Study on anti-influenza virus activity of adlay tea

ハトムギ茶の抗インフルエンザウイルス作用

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Contents

List of Abbreviations

BSA	bovine serum albumin
CDC	Center for Disease Control and Prevention
DCs	dendritic cells
DMEM	Dulbecco’s modified Eagle’s medium
FBS	fetal bovine serum
FFRA	focus-forming reduction assays
FFU	focus-forming units
HA	hemagglutinin
HI	hemagglutination inhibition
IgA	Immunoglobulin A
IgG	Immunoglobulin G
LC	liquid chromatography
M	matrix protein
m.o.i.	multiplicity of infection
MDCK	Malin Darby Canine Kidney
MEM	Eagle's minimum essential medium
NA	neuraminidase
NK cells	natural killer cells
NLF	nasal lavage fluid
NP	nucleoprotein
p.i.	post-infection
PR/8/34	Puerto Rico/8/34
PVPP	polyvinylpolypyrrolidone
qPCR	Quantitative real-time PCR

General Introduction

Influenza is a respiratory infection, and reaches epidemic levels worldwide every year. Influenza causes symptoms, such as headache, myalgia and fever, and some cases it develops complications[1, 2]. Especially, pneumonia and encephalitis induce serious illness, and about 290,000 to 650,000 deaths by these complication each year worldwide[3]. It is known that this risk is high in the pregnant, under 5 years old and 65 years of age or older[3]. Moreover, such as lung disease, heart disease and kidney disease and so on are also high risk groups[3].

Influenza is caused by influenza viruses of the family Orthomyxoviridae. Humans can be infected by type A, B, or C influenza virus; type A and B viruses cause seasonal epidemics[3], type C virus causes mild symptoms in children from 2 to 6 years old[3, 4]. These classification are based on the antigenicity of matrix protein (M) and nucleoprotein (NP)[5]. The type A virus further classified from antigenicity of haemagglutinin (HA) and neuraminidase (NA)[5]. Influenza virus has HA and NA of spike proteins, and AM2 protein for A type viruses or BM2 protein for B type viruses on the cell membrane[6, 7], and virus gene is composed of eight segments (Fig. 1A). Growth process of influenza virus; HA of the virus is binding to sialic acid on host cell and taken in a cell, after that uncoating. Next the virus is replicated viral RNA and synthesized proteins, finally reconstructed and released (Fig. 1B)[8]. M2 and NA are involved in the uncoating and release process, respectively.

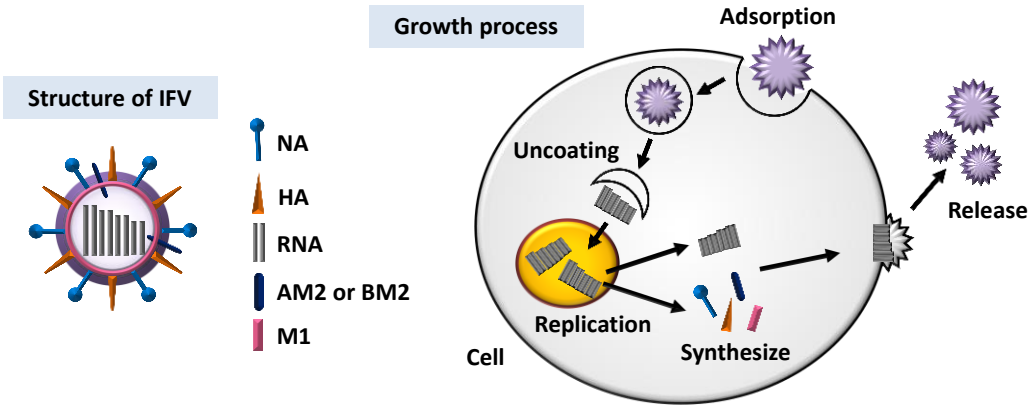

Fig. 1 Structure of virus and growth process.
IFV has several proteins on the cell membrane. The virus adsorb to cell, and taken in cell by endocytosis. After that, viral constituents are reconstructed and released. HA, M2 and NA are involved adsorption, uncoating and release, respectively.

There are sixteen HA (H1 to H16) and nine NA (N1 to N9) in type A virus, thus these combinations are 144 patterns (H1N1 to H16N9). Especially, H1N1 and H3N2 cause seasonal influenza. Subtype H5, H7 and H9 are highly pathogenic avian one[1, 9], and H2N2 caused pandemic in the past[10]. Type B virus, there is no subtype, and only two lineages of Victoria and Yamagata. As type A virus has many subtypes, so it mutate easily and more great influence to people than type B virus. Type A viruses occurred three notable pandemics for the past hundred years. In 1918, pandemic strain was H1N1, and deaths caused by the infection were around 50 to 100 million[11]. In 1957, pandemic strain was H2N2, and deaths caused by the infection were around 1 million[10]. In 1968, pandemic strain was H3N2, and deaths caused by the infection were around 1 million[12]. Moreover, in 2009, novel H1N1 strain appeared, and occurred pandemic[13].

Vaccine and an antiviral reagents are used to prevent and treat influenza. Quadrivalent vaccine is used in Japan from 2015/2016 season. This vaccine is composed H1N1, H3N2, B/Yamagata and B/Victoria. It is expected that the new vaccine can be more effective to

seasonal influenza. However, Centers for Disease Control and Prevention (CDC) reported that the adjusted vaccine effectiveness estimates for influenza seasons from 2005-2016 are 10 to 60% (CDC 2017)[14]. The research indicates that the effectiveness against the virus is unstable. In antiviral reagents, there are two types inhibiters of M2 inhibiter and neuraminidase inhibitor. M2 inhibiter is only effective against type A viruses, and almost influenza viruses are resistant strain against this inhibiter[15, 16]. Thus NA inhibiters are used to as a principal reagents currently. There are four types, which are oseltamivir, zanamivir, laninamivir and peramivir[17], but emergence of drug-resistant strain has become a serious problem in recent years[18-20].

The immune system such as innate immunity and acquired immunity protects our body from pathogens. Although these immune systems are still unknown in many parts, here I showed a basic response. In innate immune system, macrophages, dendritic cells (DCs) and natural killer (NK) cells reacts pathogens, and disturbances infection. NK cells contain component protein such as perforin and granzyme. Perforin and granzyme induce cell death of infection cells[21-23]. Macrophages and DCs take in antigen by phagocytose or micropinocytose, and presentation of the antigen to Th0 cells. In acquired immune system, Th0 cells differentiate to Th1 cells or Th2 cells, which can be activated cellular immune responses and humoral immune response, respectively (Fig. 2)[24-27]. B cells produce pathogen specific antibodies, and block virus entry to cells. Especially Immunoglobulin G (IgG) and Immunoglobulin A (IgA) are effective in influenza infection[28]. We get immunity via these processes, and can be protect when infected with same antigen. Although, influenza virus changes antigenicity frequently, and infect again. Thus, it is necessarily to preparation of vaccines every year.

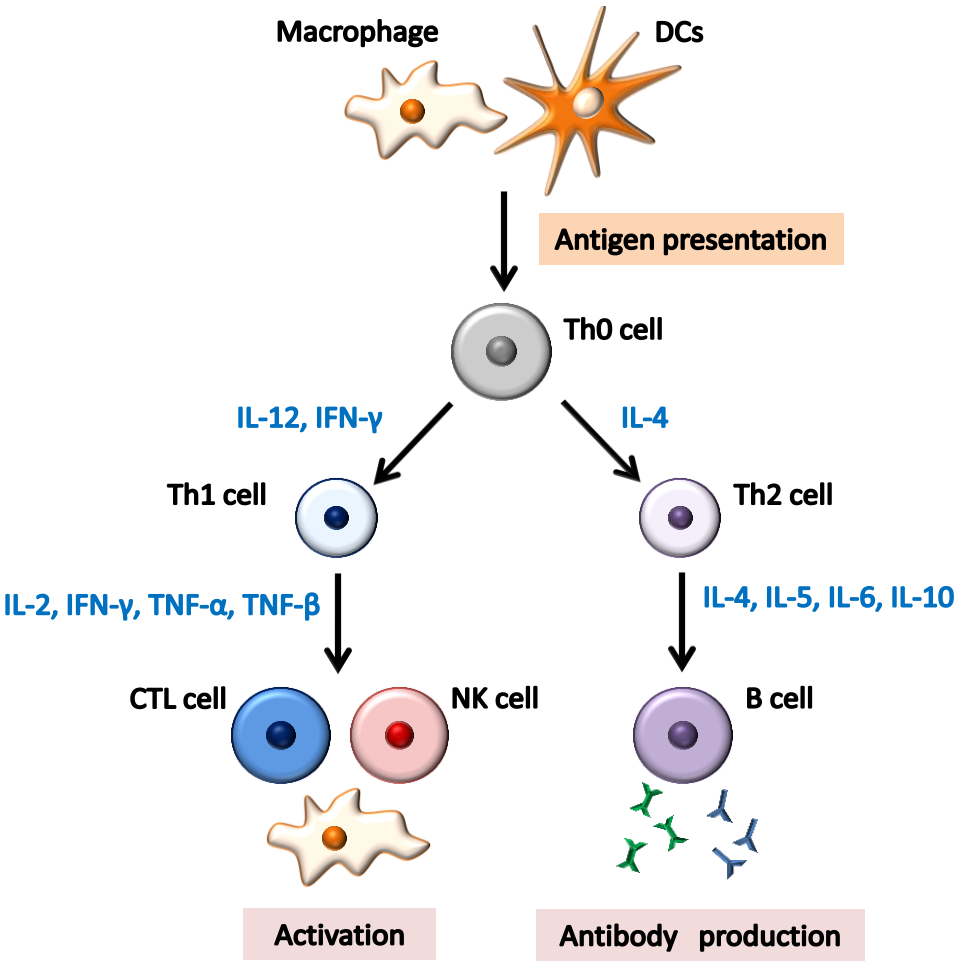

Fig. 2 Relationship between immune response and cytokines.
Th0 cells are stimulated by macrophages and DCs, and differentiate into Th1 or Th2 cell. Th1 and Th2 pathway induce cellular immune responses and humoral immune response, respectively. These responses are carried out via cytokines.

To solve problems in countermeasures for influenza, functional foods are attracting attention, and are studied anti-influenza virus activity a lot. Among them, there are many reports about anti-influenza virus activity by lactic acid bacteria and polyphenols. Lactic acid bacteria affects immune responses and control inflammatory reaction[29-32]. Polyphenols inhibited viral functions, which are HA activity and NA activity[33-36]. As shown in these studies, it is expected that functional food will be new protection manner.

I first examined the anti-influenza virus activity of a food extract library made by our

laboratory, and found that adlay tea has strong the activity. Adlay tea is a traditional drink in Japan, and some groups drink it to prevent colds. Adlay seed is the main raw material in adlay tea which used for this study, and soybeans, naked barley seeds, and cassia seeds are additionally blended. Other study has been reported that adlay seeds may be effective against viral infections by increasing the peripheral cytotoxic lymphocytes[37], but the antiviral activities of adlay seeds and adlay tea are not confirmed. In the present theses, I studied anti-influenza virus activity of adlay tea, and revealed the action mechanism *in vitro* and *in vivo* and its functional ingredients.

Chapter I

Anti-influenza virus activity of adlay tea and analysis functional ingredients.

1.1 Introduction

Influenza spreads every winter, it caused by influenza viruses which are H1N1, H3N2, B/Yamagata and B/Victoria viruses. Thus, quadrivalent vaccine and NA inhibitors are used as prevent and treatment, respectively in Japan. However, these treatments has problems such as unstabilized effect, existence of resistant viruses.

To solve these problems, I have focused on functional food. A combination of antiviral reagent therapies is more effective than a single antiviral reagent, and can prevent the emergence of drug-resistant strains[25]. Food contains a variety of ingredients, and combinations of ingredients with antiviral activities might have the same advantages as combination therapy. In addition, combining an antiviral reagent with food might have a synergistic effects.

