Inhibitory Effects of Goishi Tea against Influenza Virus Infection

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

Keywords: influenza virus, microbial fermented tea, polymeric phenol, inhibition

Introduction

Influenza virus (Flu), especially influenza virus type A, has multiple subtypes and mutates antigenicity every year. For this reason, it is hard to prevent infection. To make matters worse, safe and effective drugs are lacking, 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 influenza deaths in these segments of the population.

It has been reported that some kinds of tea have anti-Flu activity. Tea has been enjoyed by people worldwide for a very long time. 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 many differences in components among these teas. These differences are affected not only by the variety of tea leaves but also by the manufacturing process (Horie et al., 2002).

Green tea and black tea have been studied for a long time for antiviral effects. It has been proven, using the cell culture method, that (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate, and (-)-epigallocatechin in green tea have the ability to inhibit adeno virus and Flu replication 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 determined that theaflavins such as flavan-3-ol in black tea have the ability to inactivate both rotavirus and coronavirus in an 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 flavonoids, 5,7,4’-trihydroxy-8-methoxyflavone, and tannic 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 unusual on a global scale because of the use of both fungus and lactic acid bacteria (LAB). This tea uses the same leaves as green tea, Camellia sinensis. However, there is a difference in the manufacturing processes. Green tea is produced by steaming and dehydrating the leaf, whereas Goishi tea is produced by fermentation with a fungus as the first step and with LAB as the second step. It has been reported that Goishi tea extracted by ethyl alcohol improves lipid metabolism (Oyaizu et al., 2005). However,
an anti-Flu function of Goishi tea has not yet been reported. In this study, we investigated the possible anti-Flu function of Goishi tea hot water extract (GTE) in in vitro and in vivo tests that 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 found that high-molecular-weight substances in GTE inhibited viral adsorption and low-molecular-weight substances inhibited subsequent stages after adsorption.

Materials and Methods

Preparation of tea hot water extracts Various tea extracts were prepared by boiling 20 g of tea leaves in 1000 ml distilled water at 100°C for 30 min. The tea extracts were quickly separated from the 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°C by a previously reported method (Yasui et al., 1999). The allantoic fluid was removed and stored at -80°C. The titer of virus in allantoic fluid was expressed as the 50% tissue-culture infectious dose (TCID50/ml) and 50% egg-infecting dose (EID50/ml) for PR8 and Guizhou, respectively. The titer of virus in allantoic fluid of PR8 and Guizhou was 10^7.4 TCID50/ml and 10^10.6 EID50/ml, respectively.

Chicken erythrocytes and MDCK cells Chicken erythrocytes were prepared from chicken blood samples by centrifugation, suspended in Alsever solution and stored at 4°C. Mardin-Darby canine kidney (MDCK) cells were cultured in 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 weeks old) were purchased from Japan SLC Co., Ltd (Hamamatsu, Japan). The animals were housed in an air-conditioned room maintained at 24 ± 2°C 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 according to the Guidelines for Animal Experiments 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). Fifty-microliter samples in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) were mixed with 50 µl of PR8 (10^6.5 TCID50/ml) or Guizhou (10^6.9 EID50/ml) solution at room temperature for an hour in the first row of a 96-well disposable plate. After the reaction, serial 2-fold dilutions were made by transferring 50 µl from the first well to the successive right well with 50 µl of PBS containing 0.1% BSA. Next, 50 µl of 0.5% chicken erythrocyte solution with PBS containing 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 caused complete hemagglutination (HA) was determined as the HA titer value (Kiyoshima et al., 2001; Nakayama et al., 1993).

