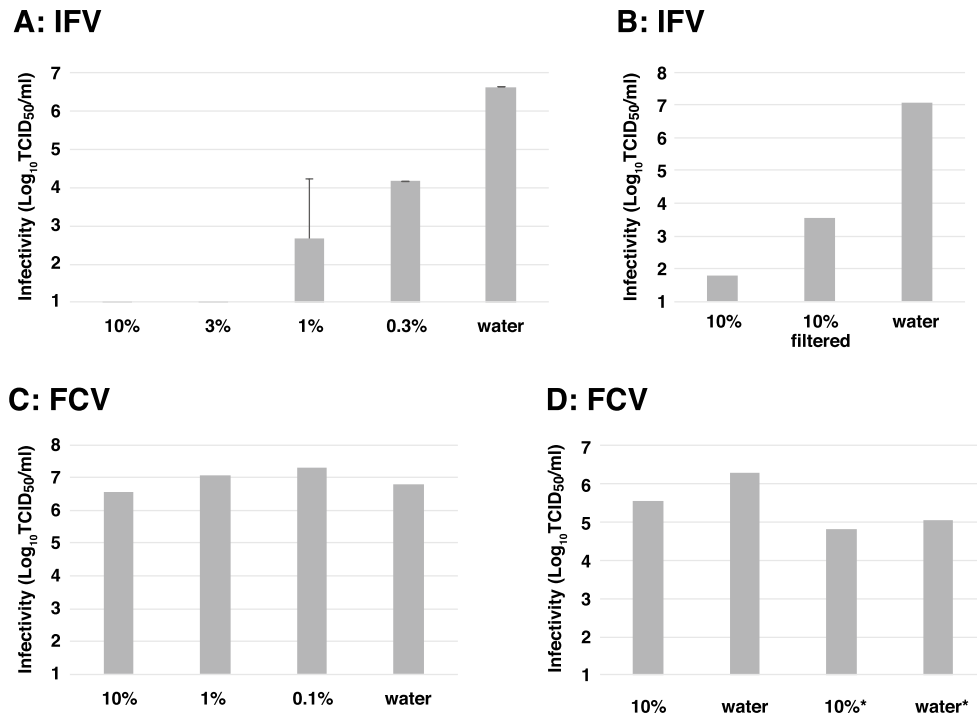


Figure 1. Effects of the FPP on pathogenic virus particles. (A) The FPP at different concentrations was incubated with IFV for 3 min. The remaining infectivity was determined using the TCID₅₀ method. Experiments were repeated three times, and the average values are shown in the bar graph. Error bars indicate standard deviation. (B) Anti-IFV assays were performed for 10% FPP with or without filtration with a 0.22 μm filter as pre-treatment. (C) Anti-FCV assays were performed for the FPP at different concentrations. (D) FCV was diluted 10 times with PBS to eliminate the effects of cellular proteins and was used for an anti-virus assay (10%*, water*).