I first examined the anti-influenza virus activities of a food extract library prepared by our laboratory, and it was suggested that adlay tea, which blended adlay seed, naked barley seeds, soybeans and cassia seeds has strongest anti-influenza virus activity. Adlay seeds has anti-inflammatory[43], anti-tumor[44], and antioxidant activities and suppress serum uric acid[45]. Thus, adlay seeds are used as a traditional medicine in many countries in East Asia. Studies have shown that adlay seeds may be effective against viral infections by increasing the peripheral cytotoxic lymphocytes[46], but the antiviral activities of adlay seeds and adlay tea have not been studied.

In this chapter, the anti-influenza virus activity of adlay tea amd its components were examined.

1.2 Materials and Methods

1.2.1 Cells and viruses.

Malin Darby Canine Kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 7% fetal bovine serum (FBS). CV-1 (monkey kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. The following influenza A H1N1 viruses were used: Puerto Rico/8/34 (PR/8/34), New Caledonia/20/99, Beijing/262/95, Suita/6/2007, Suita/1/2009, and Suita/114/2011, and the oseltamivir-resistant viruses Osaka/2024/2009 and Osaka/71/2011 (provided by the Osaka Prefectural Institute of Public Health). The influenza A H3N2 viruses Aichi/2/68 and Sydney/5/97, and the influenza B viruses Nagasaki/1/87 and Shanghai/261/2002 were also used. To infect cells, the virus was diluted in serum-free MEM supplemented with 0.4% bovine serum albumin (BSA, fraction V; Sigma-Aldrich Co. LLC., St. Louis, MO) and adsorbed to cells at a multiplicity of infection (m.o.i.) of 0.0001 focus-forming units (FFU)/cell for 1 h at 37 °C. The inoculum was then removed and replaced with serum-free DMEM supplemented with 0.4% BSA and acetyltrypsin (2 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) for the remainder of the infection period.

1.2.2 Hot-water extracts of adlay tea and tea components.

Adlay tea (Sanyo, Kobe, Japan) containing roasted adlay seeds (*Coix lacryma-jobi* var. *ma-yuen*), soybeans (*Glycine max*), naked barley seeds (*Hordeum vulgare* var. *nudum*), and cassia seeds (*Cassia obtusifolia* L.) was used. Adlay seeds are the main raw material in adlay tea, and a small amount of other components which are soybeans, naked barley seeds, and cassia seeds are blended. The commercial tea was manufactured by extracting with hot-water and powderized by freeze-dried. The tea powder was dissolved in water,

filtered through a Millex GX membrane (24 mm diameter and 0.45 μm pore size, Millipore, Billerica, MA, UK), and stored at −30 °C until use.

To test the individual components of the adlay tea, I prepared four grinds of roasted adlay seeds (Akishizuku brand), naked barley seeds (Ichibannboshi brand), soybeans (Toyomasari brand), and cassia seeds. The beans and seeds were first roasted at 180 °C for 4 h, and then hot-water extracts were prepared by adding 2.0 g of each ground substance to 50 ml hot water in a hot-water bath (80 °C) for 60 min. The extracts were then filtered through No. 2 filter paper followed by a Millex GV membrane, Millex GX membrane (24 mm diameter and 0.45 μm pore size, Millipore, Billerica, MA, UK). Finally, each extract was freeze-dried and stored at −30 °C until use.

1.2.3 Antiviral assay.

MDCK cells in MEM-FBS were seeded into a 24-well plate (Thermo Fisher Scientific, MA, USA) at 1×10^5 cells/well, and incubated for 24 h at 37 ℃. The cells were then washed twice with serum-free MEM. The virus was diluted in serum-free MEM supplemented with 0.4% BSA and adsorbed to cells at an m.o.i. of 0.0001 FFU/cell for 1 h at 37 °C. The cells were then washed once with serum-free MEM, and the medium was replaced with DMEM (500 μl) as described in “Section 1.2.1 Cells and viruses”, and supplemented with hot-water extract. After 24 h, the supernatants were harvested, and the virus titers were measured by focus-forming reduction assays (FFRA). Oseltamivir acid (Toronto Research Chemical, Toronto, Canada) was used as a positive-control viral-replication inhibitor.

1.2.4 FFRA for measuring virus titer.

Focus formation was broght about by the method of Okuno et al.[41], with slight modifications. MDCK cells in MEM-FBS were seeded into a 96-well flat-bottom plate (Corning, NY, USA) at 2×10^4 cells/well, and incubated in 5% CO_2 at 37 ℃. After 24 h, the cells

were washed three times with serum-free MEM. The virus samples collected in the anti-viral assay were serially diluted in MEM supplemented with 0.4% BSA, then 30 µL of each diluted virus sample was added to each well, and the plates were incubated in 5% CO_2 at 37 ℃. After 1 h, the cells were washed with serum-free MEM. The infected cells were then cultured in MEM supplemented with 0.4% BSA for 18 h at 37 ℃. The medium was removed, and the infected cells were washed with PBS and fixed by adding 100 µL of ethanol to each well.

The fixed cells were dried and then washed with PBS. Then, 50 µL of primary antibody, which was a mouse monoclonal anti-HA antibody: C179 for H1[42], F49 for H3[43], and 7B11 for B[44], was added to each well, and the samples were incubated for 30 min at 37 ℃. The cells were washed twice with PBS, then 50 µL of secondary antibody, which was horse-radish peroxidase-labeled goat anti-mouse immunoglobulin G (Merck KGaA, Darmstadt, Germany), was added to each well, and the samples were incubated for 30 min at 37 ℃. The cells were washed twice with PBS and stained with PBS containing 0.2 µl/ml H_2O_2 and 0.3 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan). The cells were washed in tap water and dried, and the stained infected cells were counted under a microscope.

1.2.5 Time-of-addition assay.

Time-of-addition assay was broght about by the method of Furuta et al.[45], with slight modifications. The virus was diluted in MEM supplemented with 0.4% BSA, and infected cells were cultured in DMEM containing 0.4% BSA and 2 µg/ml acetyltrypsin. The infected cells were cultured in five patterns. Pretreatment before virus adsorption was performed as follows (pattern 1): MDCK cells were seeded into 24 well plate. Hot-water extract was added to the culture medium of the cells, and cultured for 12 h at 37 °C in 5% CO_2. The cells were washed twice with serum-free MEM, and influenza virus solution (PR/8/34: MOI of 0.01) was added to the MDCK cells, and incubated for 1 h at 37 °C in 5% CO_2. The infected cells were washed twice with serum-free MEM, and cultured for 8 h at 37 °C in 5% CO_2. As to virus adsorption (pattern 2): hot-water extract and virus solution were mixed, and the mixture was adsorbed to the MDCK cells for 1 h at 37 °C in 5% CO_2. The infected cells were washed twice with serum-free MEM, and cultured as with Pattern 1. As to virus replication (pattern 3): the virus solution was added as with

Pattern 1. The infected cells were washed twice with serum-free MEM. The infected cells were cultured in medium containing hot-water extract for 8 h at 37 °C in 5% CO_2. As to virus replication at early stage or late stage (pattern 4 and 5): the influenza virus solution was added as with Pattern 1. In the case of early stage, the infected cells were cultured in medium containing hot-water extract for 4 h. Then, the culture medium was replaced with new one not containing hot water extract, and cultured for 4 h again. Late stage is reverse order, the infected cells were cultured in normal medium, and replaced with new one containing hot-water extract. After the culture, the infected cells in all patterns were frozen at −80 °C and subjected to two freeze-thaw cycles prior to measure virus titer by FFRA.

1.2.6 Cytotoxic test of hot-water extracts.

Cytotoxic test was performed by Cell Porliferation Kit (Roche Diagnostics, Basel, Switzerland) following the protocol provided by the manufacturer.

MDCK cells in MEM-FBS were seeded into a 96-well flat-bottom plate (Corning, NY, USA) at 2×10^4 cells/well, and incubated in 5% CO_2 at 37 ℃ for 24 h. The cells were washed three times with serum-free MEM, and replaced with DMEM as described in "1.2.1 Cells and viruses", and supplemented with hot-water extract. After 24 h, the medium was replaced with 100 µl/well of fresh DMEM, and 10 µl/well of the MTT labeling reagent was added and incubated for 4 h. Finally, 100 µl/well of the solubilization solution was added and incubated at overnight. After the incubation period, absorbance was measured by microplate reader. The wavelength and reference wavelength were 600 nm and 650 nm, respectively.

1.2.7 Hemagglutination inhibition (HI) test.

The HI test was performed with receptor-destroying, enzyme-treated guinea-pig ascitic fluid using a standard microtiter assay[46].

The chicken erythrocytes were suspended in PBS, separated by centrifugation (2,000 rpm, 10 min, 25 ℃), and washed three times. Influenza viruses (PR/8/34) were diluted 8 hemagglutinin unit (HAU) by PBS. The samples were diluted by serial two-fold dilution (2 to 124 fold). 25 µl/well of diluted sample and 25 µl/well of virus solution were mixed in a 96-well round bottom plate, and incubated for 1 h at 37 ℃. After incubation, 50

μl/well of 0.75% chicken erythrocytes were added and incubated for 1 h at room temperature, and determined HI titer.

1.2.8 Viral binding inhibition assay.

The amount of viral attachment to cells was assessed by measuring the M protein (MP) vRNA. Viral RNA was extracted from virus-bound cells, and cDNA was synthesized as described by Sawai-Kuroda et al.[47], with slight modification. After adsorption (m.o.i. 10) for 1 h with PBS as a control, or with diluted adlay tea as an inhibition test, the cells were washed twice with PBS. The SuperPrep^{TM} Cell Lysis & RT Kit for qPCR (Toyobo, Osaka Japan) was used as instructed by the manufacturer to extract RNA from the infected cells and synthesize cDNA. Quantitative real-time PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Life Technologies Japan) for MP RNA with a cDNA template, using the following primer sets: for MP mRNA: F 5'-CCMAGGTCGAAAC-GTAYGTTCTCTCTATC-3' (MP39-67For) and R 5'-TGACAGRATYGGTCTT-GTCTTTAGCCAYTCCA-3' (MP183-153Rev); the TaqMan probe, 5'-(FAM)ATYTCGGCTTTGAGGGGGCCTG(TAMURA)-3'; and the Thunderbird probe qPCR mix (Toyobo, Osaka, Japan). Each 20 μl of the PCR mixture contained 2 μl cDNA, 500 nM concentrations of both forward and reverse primers, and 150 nM probe. The amplification conditions were: one cycle at 95 °C for 15 min, 45 cycles at 94 °C for 15 s, and 56 °C for 75 s. Copy numbers were estimated from a standard curve obtained using serial 10-fold dilutions of the previously quantified PCR product as the template.

1.2.9 Cell-fusion inhibition test.

Cell-cell fusion was performed as described by Okuno et al.[42], with slight modifications. Monolayers of CV-1 cells in DMEM-FBS were infected with the influenza A/PR/8/34 strain (m.o.i. 0.001), incubated for 24 h, washed twice with serum-free DMEM, and incubated for 15 min at 37°C in DMEM supplemented with 10 μg/ml trypsin. The cells were then washed twice with serum-free DMEM and incubated for 30 min with DMEM containing 100 μg/ml C179 as a positive control, or with DMEM containing appropriately diluted adlay tea. Thereafter, the cells were treated for 2 min at 37 °C with a fusion medium (RPMI, 0.2% BSA, 10 mM citric acid–Na_2HPO_4 buffer) adjusted to pH 5.0. The cells were then washed twice with serum-free DMEM to completely remove the other

reagents, and incubated for 3 h in DMEM supplemented with 2% FBS. Finally, the cells fixed with absolute methanol were stained with Giemsa, and observed under a light microscope.

1.2.10 Polyvinylpolypyrrolidone (PVPP) treatment of adlay tea.

Polyphenols were removed from the adlay tea by PVPP treatment[51]. One gram of PVPP was added to 30 ml of adlay tea (20 mg/ml). The mixture was stirred for 1h, filtered through No. 2 filter paper followed by a Millex GV membrane, and collected. PVPP was added to the filtrate (1:30 w/v) again, and the mixture was filtered. Each filtrate was freeze-dried, and the dried samples were weighed. The samples were then reconstituted to their pre-freeze-drying volume. The antiviral activity of the PVPP-treated adlay tea was examined as described in "1.2.3 Antiviral assay".

