Figure 8. ISRE reporter assay in the presence of the C and Y mutants. 293T cells were co-transfected with a series of deletion mutants of the C protein (A), deletion mutants of the Y1 protein (B), or the Y1 mutants possessing triple alanine substitutions (C) together with a reporter plasmid, pISRE-EGFP. At 18 h p.i., cells were treated with IFN-α (1,000 IU/ml) for 8 h, and then expression level of EGFP and the transfected C and Y1 proteins were analyzed by Western blotting using anti-GFP and anti-C antibodies.

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plasma membrane, since the N-terminal 23 amino acid region has been shown to serve as membrane-targeting and membrane-anchoring signals [19,23]. The localization of the C protein at the plasma membrane has been reported to be important for the IFN-independent phosphorylation of STAT1, although the significance of this phosphorylation is unknown, and for promoting budding of viral and virus-like particles by recruiting cellular ESCRT machinery along with Alix to the site of budding on the plasma membrane [19,23].

In this paper, we showed that the smaller Y1 protein, lacking the N-terminal membrane-targeting and membrane-anchoring signal region, was able to actively localize to the nucleus in a Ran GTPase-dependent manner. The clustered basic amino acids within the 149-157 region were critical for the nuclear localizing function, consistent with the notion that the basic residues are critical for the function of known NLSs, although no potential NLS was found throughout the protein. Nuclear localization of the Y1 protein seems to be relevant to its ability to inhibit IFN-stimulated activation of ISGs, since these two abilities almost paralleled each other with an exception for Y1-WLR155A3, which retained the ability to inhibit the Jak/STAT signaling pathway despite the loss of nuclear localization. However, it might be impossible to connect a single region with a single function of the C proteins, since it has been reported that multiple amino acids widely spread in the C protein are involved in its inhibitory effect against the IFN-signaling pathway, although the C-terminal half is enough and the C-terminal end of the C protein is essential for the function [12,13,17,20].

Interestingly, the mechanism for inhibition of the IFN-signaling pathway seems to be different between the C and Y1 proteins, since the C-terminal deletion of 12 amino acids resulted in the loss of IFN-antagonizing ability for the C, but not Y1, protein (Figure 8). It has been reported that the C-terminal half (a.a. 85 -204) of the C protein is enough for the interaction with STAT1 as well as inhibition of IFN-mediated induction of ISGs and subsequent establishment of the antiviral state, and thus, both the C and Y1 proteins retain all of these abilities [12,13]. Blockade of the INF-α/β-signaling pathway might be achieved by both interaction of the C protein with STAT1 in the cytoplasm and that of the Y1 protein in the nucleus.

Conflicting with the above observations, there are other reports using MEFs and 2fTGH cells, that the Y1 protein possesses the ability to inhibit IFN-signaling, but not the establishment of an antiviral state, while only the full-length C protein is able to inhibit both [11]. This conflict might imply the presence of a parallel pathway independent of the Jak/STAT pathway, and the different subcellular localization of the C and Y1 proteins might reflect differences in their targeting pathways.

As described above, the C protein is able to block not only the IFN-α/β but also the IFN-γ signaling pathways [3,5,13,16]. Although the detailed mechanism has not been revealed yet, it has been reported that the C protein is not able to prevent IFN-γ-mediated tyrosine and serine phosphorylation of STAT1 and subsequent translocation of the phosphorylated STAT1 into the nucleus, but is able to prevent GAF-GAS interaction in the nucleus [16,41]. Since the Y1 protein is able to bind to STAT1, the blockade of the IFN-γ pathway might result from the Y1-GAF interaction in the nucleus.

Experiments to elucidate the detailed mechanism for blockade of the IFN-signaling pathways by the C and Y1 proteins are currently underway using in vitro cDNA-based expression systems as well as SeV recombinants possessing amino acid substitutions within the C protein which cause a defect of the nuclear-localizing ability. Further understanding of viral strategies for escape from host innate immunity will hopefully lead to a better understanding of viral pathogenesis and the development of novel therapeutics to target important steps in viral replication.

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Author Contributions

Conceived and designed the experiments: TI AY TS. Performed the experiments: TI AY. Analyzed the data: TI AY. Contributed reagents/materials/analysis tools: TI AY TS. Wrote the manuscript: TI TS.

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Inactivation of Pathogenic Viruses by Plant-Derived Tannins: Strong Effects of Extracts from Persimmon (*Diospyros kaki*) on a Broad Range of Viruses