Viral growth inhibition test. A viral cytopathic inhibition assay was employed to test the effect of tea extracts on various stages of viral infection of target cells (Imanishi et al., 2002; Nakayama et al., 1993; Sidwell 2000; Song et al., 2005). The culture supernatant of a confluent monolayer of MDCK cells cultured in a 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°C with 5% CO2 for an hour. After that, the supernatant was removed and the cells were infected with 25 µl of PR8 solution (10^7.4 TCID50/ml) for an hour. Each sample, diluted from 1.0 mg/ml to 0.1 mg/ml with PBS containing 0.1% BSA serially, was added at various stages of infection to MDCK cells; for testing the inhibition of the adsorption stage, various concentrations of samples mixed with an equal volume of virus solution at room temperature for an hour before infection were added to cells, and for testing the inhibition at subsequent stages, 100 ml of maintenance medium (MEM containing 0.2% BSA and 5 µg/ml acetylated trypsin) containing various concentrations of the sample was added to cells 1, 9, and 17 h after infection. For testing the cytotoxic effect of each sample, each sample at a concentration of 1.0 mg/ml was added to MDCK cells. After virus adsorption for an hour, 200 µl or 100 µl of maintenance medium was added to the cells for the inhibition assay at the adsorption stage or subsequent stages, respectively, and the cells were incubated at 37°C with 5% CO2 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 erythrocyte solution with PBS containing 0.1% BSA was added to each well on the plate. The plate was settled at room temperature for an hour. The required concentration to show 50% inhibition (IC50) was calculated by regression analysis of the dose-response curves generated from these data.

In vivo test Male BALB/c mice (8 weeks old) were anesthetized by an intraperitoneal injection of pentobarbital anesthesia (65 µg/g body weight). One hundred ninety-eight microliters of 0 (control group), 0.5 (GTE-0.5 group), or 2.0...
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 fractions of GTE were recovered consecutively with 20 ml of 8%, 25%, and 45% methanol, yielding separated fraction 1 (F1), fraction 2 (F2), and fraction 3 (F3), respectively. Each fraction was evaporated under reduced pressure at 40°C 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 m C18 column (150 × 4.6 mm, Phenomenex, Inc., Torrance, CA) at 40°C. 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. Five hundred microliters of sample solution was mixed with 500 µl of diluted Folin-Ciocalteu reagent (1N) in a test tube. After 3 min of reaction, 500 µl of 10% Na₂CO₃ was added, and the mixture was incubated for 60 min at room temperature. The absorbance was measured at 700 nm with a 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 cutoffs: 3,000, 10,000, 30,000, and 50,000; Millipore Corporation), and after 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 of the difference of accumulated morbidity rate and survival rate between the control group and the 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 erythrocytes  Flu has the ability to adsorb to chicken blood erythrocytes, resulting in HA. 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 viral adsorption to chicken blood erythrocytes resulting in HI. GTE and BTE at concentrations of 2.0 mg/ml significantly inhibited HA caused by PR8 (P<0.01) (Fig. 1A). GTE and BTE at concentrations of 2.0 mg/ml were also significantly effective against Guizhou virus (P<0.05) (Fig. 1B).
Effects of GTE on cytopathy of MDCK cells by PR8  Flu multiplies through four infecting steps: adsorption, invasion of 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 pre- or post-infection in MDCK cells, and the inhibition of the cytopathic effect (CPE) was observed. The 50% inhibitory concentrations (IC<sub>50</sub>) of each sample are summarized in Fig. 2. Virtual reduction in virus yields was observed for all samples and all times of sample addition. GTE showed the highest inhibitory activity among all samples at all stages and exhibited maximum inhibitory activity at the adsorption stage (IC<sub>50</sub>: 0.04 mg/ml). At subsequent stages after adsorption, the effectiveness of GTE was about 2-fold higher than that of other samples (IC<sub>50</sub>: 0.23, 0.21, 0.25 mg/ml) when the sample was added 1, 9, or 17 hours after infection, respectively (Fig. 2).