1.2.11 Polyphenol quantification of adlay tea

Polyphenols were measured by the Folin-Ciocalteu method[54], with slight modifications. The samples (30 μl/well), 150 μl/well of 4% sodium carbonate solution and 30 μl/well of phenol reagent were mixed in a 96-well flat-bottom plate (Corning Japan, Osaka, Japan) and incubated for 30 min at 30 °C. The absorbance was then measured at 660 nm using a microplate reader. The polyphenol concentration was calculated using a gallic acid as a standard.

1.2.12 Statistical analysis

Viral titers in time of addition assay and antiviral assay were compared by Student's *t*-test using the EXCEL TOUKEI system, ver. 6.0 (Esumi, Tokyo, Japan). The values are showed as mean ± standard deviation (S.D.), and $p < 0.05$ was considered significant.

1.3 Results

Antiviral activities of adlay tea. This study was conducted toward the goal of finding foods with antiviral activity against influenza viruses. I found that adlay tea inhibited influenza virus multiplication (Fig. 1.1), with an IC_{50} of 2.11 mg/ml (Table 1.1). No cell damage was observed at higher concentrations of adlay tea, as determined by MTT assay performed using the Cell Proliferation Kit (Roche Diagnostics) (Table 1.1).

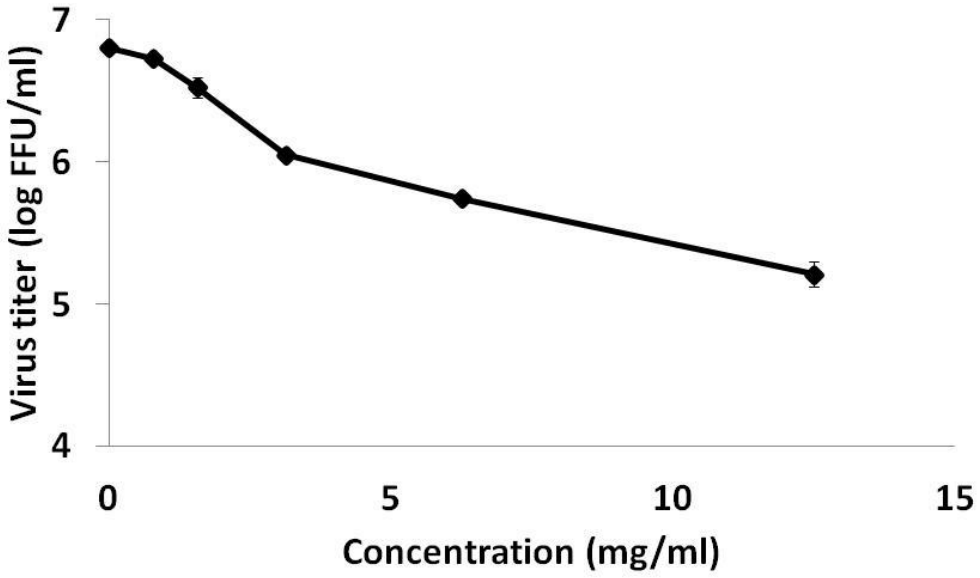

Fig. 1.1 Effect of adlay tea on the yield of influenza virus.
MDCK cells were inoculated with influenza A/PR/8/34 virus at a multiplicity of infection of 0.0001. Viral yields were determined at 24 h post-infection by focus-forming assays. Vertical lines show standard deviations (n = 3).

Table 1.1 Effect of adlay tea on the multiplication of various influenza virus types and strains.

Virus type and strain	IC_{50} (mg/ml or ng/ml)[a]		CC_{50} (mg/ml or μg/ml)[b]		SI[d]	
	Adlay tea	Oseltamivir acid	Adlay tea	Oseltamivir acid	Adlay tea	Oseltamivir acid
A(H1N1)						
PR/8/34	2.11 ± 0.03	0.45 ± 0.01	>40	>200	>19	>445521
New Caledonia/20/99	3.14 ± 0.09	0.38 ± 0.18	>40	>200	>13	>526327
Beijing/262/95	2.58 ± 0.30	ND	>40	ND	>16	ND
Suita/6/2007	3.22 ± 1.25	ND	>40	ND	>12	ND
Suita/1/2009	3.32 ± 0.21	ND	>40	ND	>12	ND
Suita/114/2011	4.12 ± 0.43	ND	>40	ND	>10	ND
Osaka/2024/2009[c]	4.39 ± 0.18	180 ± 59	>40	>200	>9	>1114
Osaka/71/2011[c]	4.83 ± 0.77	259 ± 75	>40	>200	>8	>771
A(H3N2)						
Sydney/5/97	4.40 ± 0.43	ND	>40	ND	>9	ND
Aichi/2/68	4.53 ± 0.33	0.93 ± 0.78	>40	>200	>9	>215824
B						
Nagasaki/1/87	2.91 ± 0.66	6.10 ± 1.19	>40	>200	>13	>32813
Shanghai/261/2002	4.61 ± 1.79	ND	>40	ND	>9	ND

[a]The values are averages of results obtained using the final concentrations of adlay tea, from two independent experiments. The IC50 of adlay tea and oseltamivir acid are given in mg/ml and ng/ml, respectively.
[b]The values are averages of results obtained using the final concentrations of adlay tea, from three independent experiments. The CC_{50} of adlay tea and oseltamivir acid are given in mg/ml and μg/ml, respectively.
[c]Oseltamivir-resistant virus
[d]Selectivity index=CC_{50}/IC_{50}
ND: not determined

Antiviral activities of the adlay tea components. The adlay tea used in this study was composed of adlay seeds, naked barley seeds, soy beans, and cassia seeds. I tested the effect of hot-water extracts of each components on influenza virus multiplication (Fig. 1.2), and found that all of the components had antiviral activity against the influenza virus A/PR/8/34. There was no cell damage at the higher concentration of each components, as determined by MTT assay. These results suggested that the antiviral activity of adlay tea represents the total activity of the active compounds contained in all of its components.

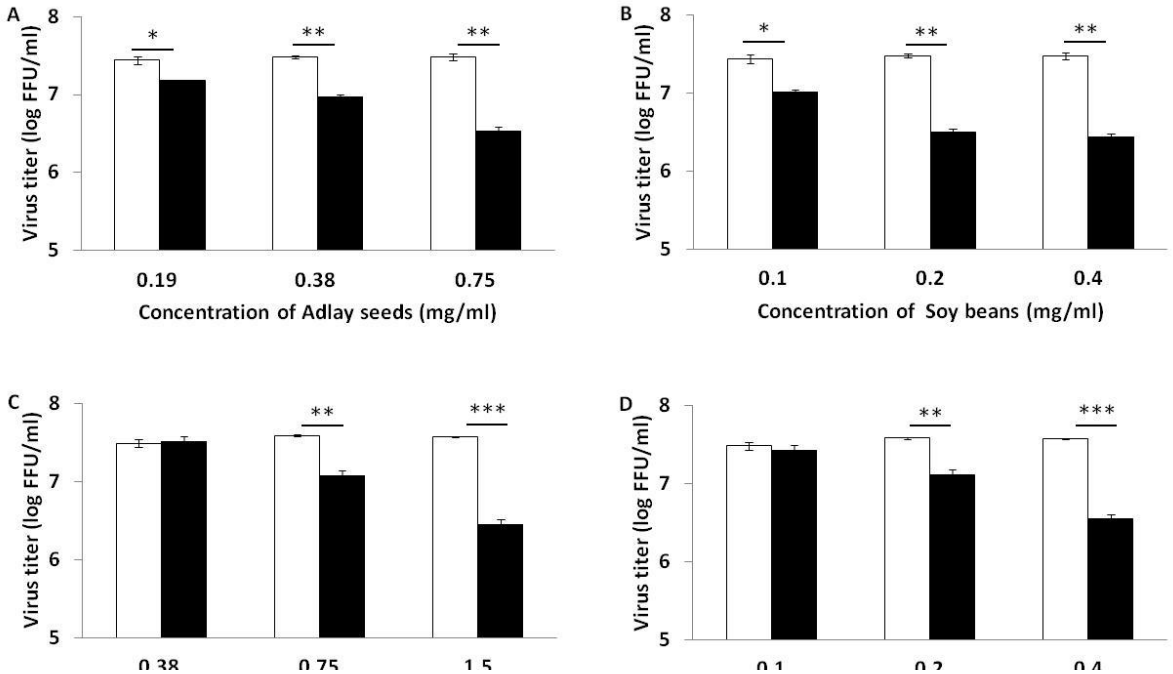

Fig. 1.2. Effect of extracts of adlay tea components on the yield of influenza virus: (A) adlay seeds; (B) soy beans; (C) naked barley seeds; and (D) cassia seeds.
The conditions of influenza virus infection and detection were the same as in Fig. 1. Open bars: mean viral yield for control cells; closed bars: mean viral yield for cells treated with three different concentrations of adlay tea components. Vertical lines show standard deviations (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

Antiviral activity of adlay tea against various influenza virus types and strains. The results of assays for the antiviral activity of adlay tea for various influenza viruses are

summarized in Table 1.1. Adlay tea inhibited the viral replication of all of the laboratory-adopted and clinical isolated influenza A and B viruses. The IC_{50}s ranged from 2.11 to 5.13 mg/ml for the influenza A viruses including the oseltamivir-resistant viruses, and from 3.23 to 4.23 mg/ml for the influenza B viruses.

Oseltamivir inhibited the influenza A viruses (IC_{50}s, 0.45 to 0.51 ng/ml), although the oseltamivir-resistant viruses (dmp: A/Osaka/2024/2009, A/Osaka/71/2010) were less susceptible to it (IC_{50}, 140 to 210 ng/ml). Adlay tea was similarly active against the oseltamivir-resistant and oseltamivir-sensitive viruses, suggesting that the reaction targets of adlay tea and oseltamivir are different.

The sensitive step for adlay tea's blocking of virus multiplication. I carried out a time-of-addition assay to investigate about when is the inhibition stage of the tea components in virus infection. The infection period was partitioned five patterns, pretreatment before virus adsorption (pattern 1), virus adsorption (pattern 2), virus replication (pattern 3) virus replication at early stage (pattern 4) and late stage (pattern 5). Although it usually took about 8 h to detect the progeny virus in the cells after inoculation with the influenza A/PR/8/34 virus, I did not detect free viruses in the medium within 10 h in my experiments. Adlay tea added 12 h before infection exerted antiviral activity (Fig. 1.3). Adlay tea inhibited the influenza virus at both the adsorption and replication time periods; virus replication was significantly inhibited when adlay tea was added to the cells before or within 4 h after viral infection (Fig. 1.3).