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Abstract

Tannins, plant-derived polyphenols and other related compounds, have been utilized for a long time in many fields such as the food industry and manufacturing. In this study, we investigated the anti-viral effects of tannins on 12 different viruses including both enveloped viruses (influenza virus H3N2, H5N3, herpes simplex virus-1, vesicular stomatitis virus, Sendai virus and Newcastle disease virus) and non-enveloped viruses (poliovirus, coxsachievirus, adenovirus, rotavirus, feline calicivirus and mouse norovirus). We found that extracts from persimmon (*Diospyros kaki*), which contains ca. 22% of persimmon tannin, reduced viral infectivity in more than 4-log scale against all of the viruses tested, showing strong anti-viral effects against a broad range of viruses. Other tannins derived from green tea, acacia and gallnuts were effective for some of the viruses, while the coffee extracts were not effective for any of the virus. We then investigated the mechanism of the anti-viral effects of persimmon extracts by using mainly influenza virus. Persimmon extracts were effective within 30 seconds at a concentration of 0.25% and inhibited attachment of the virus to cells. Pretreatment of cells with the persimmon extracts before virus infection or post-treatment after virus infection did not inhibit virus replication. Protein aggregation seems to be a fundamental mechanism underlying the anti-viral effect of persimmon tannin, since viral proteins formed aggregates when purified virions were treated with the persimmon extracts and since the anti-viral effect was competitively inhibited by a non-specific protein, bovine serum albumin. Considering that persimmon tannin is a food supplement, it has a potential to be utilized as a safe and highly effective anti-viral reagent against pathogenic viruses.

Introduction

Animal viruses, which are causative agents of human and animal diseases, can be divided into two types based on their physical properties: enveloped virus, which has a viral envelope composed of a lipid bilayer on its surface, and non-enveloped virus, which lacks an envelope with its protein shell usually being exposed to the environment [1,2].

Influenza virus, an enveloped virus, is a pathogen that causes respiratory infection. Outbreaks of influenza virus infection occur every year and sometimes cause fatal diseases including pneumonia, secondary bacterial pneumonia and encephalopathy [3]. In 2009, a novel swine-derived influenza virus emerged and caused a worldwide pandemic. Influenza virus is sensitive to detergents or ethanol-based reagents. Human norovirus, a non-enveloped virus, is a causative agent of acute gastroenteritis, affecting many people all over the world [4]. Since a cell culture system for human norovirus has not been fully established, feline calicivirus and mouse norovirus are used as surrogates of human norovirus in anti-virus tests. Human norovirus is generally resistant to usual detergents as well as ethanol-based reagents, causing difficulty in sanitation [4].

Tannins are basically derived from plants and defined as "a material which produces leather from hide" [5]. Tannins prevent plants from being edible for worms and confer resistance to microbes. They have been exploited as food and medicine for their effects against tumors, oxidants or microbes [6]. Tannins include hydrolyzable tannins, which can be hydrolyzed to glucose and gallic acid, and proanthocyanidins or condensed tannins, which are compounds composed of flavonoids. Persimmon tannins, which are condensed tannins from persimmon (*Diospyros kaki*), have been widely utilized for anti-septics, folk medicine and a clarifier for brewing Japanese sake. Green tea tannin, which is a hydrolyzable tannin, has been found to restrict the growth of viruses such as influenza virus and herpes simplex virus type 1 [7,8], reviewed in [6,9].

In the present study, we investigated the effects of seven extracts and chemical compounds containing tannins against twelve enveloped and non-enveloped viruses, and we found that

Materials and Methods

Reagents

Persimmon extracts (PE) were prepared by serial procedures of squeezing immature green persimmon fruit, clarification with filtration, and freeze-drying. A batch of PE was shown to contain 21.8% of persimmon tannins (tested by the Japan Food Research Laboratories, Tokyo, Japan, with the Folin-Denis method). Pentagalloyl glucose (PGG) was prepared by crushing gallnuts followed by extraction with water, fractionation using an Octa Docyl Silyl column and freeze-drying. Wattle extracts (WE) from an acacia species, coffee extracts (CE, Caffenol P-100 raw coffee bean extracts), propyl galate (PRG, a synthetic compound), and green tea extracts (GTE, Catechin FP95 green tea extracts) were provided by Fuji Chemical Industry Co., Ltd. (Toyama, Japan). Pyrogallol (PYG, a synthetic compound) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). These reagents were individually dissolved to make a 1% (w/w) solution in 10% (w/w) ethanol and kept at 4°C in a dark room as stock solutions. Tannin samples, their abbreviations, and the origins and types of tannin are summarized in Table 1.

Cells and viruses

FL cells (HeLa cell contaminant, a gift from S. Takao, originally purchased from ATCC), Vero cells (African green monkey kidney-derived cells, a gift from S. Takao, originally purchased from JCRB Cell Bank), MA104 cells (macaque monkey fetus kidney-derived cells, a gift from M. Kuzuya, described in [10]) and RAW264.7 cells (mouse macrophage-derived cells, purchased from RIKEN Cell Bank) were propagated in Dulbecco's modified Eagle's minimum essential medium (DMEM, Invitrogen) supplement with 10% fetal calf serum (FCS), penicillin G (100 units/ml, Meiji Seika Pharma, Tokyo, Japan) and streptomycin (100 μg/ml, Meiji Seika Pharma). LLC-MK₂ cells (macaque monkey kidney-derived cells, described in [11]) and CRFK cells (feline kidney-derived cells, a gift from M. Noda, originally purchased from

JCRB Cell Bank) were propagated in Eagle's minimum essential medium (MEM, Invitrogen) supplemented with 10% FCS, penicillin G and streptomycin. MDCK(+) cells (canine kidney-derived cells described in [12]) were propagated in MEM supplemented with 5% FCS, 5% newborn calf serum, penicillin G and streptomycin.