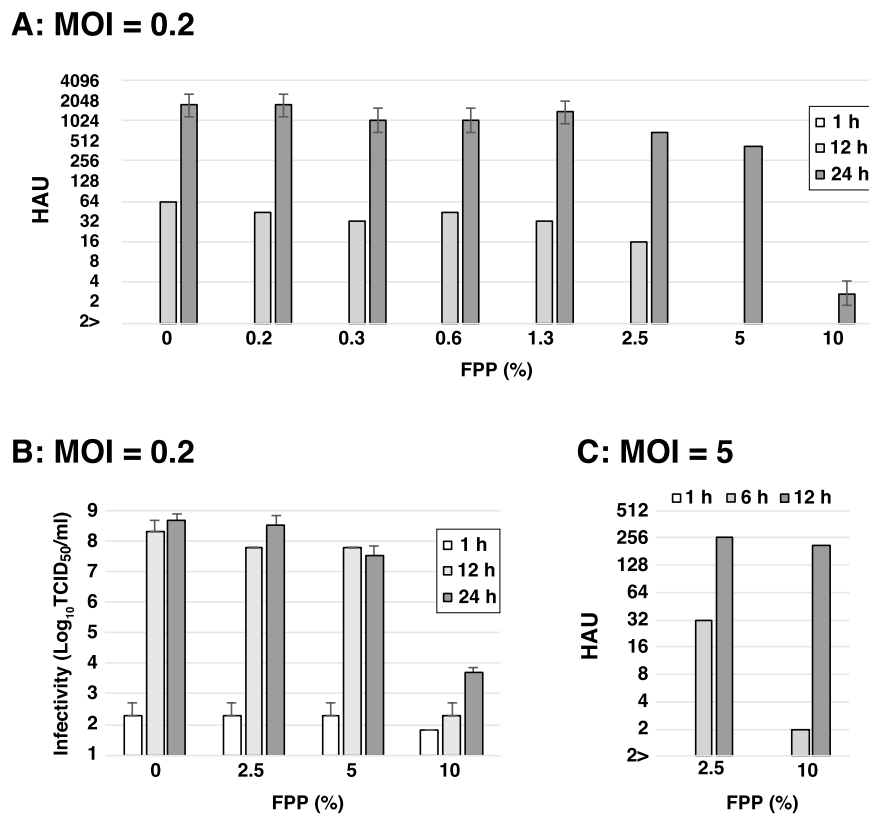


Figure 2. Effects of the FPP on influenza virus (IFV)-infected cells. MDCK cells were infected with IFV at an input MOI of 0.2 and maintained for 1 hr in a CO₂ incubator. Then, the cells were incubated in DMEM containing the FPP at different concentrations and trypsin. The culture medium was harvested at 1 hr, 12 hr, and 24 hr post infection, and HA (A) and infectivity (B) were measured. (C) MDCK cells were infected with IFV at an MOI of 5, and HA activity in the culture medium was measured. The average values are shown in the bar graph, and the error bars indicate standard deviation.

Thus, we diluted FCV 10-fold with PBS and mixed it with 10% FPP, and we then investigated the anti-viral activity. However, we observed no difference in virus inactivation between 10% FPP and water (Fig. 1D). These results indicate that the FPP can inactivate IFV but not FCV.

Effects of the FPP on infected cells

To investigate the effects of the FPP on virus-infected cells, MDCK cells were infected with IFV at an input MOI of 0.2, and the FPP was added to the maintenance medium. The culture medium was harvested at 1 hr, 12 hr, and 24 hr post infection, and HAUs in the samples were measured. The HAUs were lower in the samples treated with 5% and 10% FPP at 12 hr and 24 hr post infection (Fig. 2A), indicating that virus accumulation in

the culture medium was suppressed. Virus infectivity in the samples also indicated inhibition of virus growth, especially in the case of treatment with 10% FPP (Fig. 2B).

When the cells were infected with the virus at an input MOI of 0.2, transmission of the virus to neighbouring cells and virus replication in the infected cells were investigated. To determine the reason for the inhibition, we performed a similar experiment after IFV infection at an MOI of 5. Under this condition, all the cells were infected simultaneously, and only virus replication in infected cells was evaluated. Ten percent FPP did not strongly inhibit IFV replication (Fig. 2C), indicating that the one-step replication in the infected cells was not affected. Thus, the FPP did not inhibit virus multiplication in the infected cells, but it inhibited transmission of the virus to neighbouring cells. This result is consistent with the finding that the FPP can inactivate IFV particles.

Cytotoxicity of the FPP to MDCK cells

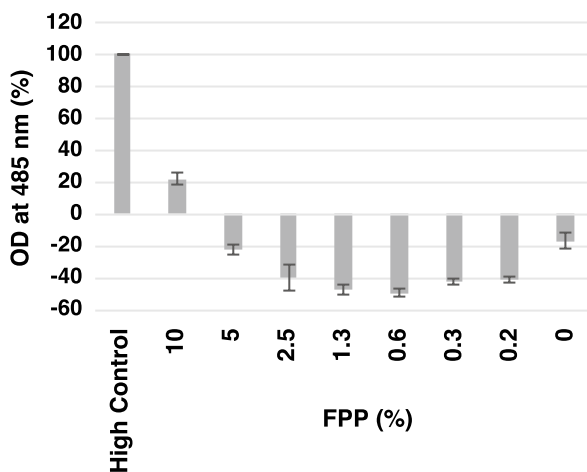
We examined the cytotoxicity of the FPP to examine the mechanism underlying the inhibition of IFV replication. MDCK cells were incubated with the FPP at different concentrations for 24 hr, and LDH level in the culture supernatant was measured (Fig. 3A). Ten percent FPP caused an approximately 20% increase in LDH, suggesting disturbance of cell membrane integrity. The MTT assay was also performed; in the presence of 10% FPP, MTT staining decreased to approximately 50% (Fig. 3B). These results indicate that 10% FPP caused considerable cytotoxicity. However, in the presence of 5% FPP or less, almost no cytotoxicity was observed.