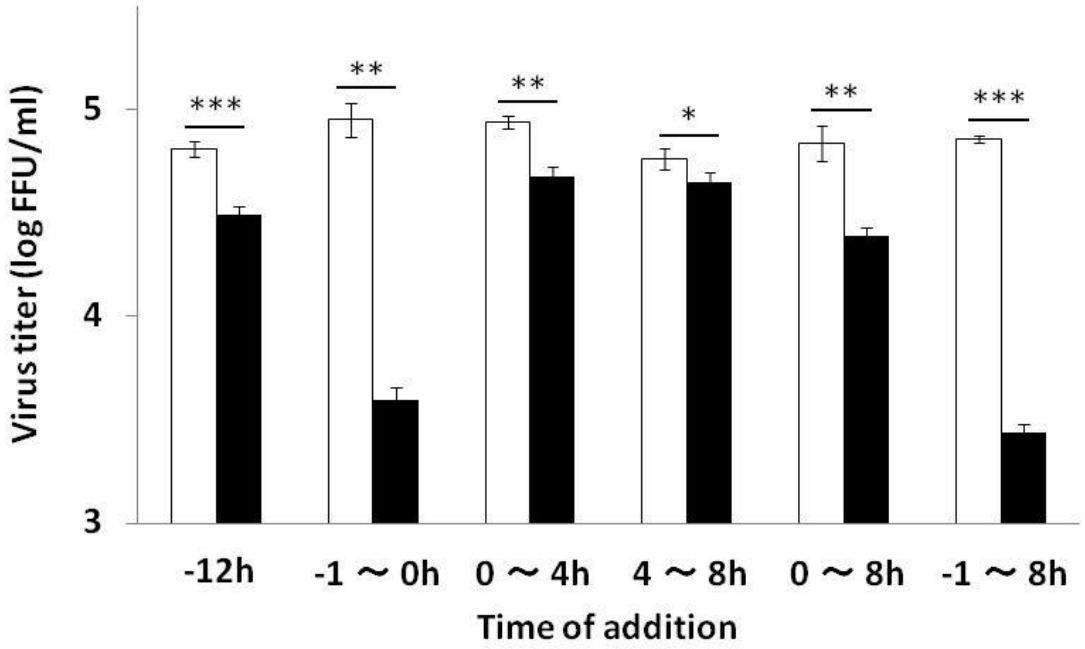

Fig. 1.3. Effectof delayed addition of adlay tea on the virus yield.
The conditions of influenza infection were the same as in Fig. 1. Adlay tea was added for the durations indicated, and viral yields were determined 8 h postinfection by focus-forming assays. Open bars: mean viral yield for control cells; filled bars: mean viral yield for cells treated with adlay tea (12.5 mg/ml). Vertical lines show standard deviations (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

Adlay tea inhibited virus adsorption. Adlay tea significantly inhibited virus adsorption (Fig. 1.3). Although it did not react with the A/PR/8/34 virus in the HI test, adlay tea slightly inhibited the ability of this influenza virus to bind to the MDCK cells (Fig. 1.4A). The time-of-addition assay showed that adlay tea (12.5 mg/ml) suppressed virus adsorption with an inhibition ratio of 95.6% (Fig. 1.3), and a viral binding inhibition assay showed that adlay tea (10 mg/ml) blocked virus binding to the cells with an inhibition ratio of 32.6% (Fig. 1.4A). Here, the inhibition ratio was defined as [(A-B)/A x 100, where A was the virus quantity in mock-treated cells and B was the quantity in adlay tea-treated cells]. These results suggested that adlay tea neutralized the virus by other mechanisms

in addition to preventing the virus from attaching to the cells. The tea did not inhibit cell-cell fusion, because the fusion index was more than 0.84 and did not depend on the concentration of adlay tea (Fig. 1.4B). Thus, the adlay tea did not affect the denaturation of the hemagglutinin (HA) protein.

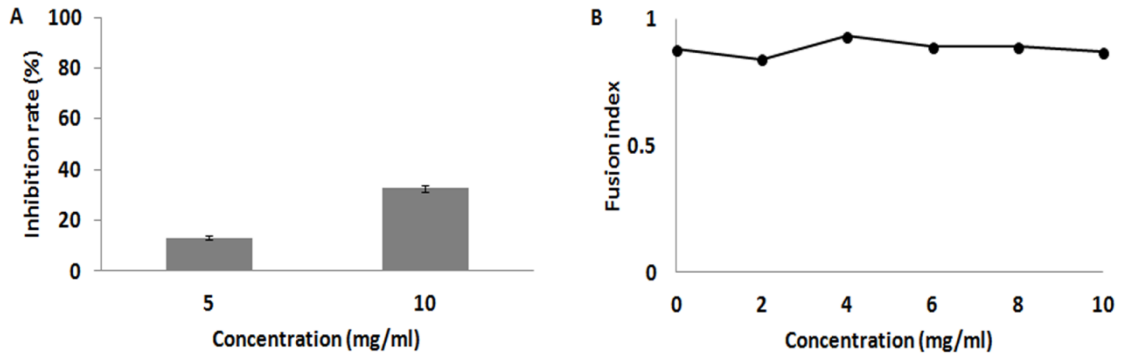

Fig. 1.4. Effect of adlay tea on virus adsorption.
(A) Adlay tea inhibited the binding of the influenza A/PR/8/34 virus to MDCK cells. Closed bars: mean viral yield for cells treated with two different concentrations of adlay tea components. Vertical lines show standard deviations (n = 3). (B) Lack of an inhibitory effect of adlay tea on cell-cell fusion mediated by influenza A/PR/8/34 virus. The fusion index was obtained by the following formula: fusion index = 1 − (number of cells/number of nuclei).

Effect of polyphenols against antiviral activities. Several studies have reported that polyphenols such as catechin and other flavonoids, and cocoa polyphenols have anti-influenza virus activity[55-57]. The used adlay tea contains adlay seeds, naked barley seeds, soy beans, and cassia seeds, all of which contain polyphenols. Therefore, I investigated whether the polyphenols in adlay tea have antiviral activities. The polyphenol concentration of the normal adlay tea was 0.99 mg/ml, and the polyphenol concentrations in the first and second filtrates of adlay tea treated with PVPP were 0.71 mg/ml and 0.63 mg/ml, respectively. The results showed that about 40% of the polyphenols in adlay tea was ab-

sorbed by PVPP, and about 60% of the polyphenols remained in the filtered sample. However, the antiviral activity was similar in normal adlay tea and in the filtrates of adlay tea treated with PVPP (Table 1.2).

Sample	Polyphenol concentration (mg/ml)	IC_{50} (mg/ml)	CC_{50} (mg/ml)	SI[a]
Nomal adlay tea	0.99	4.48 ± 0.62	>40	>9
First PVPP filtrate	0.71	4.27 ± 0.02	>40	>9
Second PVPP filtrate	0.63	4.07 ± 0.92	>40	>10

Table 1.2. Antiviral activity of PVPP-treated adlay tea.

[a]Selectivity index=CC_{50}/IC_{50}

1.4 Discussion

Influenza infections can cause severe complications and increase hospitalization and mortality rates, particularly in young children and the elderly[3]. Although the therapeutic drugs amantadine, rimantadine, zanavir, and oseltamivir reduce the severity and duration of illness[52-54], the occurrence of resistant influenza A viruses is inevitable[55, 56]. The new antiviral drug T-705 (Favipiravir), which is an RNA-dependent RNA polymerase inhibitor, has demonstrated potent inhibitory activity against influenza A, B, and C viruses[45, 57]. In addition, plant-derived isoquercetin inhibits the replication of the influenza virus[58]. Here I showed that adlay tea, which includes the hot-water extracts of adlay seeds, naked barley seeds, soy beans, and cassia seeds, inhibited influenza A and B viruses replication in MDCK cells (Table 1). Various other plants are also reported to inhibit virus replication[58, 59]. Thus, it is possible that many plants contain general inhibitors against various

kinds of viruses, including influenza virus. Within the plant, these inhibitors might prevent infection by harmful plant viruses.

In growth process of influenza virus, HA is binding to sialic acid on host cell and taken in the cell, after that uncoating. Next, synthesis of viral RNA and proteins are done, and the viruses are reconstructed and released[8] (Fig. 1). In time-of-addition assay, adlay tea extracts inhibited viral activity at each stage: pretreatment (−12 h), adsorption (−1 to 0 h), and replication (0 to 8 h). Adlay tea weakly inhibited virus binding to the cells in the binding-inhibition assay (Fig. 1.4A), although it did not prevent the virus from attaching to target cells in the HI test. It is a possibility that the adlay tea has a HA activity, thus results of HI test and binding-inhibition assay did not agree. Furthermore, a cell-fusion inhibition test showed that adlay tea did not denature HA protein (Fig. 1.4B). These results suggested that the antiviral activity of adlay tea is not related to HA, probably the tea has other inhibition mechanism at virus adsorption.

Polyphenols interact with PVPP by hydrogen bonds. The binding properties depend on polyphenols; for example, quercetin, catechin, and epicatechin show high binding at pH 6.5, whereas 4-methylcatechol and caffeic acid show low binding at this pH[60]. Therefore, I assumed that PVPP would show high binding with at least some of the flavonoid phenols in the adlay tea.

The anti-influenza virus activity of tea polyphenols such as catechin, theaflavin, and procyanidin has been reported[61]. In addition, quercetin inhibits influenza infection[62], and tea contains quercetin[63]. Another study suggested that anthocyanin pigments in hibiscus tea are associated with anti-influenza virus activity[64]. PVPP removes the polyphenols from cocoa, and the inhibitory effect of cocoa against influenza virus infection was shown to depend on the polyphenol concentration. Reported materials like tea, cocoa contain

procyanidins[51]. Based on these previous studies, I expected that the polyphenols in adlay tea would have the antiviral activity, and I compared the activity in normal adlay tea or adlay tea in which polyphenols were removed by PVPP treatment. However, against my expections, there was no difference between anti-influenza virus activities treated with and without PVPP, suggesting that the antiviral activity of adlay tea is independent of its PVPP-binding polyphenols. Thus, the polyphenols in adlay tea may not include quercetin, catechin, epicatechin, or procyanidins, which are reported to have antiviral activity against influenza virus. I concluded that two possibilities exist for the antiviral activity of adlay tea: either the polyphenols are non-flavonoid phenols and/or the antiviral activity is elicited by other kinds of compounds.

The IC_{50}s of extracts cannot be compared directly, due to variations in extraction efficiency, material stability, and inhibitors for the active ingredients, such as degrading enzymes or oxidants. However, the IC_{50}s are still useful as baseline data for the isolation and purification of effective substances. Adlay tea inhibited the infection by influenza A and B viruses *in vitro* (Table 1.1). The IC_{50}s ranged from 2.11 to 5.13 mg/ml for the influenza A viruses, and from 3.23 to 4.23 mg/ml for the influenza B viruses. There was some variability in the inhibitory activity between types of influenza virus, which was weaker than that of oseltamivir for all type A (H1N1 and H3N2) and B viruses, including the oseltamivir-resistant viruses. However, the tea demonstrated anti-influenza effects at just one-fifth the concentration normally consumed as tea.

In Chapter I, I found that adlay tea has potential as a novel treatment against influenza viruses. Moreover, antiviral effects is not affected by polyphenol content, suggesting that there are novel functional ingredients in adlay tea.

Chapter II

Antiviral activity of tea components and analysis of functional ingredients.

2.1 Introduction

In Chapter I, I revealed that anti-influenza virus activity of adlay tea. The tea is composed with adlay seeds, naked barley seeds, soybean, and cassia seeds. The adlay tea exhibited antiviral activity in the H1N1, H3N2, and B types of influenza viruses, including oseltamivir-resistant viruses. The action mechanism of adlay tea was inhibited virus replication as well as virus adsorption to the host cells.

I showed the possibility in Chapter I that the action mechanism of adlay tea is different from oseltamivir. In this Chapter, I conducted a detailed research of anti-influenza virus activity against each tea components including identification of active ingredients against IFV.

2.2 Materials and Methods

2.2.1 Compounds.

Daidzein, glycitein, biochanin A, genistein and 6’’-O-acetylgenistin were purchased by Wako Pure Chemical Industries, Osaka, Japan.

2.2.3 Hot-water extracts of adlay tea components.

Akishizuku brand adlay seeds (*Coix lacryma-jobi* L. var. *ma-yuen*), Ichibanboshi brand naked barley seeds (*Hordeum vulgare* var. *nudum*), Toyomasari brand soybeans (*Glycine max*) and cassia seeds (*Cassia obtusifolia* L.) were provided by Sanyo, Co., Ltd., Kobe, Japan. Its components were roasted at 180 °C for 4 h, and powdered. The roasted powder

(2.0 g) was mixed water (50 ml), and extracted in hot water bath for 80 °C, 1 h. These hot-water extracts were filtered through No. 2 filter paper followed by a Millex GV membrane (24 mm diameter and 0.22 μm pore size, Millipore, Billerica, MA, USA) and freeze-dried. The each lyophilizate were dissolved again in water, filtered through a Millex GX membrane (24 mm diameter and 0.22 μm pore size, Millipore, Billerica, MA, USA), and stored at −30 °C before use.

2.2.4 Cells and viruses.

MDCK cells and CV-1 cells were cultured as described in "1.2.1 Cells and viruses".

2.2.5 Antiviral assay of hot-water extracts.

Antiviral assay was performed as described in "1.2.3 Antiviral assay".

2.2.6 Time-of-addition assay.

Time-of-addition assay was performed as described in "1.2.5 Time-of-addition assay".

2.2.7 Cytotoxic test of hot-water extracts.

Cytotoxic test was performed by Cell Porliferation Kit (Roche Diagnostics, Basel, Switzerland)and performed as described in "1.2.6 Cytotoxic test of hot water extracts".

2.2.8 Hemoagglutination inhibition (HI) test.

HI test was performed as described in "1.2.7 Hemoagglutination inhibition (HI) test ".

2.2.9 Cell-fusion inhibition test.

Cell-fusion inhibition test was performed as described in "1.2.9 Cell-fusion inhibition test".

