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Received: December 25, 2018. Accepted: July 29, 2019
Published online: August 30, 2019
DOI:10.7883/yoken.JJID.2018.522

Advance Publication articles have been accepted by JJID but have not been copyedited or formatted for publication.
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Running head: Anti-influenza viral activity of umesu phenolics

Keywords: Prunus mume; phenolics; Antiviral; Virucidal; influenza virus

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SUMMARY. Umesu phenolics were purified from the salt-extracts of Japanese apricot (Nanko-mume cultivar of *Prunus mume* Sieb. et Zucc.). Characterization of umesu phenolics revealed that they inhibited the multiplication of influenza virus and several other RNA and DNA viruses, when added to the culture media of the infected cells. In addition to these antiviral activities, the phenolics significantly decreased a plating efficiency of influenza virus, if present in the virus inoculum. More drastic effects were observed in virucidal activity; the infectivity of several strains of influenza viruses decreased less than 0.001 when they were incubated with 4 mg/ml phenolics at 30°C for 5 min. The virucidal activity of phenolics was found to be more remarkable in acidic conditions, but the activity was not merely a result of the acidity of the phenolics. These results clearly indicate the antiviral and virucidal activities of the umesu phenolics against influenza viruses and suggest their potential pharmacological usefulness as a disinfectant or preventive medicine against superficial infections, such as the respiratory infection.

INTRODUCTION

Previously we have characterized antiviral and virucidal activities of daily dietary
products and their components (1-7). Umesu phenolics are among those compounds and were chromatographically purified from salt-extracts (i.e., “Umesu”, a by-product of manufacturing Japanese “Umeboshi”) of Japanese apricot (Nanko-mume, cultivar of Prunus mume Sieb. et Zucc.) (8). The phenolics were found to have many physiological activities, including antibacterial activities (9). Characterization of alkali hydrolysate of the phenolics revealed that the umesu phenolics comprise four main phenolic acids (i.e., caffeic acid, cis- and trans-p-coumaric acid and ferulic acid), indicating that the majority of the phenolics in this mume fruit are hydrocinnamic acid derivatives (10). We have here examined their antiviral activities against influenza as well as other viruses.

MATERIALS AND METHODS

**Cells and viruses.** Vero, HEp-2, CRFK and MDCK cells were grown in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum (FBS). Herpes simplex virus type 1 strain F (HSV-1), influenza viruses (IV), feline calicivirus F4 strain (FCV), poliovirus type 1 Sabin vaccine strain (PV-1) and Coxsackie B virus type 5 (CB5) were used. For IV, three different strains were used; i.e., strain A/PR/8/34 (H1N1) (A0PR8 virus), strain A/Bankok/83 (H1N1), strain A/RI/57 (H2N2), strain A/Aichi/2/68
(H3N2) (A/Aichi virus) and strain B/Tokyo/7/66 (B/Tokyo virus). The viruses were propagated in Vero cells in MEM supplemented with 0.5% FBS for HSV-1, PV-1 and CB5, in CRFK cells in MEM with 0.5% FBS for FCV or in MDCK cells in MEM with 0.1% bovine serum albumin (BSA), acetylated trypsin (5.3 μg/ml) and 0.01% DEAE-dextran for IV. The viruses were harvested after three cycles of freezing and thawing of the infected cells along with culture medium, except IV. IV was harvested from culture medium. The harvested viruses were centrifuged at 3,500 rpm for 15 min to remove the cell debris and the supernatant was used as a conventional virus stock and stored at –80°C. The amount of infectious virus was measured by a plaque assay.

**Preparation of umesu phenolics solution.** Umesu phenolics were kindly gifted from Wakayama Industry Promotion Foundation. A stock solution (100 mg/ml) of the umesu phenolics was prepared by dissolving the phenolics powder with distilled water or 0.2 M arginine solution (pH 5.0). The antiviral activities of the stock solutions were stable, at least, for two months when stored at 4°C in a light-shielded bottle. Although the umesu phenolics were soluble in distilled water at room temperature, they formed a significant amount of precipitation during storage in a refrigerator and needed to be warmed to make homogeneous solution before use. In some experiments, we prepared the phenolics in
arginine solution to keep the compounds soluble.

**Virus yield in the presence of the umesu phenolics.** To avoid multiple step growth in the infected culture, we carried out the infection at a multiplicity of infection (MOI) of 3 or 10. Virus-infected cells were incubated at 37°C for overnight in the serum-free MEM containing 0.1% BSA and the indicated concentrations of umesu phenolics. For influenza viruses, acetylated trypsin (5.3 μg/ml) and 0.01% DEAE-dextran were added.