Viruses used in this study are summarized in Table 2. Six enveloped viruses [human influenza virus A/Udorn/72 (H3N2), avian influenza virus A/swan/Shimane/499/83 (H5N3), herpes simplex virus-1, vesicular stomatitis virus, Sendai virus and Newcastle disease virus] and six non-enveloped viruses [poliovirus, Coxsachievirus, adenovirus, rotavirus, feline calicivirus and mouse norovirus] were used. Abbreviations of the viruses, virus families, cells used for anti-virus assay and methods to determine viral infectivity are shown in Table 2. Influenza virus A/swan/Shimane/499/83 (H5N3) was provided by K. Otsuki. Human herpes simplex virus type 1 VR-3, a Vero cell-adapted clinical isolate, was provided by K. Kiyotani. Poliovirus Sabin-1 vaccine strain and coxsachie virus group B type 5 were provided by S. Takao. Feline calicivirus F9 strain was provided by M. Noda. Rotavirus Wa strain was provided by M. Kuzuya. Sendai virus expressing enhanced green fluorescent protein (EGFP) (SeV-EGFP) was generated following the method described in [13].

Cytotoxicity assay

Seven tannin samples were subjected to cytotoxicity assays by using a Cell Proliferation Kit I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Briefly, confluent cells in a 96-well plate were exposed to 50 μl/well of DMEM containing 0.05%, 0.025% or 0.005% of tannins for 24 h in a CO₂ incubator, the concentration of 0.025% being the same as that used for anti-virus tests in this study. Then 5 μl of MTT (3-[4,5-dimethylth-iazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added and the cells were further incubated for 4 h. The cells were solubilized and optical absorbance at 570 nm was measured by using a microtiter plate reader (Model 680, Bio-Rad Laboratories, Hercules, CA). Cell viability was estimated by comparing values of tannin samples with that of DMEM without tannins.

Anti-virus assay

Anti-virus assays for H3N2, H5N3, HSV, VSV, NDV, PoV, CoV, FCV and MNV were performed by a standard TCID₅₀ method. Briefly, equal volumes of 0.5% tannin solution and virus solution were mixed to make a 0.25% tannin-containing virus solution. After 3-min incubation at room temperature, the solution was 10-fold serially diluted with DMEM. Susceptible cells for a virus in a 96-well plate were inoculated with 50 μl of a diluted virus solution in quadruplicate or octuplicate. After 1-h adsorption, the inoculum was removed and cells were incubated in 100 μl/well of DMEM supplemented with penicillin G and streptomycin. Twenty μg/ml of trypsin was included for the assay of influenza viruses, H3N2 and H5N3, and 5% FCS-containing MEM was used instead of DMEM for the assay of MNV. Water, Dulbecco's phosphate-buffered saline (PBS) and 10% ethanol were used instead of a tannin solution, and the average infectivity of the three was used as virus infectivity of mock-treated control. When the cytopathic effect had fully developed after several days, cells were fixed with ethanol and acetate (5:1) and further stained with 0.5% amido black 10B in 45% ethanol and 10% acetate. The 50% endpoint of virus infection was determined by the Behrens-Kaeber method, and 50% tissue culture infectious dose (TCID₅₀) was calculated. Anti-virus effects were estimated by comparing tannin-treated infectivity with mock-treated infectivity.

Table 1. Tannins used in this study.

Sample	Abbreviation	Origin	Tannin Type
persimmon extract	PE	extracts from persimmon	condensed
wattle extract	WE	extracts from acacia	condensed
coffee extract	CE	extracts from coffee beans	(pseudo tannin)
green tea extract	GTE	extracts from green tea	hydrolyzable
pentagalloyl glucose	PGG	extracts from gallnuts	hydrolyzable
propyl gallate	PRG	synthetic compound	hydrolyzable
pyrogallol	PYG	synthetic compound	hydrolyzable

Tannin names and their abbreviations in this paper are shown in the table. Origins of tannins and type of tannin, condensed tannin or hydrolyzable tannin, are also shown.
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Table 2. Viruses used in this study.

Virus	Abbreviation	Family	Envelope	Cell	Assay Method
A/Udorn/72 (H3N2)	H3N2	Orthomyxoviridae	+	MDCK(+)	1*
A/swan/Shimane/499/83 (H5N3)	H5N3	Orthomyxoviridae	+	MDCK(+)	1
herpes simplex virus-1, VR-3	HSV	Herpesviridae	+	FL	1
vesicular stomatitis virus, New Jersey serotype	VSV	Rhabdoviridae	+	FL	1
Sendai virus, Z strain	SeV	Paramyxoviridae	+	LLC-MK$_2$	2**
Newcastle disease virus, Herts strain	NDV	Paramyxoviridae	+	FL	1
poliovirus, Sabin-1	PoV	Picornaviridae	−	Vero	1
Coxsachie virus, group B, type 5	CoV	Picornaviridae	−	Vero	1
adenovirus type 5, a non-replicating recombinant virus	AdV	Adenoviridae	−	FL	2
rotavirus, Wa strain	RoV	Reoviridae	−	MA104	2
feline calicivirus, F9 strain	FCV	Caliciviridae	−	CRFK	1
mouse norovirus, S7 strain	MNV	Caliciviridae	−	RAW264.7	1

