Inhibitory Effects of *Goishi* Tea against Influenza Virus Infection.

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Abstract

We noticed G*oishi* tea, which is a Japanese traditional microbial fermented tea, and investigated an anti-influenza virus function in *in vitro* and *in vivo* tests. As results, we found that *Goishi* tea hot water extract (GTE) inhibited hemagglutination caused by influenza A/ Puerto Rico/ 8/ 34 (PR8, H1N1) and influenza A/ Guizhou/ 54/ 89 (Guizhou, H3N2) virus, viral growth in Mardin-Darby canine kidney (MDCK) cells caused by PR8 and viral infection of mice caused by nasal inoculation of the PR8. Furthermore, we investigated the functional fractions of GTE and found that a high molecular fraction in GTE inhibited viral adsorption to MDCK cells and low molecular fractions inhibit subsequent stages, such as viral invasion, genomic multiplication and release stage on the cells, after the adsorption. These results indicate that GTE exerts an inhibitory property against influenza virus infection by inhibiting various stages of infection.

key words: influenza virus, microbial fermented tea, polymeric phenol, inhibition.

Introduction

Influenza virus (Flu), especially influenza virus A type, has multiple subtypes and mutates antigenicity every year. For this reason, it is hard for us to prevent its infection. To make matters worse, we don't have effective and safe drugs and influenza infection sometimes causes pneumonia and encephalitis in older adults and children (Kappagoda *et al*., 2000; Munoz, 2003; Watkins, 2004). It is important to reduce excess influenza deaths in these people.

It has been reported that some kinds of tea have anti-Flu activity. Tea has been loved by people in the worldwide from a long time ago. Based on the manufacturing technique, teas can be classified as non-oxidized tea, semi-oxidized tea, fully oxidized tea and microbial fermented tea, for example, green tea, oolong tea, black tea and pu-erh tea, respectively (Yamamoto *et al*., 2002). There are a lot of differences in the components among these teas. These differences are affected by not only a variety of tea leaves but also manufacturing process (Horie *et al*., 2002).

Green tea and black tea especially have been studied for a long time for antiviral effects. It has been proven that (-)-epigallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin in green tea have the ability to inhibit adenovirus and Flu replication using the cell culture method and have a potentially direct virucidal effect (Imanishi *et al*., 2002; Nakayama *et al*., 1993; Song *et al*., 2005; Weber *et al*., 2003). Furthermore, it has been evaluated that theaflavins such as flavan-3-ol in black tea have inactive function against both rotavirus and coronavirus in *in vitro* test, and inhibit the infectivity of both influenza A virus and influenza B virus in Mardin-Darby canine kidney (MDCK) cells (Clark *et al*., 1998; Nakayama *et al*., 1993). In addition, it has been reported that flavonoid, 5,7,4'-trihydroxy-8-methoxyflavone and tannin acid in some plants inactivate Flu, too (Carson *et al*., 1953; Miki *et al*., 2007; Nagai *et al*., 1990; Nagai *et al*., 1995).

Goishi tea is a traditional microbial fermented tea in Japan. Microbial fermented tea is generally fermented by fungus. However, *Goishi* tea is rare on a global scale because of the use of fungus and lactic acid bacteria (LAB). This tea uses the same leaf as green tea, *Camellia sinesis*. However,

there is a difference in manufacturing process. Green tea is produced by steaming and dehydrating the leaf, whereas *Goishi* tea is produced by fermentation of fungus on the first step and LAB on the second step. Anti-Flu function of some kinds of teas has been already reported. But *Goishi* tea has not been reported yet. Consequently, we noticed GTE and studied the anti-Flu function in *in vitro* and *in vivo* tests which used chicken blood erythrocytes, MDCK cells and mice, respectively. Black tea hot water extract (BTE) and green tea hot water extract (GRTE) were tested as controls in various experiments. Furthermore, we investigated functional materials in GTE and indicated that high molecular weight substances in GTE inhibited viral adsorption and low molecular weight substances inhibited subsequent stages after the adsorption.

Materials and Methods

Preparation of tea hot water extracts.

Various tea extracts were prepared by boiling 20 g of their leaves in 1000 ml distilled water at 100℃ for 30 min. Their tea extracts were quickly separated from their leaves by filtration, and freeze-dried.

Viruses.

Influenza A/ Puerto Rico/ 8/ 34 virus (PR8, H1N1) and influenza A/ Guizhou/ 54/ 89 virus (Guizhou, H3N2) were grown in the allantoic sacs of 11-day-old chicken embryos for 2 days at 35.5℃ by a previously reported method (Yasui *et al*., 1999). The allantoic fluid was removed and stored at -80℃. The titer of virus in allantoic fluid was expressed as the 50% tissue-culture infectious dose ($TCID_{50}$) and 50% egg-infecting dose (EID_{50}) for PR8 and Guizhou, respectively. The titer of virus of allantoic fluid of PR8 and Guizhou was $10^{7.4}$ $TCID_{50}$/ml and $10^{10.6}$ EID_{50}/ml, respectively.

Chicken erythrocytes and MDCK cells.

Chicken erythrocytes were prepared from blood samples of chicken by centrifugation,

suspended by alsever solution and stored at 4℃. Mardin-Darby canine kidney (MDCK) cells were cultured in the Earle's MEM medium (MEM; GIBCO) supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic antimycotic solution (SIGMA).

Mice.

Male BALB/c mice (8-week-old) were purchased from Japan SLC co., Ltd (Hamamatsu, Japan). The animals were housed in an air-conditioned room maintained at 24 ± 2℃ with a relative humidity of 55 ± 15%. They were given standard laboratory rodent feed (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. All procedures were conducted by the Guidelines for Animal Experiment at the Shinshu University and approved by the Ethical Committee at the Shinshu University.