**Assay for virucidal activity.** All the starting materials were stored on ice prior to experiments. As described previously (7, 11-13), a 190 μl aliquot of the buffer solutions with or without umesu phenolics received a 10 μl aliquot of the virus preparations and were incubated at 30°C for 5 min. After incubation, aliquots of these virus samples were immediately 100-fold diluted with ice-cold Dulbecco’s phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS) containing 0.1% BSA for influenza viruses or 0.5% FBS for other viruses to prevent further virus inactivation. In addition to the ice-cold temperature, both the excess dilution and the presence of proteins in diluent solution can inhibit further inactivation by the virucidal activity of phenolics. Then, the residual infectivity in the samples was determined by a plaque assay and was normalized to that of incubation in
PBS to obtain a relative infectivity.

**Effect of the umesu phenolics on the viral adsorption.** Viral adsorption to cells were examined as follows. Monolayered cells in 50 mm-dishes were incubated at room temperature with 0.5 ml PBS containing about 200 PFU of influenza virus and various concentrations of the umesu phenolics for 60 min with mechanically rocking on a rocker platform. After viral adsorption procedure, the supernatant containing free virus and phenolics was removed from dishes and the cell monolayers on the dishes were washed with 2 ml of PBS to remove the loosely bound virus and the phenolics. The cell monolayers were incubated in the MEM containing 0.8% agar and acetylated trypsin (5.3 mg/ml) and 0.01% DEAE-dextran at 37°C for 2 days, allowing the formation of plaques. The plating efficiencies of the virus in the presence of the phenolics were determined by dividing the number of plaques in the presence of the phenolics by the number in the absence of the phenolics.

**Data analysis and statistics.** All the experiments were repeated at least three times. The results shown in the figures are based on the averages, except Fig. 4. Average values and their standard deviation were calculated by Excel in Microsoft Office 2013. Error bars
represent the range of two times the standard deviation (2σ) of each average value at each x-axis point in the figures, indicating the Confidence interval of 95%. For the time course experiments (Fig. 4) we carried out the experiment more than three times under the similar, but not the same conditions and the most typical results are shown in the “Results” section. In addition, data were statistically analyzed by ANOVA followed by Dunnett’s test and considered to be significantly different when the p-value was less than 0.05. StatFxex (ver. 6.0, Artec Co. Ltd., Osaka, Japan) was used as statistical software.

RESULTS

**Antiviral activities of the umesu phenolics.** Figure 1 shows the effects of umesu phenolics on the multiplication of HSV-1 in HEp-2 cells, A0PR8 virus in MDCK cells, PV-1 and CB5 in Vero cells and FCV in CRFK cells. When the virus-infected cells were incubated in the presence of various concentrations of umesu phenolics, the final yields of these viruses decreased concentration-dependently, although the sensitivity of each virus to the phenolics differed. For example, relative progeny virus yield at 4 mg phenolics/ml of HSV-1, A0PR8 virus, PV-1, CB5 and FCV was 0.08, 0.014, 0.51, 0.25 and 0.038, respectively. These results indicate that the intracellular multiplication of both
enveloped and non-enveloped viruses and both DNA and RNA viruses in different types of cells were suppressed by the phenolics in culture media. Among these viruses, influenza virus showed the highest sensitivity.

We have reported before that accelerated death of virus-infected cells can cause abortion of the virus multiplication, resulting in the suppression of the viral growth (14-16). Thus, we examined cytotoxic effects of the umesu phenolics by incubating uninfected HEp-2 or MDCK cells in the serum-free MEM containing 0.1% BSA and various concentrations (0 to 10 mg/ml) of umesu phenolics at 37°C for 24 h. Although a small, but increasing fraction of cells showed rounding above 6 mg/ml, the viabilities of the cells, determined by a dye-exclusion test, were not significantly affected by the incubation with the phenolics even at 10 mg/ml (data, not shown), supporting that the observed antiviral effects of umesu phenolics were not a secondary result of the cytotoxic effects of phenolics.