Virus name, its abbreviation in this paper, the family that it belongs to, presence(+) or absence(−) of an envelope, cells used for infectivity assay and an assay method for the virus are shown in the table. *1: TCID$_{50}$ method, **2: Immunofluorescent infectious focus assay.
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Anti-virus assays for SeV, AdV and RoV were performed by an immunofluorescent infectious focus assay. Briefly, tannin-treated and diluted virus solutions were inoculated to susceptible cells on a glass coverslip. After 24 h, cells were fixed and stained using a specific antibody as described previously [14]. Primary antibodies used were rabbit anti-serum for SeV, mouse monoclonal antibody REMI-1 (ARGENE, Varilhes, France) for RoV and mouse monoclonal antibody MAB805 (Merck-Millipore, Darmstadt, Germany) for AdV. Infected cells were counted under a fluorescent microscope (VANOX-T, Olympus, Tokyo, Japan), and virus infectivity was determined and designated as cell-infecting units (CIU)/ml as described previously [11]. Since the virus does not make a cell-to-cell transmission in these combinations of virus and cells, an infected cell represents an infectious focus. (SeV and RoV require exogenous trypsin to spread to neighboring cells and AdV is a replication-incompetent virus lacking the early region of the genome).

Anti-virus effects were recognized when virus infectivity was decreased after tannin treatment by 4 Log$_{10}$ reduction or more (99.99% reduction or more) compared with mock-treated infectivity.

Effects of PE in steps of virus infection

PE was added in different steps of influenza virus infection in a standard TCID$_{50}$ assay. (1) MDCK cells in a 96-well plate were treated with 100 µl/well of DMEM containing 0.025% PE for 1 h before virus inoculation (pre-infection). (2) Virus solution was mixed with an equal volume of 0.5% PE and incubated for 3 min. After 10-fold serial dilution, 50 µl/well of the diluted virus inoculum was applied to cells in a well and kept at 37°C for 1 h as described above (adsorption). (3) After virus adsorption, the inoculum was replaced with 100 µl/well of DMEM containing 0.025% PE and 20 µg/ml of trypsin (post-infection). After virus infection in the three different conditions, virus infectivity was assessed as described above.

Alternatively, monolayers of LLC-MK$_2$ cells on a coverslip in a 35-mm dish were infected with SeV-EGFP at an input multiplicity of infection of 5 under PE treatment in three different conditions, (1) pre-infection, (2) adsorption and (3) post-infection, as described

above. After 24 h, EGFP fluorescence was observed under a fluorescent microscope.

Hemagglutination inhibition (HI) test

HI test was performed according to a standard procedure. Briefly, 25 µl of 0.1% PE or 0.1% bovine serum albumin (BSA) was 2-fold serially diluted in a round-bottom 96-well plate. Influenza virus H3N2 (16 HA in 25 µl) was added to each well and incubated at room temperature for 3 min. Then 0.5% (v/v) of chicken red blood cells in PBS (50 µl) was added to each well and hemagglutination was measured after 1 h.

SDS-PAGE analysis of PE-treated virus particles

Influenza virus H3N2 particles were concentrated from infected allantoic fluid of chicken eggs by ultracentrifugation, followed by purification in a continuous sucrose gradient in ultracentrifugation and by suspension in PBS as described previously [15]. An equal volume of purified virions (10 µg/µl) or BSA (10 µg/µl) was mixed with 0.5% PE and kept for 3 min. The samples were further mixed with an equal volume of 2× SDS sample buffer (0.5 M Tris-HCl [pH 6.8], 10% sodium dodecyl sulfate (SDS), 20% glycerol, one grain of bromophenol blue, 1.7 M β-mercaptoethanol) and kept at 50°C for 20 min. Proteins were analyzed by 12% SDS-polyacryl-amide gel electrophoresis (PAGE) and Coomassie brilliant blue staining as described previously [15].

Accelerated aging test of persimmon tannin

Accelerated aging was performed according to the guidelines. One percent (w/w) PE in 10% ethanol was kept for 0, 1, 4, or 6 months at 40°C±2°C and at relative humidity of 75%±5% and was subjected to anti-virus test against influenza virus.

Results

Cytotoxicity of tannins to cultured cells

Cultured cells used in this study for anti-virus tests, MDCK(+), FL, LLC-MK$_2$, Vero, MA104, CRFK and RAW264.7 cells (Table 2), were individually incubated with the seven tannin samples, PE, WE, CE, GTE, PGG, PRG and PYG (Table 1), for

24 h, and viability of the cells was examined by using the MTT assay.