Hemagglutination inhibition test.

Hemagglutination inhibition (HI) activity was measured to test the effect of samples on virus adsorption to target cells (Glaser *et al*., 2005; Robert *et al*., 2000). 50 μl of samples with phosphate buffered saline (PBS) contained 0.1% bovine serum albumin (BSA) were mixed with 50 μl of PR8 ($10^{6.5}$ $TCID_{50}$/ml) or Guizhou ($10^{9.9}$ EID_{50}/ml) solution at room temperature for an hour in the first row on a 96 well disposable plate. After the reaction, the mixture of sample and virus was made into serial 2-fold dilutions by transferring 50 μl from the first well to successive right well with 50 μl of PBS contained 0.1% BSA. Next, 50 μl of 0.5% chicken erythrocytes solution with PBS contained 0.1% BSA was added to each well on the plate. The plate was settled at room temperature for an hour. The highest dilution of virus that causes complete hemagglutination (HA) was determined as the HA titer value (Kiyoshima *et al*., 2001; Nakayama *et al*., 1993).

Viral growth inhibition test.

Viral cytopathic inhibition assay was employed to test the effect of tea extracts on various stages of viral infection to target cells (Imanishi *et al*., 2002; Nakayama *et al*., 1993; Sidwell 2000; Song *et al*., 2005). Culture supernatant of confluent monolayer MDCK cells cultured in 96 well tissue culture plate (FALCON, 200 μl of 3×10^5 cells/ml) was removed and serum-deprived MEM was

replaced, and the cells were incubated at 37℃ with 5% CO_2 for an hour. After that, the supernatant was removed and the cells were infected with 25 μl of PR8 solution ($10^{2.4}$ $TCID_{50}$/ml) for an hour. Each sample was added at various stages of infection to MDCK cells; for testing the inhibition on the adsorption stage, various concentrations of samples mixed with equal volume of virus solution at room temperature for an hour before infection were added to cells, and for testing the inhibition on subsequent stages, 100 ml of maintenance medium (MEM containing 0.2% BSA and 5 μg/ml trypsin acetylated) containing various concentrations of the sample were added to cells after 1, 9, and 17 h from infection. After virus adsorption for an hour, 200 μl or 100 μl of maintenance medium were added to these cells for the inhibition assay on adsorption stage or subsequent stages, respectively, and these cells were incubated at 37℃ with 5% CO_2 for 3 days. Then, 50 μl of culture supernatant was moved to a 96 well disposable plate (round-bottomed plate; AS ONE corporation) and 50 μl of 0.5% chicken erythrocytes solution with PBS contained 0.1% BSA was added to each well on plate. The plate was settled at room temperature for an hour. A required concentration to show the 50% inhibitory concentration (IC_{50}) was calculated by regression analysis of the dose-response curves generated from these data.

***In vivo* test**

Male BALB/c mice (8-week-old) were anesthetized by an intraperitoneal injection of nembutal anesthesia (65 μg/g body weight). 198 μl of 0 (control group), 0.5 (GTE-0.5 group), or 2.0 (GTE-2.0 group) mg/ml of GTE dissolved in PBS containing 0.1% BSA was mixed with 2 μl of PR8 solution with PBS contained 0.1% BSA ($10^{6.8}$ $TCID_{50}$/ml) at 37℃ for 3 min. Lower respiratory tract injection was done by dropping 10 μl of fluid containing the mixture of GTE and PR8 solution into each nostril (20 μl per mouse). The morbidity and mortality of infected mice was observed for 2 weeks. Determination of morbidity was measured by raising of fur, slow-movement and decrease of body weight (Kiyoshima *et al*., 2001; Yasui *et al*., 2004).

Fractionation of GTE

GTE dissolved in distilled water was applied to a BOND ELUT 500 mg C18 cartridge

(VARIAN co.), preconditioned with methanol and acidified water. The column was washed with 30 ml of acidified water, and the fraction of GTE was recovered consecutively with 20 ml of 8%, 25% and 45% methanol, and separated fraction 1 (F1), fraction 2 (F2) and fraction 3 (F3), respectively. All of the fractions were evaporated under reduced pressure at 40℃ until all organic solvent was removed. They were re-dissolved into distilled water, frozen and then freeze-dried. Then they were analyzed by HPLC. Chromatographic separation was carried out on a Luna 5 μ C18 column (150 × 4.6 mm, Phenomenex, Inc., Torrance, CA) at 40℃. Solvents were 0.1% trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The gradient program was started with 5% B and changed to obtain 15% B at 30 min, 40% B at 45 min and 75% B at 50 min. The 75% B was maintained until 65 min. The flow rate was 1.0 ml/min, and the injection volume was 20 μl. Detection was performed at 280 nm for flavan-3-ol and 325 nm for hydroxycinnamic derivatives on a Shimadzu SPD-M10Avp photodiode array detector (Hamauzu *et al*., 2007).

Total phenolic assay

Total polymeric phenol content was determined by the Folin-Ciocalteu method. 500 μl of sample solution was mixed with 500 μl of diluted Folin-Ciocauteu reagent (1N) in a test tube. After 3 min of reaction, 500 μl of 10% Na_2CO_3 was added and the mixture was incubated for 60 min at room temperature. The absorbance was measured at 700 nm with Shimadzu UV-1200 spectrophotometer (Tokyo, Japan) against a blank (500 μl distilled water, plus reagents). (-)-Epicatechin was used as the standard (r=0.999 75) (Hamauzu *et al*., 2007).

Molecular weight analysis of GTE fractions using centrifugal concentration.