To understand the mechanism of the inhibition of the multiplication by the phenolics, one-step growth curve was examined with A/Aichi virus and B/Tokyo virus. No delay in the onset of progeny virus formation was observed in the presence of phenolics, although the number of progeny viruses was lower than in the absence of phenolics at any time point of viral multiplication (data not shown).
**Effect of the umesu phenolics on the influenza virus adsorption.** Because influenza virus showed the highest sensitivity to the umesu phenolics, we further examined the effects of umesu phenolics on the initial step of virus infection. When various concentrations of the phenolics were added to the virus inoculum, the plating efficiency of A0PR8 virus was significantly reduced (Fig. 2). Less than 10% of the input virus successfully formed plaques with 10 mg/ml phenolics. In addition, Fig. 2 showed that, if the phenolics were prepared in 0.2 M arginine solution, 5 mg phenolics/ml was enough to achieve the similar level of inhibition. These results indicate that, in addition to the suppression of intracellular viral multiplication (Fig. 1), the umesu phenolics can affect the influenza virus adsorption to the cell surface. This observed inhibition does not appear to be due to irreversible binding of the phenolics to the cell surface, because pretreatment of the cells with phenolics did not so noticeably affect the plating efficiency (Fig. 2).

**Virucidal effect of the umesu phenolics.** Next, we examined a possible direct effects of the phenolics on the influenza virus infectivity. We analyzed three different strains of influenza virus; i.e., A0PR8 virus (H1N1 subtype of influenza A virus), A/Aichi virus
(H3N2 subtype of influenza A virus) and B/Tokyo virus (influenza B virus), because these types and subtypes are most commonly occurring influenza viruses among human population.

As shown in Fig. 3, the infectivity of all these three viruses markedly decreased by the umesu phenolics. When the phenolics were prepared in distilled water, the infectivity decreased to 0.01 for A0PR8 virus, 0.2 for A/Aichi virus and 0.002 for B/Tokyo virus at the concentration of 2 mg/ml. At higher concentrations (4 mg/ml or higher), all these three viruses were inactivated to the similar degree, showing that the umesu phenolics have a remarkable ability to inactivate influenza viruses with different types and subtypes.

A similar degree of inactivation was observed for other human strains, such as A/Bankok/83 (H1N1) and A/RI/57 (H2N2).

Previously we found that aqueous arginine effectively increased the solubility of organic substances and stabilized homogeneous solution (17, 18). When the umesu phenolics stock solution was prepared in 0.2 M arginine at pH 5.0, the virucidal effects of the phenolics were significantly enhanced (Fig. 3); at 4 mg/ml, the residual infectivity of A0PR8 virus was 0.0001; i.e., 10-fold less than that achieved by the phenolics preparation with distilled water.
Role of acidity in the virucidal action of umesu phenolics. It has been well-known that influenza viruses are sensitive to acidity; even mildly acidic pH, such as pH 5, may affect virus infectivity. When the umesu phenolics were dissolved with distilled water or the arginine solution, pH value of the solution showed approximately 5 at the concentration of 2 mg/ml. It is thus possible that the observed effects of the umesu phenolics are due to acidity, not the compounds themselves. Figure 4 shows the effects of umesu phenolics on time course of virus inactivation. A0PR8 virus or A/Aichi virus was incubated in the aqueous phenolics solutions or in several other solvents, such as PBS and 10 mM sodium citrate buffer (pH 4.9 or 5.4). The pH of these solutions were measured immediately after the experiments and were described in the legend of Fig. 4. As shown in Fig. 4A, A0PR8 virus infectivity was not remarkably affected by the incubation in the PBS or in the citrate buffer (pH 5.4), but decreased with time in the citrate buffer (pH 4.9). The 1.0 mg/ml phenolics solution at pH 5.4 greatly reduced infectivity compared to the pH 5.4 buffer that had limited effect. The magnitude of reduction was identical to the pH 4.9 buffer. The 1.5 mg/ml phenolics solution at pH 4.9 resulted in far greater reduction than the pH 4.9 buffer. These results clearly indicate that the observed virucidal activity of umesu phenolics is not merely the result of the acidity, but the result of intrinsic nature of the phenolics. The same conclusion was obtained from
the results with A/Aichi virus (Fig. 4B). The 1.5 mg/ml phenolics solution (pH 5.1) and the 2.0 mg/ml phenolics solution (pH 4.8) resulted in far greater reduction of infectivity than the pH 4.9 buffer despite their similar pH values.