In the anti-viral test, 0.5% tannin sample was mixed with an equal volume of virus solution and then 10-fold serially diluted. Therefore, the highest concentration of tannin to which cells were exposed was 0.025%. We thus employed 0.025% tannin samples together with 0.05% and 0.005% samples.

All of the cells showed more than 100% of MTT staining (OD_{570}) in the presence of 0.025% tannin samples compared with 0% tannin-, mock-treated individual cells (Fig. 1). In the presence of 0.05% PRG or 0.05% PGG, MTT staining decreased to ca. 85% in RAW264.7 cells, indicating mild cytotoxicity (Fig. 1). However, no cytotoxicity was observed in other combinations of cells and tannin samples (Fig. 1). It was thus concluded that tannin samples are not cytotoxic in the anti-virus test using 0.5% tannin, which was diluted to a final concentration of 0.025% in the first row of a 96-well plate in the $TCID_{50}$ assay.

Anti-virus effects of tannins

The abilities of seven different tannin reagents (Table 1) to inactivate viruses were investigated. Five of those tannin reagents were extracts from plants (PE, WE, CE, GTE and PGG) and two were tannin constituents chemically synthesized (PRG and PYG). Six enveloped viruses and six non-enveloped viruses were used for anti-virus assays (Table 2). Enveloped viruses included human and avian influenza viruses (H3N2 and H5N3), herpes simplex virus-1 (HSV), vesicular stomatitis virus (VSV), Sendai virus (SeV) and Newcastle disease virus (NDV), and non-enveloped viruses included poliovirus (PoV), Coxsachie virus (CoV), adenovirus (AdV), rotavirus (RoV), feline calicivirus (FCV), and mouse norovirus (MNV). The last two viruses were used as surrogates of human norovirus. The anti-virus test was performed by measuring virus infectivity after tannin treatment with a standard $TCID_{50}$ method or an immunofluorescent infectious focus method.

The results showed that PE treatment inactivated all of the 12 viruses, suppressing their infectivities to less than the detection limit by more than 4 log reduction (Fig. 2, Table 3). GTE inactivated 9 different viruses, the largest number of viruses next to PE, but did not restrict the infectivity of non-enveloped viruses, AdV, MNV and CoV (Fig. 2, Table 3). WE, PGG, PYG and PRG inactivated 2 to 8 viruses, showing intermediate effects on viruses. In contrast, CE had no suppressive effect on any virus, indicating that it has absolutely no effect on viruses (Fig. 2, Table 3). These results indicate that PE has potent anti-virus effects against a broad range of viruses, including both enveloped and non-enveloped viruses.

Effective concentration and incubation time of PE

We further characterized the anti-virus effect of PE on viruses by using influenza virus H3N2. Dilution of PE revealed that 0.005% PE (1/100 dilution) had lost most of its anti-virus activity (Fig. 3A). Next, we investigated incubation time necessary for virus inactivation. Influenza virus H3N2 and 0.5% PE were mixed, and after 0.5, 3, 5, 60 and 120 min, the mixture was diluted to 100 times with DMEM to stop the reaction. Measurement of infectivity revealed that even 0.5 min (30 sec) is sufficient time to inactivate the virus completely (Fig. 3B), indicating that the anti-virus effect of PE is exerted in a short time. Incubation time 0 means that the virus and PE were individually diluted and cells in a 96-well plate were infected with the diluted virus first, and then the diluted PE was added to the wells.

Effects of PE in steps of virus infection

To determine the step of virus infection in which PE exerts its effect, PE was included in different steps of influenza virus infection in a standard $TCID_{50}$ assay (Fig. 4A). PE was included in the periods of (1) pre-infection, (2) adsorption, and (3) post-infection. Measurement of virus infectivity demonstrated that PE inactivated influenza virus infection when it was applied in the period of virus adsorption (Fig. 4B). This suggests that PE suppressed virus replication in the step of virus adsorption to cells.

A similar experiment was performed in SeV infection. LLC-MK_2 cells were infected with SeV possessing the EGFP gene (SeV-EGFP) with PE treatment in the three different conditions described above. After 24 h, EGFP expression was observed under a fluorescent microscope. Fig. 4C indicates that treatment with PE in the period of virus adsorption abolished EGFP production. Treatment of cells with PE before and after infection, however, did not restrict virus infection. In a separate experiment, effects of PE on virus attachment and virus invasion were individually investigated (Fig. S1). Only in the condition that PE was included in virus attachment but not in endocytosis and the membrane fusion, virus replication was restricted almost completely (Fig. S1). These results suggest that PE works by inhibiting virus attachment to cells.

## Hemagglutinating inhibition (HI) by PE

To further support the notion that PE inhibits virus attachment to cells, HI by PE was investigated. High concentrations (0.05% and 0.025%) of PE inhibited hemagglutination by influenza virus, while the same concentrations of BSA did not (Fig. 5A). These results indicate that PE restricted attachment of influenza virus to chicken erythrocytes. Interestingly, PE at higher concentration (0.1% or more) caused hemagglutination without influenza virus (data not shown). This suggests that PE at a high concentration directly reacted with chicken red blood cells, resulting in hemagglutination. PE was presumed to bind to proteins on virus particles and the cell surface.