Molecular weights of various fractions were tested by microcon (molecular weight cut off: 3,000, 10,000, 30,000 and 50,000; Millipore corporation), and after the centrifugation all samples were analyzed by HPLC.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical evaluation for the difference of sample untreated and treated groups on the HI test was performed by the Williams test.

Statistical evaluation for the difference of accumulated symptom rate and survival rate between control group and experimental group was performed by a log rank test. A probability value of less than 0.05 was considered statistically significant.

Results

Effects of GTE on adsorption of Flu to chicken blood erythrocyte.

Flu has an ability to adsorb to chicken blood erythrocytes resulting in HA. Additionally, the action is similar to the phenomenon in which the virus adsorbs to target cells (Glaser *et al*., 2005; Kiyoshima *et al*., 2001; Nakayama *et al*., 1993; Sidwell *et al*., 2000). We investigated whether GTE could interfere with the viral adsorption to chicken blood erythrocytes resulting in HI. GTE and BTE at the concentration of 2.0 mg/ml significantly inhibited HA caused by PR8 ($P<0.01$) (Fig.1 A). Furthermore, GTE and BTE at the concentration of 2.0 mg/ml were significantly effective against Guizhou virus, too ($P<0.05$) (Fig.1 B). On the other hand, GRTE didn't inhibit HA against both PR8 and Guizhou.

Effects of GTE on cytopathy of MDCK cells by PR8.

Flu multiples through four infecting steps such as adsorption, invasion to target cells, replication of viral genome in the cells and release from the cells (Imanishi *et al*., 2002; Nakayama *et al*., 1993; Sidwell *et al*., 2000; Song *et al*., 2005). Therefore we tested which steps GTE inhibited. Some samples were added at various times of pre- or post-infection in MDCK cells and the inhibition of cytopathic effect (CPE) were observed. 50% inhibitory concentration (IC_{50}) of each sample is summarized in Fig. 2. Virtual reduction in virus yields was observed for all samples and all time added samples. GTE showed the highest inhibitory activity among the all sample on the all stages, and exhibited maximum inhibitory activity on the adsorption stage (IC_{50}: 0.04 mg/ml). On subsequent stages after the adsorption, GTE was effective about 2-fold higher than other samples (IC_{50}: 0.23, 0.21, 0.25 mg/ml when the sample was added after 1, 9 or 17 hours from infection,

respectively (Fig. 2.).

Overall, the inhibitory effect of GTE was observed throughout various stages of the virus replication cycle after initial infection. The results suggest that the antiviral effect of GTE exerts not only on the viral adsorption to the target cells but also on subsequent stages such as viral invasion, genomic multiplication, and viral release after the adsorption. This test was performed twice and the two experiments yielded similar results. Furthermore, all samples at the concentration of 1.0 mg/ml didn't indicate cytotoxic activity on MDCK cells.

Effects of GTE on accumulated symptom rate and survival rate of mice inoculated with PR8.

By using HI test and inhibitory test of CPE on MDCK cells, we have already been clear that GTE has high anti-Flu activity. Next, we investigated whether GTE protected against Flu infection on mice. Fig. 3 showed the time course of the accumulated symptom rate and the survival rate after the inoculation of GTE-treated PR8. The accumulated symptom and survival rate of GTE-2.0 group were significantly lower ($P<0.05$) and higher ($P<0.01$) than those of the control group, respectively. Furthermore, the survival rate of GTE-0.5 group were also significantly higher ($P<0.05$) than that of the control group. Although all mice of GTE-0.5 group developed the symptoms, some mice recovered during the experimental period.

Property and function of various fraction of GTE.

We searched functional materials in GTE. GTE dissolved in distilled water was applied to solid-phase extraction and separated three fractions. Fig. 4 shows the chromatogram recorded at 280 nm by HPLC. It can be observed that most quantities of GTE materials were eluted in F1, although a small quantity was also collected in F2 and F3.

Next, we determined molecular weight of all fractions in order to understand one property of GTE. The constituents of F1 and F2 samples were distributed in low molecular weight of less than 3,000. On the other hand, the constituents of F3 sample were distributed in high molecular weight from 30,000 to 50,000. Furthermore, total polymeric phenol contents in F1, F2 and F3 samples was 0.26, 0.34 and 0.42g per gram of each fraction, respectively (Table. 1). Furthermore, the quantity of

total polymeric phenols in non-fraction was 0.22 g per gram of GTE (data not shown).

Anti-influenza virus function of each fraction of GTE.

We investigated bioactivity of these fractions and non-fraction. Fig. 5 shows HI activity of various fractions and non-fraction of GTE. All samples at the concentration of 2.0 mg/ml significantly inhibited HA caused by PR8 ($P<0.01$). Furthermore, F3 sample and non-fraction at the concentration of 0.2 mg/ml also significantly inhibited the HA by PR8 ($P<0.01$ and $P<0.05$, respectively). F1 and F2 samples at the concentration of 0.2 mg/ml did not inhibit HA caused by PR8.

Additionally, Fig. 6 shows the effect of each fraction and non-fraction sample of GTE on various stages of viral infection. The inhibitory activity on the adsorption stage was very high in non-fraction and F3 samples (IC_{50}: 0.14 and 0.20 mg/ml, respectively). On subsequent stages, the inhibitory activity of F1 and F2 samples were equivalent and were higher than non-fraction sample. However, F3 sample was lower in activity than non-fraction sample when it was added at 9 or 17 hours after infection. In these tests, F3 sample inhibited mainly the adsorption stage. Furthermore, F1 and F2 samples showed a high effect on subsequent stages after the adsorption. This test was performed twice, and the two experiments yielded similar results.