2.2.10 Purification and identification of the antiviral compounds in hot-water extract of soybean.

The roasted powder of soybeans (2.0 g) was mixed water (50 ml), and extracted in hot water bath for 80 °C, 60 min. The hot-water extract was filtered through No. 2 filter paper followed by a Millex GV membrane and freeze-dried.

Flash column chromatography was done by using Isolera Spektra (Biotage, Sweden). The lyophilizate (1.0 g) was suspended in H_2O (2 ml) and was subjected to reversed-phase flash chromatography eluting with a mixture of water and acetonitrile. The water-soluble fraction was developed on a pre-packed C18 cartridge (“SNAP Ultra C18”, 16mm×100mm) eluted in series with 264 ml of H_2O, 132 ml each of a mixture of H_2O:acetonitrile (v/v, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, or 1:9) and 198 ml of 100% acetonitrile to yield 50 fractions, the flow rate was 12 ml/min. Accordingly, fractions 1 to 8 eluted with water, fractions 9 to 12 eluted with a mixture of water:acetonitrile (v/v, 9:1), fractions 13 to 16 eluted with a mixture of water:acetonitrile (v/v, 8:2), fractions 17 to 20 eluted with a mixture of water:acetonitrile (v/v, 7:3), fractions 21 to 24 eluted with a mixture (v/v, 6:4), fractions 25 to 28 eluted with a mixture (v/v, 5:5), fractions 29 to 32 eluted with a mixture (v/v, 4:6), fractions 33 to 36 eluted with a mixture (v/v, 3:7), fractions 37 to 40 eluted with a mixture (v/v, 2:8), fractions 41 to 44 eluted with a mixture (v/v, 1:9), and fractions 45 to 50 eluted with 100% acetonitrile were collected. The eluate was detected with PDA monitor using 200 nm and 250 nm. The antiviral activity of each fraction was decided by antiviral assay and the active fractions were analyzed by LC/qTOFMS.

Analysis was carried out according to the method described by Kammerer et al.[65] with some modifications. The active fractions separated by preparative liquid chromatography (LC) as described above were subjected to an LC20ADXR high performance liquid chromatographyv (HPLC) system (Shimadzu, Kyoto, Japan) and a microTOF-QII quadrupole-time-of-flight tandem mass spectrometer fitted with an ESI ion source (Bruker Daltonics, Billercia, MA, USA). Synergi Hydro RP- column (particle size = 2.5 µm; 100mm × 3 mm i.d., Phenomenex, Torrance, CA, USA) was employed for the separation of the compounds. The mobile phase was (A) 2% acetic acid and (B) 0.5% acetic acid / acetonitrile = 1:1. The gradient began with 10% B and was varied to 24% B at 8 min, 30% B at 16 min, 55% B at 24 min, 100% B at 30 min, 100% B isocratic from 30 to 33.2 min, and 10% B from 34 to 36 min. The flow rate of mobile phase was 0.4 min/min. The temperature of the column oven was 40 ℃, and the sample injection volumn was 5 µL. Mass spectrometry was carried out using the following conditions: mass range 50-3000; spectra rate, 2 Hz, nebulizing gas, nitrogen (1.6 bar); drying gas, nitrogen (200℃, 8 L/min); capillary voltage,-4500 V for positive ion and +2800 V for negative ion; hexapole RF, 200 Vpp; quadrupole ion energy, 5 eV; collision gas nitrogen (1.6 bar); collision energy, 10 eV; collision RF, 200 Vpp. The antiviral activity of detected compounds were checked by antiviral assay.

2.2.11 Statistical analyses

Viral titers in time-of-addition assay and antiviral assay of soybeans fractions were analyzed by Student's *t*-test using the EXCEL TOUKEI ver. 6.0 (Esumi, Tokyo, Japan) system. The values are displayed as mean ± standard deviation (S.D.), $p < 0.05$ was considered significant.

2.3 Results

Antiviral activities of hot-water extracts. The all tea components showed antiviral activity against PR/8/34 (Fig. 2.1), the IC_{50}s were 356.9 μg/ml (adlay seeds), 571.8 μg/ml (naked barley seeds), 131.5 μg/ml (soybeans) and 335.4 μg/ml (cassia seeds).

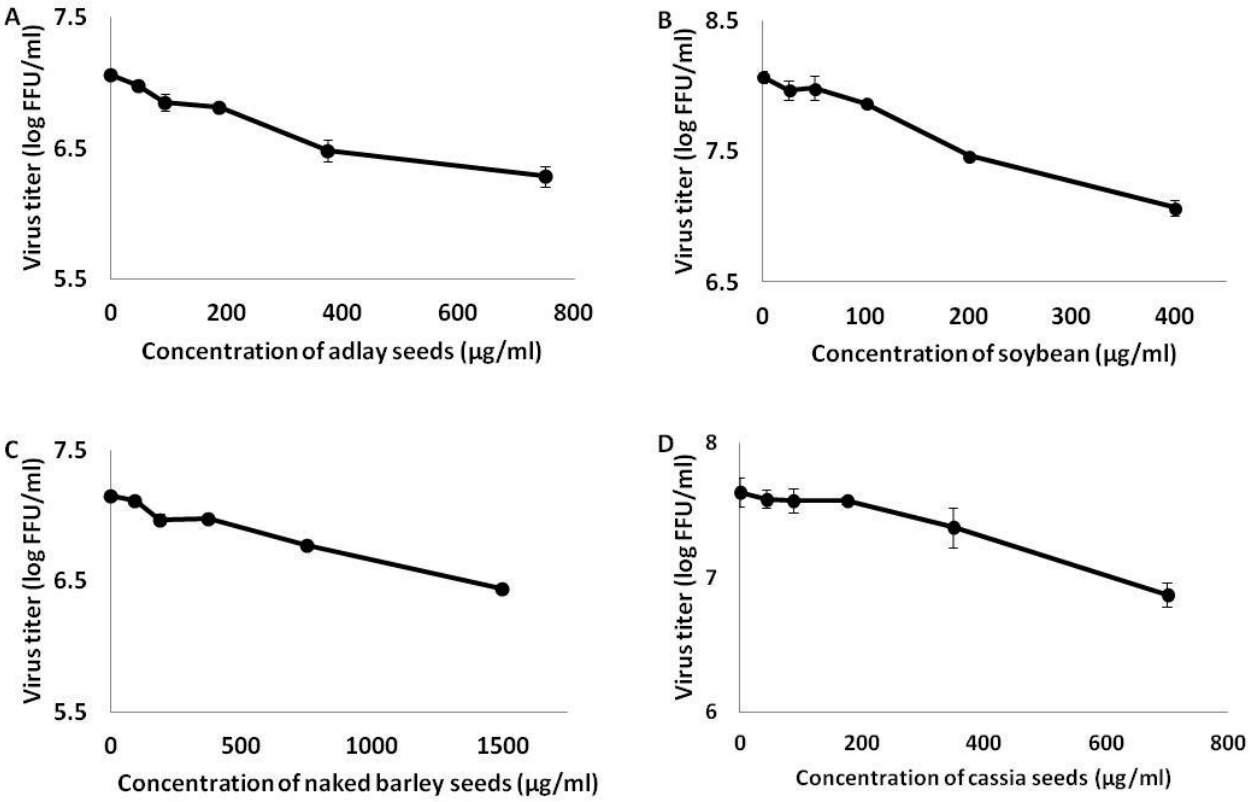

Fig. 2.1. Antiviral activity of tea components. (A) adlay seeds (Akishizuku); (B) soybeans (Toyomasari); (C) naked barley seeds (Ichibanboshi); and (D) cassia seeds. A/PR/8/34 (m.o.i. 0.0001) was infected MDCK cells, and cultured in medium containing hot-water extract for 24 h. The virus titers were measured by focus-forming assays.

Table 2.1. Effect of tea components on the multiplication of various influenza virus types and strains.

Virus type and strain	IC_{50} (μg/ml)[a]				SI[b]			
	Adlay seeds (Akishizuku)	Naked barley seeds (Ichibanboshi)	Soybean (Toyomasari)	Cassia seeds (From indian)	Adlay seeds (Akishizuku)	Naked barley seeds (Ichibanboshi)	Soybean (Toyomasari)	Cassia seeds (From indian)
A(H1N1)								
PR/8/34	356.9 ± 51.9	571.8 ± 47.6	131.5 ± 7.2	335.4 ± 68.5	26	25	56	9
New Caledonia/20/99	338.6 ± 41.6	636.3 ± 1.2	329.0 ± 46.1	114.3 ± 32.4	28	22	23	26
Beijing/262/95	422.1 ± 54.9	1264.4 ± 426.3	71.5 ± 5.9	115.3 ± 41.1	22	11	104	27
Osaka/2024/2009[c]	499.4 ± 77.9	544.5 ± 12.2	78.6 ± 7.3	210.5 ± 88.5	19	26	94	14
Osaka/71/2011[c]	333.5 ± 96.7	274.0 ± 238.3	83.6 ± 19.0	98.1 ± 22.9	28	52	88	30
A(H3N2)								
Sydney/5/97	158.1 ± 44.4	470.9 ± 88.8	90.5 ± 30.4	76.7 ± 15.4	80	88	62	14
Aichi/2/68	117.9 ± 58.7	161.3 ± 99.9	118.5 ± 13.4	215.0 ± 96.8	60	30	81	39
B								
Nagasaki/1/87	59.4 ± 15.1	724.1 ± 81.0	35.4 ± 18.6	837.6 ± 147.3	137	20	212	4
Shanghai/261/2002	69.5 ± 3.4	494.3 ± 79.6	32.1 ± 16.7	376.0 ± 181.3	160	29	232	8

[a]The values are averages of results obtained using the final concentrations of tea components, from two independent experiments.
[b]The values are averages of results obtained using the final concentrations of adlay tea, from three independent experiments.
[c]Oseltamivir-resistant virus
[d]Selectivity index=CC_{50}/IC_{50}
ND: not determined

components inhibited virus multiplication of all laboratory-adopted and clinical isolates of influenza A and B viruses (Table 2.1), and effectiveness against oseltamivir resistant viruses such as Osaka/2024/2009 and Osaka/71/2011. The samples did not show cytotoxicity in the range used for the antiviral assay. The CC_{50}s of adlay seeds, naked barley seeds, soybeans and cassia seeds were 9.5 mg/ml, 14.2 mg/ml, 7.4 mg/ml and 3.0 mg/ml, respectively. The result of calculating the SI value, soybeans showed strongest effects than other components.

Elucidation of the action mechanism of hot-water extracts. I carried out a time-of-addition assay to investigate about when is the inhibition stage of the tea components in virus infection. The infection period was partitioned five patterns, pretreatment before virus adsorption (pattern 1), virus adsorption (pattern 2), virus replication (pattern 3) virus replication at early stage (pattern 4) and late stage (pattern 5). The results of time-of-addition assay are shown in Fig. 2.2. The all tea components blocked virus adsorption most strongly, and the inhibition rate is more than 90% in any components. The virus replication was also inhibited by its components. However, the naked barley seeds and cassia seeds were ineffective in virus replication at late stage.

The tea components inhibited both the virus adsorption and replication in time-of-addition assay. Thus, I examined the more detail action mechanism by HI test and cell fusion inhibition test. However, viral neutralization and inhibition of cell fusion were not confirmed in any components (date not shown).