Effect of PE on virions

We next investigated interaction of PE with virions (virus particles). Purified virions of influenza virus H3N2 were incubated with PE at room temperature for 10 min, and viral proteins were analyzed by SDS-PAGE (Fig. 5B). Viral protein bands disappeared from the original positions, except for the M1 protein band, with PE treatment, but the protein band at the top of the lane became denser (Fig. 5B, lane 4). This band at the top of the lane was slightly denser than that of PE treatment alone (Fig. 5B, lane 5). This result suggested that viral proteins formed large aggregates and were trapped at the well of the gel. Treatment of BSA instead of purified virions with PE caused a similar phenomenon: reduction of the BSA protein band from the original migrating position and appearance of a thick protein band around the top of the gel (Fig. 5B, lane 2). We further investigated aggregation of H3N2 virions by other tannin reagents. WE, GTE, CE, PGG, PRG and PYG did not appear to form protein aggregation in a similar SDS-PAGE analysis, while PE caused aggregation of virus proteins (Fig. S2). Similar results were obtained when SeV particles were used instead of H3N2 virions (data not shown). These results suggest that PE, which has binding capacity with proteins, causes aggregation of proteins.

To confirm this notion, competition of BSA with PE in anti-virus effects was investigated. PE and different concentrations of BSA were incubated, and then the PE solution was subjected to an anti-virus test for influenza virus H3N2 (Fig. 5C). Increasing amounts of BSA caused increasing virus infectivity, and 0.2% BSA
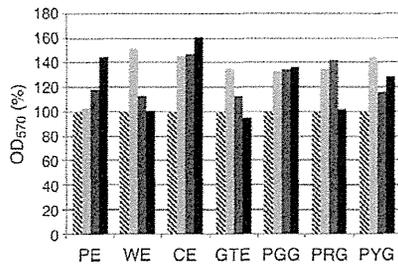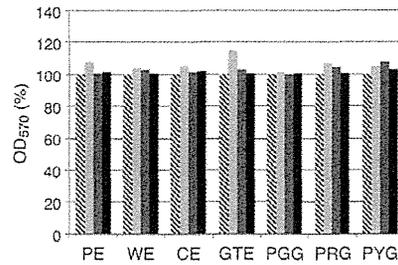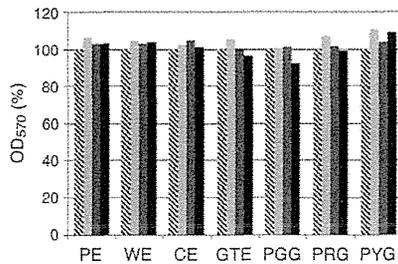
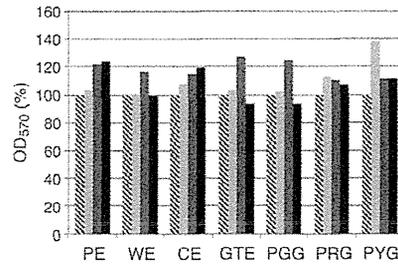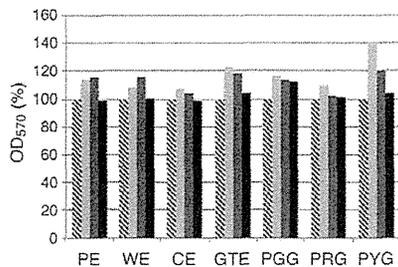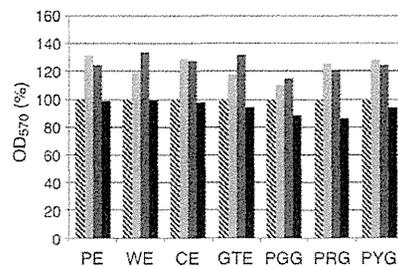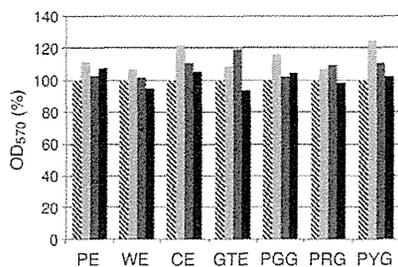**Figure 1. Cytotoxicity of tannins to cultured cells.** Seven cultured cell lines, (A) MDCK(+), (B) FL, (C) LLC-MK$_2$, (D) Vero, (E) MA104, (F) CRFK and (G) RAW264.7 cells, used for anti-virus tests (Table 2) were individually incubated with 0%, 0.005%, 0.025% or 0.05% of one of the tannin samples (Table 1) in DMEM for 24 h, and viability of the cells was examined by using the MTT assay. The OD$_{570}$ value of 0% tannin was set to 100%, and the OD$_{570}$ values in the presence of tannin samples are shown in percent for each tannin sample.
doi:10.1371/journal.pone.0055343.g001

almost completely abrogated the anti-virus ability of PE (Fig. 5C). These results suggest that PE exerts if anti-virus effect by binding to viral proteins in virions.