Discussion

Phenomenal epidemic of influenza began from killing about 50 million people in Spain in 1918 (Spanish Flu) and we are threatened by the emergence of a new type of influenza such as avian Flu (antigenic shift) (Ansaldi *et al*., 2005; Glaser *et al*., 2005; Hampson *et al*., 2006). To make matters worse, the strain that once broke out such as Spanish Flu has gone through genomic change (antigenic drift) (Hampson *et al*., 2006). As results, it is difficult for us to protect perfectly against the Flu by vaccine. On the other hand, anti-Flu drugs are developed by many researchers, but it is problematic because the drugs show side effects and viruses tolerant against the drugs appear.

Hence, it has been observed that black tea and green tea have an anti-Flu function (Imanishi *et al*., 2002; Clark *et al*., 1998; Nakayama *et al*., 1993; Song *et al*., 2005). It has been reported that the functional materials to inactivate Flu are included in these tea extracts (Imanishi *et al*., 2002; Song *et al*., 2005; Weber *et al*., 2003). However the anti-Flu function of *Goishi* tea has not yet been studied. Therefore, we investigated the anti-Flu function and the functional materials of GTE.

Since Hirst observed hemagglutination (HA) by influenza A viruses (Hirst, 1941), it has been proven that HA is an extremely valuable technique for identification, quantification and purification of Flu. The hemagglutinin of Flu is a surface protein binding with sialic acid of host cell on early stages of infection (Glaser *et al*., 2005; Sidwell *et al*., 2000). HI test was employed to determine the adsorption inhibition of various samples. It has been reported that BTE show the high HI activity against various subtypes of Flu. However, our results suggested that the antiviral effect of GTE was higher than that of BTE and the activity was not specific to various subtypes of Flu (Fig. 1).

We investigated which step through Flu infection was inhibited by GTE, using MDCK cells that PR8 is able to infect. On the test using the cells, it has been reported that BTE and GRTE inhibit an adsorption stage and scarcely gave the effect on subsequent stages after viral adsorption (Imanishi *et al*., 2002; Nakayama *et al*., 1993). In our studies, BTE showed high inhibitory activity on the adsorption stage and GRTE showed low inhibitory activity. Furthermore, both BTE and GRTE showed low inhibitory activity on subsequent stages (Fig. 2). Our results were similar to reports of Nakayama *et al* (Nakayama *et al*., 1993). On the other hand, GTE showed the highest inhibitory activity among these samples on adsorption stage. In addition, GTE inhibited subsequent stages such as viral invasion, genomic multiplication and viral release stages after viral infection, too (Fig. 2).

Next, we investigated whether GTE that showed HI activity and inhibitory effects of CPE protected against Flu infection of mice. We compared the Flu infective rates of GTE-0.5 and GTE-2.0 groups with that of the control group. Accumulated symptoms and survival rate of GTE-2.0 group were significantly lower and higher than those of the control group, respectively. In

addition, GTE-0.5 group showed a significantly higher survival rate compared with the control group. Although all mice of GTE-0.5 group developed the symptoms, some mice recovered during the experimental period (Fig. 3). This result indicated that although the anti-Flu function of GTE had been demonstrated in *in vitro*, the effect was demonstrated in *in vivo*, too.

It has been reported that the anti-Flu function of BTE and GRTE relate with their polymeric phenols (Clark *et al*., 1998; Horie *et al*., 2002; Imanishi *et al*., 2002; Nakayama *et al*., 1993; Song *et al*., 2005). We measured the quantity of total polymeric phenols in GTE, and found that total polymeric phenols of GTE, which could expect to show the anti-Flu function, contained about 22% (data not shown). Furthermore, we searched functional materials by fractionating GTE (Fig. 4). In this study, we showed that F3 sample contained the greatest quantity of total polymeric phenols among all fractions and it was the highest molecular weight substance (Table. 1). On the adsorption stage, the inhibitory effect of F3 sample was very high and was equivalent to that of non-fraction sample (Fig. 5, 6). On the other hand, F1 or F2 samples were low molecular weight substance (Table. 1) and showed the high inhibitory effects on subsequent stages after viral adsorption (Fig. 6). Consequently, these results suggest that the high molecular weight substances in GTE inhibit the adsorption stage and the low molecular weight substances inhibit subsequent stages such as viral invasion, genomic multiplication and viral release stage after the adsorption.

Goishi tea is microbial fermented tea. It has been reported that GTE include (-)-epigallocatechin gallate, and high molecular weight substances of *Goishi* tea extracted by ethyl alcohol improve lipid metabolism (Oyaizu *et al*., 2005). In our test, GTE showed remarkable inhibition not only on the adsorption stage but also on subsequent stages after the adsorption during Flu infection, compared with BTE and BRTE. It was interesting that GTE showed different effects from GRTE which was produced by same leaf. We are thinking that the functional materials of GTE may be generated through the production process. *Goishi* tea is produced by fermentation of fungus on the first step and LAB on the second step. It has been reported that these microorganisms resolved plant materials into various components. For example, *Bacillus licheniforis*, *Rhizopus oryza*, *Aspergillus*

foetidus, *Lactobacillus plantrum, Lactobacillus paraplantrum* and *Lactobacillus pentosus* produce tannase and specially break the galloyl ester bonds of tannins (Banerjee *et al*., 2005; Kostinek *et al*., 2007; Mondal *et al*., 2000; Osawa *et al*., 2000). In our study, low molecular weight substances in GTE showed inhibitory effects on subsequent stages after viral adsorption, but BTE and GRTE showed very few effects on the same stages. We guessed that the low molecular weight substances in GTE, which showed inhibition on subsequent stages, were produced by fungus or LAB during fermentation. On the other hand, it has been already reported that GRTE has an anti-Flu function and the inhibitory activity is lower than that of BTE (Nakayama *et al*., 1993). The antiviral effect of GTE which used the same leaf as GRTE was higher than that of BTE on the adsorption stage. On the adsorption stage, the inhibitory materials of GTE were high molecular weight substances. Therefore, although microorganisms which polymerize the components have not been reported yet, we guess that microorganisms which polymerize polymeric phenol appear during the production process of *Goishi tea*.