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

Effects of GTE on accumulated morbidity rate and survival rate of mice inoculated with PR8  By using the HI test and inhibitory test of CPE on MDCK cells, we have already clarified that GTE has high anti-Flu activity. Next, we investigated whether GTE protected against Flu infection in mice. Fig. 3 shows the time course of the accumulated morbidity rate and the survival rate after inoculation with GTE-treated PR8. The accumulated morbidity and survival rates of the 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 the GTE-0.5 group was also significantly higher (P<0.05) than that of the control group.
Although all mice of the GTE-0.5 group developed morbid-
ity, some mice recovered during the experimental period.

Property and function of various fractions of GTE. We
searched for functional materials in GTE. GTE dissolved in
distilled water was applied to solid-phase extraction and sep-
arated into three fractions. Fig. 4 shows the chromatogram
recorded at 280 nm by HPLC. We hypothesized the identity
of materials in each fraction from retention time, spectral
features, or conformity to standard materials. It was suggest-
ed that the F1 sample contained a caffeine peak at 13.5 min
(Fig. 4 II), the F2 sample contained an EGCG peak at 21.4
min (Fig. 4 III), and the F3 sample contained a theaflavin
peak at 38 min of retention time (Fig. 4 IV). Caffeine in the
F2 sample at 13.6 min was considered to reflect a trace of the
F1 sample because the absorbance unit (AU) of caffeine in
the F2 sample was about one tenth of that in the F1 sample.

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

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

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
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</thead>
<tbody>
<tr>
<td>3000</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10000</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>300000</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>500000</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
</tr>
</tbody>
</table>

+ : Passed the membrane
- : Did not pass the membrane
N.D. : Not done
parenthesis (): total polymeric phenol content

Anti-influenza virus function of each fraction of GTE. We
investigated the bioactivity of these fractions and the non-
fractionated sample. Fig. 5 shows the HI activity of various
fractions and the non-fractionated sample of GTE. All sam-
pies at a concentration of 2.0 mg/ml significantly inhibited
HA caused by PR8 (P<0.01). Furthermore, the F3 sample
and the non-fractionated sample at a concentration of 0.2
mg/ml also significantly inhibited HA by PR8 (P<0.01 and
P<0.05, respectively). F1 and F2 samples at a concentration
of 0.2 mg/ml did not inhibit HA caused by PR8.
Fig. 5. Inhibitory effects of each fraction and the non-fractionated sample of GTE on adsorption of PR8 to chicken red blood cells. Each fraction (F1 – F3) and the non-fractionated sample were mixed with PR8 solution and incubated for an hour at room temperature. HA titters 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 (n=3). The asterisks indicate a significant difference versus controls (each sample: 0 mg/ml), *P<0.05 and **P<0.01.

Additionally, Fig. 6 shows the effect of each fraction and the non-fractionated sample of GTE on various stages of viral infection. The inhibitory activity on the adsorption stage was very high in the non-fractionated and F3 samples (IC₅₀: 0.14 and 0.20 mg/ml, respectively). At subsequent stages, the inhibitory activities of the F1 and F2 samples were equivalent and were higher than that of the non-fractionated sample. However, the F3 sample was lower in activity than the non-fractionated sample when it was added at 9 or 17 hours after infection. In these tests, the F3 sample inhibited mainly the adsorption stage. Furthermore, the F1 and F2 samples showed a strong effect on subsequent stages after adsorption. This test was performed twice, and the two experiments yielded similar results.

Discussion

A phenomenal epidemic of influenza that began in Spain killed about 50 million people 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, strains that once broke out, such as Spanish Flu, have gone through genomic change (antigenic drift) (Hampson et al., 2006). As a result, it is difficult to protect perfectly against Flu by vaccination. Anti-Flu drugs have been developed by many researchers, but they are problematic because the drugs cause side effects, and viruses tolerant to the drugs appear. It has been observed that BTE and GRTE have anti-Flu functions (Imanishi et al., 2002; Clark et al., 1998; Nakayama et al., 1993; Song et al., 2005; Weber et al., 2003). However the anti-Flu function of Goishi tea has not been previously 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 the host cell during the early stages of infection (Glaser et al., 2005; Sidwell et al., 2000). The HI test was employed to determine the adsorption inhibition of various samples. It has been reported that BTE has high HI activity against various subtypes of Flu. However, our results suggested that the antiviral effect of GTE was greater than that of BTE, and the activity was not specific to various subtypes of Flu (Fig. 1).