Effects of PE on virus after accelerated aging

Polyphenols are strong anti-oxidants, and oxidation changes at least their color with time [17]. PE gradually undergoes color
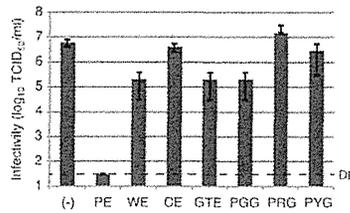
A. H3N2
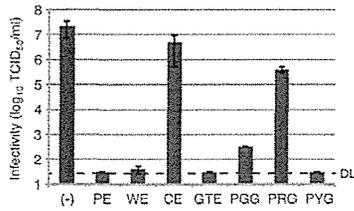
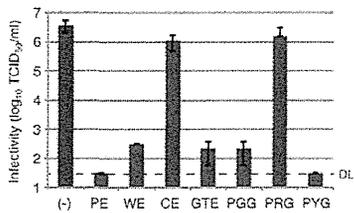
G. PoV
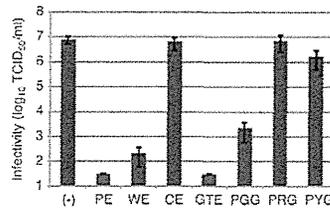
B. H5N3
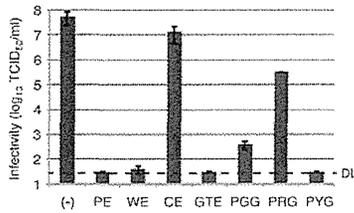
H. CoV

C. HSV

I. AdV
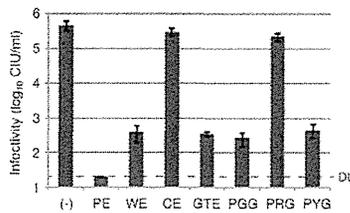
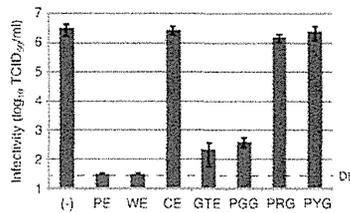

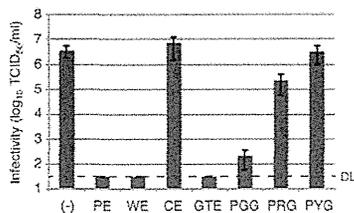## D. VSV
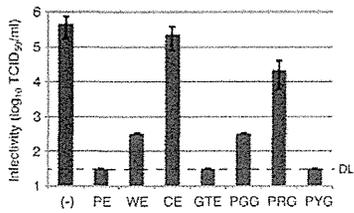
J. RoV
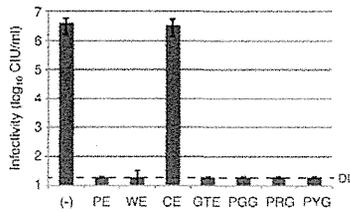
E. SeV

K. FCV
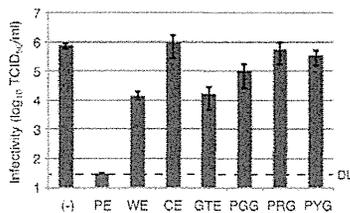

F. NDV

L. MNV

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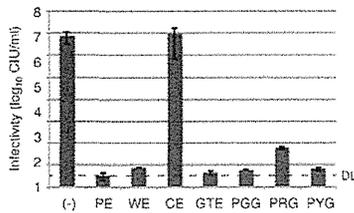
Figure 2. Anti-virus effects of tannins on a broad range of viruses. Each virus was incubated with a 0.5% tannin solution (PE, WE, CE, GTE, PGG, PRG or PYG; summarized in Table 1) for 3 min, and the remaining infectivity was measured by a $TCID_{50}$ method or an immunofluorescent focus assay. Average infectivities of three independent experiments are shown in the graphs and error bars indicate standard deviations. (−), virus was treated with each of water, PBS and 10% ethanol, and the average infectivity of the three is shown as mock-treated control infectivity. A-L, infectivities of twelve different viruses as indicated in the graphs are shown.
doi:10.1371/journal.pone.0055343.g002

change by oxidation from clear to brown. To investigate the change in anti-virus effects associated with color change, we prepared aged PE samples. According to a guideline of stability tests of medicine, PE solution was kept under the condition of 40°C and relative moisture of 75% in a dark room for 1, 4, or 6 months. By this procedure, PE solution gradually became brown (Fig. 6A). Anti-virus activity against influenza virus, however, did not change (Fig. 6B). With a 10-times dilution of PE, in which the virus was partially inactivated and a subtle difference was observed (Fig. 3A), there was also no difference in anti-virus effect (data not shown). These results indicate that the anti-virus activity of PE is stable in an accelerated aging test.