In conclusion, we found that GTE inhibited various infectious stages of the Flu. On the adsorption stage, the functional materials were high molecular weight substance. Furthermore, on subsequent stages after the adsorption, the functional materials were low molecular weight substance. These activities of GTE were higher than that of BTE and GRTE. These results indicated that functional materials of GTE appeared through polymerizing or resolving by some microorganisms. Further, search of microorganisms to polymerize polymeric phenols and detailed analysis of these functional polymeric phenols in GTE are now in progress.

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Legands for figures

Fig. 1. Inhibitory effects of GTE on adsorption of influenza virus to chicken red blood cells. 50 μl of PR8 (A) and Guizhou (B) virus solution were mixed with an equal volume of samples (0, 0.2, 2.0 mg/ml) and incubated for an hour at room temperature. After that, the virus titer of the reaction mixture was measured by hemagglutination inhibition test. Results were expressed as mean ± S.D. of three independent experiments. The asterisks indicate a significant difference against control (each sample: 0 mg/ml), *P<0.05 and **P<0.01.

Fig. 2. Inhibitory effects of GTE on viral cytopathic effect. Monolayer of MDCK cells on 96 well plate was washed with serum-deprived MEM and infected with 25 μl of PR8 solution ($10^{2.4}$ $TCID_{50}$/ml) for an hour. Various samples were added to each stage of infection on MDCK cells; for testing the inhibition on the adsorption stage, samples were mixed with an equal volume of virus solution for an hour at pre-infection and the mixture was used for adsorption, and for testing the inhibition on subsequent stages after the adsorption, maintenance medium containing various concentrations of samples were added to cells after 1, 9, and 17 h from post-infection. The cells were incubated at 37℃ with 5% CO_2 for 3 days. Next, detection of the virus in culture supernatants was performed by hemagglutination reaction. 50% inhibiting concentration (IC_{50}) of each sample was calculated by regression analysis of the dose-response curves generated from these data. This test was performed twice and the similarity of both results was confirmed.

Fig. 3. Protective effects of GTE on morbidity and mortality due to influenza virus infection on mice. 198 μl of GTE solution (0, 0.5 and 2.0 mg/ml) was mixed with 2 μl of PR8 ($10^{6.8}$ $TCID_{50}$/ml) at 37℃ for 3 min. Lower respiratory tract injection was done by dropping 10 μl of the reaction mixture into each nostril (20 μl per mouse). These mice were observed for 14 days to assess the accumulated symptom rate and survival rate. The asterisks indicate a significant difference against

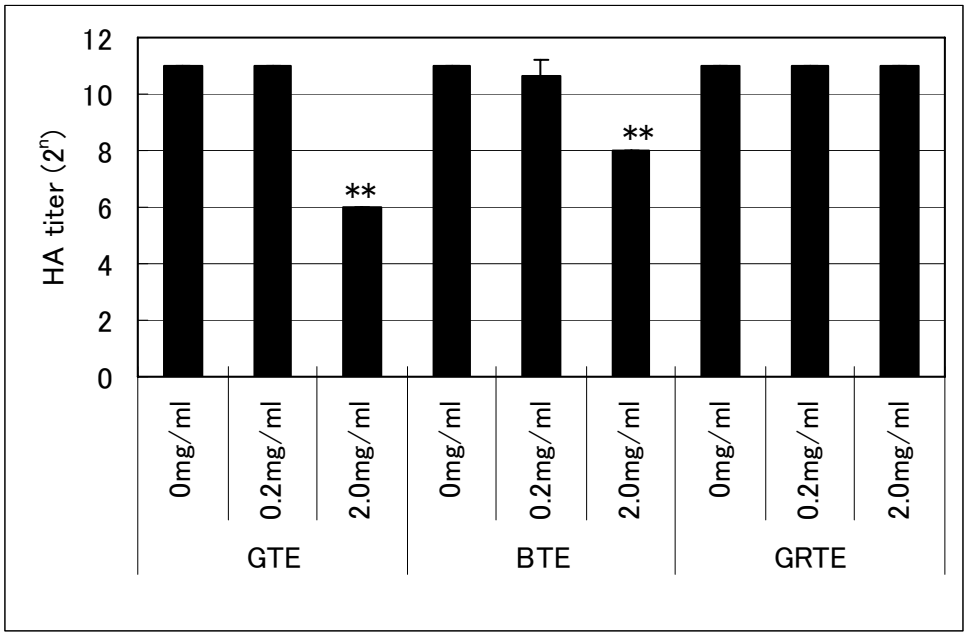
control (GTE: 0 mg/ml), *P<0.05 and **P<0.01.

Fig. 4. Chromatogram of solid-phase extraction fractionation of GTE by HPLC. GTE were applied to a BOND ELUT 500 mg C18 cartridge. The fraction of GTE was recovered consecutively with 20 ml of 8%, 25% and 45% methanol, and separated F1, F2 and F3, respectively. All of the fractions were evaporated and re-dissolved into distilled water, frozen and then freeze-dried. Then they were analyzed by HPLC. Chromatographic separation was carried out on a Luna 5 μ C18 column. Solvents were 0.1% trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The flow rate was 1.0 ml/min, the injection volume was 20 μl, and detection was performed at 280 nm.