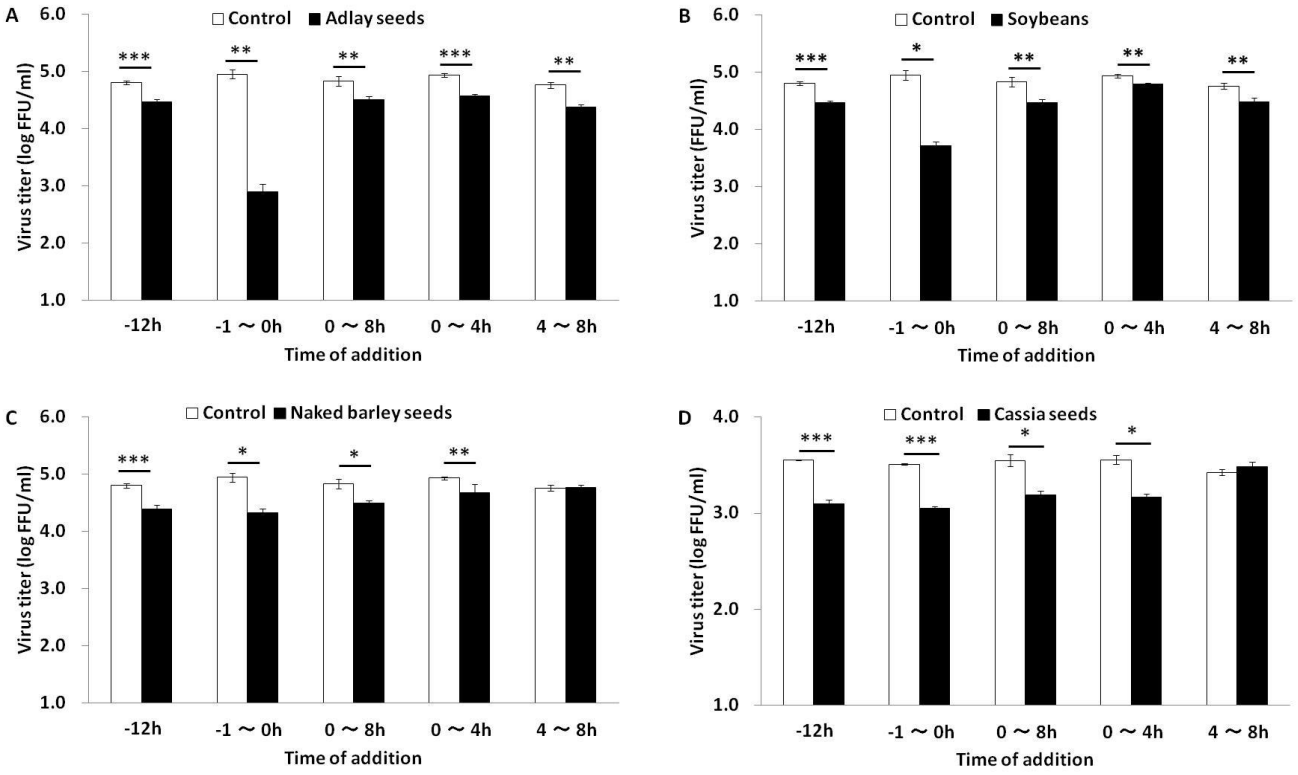

Fig. 2.2. The inhibition stage of tea components in the virus multiplication. (A) adlay seeds (Akishizuku); (B) soybeans (Toyomasari); (C) naked barley seeds (Ichibanboshi); and (D) cassia seeds. A/PR/8/34 (m.o.i. 0.0001) was infected MDCK cells, the infected cells were cultured under the five patterns. Pattern 1 (-12 h); Pattern 2 (-1~0 h); Pattern 3 (0~8 h); Pattern 4 (0~4 h); Pattern 5 (4~8 h). The addition content is as follows: adlay seeds (400 μg/ml), soybeans (300 μg/ml), naked barley seed (1500 μg/ml), cassia seeds (700 μg/ml). The virus titers were measured by focus-forming assays. *P < 0.05; **P < 0.01; ***P < 0.001.

Identification of the antiviral compounds in hot-water extract of soybeans. Each collected fractions of soybeans extract were checked antiviral activity against PR/8/34 (Fig. 2.3A). Factions 1 to 2 and fraction 12 to 23 showed antiviral activity, thus the active fractions (fraction 12 to 23) and inactive fractions (fraction 10 and 24) were analyzed by LC/qTOFMS. In fractions 16 and 17, daidzein which are molecular weight (MW) 254 was speculated. In fraction 18 to 20, glycitein or biochanin A which are MW 284 were speculated. In fractions 19 to 22, 6''-O-acetylgenistin which are MW 474, and genistein which are MW 270 were speculated.

The antiviral activities of the isoflavones were checked, and confirmed activity against PR/8/34 by daidzein and glycitein (Table 2.2), the IC_{50}s were 143.6 μM and 204.7 nM, the CC_{50}s were more than 3.9 mM and 3.5 mM, respectively. The other isoflavones did not show the antiviral activity (date not shown).

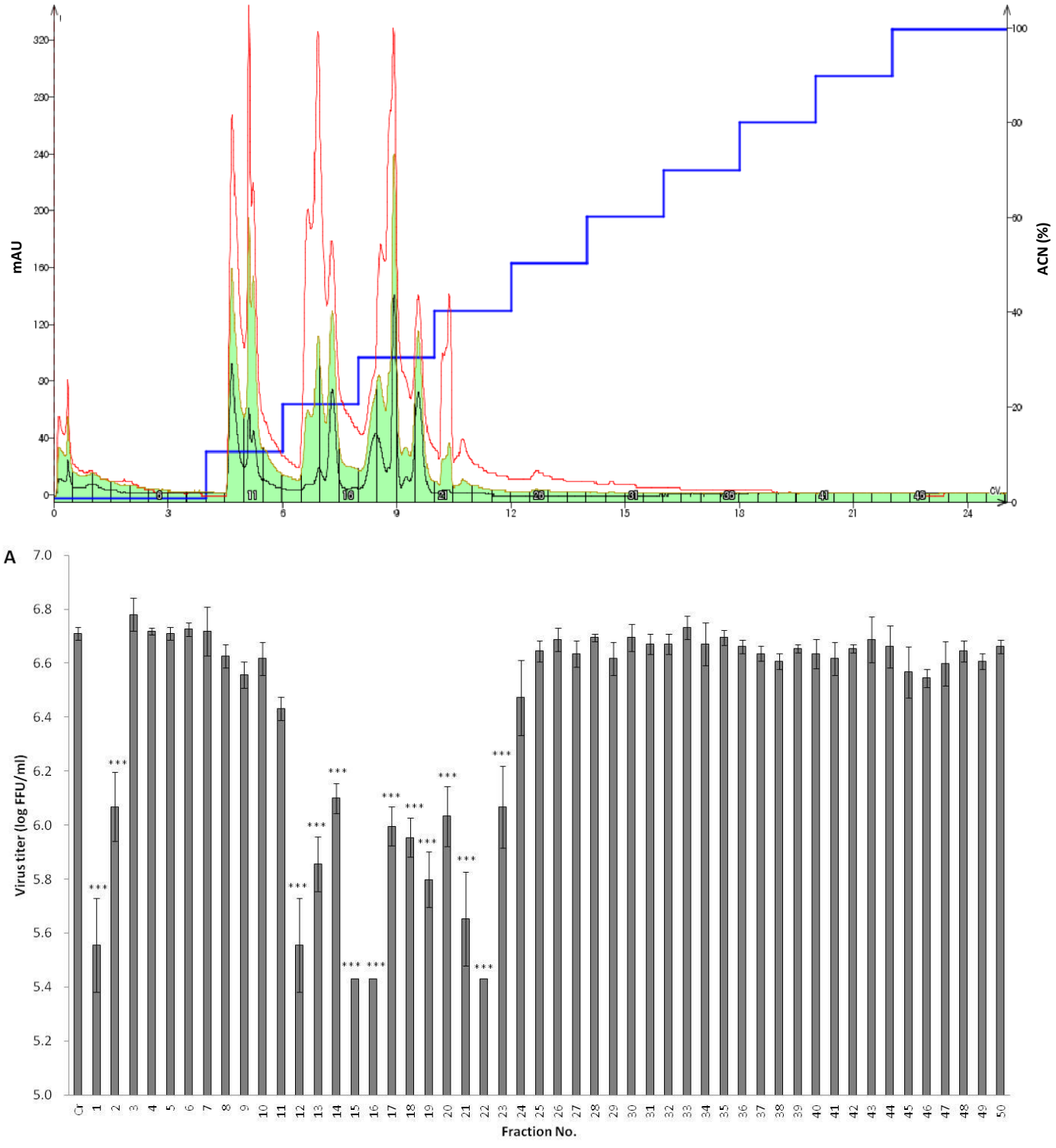

Fig. 2.3 Fractionation and virus-inactivating ability of the hot-water extract from roasted soybeans. (A) peak detection in flash column chromatography; (B) antiviral assay of fractions.
The soybeans extract was fractionated by preparative LC using SNAP C_{18} column chromatography. Antiviral activity of fractions (Fr.) 1-50 were checked by antiviral assay. A/PR/8/34 (m.o.i. 0.0001) was infected MDCK cells and incubated for 24 h. The virus titers were measured by focus-forming assays. Statistical test vs control (Cr): ***, $P<0.001$.

Table 2.2. Antiviral activity of daidzein and glycitein.

Compound	IC_{50} (μM or nM)[a]	SI[b]
Daidzein	143.6 ± 78.9	>27
Glycitein	204.7 ± 21.0	>17182

[a]The values are averages of results in antiviral assay against A/PR/8/34, from two independent experiments. The IC_{50} of daidzein and glycitein are given in μM and nM, respectively.
[b]Selectivity index=CC_{50}/IC_{50}

2.4 Discussion

In antiviral assay, all tea components showed antiviral activity to type A and B influenza viruses. Adlay seeds and soybeans were also especially effective against type B viruses, suggesting that there is a relation between antiviral activity of tea components and antigenic structure of IFV. Moreover, all components showed the activity to oseltamivir resistance viruses, so action mechanism of its components is different from the inhibitor.

There are many isoflavones in soybeans [66], and daidzein, genistein, formononetin and sophorico side has NA inhibition activity[36]. Also in my study, soybeans exhibited strong anti-influenza virus activity. The reason is probably related to isoflavones contained. However, the antiviral activity of adlay tea did not correlate with the polyphenol content in Chapter I. The content ratio of soybeans is as low as 12 % of the whole in adlay tea. Therefore it is assumed that adlay tea has many active ingredients other than polyphenols, and that polyphenols are not principal active ingredients.

In time-of-addition assay, all tea components showed inhibition effect at pretreatment, adsorption and replication. The action mechanism of adsorption inhibition is a possibility that binding block between HA and sialic acid, or virucidal effects. However, any components did not show inhibition effect in HI test. Thus it is possibility that these components has other mechanism that inhibit the uptake into the cells like a endocytosis inhibitors. Moreover, all components did not show the activity in fusion inhibition assay, so

antiviral activity of components is unrelated with HA.

It is remarkable that the all components showed inhibitory effects in pretreatment before virus adsorption. This results suggested that these components might affect function of the cell such as signaling pathway. It has been reported that replication process of influenza virus and signaling pathway are related [67, 68]. From these result, it is expected that tea components has novel action mechanism against influenza virus. In addition, it is considered that each component probably has several active ingredients, and showed the antiviral activity at various stages.

It has reported that anti-influenza virus activity and neuraminidase inhibitory effect in daidzein[36]. In this report, they obtained strong neuraminidase inhibitory effect of daidzein but it showed weak activity against A/Jinan/15/90 (H3N2) that IC_{50} is more than 787 μM in CPE reduction assay. The differences between my results and reported one might be due to different type of the virus and evaluation method. The antiviral activity of glycitein has not been reported. I discovered the activity for the first time. Glycitein showed the activity even in small amount, and it has more strong effect than that of daidzein. Moreover, soybeans has several active ingredients because other fractions showed antiviral activity. Thus, the antiviral effect of soybeans is probably combined effect by many ingredients.

In Chapter II, I revealed that all tea components exhibited antiviral activity thus the antiviral activity of adlay tea is composite effect from a lot of active ingredients. Moreover, as adlay tea and tea components has several action mechanism, it is expected that the anti-influenza effect of adlay tea *in vivo*.

Chapter III

Anti-influenza effect of adlay tea in mice.

3.1 Introduction

Influenza virus infects to the columnar epithelial cells of respiratory tract. In this process, viral HA is combine with sialic acid on host cells. Next, HA structure changed by the trypsin-like protease on respiratory tract, and virus proliferate[69, 70]. NA mutilate HA from host cell at release of progeny virus particles[8], thus NA inhibitors are effective for inhibition of viral multiplication. The cytokines and chemokines produced at virus infection, and regulate immune system[71, 72]. However, excessive cytokine and chemokine production is related to inflammatory response, and cause serious complications such as pneumonia and encephalitis[1, 2].