Discussion

It is important for prevention of virus infection to inhibit transmission of pathogens. One of the means is the use of an anti-microbial reagent. However, some viruses, especially non-enveloped viruses such as human norovirus, have high levels of resistance to anti-microbial reagents such as detergents and ethanol. In the present study, we searched for an anti-viral reagent to efficiently inactivate both enveloped and non-enveloped viruses. We investigated plant-derived tannin samples, since catechins, tannins from green tea or black tea, were reported to inhibit pathogenic viruses such as influenza virus, herpes simplex virus type 1 and human immunodeficiency virus [7,8,18–21]. We employed seven different tannin samples at a non-cytotoxic concentration and investigated their anti-virus effects against six enveloped and six non-enveloped viruses. Since some tannin samples such as PE and WE contain complex constituents, we employed crude extracts for those. The results clearly showed that PE alone inactivated all of the viruses to an undetectable level

(Fig. 2, Table 3). We then characterized the inhibition of virus infection by PE mainly by using influenza virus.

When PE was applied to cells before or after infection, it did not suppress virus infection. However, PE abrogated virus infection when it was applied in the period of virus adsorption. PE appeared to react with virus particles and inactivate virus attachment to cells. Inhibition of adsorption was reported in inactivation of herpes simplex virus by tannic acid by using radiolabeled virus particles [19]. On the other hand, the inhibition of HIV-1 infection by epigallocatechin gallate was shown to be due to the binding of the catechin with the CD4 receptor on the cell surface [22,23]. In the present study, high concentrations of PE (0.1% or more) caused aggregation of chicken blood cells without influenza virus (data not shown). Thus, a high concentration of persimmon
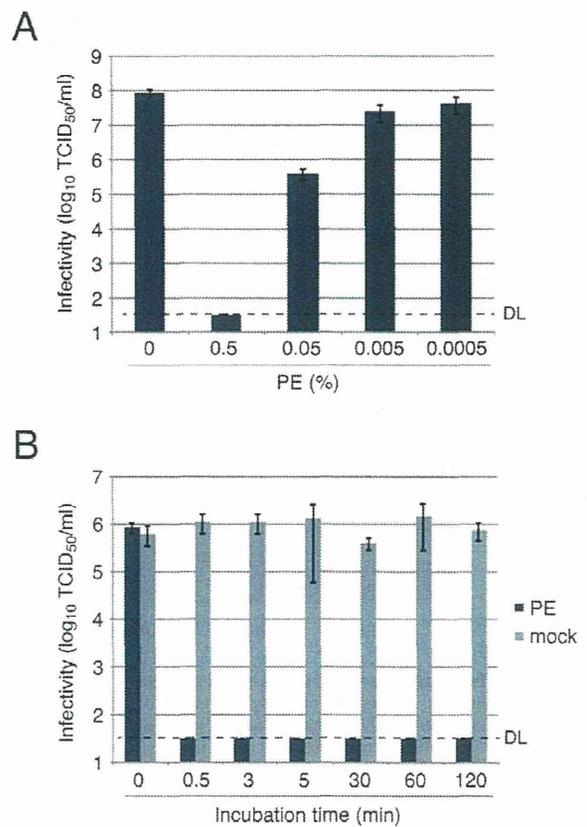
A

B

Figure 3. Concentration dependency and time dependency of effects of PE on influenza virus. Influenza virus H3N2 was treated with different concentrations of PE for 3 min (A) or with 0.5% PE for different incubation times (B), followed by measurement of virus infectivity with the standard $TCID_{50}$ assay. PE-treated virus infectivity is plotted in the black bar and mock-treated infectivity is shown in the gray bar. Average infectivities of three independent experiments are shown in the graph. Error bars indicate standard deviations. DL, detection limit of virus infectivity with the method.
doi:10.1371/journal.pone.0055343.g003

Table 3. Summary of log_{10} reductions of virus infectivity by tannin treatment.

Virus	Tannins						
	PE	WE	CE	GTE	PGG	PRG	PYG
H3N2	**5.8**	**5.7**	0.7	**5.8**	**4.8**	1.8	**5.8**
H5N3	**6.2**	**6.1**	0.7	**6.2**	**5.1**	2.3	**6.2**
HSV	**5.1**	**4.1**	0.6	**4.2**	**4.2**	0.4	**5.1**
VSV	**4.2**	3.3	0.4	**4.2**	3.3	1.4	**4.2**
SeV	**6.6**	**6.0**	0	**6.2**	**6.1**	**5.1**	**6.0**
NDV	**5.1**	**5.1**	0	**5.1**	**4.2**	1.3	0.06
PoV	**5.4**	**4.5**	0.09	**5.4**	3.6	0.03	0.6
CoV	**5.2**	1.4	0.1	1.5	1.5	0	0.4
AdV	**4.3**	3.2	0.2	3.2	3.3	0.4	3.1
RoV	**5.3**	**5.3**	0.06	**5.3**	**5.3**	**5.3**	**5.3**
FCV	**4.9**	**4.9**	0.05	**4.1**	3.9	0.3	0.1
MNV	**4.3**	1.6	0	1.7	0.9	0.2	0.4

Mean values of log_{10} reduction are shown in the table. Significant reduction, 4 or more, of log_{10} reduction is marked by bold and underline.
doi:10.1371/journal.pone.0055343.t003

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