Fig. 5. Inhibitory effects of each fraction and non-fraction of GTE on adsorption of PR8 to chicken red blood cells. Each fraction (F1 – F3) and non-fraction were mixed with PR8 solution and incubated for an hour at room temperature. HA titers were measured in the same manner as described in the legend to Fig. 1. Results were expressed as means ± S.D. of three independent experiments. The asterisks indicate a significant difference against control (each sample: 0mg/ml), *P<0.05 and **P<0.01.

Fig. 6. Inhibitory effects of each fraction and non-fraction of GTE on viral cytopathic effect. Each fraction and non-fraction were added to each stage of PR8 infection on MDCK cells. IC_{50} of each sample was measured in the same manner as described in the legend to Fig. 2.

(A)


HA titer (2^n)
12
10
8
6
4
2
0
**
**
0mg/ml
0.2mg/ml
2.0mg/ml
0mg/ml
0.2mg/ml
2.0mg/ml
0mg/ml
0.2mg/ml
2.0mg/ml
GTE
BTE
GRTE

(B)

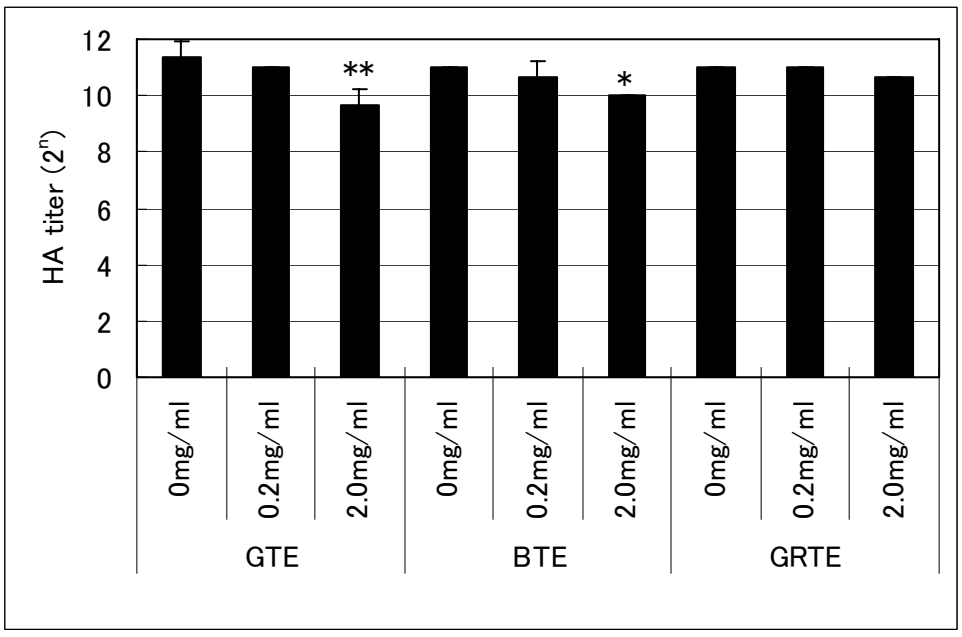

HA titer (2^n)
12
10
8
6
4
2
0
**
*
0mg/ml
0.2mg/ml
2.0mg/ml
0mg/ml
0.2mg/ml
2.0mg/ml
0mg/ml
0.2mg/ml
2.0mg/ml
GTE
BTE
GRTE

Fig. 1.

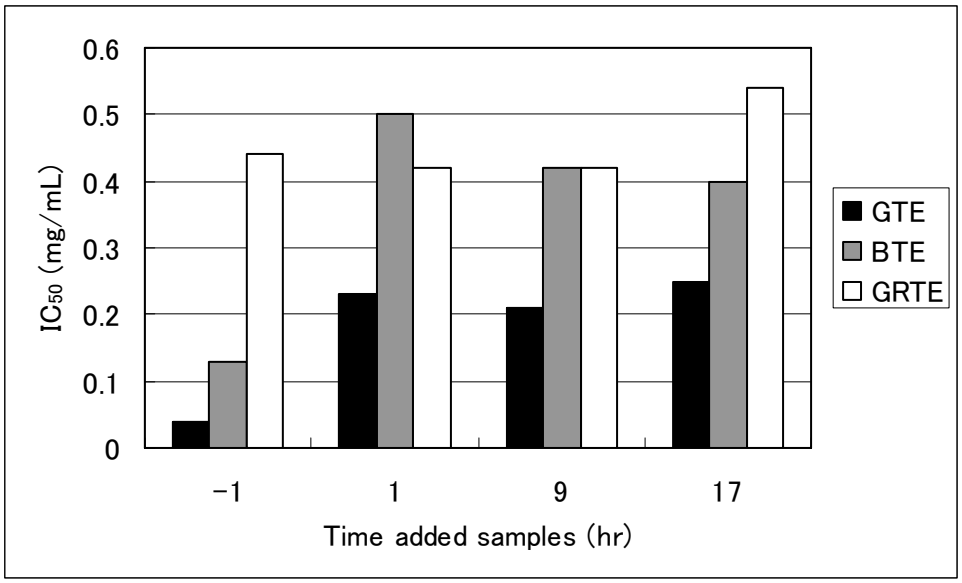
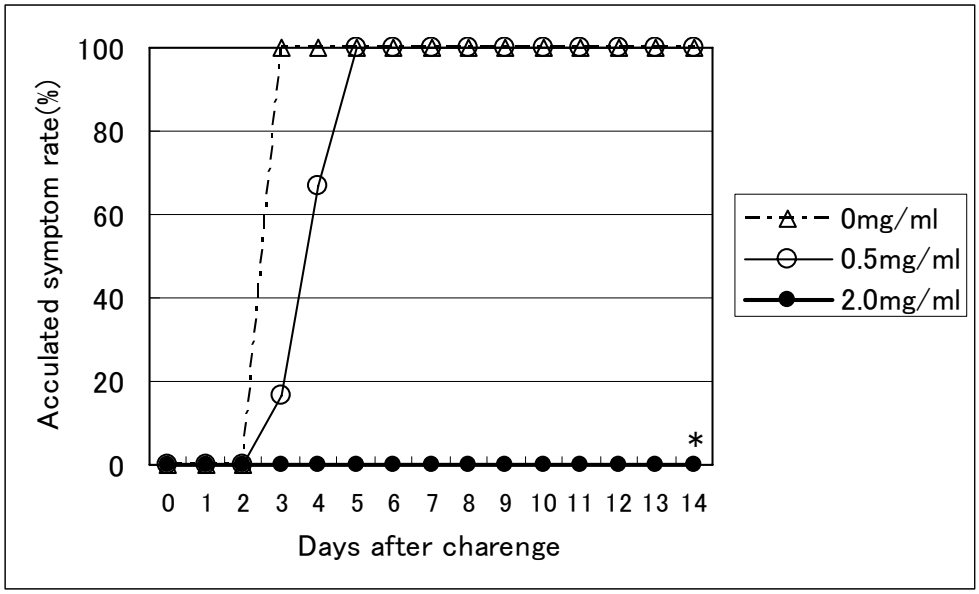