DISCUSSION

In the present study, we showed that the FPP could inactivate virus particles of IFV but not those of FCV. The FPP inhibited IFV replication in MDCK cells in low MOI experiments. Thus, the FPP may affect the infected cells to disturb virus growth. However, the results showed that the inhibition was due to the inhibition of IFV transmission from cell to cell, and not due to the inhibition of virus replication in the infected cells. The mechanism underlying the inactivation of IFV particles is unknown; however, our results suggest that the mechanism underlying the inhibition was not virus-particle aggregation by the tannins of the FPP, as shown by persimmon or green tannins, because FCV was resistant to the FPP^{10,11}.

It is also unclear why IFV, but not FCV, was inactivated. Detergents are known to inactivate envelope viruses such as IFV by impairing the lipid envelope encasing the viral genes. In contrast, detergents are generally not effective in inactivating non-envelope viruses such as FCV. Thus, there is a possibility that the FPP acted as a detergent; however, the FPP is not reported to have any detergent ac-

A: LDH



B: MTT

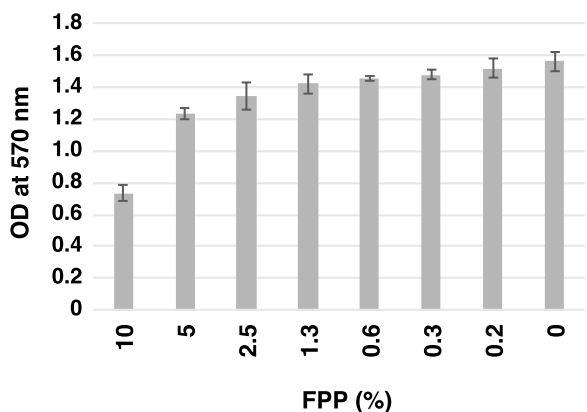


Figure 3. Cytotoxicity of the FPP. (A) MDCK cells were incubated with DMEM containing the FPP at different concentrations for 24 hr, and LDH level in the culture medium was evaluated. The value from High Control, incubated with NP-40, was set to 100%, and the relative values were plotted. Mean values from eight wells are plotted, and the error bars indicate standard deviation. (B) After incubation for 24 hr, the cells were treated with MTT for 4 hr, and OD at 570 nm was measured after cell lysis.

tivity.

One possible mechanism underlying the inhibition of IFV replication is that the binding of abundant sialic acids or their analogues in the FPP to the receptor-binding pocket of the IFV HA protein causes competition to the attachment of the virus to cells. Alternatively, some components of the FPP may bind to IFV particles specifically and block the infection by IFV. However, we have no evidence to confirm these hypotheses.

Another possibility is the low pH of the FPP. We found that the pH value of 10% FPP is approximately 4 (data not shown). The HA protein of an A-type IFV undergoes low-pH-induced conformational change to cause membrane fusion in endosomes, which results in the initiation of virus infection with the assumption that the HA protein is activated by limited proteolysis¹²⁾. The pH threshold is thought to be 5 or 5.5. The IFV particles used in this study had already been activated, and the HA proteins might have undergone irreversible conformational change when exposed to low pH, leading to the loss of infectivity. In contrast, FCV is thought to be stable at pH 5. Although extremely low pH is known to inactivate FCV, the pH for 50% FCV inactivation is approximately 3.9 (our unpublished observation).

The cytotoxicity of the FPP was examined using two methods: LDH leakage to examine membrane integrity and MTT test to examine mitochondrial reductase activity. The results obtained using both methods showed that 10% FPP apparently had cytotoxicity. However, IFV could replicate in MDCK cells in the presence of 10% FPP, as revealed by the results of the high MOI (=5) experiment.

In conclusion, the FPP *Manda Koso* has anti-IFV activity and could be beneficial in preventing IFV infection. However, because the FPP is not sufficient for use as an anti-viral reagent, it is necessary to develop a reagent based on the FPP that would be more effective and have less cytotoxicity.

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