In protection against influenza, it is important that regulate of inflammatory response and produce of virus specific IgA antibodies, which is main system of mucosal immunity in respiratory tract. Thus, the functional foods which has these effects are attention to control influenza. Especially, *Lactococcus* strains has many activity such as regulation cytokine response and activation NK cells[73-76], and it effective against influenza. Also adlay seeds has anti-inflammatory effect and increasing the peripheral cytotoxic lymphocytes[43, 45].

I revealed anti-influenza virus activity of adlay tea *in vitro* in Chapter I and II. In this Chapter, I validated anti influenza effects *in vivo*, and confirmed relevance between antiviral effect and immune responses that production of cytokines and IgA antibody.

3.2 Materials and Methods

3.2.1 Hot-water extract of adlay tea

The adlay tea components (adlay seeds, naked barley seeds, soybeans, cassia seeds) were roasted at 180 ℃ for 4 h, and powdered. The roasted powders were blended, and its powder (2.0 g) was mixed H_2O (50 ml), and extracted in hot water bath at 80 °C, for 1 h. The hot-water extract were filtered through No. 2 filter paper followed by a Millex GV membrane (24 mm diameter and 0.22 μm pore size, Millipore, Billerica, MA, UK) and freeze-dried. The each lyophilizate were dissolved again in H_2O, filtered through a Millex GX membrane (24 mm diameter and 0.22 μm pore size, Millipore, Billerica, MA, UK), and stored at −30 °C before use.

3.2.2 Cells and viruses

MDCK cells were cultured as described in "1.2.1 Cells and viruses". PR/8/34 strain was passaged in mice to adapt.

3.2.3 Mice

BALB/c mice (5 weeks old, female) were purchased from Japan SLC and were acclimatized for 7 days. The mice were housed in plastic cages (5-6 mice/cage), and kept as follows: light/dark cycle (12 h each), temperature (23 ± 2 ℃), pellet diet (AIN-76) and sterile water *ad libitum*. I performed animal experiments in Hyogo College of Medicine according to the animal experimentation guidelines of the College and Science Council of Japan.

3.2.4 Animal test.

I performed 2 experiments, the time schedule is displayed in Fig. 3.1. In experiment 1, the mice were separated to PBS group (control) and adlay tea group. Adlay tea was nasal administered (2 mg/ 20 µl/ mouse), and PBS was administered in the same way (20 µl/ mouse). Mouse-adapted PR/8/34 strain was intranasally infected (100 PFU/ 20 µl/ mouse), and monitored body weight, and mortality for 14 days after infection. In experiment 2, the test was performed under the same conditions as experiment 1. Five to six mice of each group were killed on day 3 and 4 post-infection (p.i.) to collect samples such as lung, nasal lavage fluid (NLF) and blood.

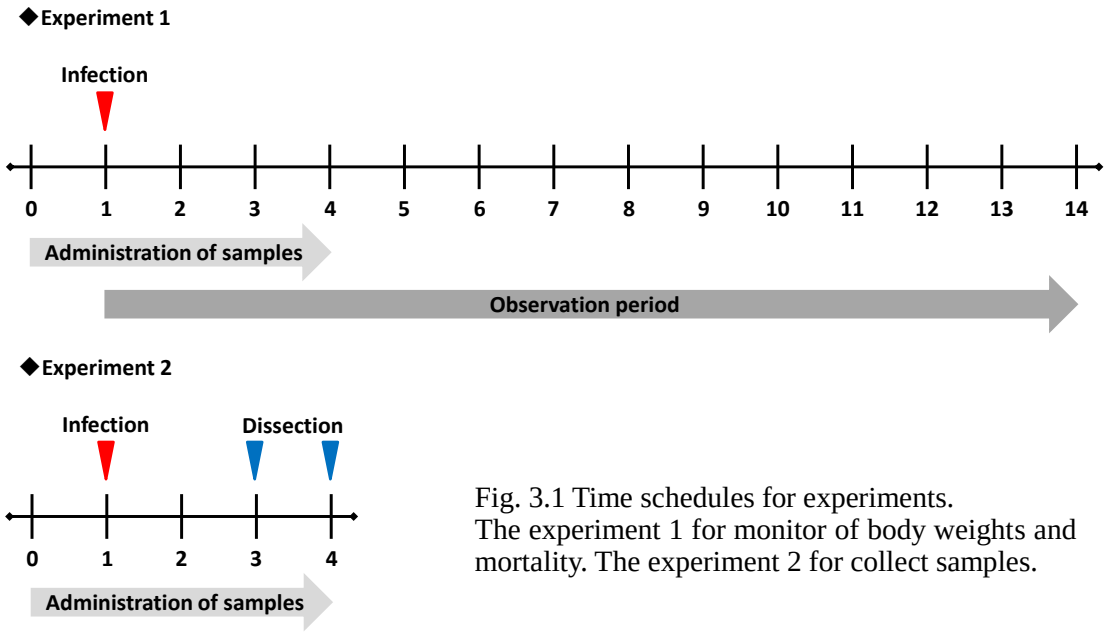

Fig. 3.1 Time schedules for experiments.
The experiment 1 for monitor of body weights and mortality. The experiment 2 for collect samples.

3.2.5 Quantification of virus titer in lung

The lung samples were homogenized with 5 ml of cold MEM, and centrifuged at 2,000 rpm, 4℃ for 20 min. The supernatants were collected to quantification of virus titer by FFRA. FFRA was performed as described in "1.2.4 FFRA for measuring virus titer"

3.2.6 Measurement of Cytokines in serum

The blood samples were incubated at room temperature for 30 min, than allowed to stand at 4 ℃ for 18 h, and centrifuged at 3,000 rpm, 4 ℃ for 20 min. The supernatants were collected, and measurement each cytokine by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

3.2.7 Measurement of IgA in NLF

NLF was centrifuged at 2,000 rpm, 4 ℃ for 20 min, and measurement by ELISA kit (Bethyl Laboratories, Montgomery, AL, USA).

3.2.8 Histopathologic analysis

The lung samples were fixed by folmalin for 24 h, dehydrated by ethanol, and embedded in paraffin. The lung sections (2 μm) were stained by hematoxylin and eosin.

3.2.9 Statistical analysis

A survival rates, body weight losses, and other data were analyzed by Kaplan-Meier method, two-way ANOVA and Student's *t*-test, respectively, by using EXCEL TOUKEI ver. 6.0 (Esumi, Tokyo, Japan) system. The values are displayed as mean ± standard deviation (S.D.), and $p < 0.05$ was considered significant.

3.3 Results

Influence on body weight loss and survival rate. In adlay tea group, the body weight losses were suppressed from day 3 p.i., and a significant difference on day 4 and 5 p.i. (Fig. 3.2). The comparison was impossible from day 6 p.i., because the mice of PBS group

almost dead. The body weights of all mice were recovered on day 14 p.i. in adlay tea group. Although the all mice dead day 7 p.i. in PBS group, the survival rate of adlay tea group was around 38 %, which are significant higher than PBS group (Fig. 3.3). This experiment was carried out again, and obtained similar result.

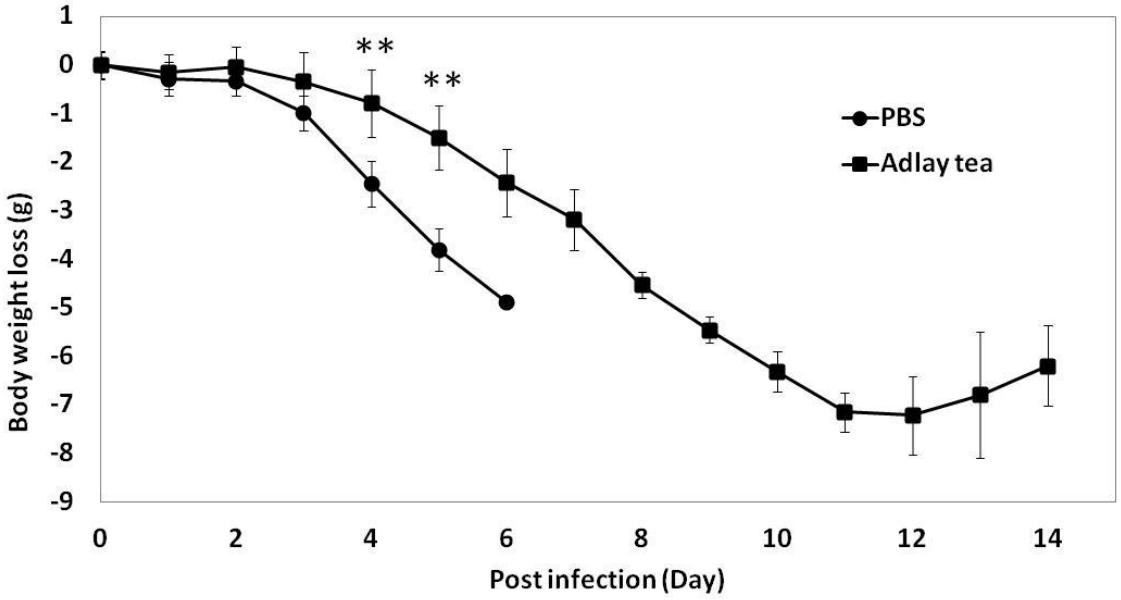

Fig. 3.2 Body weight loss from post infection.
The body weights were monitored for 14 days after infection. PBS group (n =10), Adlay tea group (n =11). **P < 0.01, by two-way ANOVA.

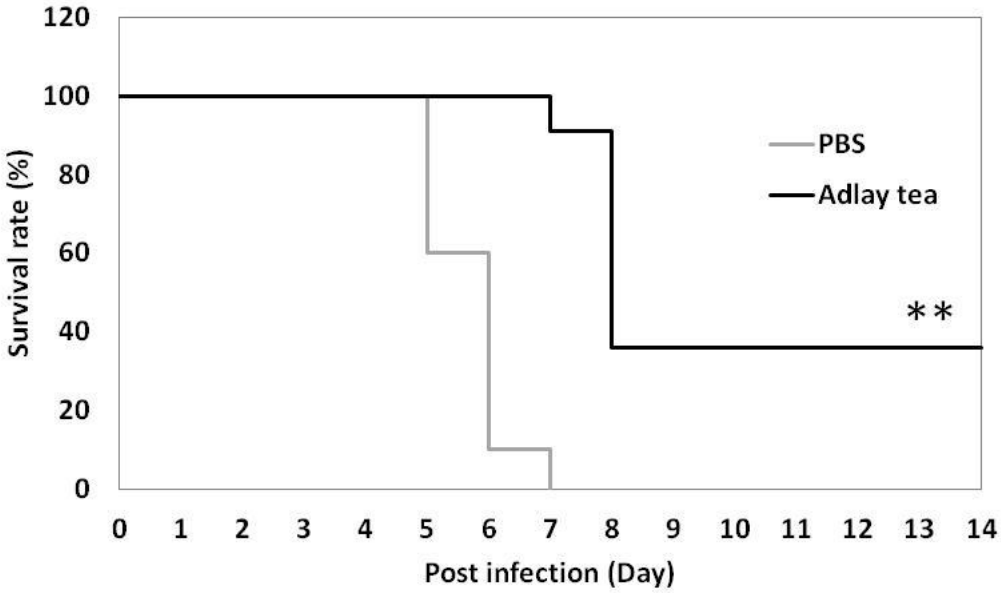

Fig. 3.3 Comparison of survival rate.
The body weights were monitored for 14 days after infection. PBS group (n =10), Adlay tea group (n =11). **P < 0.01, by Kaplan-Meier method.

Analysis of samples. Adlay tea inhibited virus growth in lungs (Fig. 3.4), and decreased production of interleukin (IL)-6 (Table 3.1). In both groups, IL-4 increased on day 4 p.i., and interferon (IFN)-γ decreased on day 4 p.i. Furthermore, tumor necrosis factor (TNF)-α was a minuscule amount, and it was difficult to detect (Table 3.1). There was no difference to production of IgA antibodies between both groups (Table 3.2).