0.6
0.5
0.4
0.3
0.2
0.1
0
IC50 (mg/mL)
-1
1
9
17
Time added samples (hr)
GTE
BTE
GRTE

Fig. 2.

(A)


Acculated symptom rate(%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days after charenge
*
0mg/ml
0.5mg/ml
2.0mg/ml

(B)

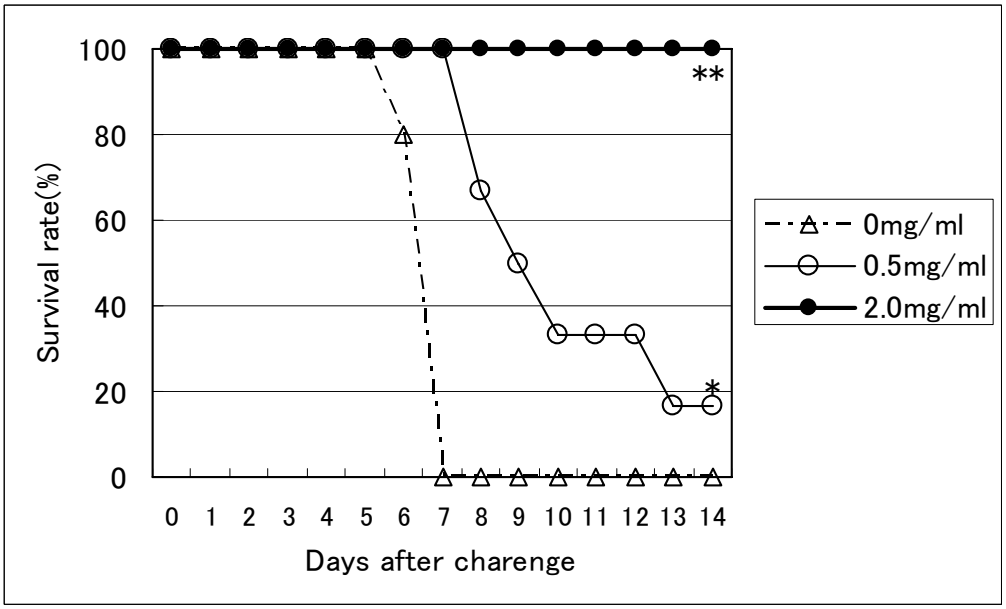

Survival rate(%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days after charenge
**
*
0mg/ml
0.5mg/ml
2.0mg/ml

Fig. 3.