This conclusion was further confirmed by examining the effect of pH on the virucidal activity of umesu phenolics. As shown in Fig. 5, A0PR8 virus was incubated in the citrate buffer at various pH containing 0 or 2 mg/ml phenolics. In the absence of the phenolics, infectivity was not reduced up to pH 5.6, followed by a sharp reduction below the pH. The 2.0 mg/ml phenolics solution augmented the effects of acid, resulting in significant reduction even at pH 5.8. At each point below pH 5.8, infectivity reduction was greater in the presence of 2.0 mg/ml phenolics. Similar results were observed with B/Tokyo virus (data not shown). Characterization of acid-sensitivity of the viruses (Figs. 4 and 5) confirmed that this virucidal effect is not simply a result of acidic pH of aqueous phenolic solution.

Because suppression of microbicidal activity of disinfectants by proteins has been generally recognized, we examined an effect of the addition of BSA on the virucidal activity of the phenolics. As shown in Fig. 6, when A0PR8 virus was incubated with umesu phenolics in the presence or absence of 0.25% BSA, the reduction of virus infectivity was apparently suppressed by BSA. However, when we measured the pH of
the incubation mixtures, the mixture containing 2.0 or 4.0 mg phenolics/ml showed pH 4.7 or 4.4 in the absence of BSA but pH 5.1 or 4.9 in the presence of BSA, suggesting that the pH values at any phenolics concentrations in the presence of 0.25% BSA were about 0.5 pH unit higher than those in the absence of BSA and that the apparent suppression of virucidal activity by BSA can be explained by buffering effect of BSA on acidic pH of the phenolics solutions.

DISCUSSION

Umesu phenolics are abundant ingredient of Japanese apricot which is very familiar to Japanese people. The phenolics content of mume fruit is about 1% of the flesh of matured fruit on a dry weight basis (10). The present study demonstrated the antiviral and virucidal activities of the umesu phenolics against influenza viruses; i.e., the inhibition of both intracellular multiplication (Fig. 1) and adsorption step (Fig. 2) of the virus and the inactivation of viral infectivity (Fig. 3). Although the virucidal effect was the most prominent, this effect does not likely affect the results of other two processes, because the virucidal effect was strongly dependent on acidic pH (Figs. 4 and 5) while the other two effects (on multiplication and adsorption) were observed at neutral pH. This difference in pH-dependence may imply that the mechanism of the observed
antiviral effects and that of virucidal effect are different.

Although umesu phenolics are soluble in water, stock phenolics solution, prepared in 0.2 M arginine, was significantly more effective in viral inactivation (Fig. 3) and suppression of viral adsorption (Fig. 2). This enhancement of phenolics action is likely due to increased dissolution or dispersion of phenolics molecules by arginine (18). Considering that arginine is a safe material, as one of digestive amino acids, this role of arginine in effective preparation of the phenolics would be a practically important and useful finding for the application of the phenolics as a disinfectant or preventive medicine against viral infection on the body surface.

Although we cannot elucidate the mechanism of action of umesu phenolics, polyphenols have been shown to bind to proteins and, in the case of enzyme, can inactivate some (19, 20). We speculated that binding of the phenolics to certain component(s) of virus particle and cellular proteins is likely the cause of the virus inactivation and changes in physiological status of the cells, leading the cells to antiviral state.

**Acknowledgements** This work was supported in part by research grants from the Kishu Tanabe Ume Promotion Committee, Wakayama and by those from “The Consortium of
High Education Institutions” in Wakayama Prefecture. The authors thank Dr. Tsutomu Arakawa (Alliance Protein Lab. CA, USA) for his stimulative discussion and Dr. Tomomi Kuwahara (Kagawa Univ. Kagawa, Japan) for the statistical processing.

**Conflict of Interest.** T.M. and A.H.K. have a patent JP6049533, licensed to JA-Kinan and Tanabe City.

**REFERENCES**

1. Arakawa T, Yamasaki H, Ikeda K, et al. Antiviral and virucidal activities of natural products. Curr Med Chem. 2009;16:2485-97.

2. Ikeda K, Tsujimoto K, Uozaki M, et al. Inhibition of multiplication of herpes simplex virus by caffeic acid. Int J Mól Med. 2011;28:595-8.

3. Murayama M, Tujimoto K, Uozaki M, et al. Effect of caffeine on the multiplication of DNA and RNA viruses. Mol Med Rep. 2008;1:251-5.

4. Uozaki M, Yamasaki H, Katsuyama Y, et al. Antiviral effect of octyl gallate against DNA and RNA viruses. Antiviral Res. 2007;73:85-91.

5. Utsunomiya H, Ichinose M, Ikeda K, et al. Inhibition by caffeic acid of the influenza A virus Multiplication *in vitro*. Int J Mol Med. 2014;34:1020-1024.
6. Utsunomiya H, Ichinose M, Uozaki M, et al. Antiviral activities of coffee extracts in vitro. Food Chem Toxicol. 2008;46:1919-24.