We investigated which step in Flu infection was inhibited by GTE, using MDCK cells that PR8 is able to infect. The inhibitory activity of GTE was higher than that of BTE and BRTE at the adsorption stage. In addition, GTE also inhibited subsequent stages such as viral invasion, genomic multiplication, and viral release. It has been reported that BTE and GRTE inhibit the adsorption stage and have little effect on subsequent stages after viral adsorption (Imanishi et al., 2002; Nakayama et al., 1993). In our studies, BTE and GRTE showed high inhibitory activity and low inhibitory activity, respectively, at the adsorption stage. Furthermore, both BTE and GRTE showed low inhibitory activity against subsequent stages. Our results are similar to a report by Nakayama et al. (Nakayama et al., 1993) (Fig. 2).

Next, we investigated whether GTE protected against Flu infection of mice. We compared the Flu infective rates of GTE-0.5 and GTE-2.0 groups with that of a control group.
The accumulated morbidity and survival rate of the GTE-2.0 group were significantly lower and higher than those of the control group, respectively. In addition, the GTE-0.5 group showed a significantly higher survival rate compared with the control group. Although all mice of the GTE-0.5 group developed morbidity, some mice recovered during the experimental period (Fig. 3). This result indicated that the anti-Flu function of GTE can be demonstrated in vivo.

It has been reported that the anti-Flu function of BTE and GRTE relates to 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 about 22% (data not shown). Furthermore, we searched for functional materials by fractionating GTE using a solid-phase extraction method (Fig. 4). F1 or F2 samples were low-molecular-weight substances (Table 1). The likelihood that the F1 sample contained caffeine and the F2 sample contained (-)-epigallocatechin gallate (EGCG) was suggested from the retention time, spectral features, or conformity to standard materials by HPLC. These samples showed high inhibitory effects on subsequent stages after viral adsorption (Fig. 6). However, GRTE, which was produced from the same leaves as GTE and contained EGCG, showed very few effects on the same stages (Fig. 2). Therefore, it was hypothesized that these active materials differed from EGCG. We hypothesize that the functional materials of GTE may be generated through the production process. Goishi tea is produced by fermentation by a fungus as the first step and LAB as the second step. It has been reported that these microorganisms resolve plant materials into various components. For example, Bacillus licheniformis, Rhizopus oryzae, Aspergillus foetidus, Lactobacillus plantarum, Lactobacillus paraplantarum, and Lactobacillus pentosus produce tannase and specifically break the galloyl ester bonds of tannins (Banerjee et al., 2005; Kostinek et al., 2007; Mondal et al., 2000; Osawa et al., 2000). From these reports, we hypothesized that the materials active against subsequent stages after adsorption were substances segmentalized by fungus and/or LAB.

On the other hand, we showed that the F3 sample contained the greatest quantity of total polymeric phenols among all fractions (Table 1). The inhibitory effect of the F3 sample was very high at the adsorption stage and was equivalent to that of the non-fractionated sample (Fig. 5, 6). The F3 sample contains substances of high molecular weights from 30,000 to 50,000 (Table 1). Therefore, although microorganisms that polymerize the components have not been reported yet, we surmise that microorganisms that polymerize polymeric phenol appear during the production process of Goishi tea.

In conclusion, we found that GTE inhibited various infectious stages of Flu. The materials functional against the adsorption stage were high-molecular-weight substances. The materials active against subsequent stages after adsorption were low-molecular-weight substances. These activities of GTE were higher than those of BTE and GRTE. These results indicated that functional materials of GTE appeared through polymerization or resolution by some microorganisms. The search for microorganisms to polymerize polymeric phenols and the detailed analysis of these functional polymeric phenols in GTE are now in progress.

References