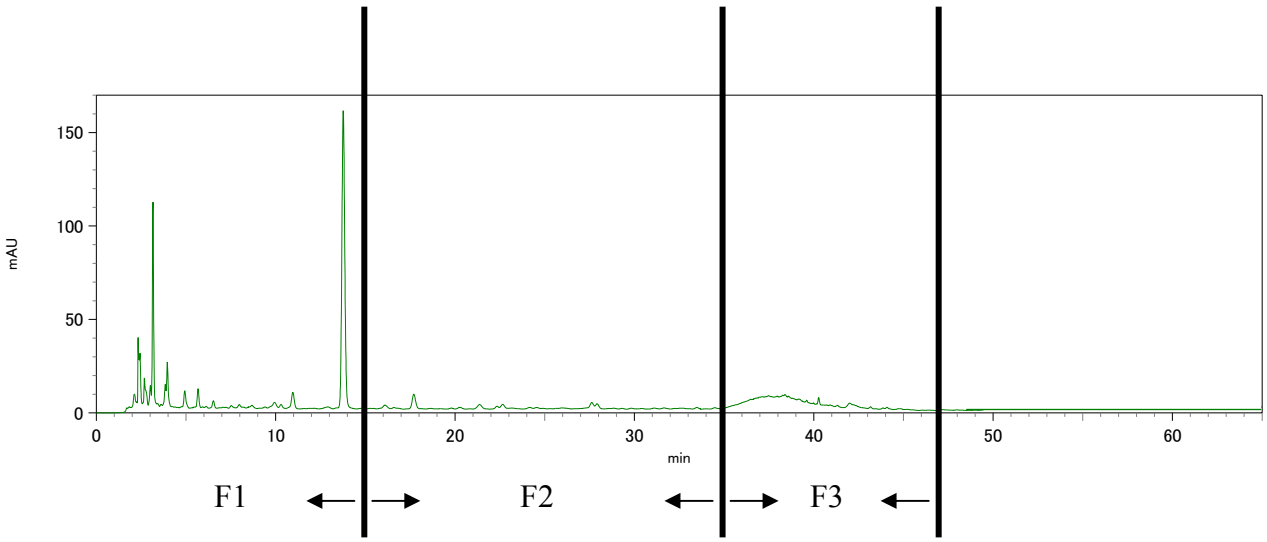


Fig. 4

Table. 1. Molecular weight and total polymeric phenol volume of each fraction of GTE.

molecular weight cut off	F1 (0.26g/g)	F2 (0.34g/g)	F3 (0.42g/g)
3,000	+	+	-
10,000	+	+	-
30,000	+	+	-
50,000	**N.D.**	**N.D.**	+

+ : Pass the membrane
- : Not pass the membrane
N.D. : Not done

parenthesis (): total polymeric phenol content

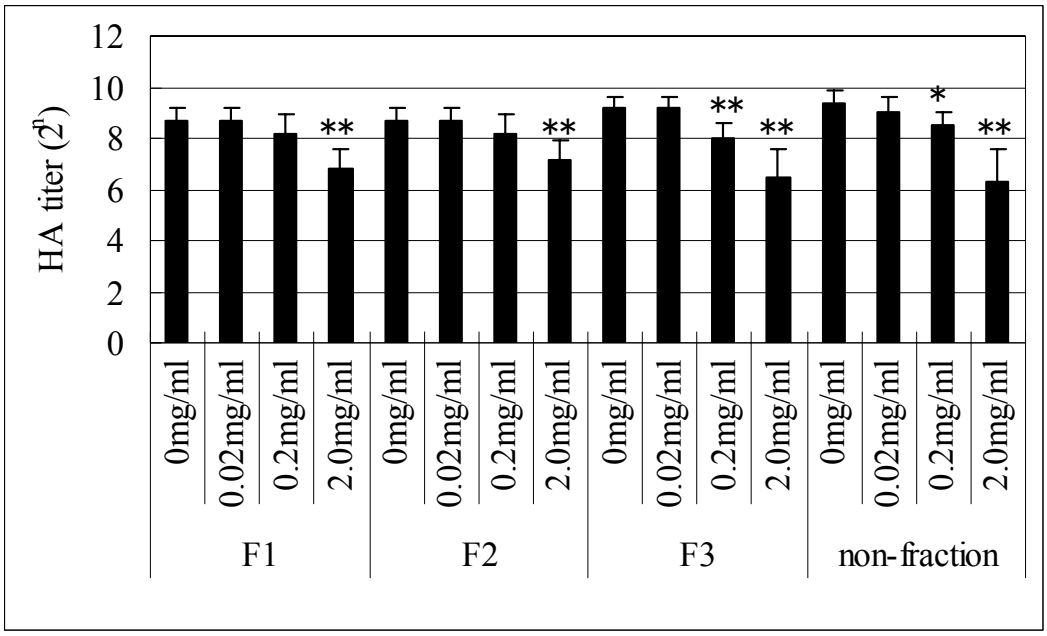

HA titer (2^n)
12
10
8
6
4
2
0
**
**
**
**
*
**
0mg/ml
0.02mg/ml
0.2mg/ml
2.0mg/ml
F1
F2
F3
non-fraction

Fig. 5

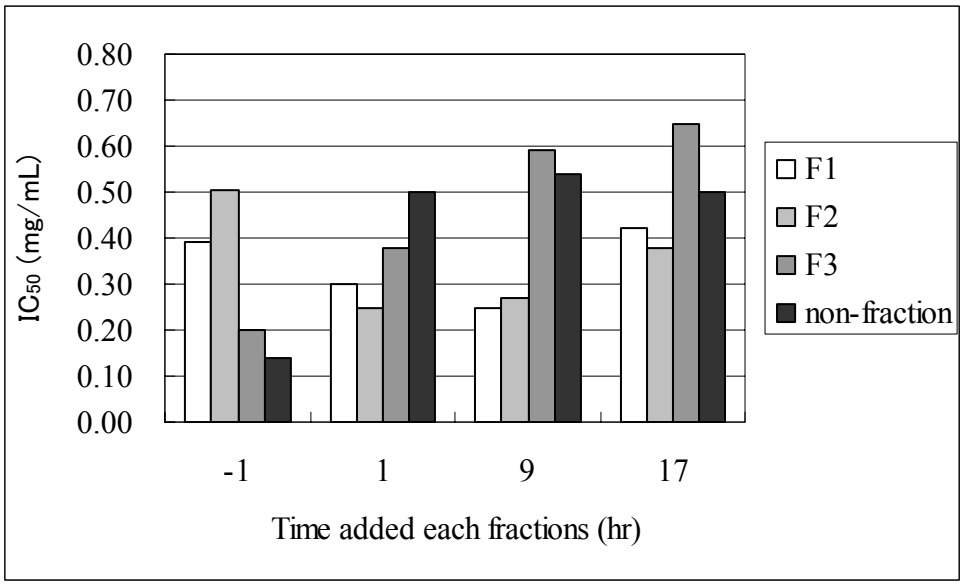

0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
IC50 (mg/mL)
-1
1
9
17
Time added each fractions (hr)
F1
F2
F3
non-fraction

Fig. 6