7. Yamasaki H, Tsujimoto K, Koyama AH, et al. Arginine facilitates inactivation of enveloped viruses. J Pharm Sci. 2008;97:3067-73.

8. Mitani T, Mimura H, Horinishi A, et al. Chemical futures of phenolic extracts prepared on an industrial scale from a processing byproduct of the Japanese apricot, Mume fruit (Prunus mume Sieb et Succ.). Japan Journal of Food Engineering 2017;18:147-52.

9. Mitani T, Ota K, Inaba N, et al. Antimicrobial Activity of the Phenolic Compounds of Prunus mume against Enterobacteria. Biol Pharm Bull. 2018;41:208-12.

10. Mitani T, Horinishi A, Kishida K, et al. Phenolics profile of mume, Japanese apricot (Prunus mume Sieb. et Zucc.) fruit. Biosci Biotechnol Biochem. 2013;77:1623-27.

11. Tsujimoto K, Uozaki M, Ikeda K, et al. Solvent-induced virus inactivation by acidic arginine solution. Int J Mol Med. 2010;25:433-7.

12. Ikeda K, Yamasaki H, Minami S, et al. Arginine inactivates human herpesvirus 2 and inhibits genital herpesvirus infection. Int J Mol Med. 2012;20:1307-12.

13. Goda H, Ikeda K, Nishide M, et al. Characterization of virucidal activities of chlorous Acid. Jpn J Infect Dis. 2018;71:333–7.
14. Koyama AH, Miwa Y. Suppression of apoptotic DNA fragmentation in herpes simplex virus type 1-infected cells. J Virol. 1997;71:2567-71.

15. Koyama AH, Arakawa T, Adachi A. Acceleration of virus-induced apoptosis by tumor necrosis factor. FEBS Lett. 1998;426:179-82.

16. Koyama AH, Fukumori T, Fujita T, et al. Physiological significance of apoptosis in animal virus infection. Microbes Infect. 2000;2:1111-7.

17. Arakawa T, Kita Y, Koyama AH. Solubility enhancement of gluten and organic compounds by arginine. Int J Pharm. 2008;335:220-3.

18. Arakawa T, Uozaki M, Koyama AH. Modulation of small molecule solubility and protein binding by arginine. Mol Med Rep. 2010;3:833-6.

19. Kang NJ, Lee KW, Shin BJ, et al. Caffeic acid, a phenolic phytochemical in coffee, directly inhibits Fyn kinase activity and UVB-induced COX-2 expression. Carcinogenesis 2009;30:321-30.

20. Trnkova L, Bousova I, Kubicek V, et al. Binding of naturally occurring hydroxycinnamic acids to bovine serum albumin. Natural Science 2010;2:563-70.
Figure 1. Effects of umesu phenolics on the virus yields. Confluent monolayers of HEP-2 (for HSV-1, PV-1 and CB5), MDCK (for A0PR8 virus) or CRFK (for FCV) cells were infected with each of these viruses at an MOI of 10 for HSV-1, PV-1 and FCV, 3 for A0PR8 virus and CB5. The infected cells were incubated for overnight in the medium containing varying concentrations of umesu phenolics at 37°C for HSV-1, A0PR8 virus, FCV and CB5 or at 35.5°C for PV-1. At the end of incubation, the amounts of infectious progeny viruses were determined and normalized to the virus yield in the absence of the phenolics. ○, HSV-1; △, A0PR8 virus; □, FCV; ◇, PV-1; ◆, CB5. Compared to the sample without the phenolics (i.e., 0 mg/ml), P value was below 0.01 for the measured points of all viruses except for those of CB5. P value for the points of CB5 was below 0.05.
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Figure 2. Effect of umesu phenolics on the adsorption of A0PR8 virus. The virus was diluted in PBS containing the indicated concentrations of the phenolics, inoculated onto monolayer of MDCK cells and allowed the adsorption to the cells. The number of plaques was normalized to that in the absence of the polyphenols. The stock phenolics solution was prepared with distilled water (○) or 0.2 M arginine, pH 5.0 (●). The plating efficiency on phenolics-pretreated cells (△) was determined after cell monolayers were incubated in MEM containing indicated concentrations of the phenolics at 37°C for 60 min, followed by removal of the medium, by washing with PBS and by the virus infection as above in the absence of the phenolics. Compared to the sample without the phenolics (i.e., 0 mg/ml), P value was below 0.01 for all measured points except those of the pretreated cells (△) and treated cells with 0.5 mg/ml with the phenolics dissolved in water. For the pretreatment experiment, P value showed NS (no significant difference) for the points at 2 or 4 mg/ml, below 0.05 at 6 mg/ml and below 0.01 at 8 mg/ml.
efficiency on phenolics-pretreated cells (△) was determined after cell monolayers were incubated in MEM containing indicated concentration of phenolics, followed by removal of the medium, by washing with PBS and by the virus infection as above in the absence of the phenolics. Compared to the sample without the phenolics (i.e., 0 mg/ml), P value was below 0.01 for all measured points except those of the pretreated cells (△) and treated cells with 0.5 mg/ml with the phenolics dissolved in water. For the pretreatment experiment, P value showed NS (no significant difference) for the points at 2 or 4 mg/ml, below 0.05 at 6 mg/ml and below 0.01 at 8 mg/ml.