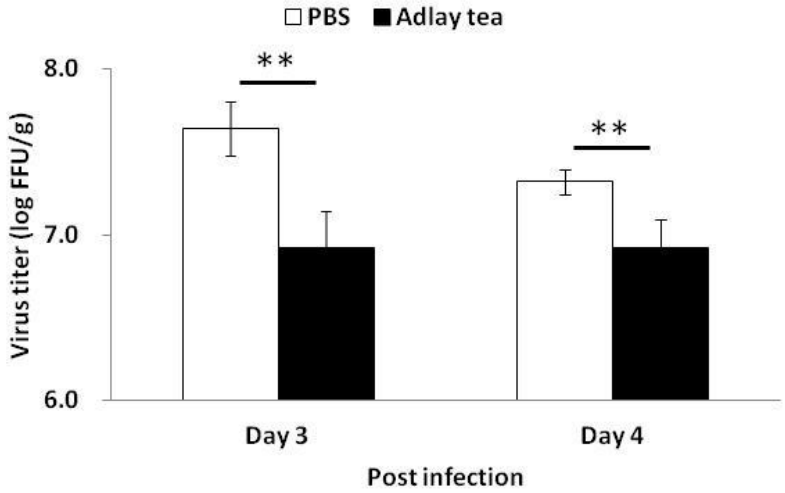

Fig. 3.4 Comparison of virus titer in lung.
The virus titers of lung were compared on day 3 and 4 p.i. PBS groups (n =5 on day 3 and 4 p.i.), Adlay tea groups (n =6 on day 3 p.i., n =5 on day 4 p.i.). **P < 0.01, by Student's *t*-test.

Table 3.1. Cytokines in serum.

Cytokine	Day 3 p.i.		Day 4 p.i.	
(pg/ml)	PBS (n =5)	Adlay tea (n =6)	PBS (n =5)	Adlay tea (n =5)
IL-4	79.2[a]	0	161 ± 120	57 ± 43
IL-6	646 ± 282	283 ± 87	649 ± 286	311 ± 94
IFN-γ	387 ± 310	189 ± 88	118 ± 41	107 ± 49
TNF-α	27 ± 23	33 ± 18	21 ± 8	0

[a] Only one animal was detected.

Table 3.2. IgA antibody in NLF.

(ng/ml)	Day 3 p.i.		Day 4 p.i.	
	PBS (n =5)	Adlay tea (n =6)	PBS (n =5)	Adlay tea (n =5)
IgA antibody	678 ± 363	796 ± 628	1232 ± 582	1133 ± 285

Anti-inflammatory effect in lung. The histopathologic analyses was compared between mock infected group, PBS group and adlay tea group on day 3 p.i. This assay observed the inflammatory responses and haemorrhage in PBS group (Fig. 3.6A). It also confirmed necrose of epithelial cells on respiratory tract (Fig 3.6B), and infiltration of immune cells such as monocytes, lymphocytes and neutrophils (Fig 3.6A, B). Adlay tea alleviated these response, and show protection effect against influenza (Fig 3.6 C, D).

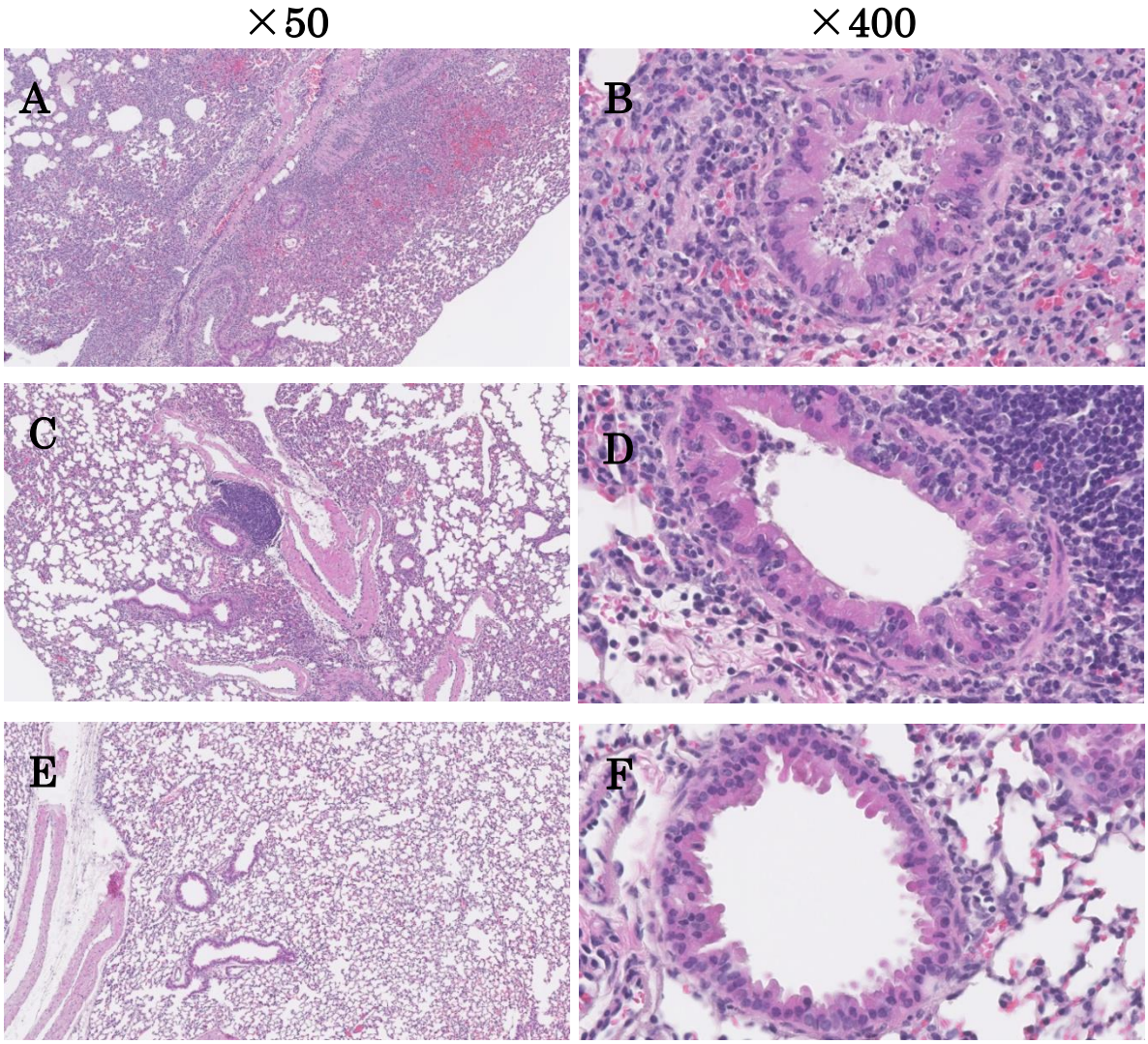

Fig. 3.4 The lung lesion involved in viral infection: (A) Lung tissue of PBS group; (B) Respiratory tract of PBS group; (C) Lung tissue of Adlay tea group; (D) Respiratory tract of Adlay tea group; (E) Lung tissue of Mock infected group; (F) Respiratory tract of Mock infected group.
Histopathologic analyses was performed on day 3 p.i. The lungs were stained by hematoxylin and eosin. n =3/animal group.

3.4 Discussion

There are two approaches for prevent influenza. One is act to the virus, and the other is act to immune system of the host. NA inhibitors and vaccines corresponds these approaches, respectively. *In vitro* studies in Chapter I and II revealed that adlay tea inhibited the virus adsorption to the cells and growth process in the cells. Moreover, it has reported that adlay seeds increasing the peripheral cytotoxic lymphocytes[45]. Thus, it expected that adlay tea acts to the virus and immune system.

I revealed anti-influenza effect of adlay tea in this chapter. Adlay tea suppressed the body weight losses from day 3 p.i., and inhibited the virus growth in lungs on day 3 and 4 p.i. These results suggested that the adlay tea exert anti influenza virus effects at early stage of infection, and its effects are probably due to activation of cellular immunity and/or inhibition of virus multiplication in a cell. Measurement of cytokines was carried out to confirm which pathway is activated by adlay tea. Then IL-6 concentration in adlay tea group was lower than that of PBS group. However, it seems that the cause of low IL-6 concentration is follow reduce influenza virus, and not reflective of immunoregulation activity by adlay tea. The other cytokines were no difference or minuscule amount. This assay was not able to exact comparison, so other assays are necessarily to reveal the action mechanism. There was no difference to IgA production on nasal cavity between PBS and adlay tea groups. Thus, the anti-influenza effect by adlay tea is probably low relevance with protection effect by IgA antibodies.

The lactic acid bacteria which acts to enhancement of antibodies production shows protection effects at late stage during infection[77-79] . The reasons is probably that the pathogen specific antibodies are generally induced from about day 10 p.i.[78-81]. On the

other hands, the plants having anti-influenza virus activity such as HA inhibition activity, NA inhibition activity and blocked viral replication are show anti-influenza effects at early stage during infection[82-85], and it is similary to anti-influenza effect by adlay tea in mice. Moreover, adlay seeds increasing the peripheral cytotoxic lymphocytes[45], so adlay tea may remove infected cells.

In this Chapter, I revealed anti-influenza effects of adlay tea in mice. In serious infection, it important to remove infected cells quickly, but the antibody production takes time. All mice in PBS group died at day 7 p.i. in animal tests, so antibodies are probably not able to full ability in this condition. The adlay tea may has activities on viral growth in intracellular of the host and enhance early immune responses, and show anti-influenza effect in serious infection. These show possibility that adlay tea is useful to novel influenza viruses and resistant one.

Conclusion

The countermeasures against influenza still has problems, so I am researching anti-influenza virus activity of foods and its components as a novel approach to influenza. At first, I examined food extract, which has anti-influenza virus activity. As a results, I found that the adlay tea inhibits growth of influenza virus, and decided it as a subject of study for Ph. D. thesis.

Adlay tea showed antiviral activity against type A and B influenza viruses *in vitro*. The action mechanisms were block of virus adsorption to the host cells, and inhibition of viral growth in the cells, but adlay tea did not affect to action of HA. In addition, *in vitro* study indicated that adlay tea may affect to cell function, and show the antiviral activity. These action mechanisms are different to anti-influenza agents, and it is remarkable characteristics for development novel approach.

The adlay tea, which use this study composed adlay seeds, naked barley seeds, soybeans and cassia seeds. I confirmed the antiviral activity of its components, and all components were effective against type A and B influenza viruses. The action mechanism were block of virus adsorption, and inhibition of viral growth in the cells. However, naked barley seeds and cassia seeds did not show the activity at late stage in viral growth process, thus the kind of active ingredients are different for each components constituting adlay tea. Moreover, it is assumed that anti-influenza virus activity of adlay tea is multiple effect from many active ingredients, and it is reason why adlay tea has several action mechanisms.

Soybeans showed the strongest anti-influenza virus activity among the raw materials used for adlay tea. Accordingly, I analyzed active ingredients by LC/qTOFMS. As a results, I found daidzein and glycitein for the active ingredient. The anti-influenza virus

activity of daidzein has been reported, but about glycitein is unreported result. I revealed anti-influenza virus activity of glycitein for the first time. Other researcher has shown that daidzein has NA inhibition activity. However, soybeans inhibited the virus adsorption and replication in this study. It is suggested that there are many active ingredients other than daidzein and glycitein.

In vitro study against inhibition of influenza virus growth showed that the adlay tea probably involve to the viral growth process and function of the host cells, and exert the antiviral activity. Then it is confirmed that whether the adlay tea show the activity in mice infected influenza, and proved effectiveness. The adlay tea reduced body weight losses, and raised survival rates. Moreover, the virus titers of adlay tea group was lower than control group (PBS). Histopathologic analyses revealed that the adlay tea inhibited inflammation and infiltrate immune cells in lungs. The virus titer was different between PBS group and adlay tea one at day 3 p.i. From these results, adlay tea exert the antiviral activity at quite early stage during infection. *In vivo* study showed that the adlay tea alleviate symptom of influenza, and the action mechanism may be due to activation of cellular immunity and/or inhibition of virus multiplication in a cell.

I proved that it is possible to prevent serious influenza by functional food. The foods are few restriction of use, and can allows daily continuously be taken, so it seems effective to prevention of infection, it is the most different point from antiviral agents. Moreover, the functional foods are often enhance defense capacity of the host, and show anti-influenza effect. This function is particularly important for young children and the elderly with poor immunocompetence. Thus, the countermeasures against influenza by functional foods will be establish as a novel approach in the future.

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