Figure 3. Virucidal effects of umesu phenolics on influenza viruses. Aliquots of virus preparation were incubated at 30°C for 5 min in the presence of various concentrations of umesu phenolics. The phenolics were prepared with distilled water (○, △, □) or with 0.2 M arginine, pH 5.0 (●). The number of infectious virus after the incubation was determined by a plaque method. ○ and ●, A0PR8 virus; △, A/Aichi virus; □, B/Tokyo virus. Compared to the sample without the phenolics (i.e., 0 mg/ml), P value was below 0.01 for all measured points except at 0.5 mg/ml of B/Tokyo virus (P value showed no significant difference) and 1 mg/ml of A/Aichi virus (p < 0.05).
Figure 4. Time course of virus inactivation in the presence or absence of umesu phenolics at different pHs. (A) A0PR8 virus was incubated at 25°C for 0, 4, 8, 12 or 16 min in distilled water, containing the indicated concentration of the phenolics, or in 10 mM sodium citrate buffer. The pH was measured immediately after the incubation, as shown in the brackets below. The number of infectious viruses after the incubation was determined and normalized to that incubated in PBS. ○, 1.0 mg/ml (pH 5.4); △, 1.5 mg/ml (pH 4.9); ■, 0 mg/ml (pH 4.9); ●, 0 mg/ml (pH 5.4); ▲, PBS (pH7.2). (B) A/Aichi virus was used instead of A0PR8 virus and the incubation temperature was at 30°C. △, 1.5 mg/ml (pH 5.1); □, 2.0 mg/ml (pH 4.8); ■, 0 mg/ml (pH 4.9); ●, 0 mg/ml (pH 5.4); ▲, PBS (pH7.3).
**Figure 4.** Time course of virus inactivation in the presence or absence of umesu phenolics at different pHs. (A) A0PR8 virus was incubated at 25°C for 0, 4, 8, 12 or 16 min in distilled water, containing the indicated concentration of the phenolics, or in 10 mM sodium citrate buffer. The pH was measured immediately after the incubation, as shown in the brackets below. The number of infectious viruses after the incubation was determined and normalized to that incubated in PBS. ○, 1.0 mg/ml (pH 5.4); △, 1.5 mg/ml (pH 4.9); ■, 0 mg/ml (pH 4.9); ●, 0 mg/ml (pH 5.4); ▲, PBS (pH 7.2). (B) A/Aichi virus was used instead of A0PR8 virus and the incubation temperature was at 30°C.

**Figure 5.** Effects of pH on the virus inactivation by umesu phenolics. A0PR8 virus was incubated at 30°C for 5 min in 10 mM citrate buffer at the indicated pH in the presence (●) or absence (〇) of 2.0 mg/ml umesu phenolics. The number of infectious virus after the incubation was determined and normalized to the virus infectivity when incubated in PBS. Compared to the sample without the phenolics (i.e., 0 mg/ml), P value was below 0.01 for the measured points below pH 5.6 and showed NS (no significant difference) for the points above pH 5.8, in the buffer solutions containing or not the phenolics.
Figure 6. Effects of protein on the virucidal activity of umesu phenolics. Virucidal effects of umesu phenolics on A0PR8 virus were examined in the presence (△) or absence (○) of 0.25% BSA. Aliquots of virus preparation were incubated at 30°C for 5 min with various concentrations of umesu phenolics. The number of infectious virus after the incubation was determined by a plaque method. Compared to the sample without the phenolics (i.e., 0 mg/ml), P value was below 0.01 for all measured points.