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Antiviral effects of *Psidium guajava* Linn. (guava) tea on the growth of clinical isolated H1N1 viruses: Its role in viral hemagglutination and neuraminidase inhibition

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ABSTRACT

Rapid evolution of influenza RNA virus has resulted in limitation of vaccine effectiveness, increased emergence of drug-resistant viruses and occurrence of pandemics. A new effective antiviral is therefore needed for control of the highly mutative influenza virus. Teas prepared by the infusion method were tested for their anti-influenza activity against clinical influenza A (H1N1) isolates by a 19-h influenza growth inhibition assay with ST6Gal I-expressing MDCK cells (AX4 cells) using fluorogenic quantification and chromogenic visualization. Guava tea markedly inhibited the growth of A/Narita/1/2009 (amantadine-resistant pandemic 2009 strain) at an IC_{50} of 0.05% and the growth of A/Yamaguchi/20/06 (sensitive strain) and A/Kitakyushu/10/06 (oseltamivir-resistant strain) at similar IC_{50} values ranging from 0.24% to 0.42% in AX4 cells, being 3.4- to 5.4-fold more potent than green tea (IC_{50} values: 0.27% for the 2009 pandemic strain and 0.91% to 1.44% for the seasonal strains). In contrast to both teas, oseltamivir carboxylate (OC) demonstrated high potency against the growth of A/Narita/1/09 (IC_{50} of 3.83 nM) and A/Yamaguchi/20/06 (IC_{50} of 11.57 nM) but not against that of A/Kitakyushu/10/06 bearing a His274-to-Tyr substitution (IC_{50} of 15.97 μM). Immunofluorescence analysis under a confocal microscope indicated that both teas inhibited the most susceptible A/Narita/1/2009 virus at the initial stage of virus infection. This is consistent with results of direct inhibition assays showing that both teas inhibited viral hemagglutination at concentrations comparable to their growth inhibition concentrations but inhibited sialidase activity at about 8-times higher concentrations. Guava tea shows promise to be efficacious for control of epidemic and pandemic influenza viruses including oseltamivir-resistant strains, and its broad target blockage makes it less likely to lead to emergence of viral resistance.

1. Introduction

None of the 17 known hemagglutinin (HA) and 10 known neuraminidase (NA) subtypes of influenza A viruses usually cross the host species barrier, but they are occasionally transmitted to other animal species, resulting in devastating outbreaks in that naïve animal population (Tong et al., 2012; Kuiken et al., 2006; Sriwilaijaroen et al., 2011b). There are currently two circulating H1N1 viruses in humans: one originates from the 1918 Spanish flu widely spread in humans until 1957 and restarted in 1977 and the other is derived from swine flu 2009. After the pandemic period, the viruses still occur at high levels in winter as seasonal flu strains. The repetitive occurrence of influenza continues annually due to antigenic mutations in viral surface proteins HA and NA, resulting in morbidity and mortality globally in humans of all ages (WHO, 2009). The high mutation rate of influenza RNA virus also limits the effects of influenza vaccine. Only two classes of licensed anti-influenza drugs acting at different stages of the influenza life cycle have so far been available for influenza therapy (De Clercq, 2006). The first class targets M2 ion-channel protein, which plays a crucial role in uncoating of the virus, resulting in release of viral content to the host cytoplasm; however, M2 inhibitors including oral amantadine and its derivative rimantadine have encountered widespread resistance. The other class interacts with the active site of influenza NA enzyme rendering progeny virions that are unable

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to escape from infected host cells to new host cells. Although inhaled zanamivir (Relenza), an NA inhibitor, has rarely been reported to cause influenza virus resistance in clinical use (Hurt et al., 2009), its adverse effects on respiratory function of patients with underlying respiratory disease have restricted its use (FDA). Unlike inhaled zanamivir, oral oseltamivir (Tamiflu, Roche) has been widely used worldwide for prophylaxis and treatment of influenza A viruses, and resistance to oseltamivir has a tendency to increase yearly in circulating H1N1 viruses (Lackenby et al., 2011; WHO, 2011).

Plants are natural sources of valuable materials for medicine. Shikimic acid, originally extracted from fruits of Chinese star anise (*Illicium verum* in Illiciaceae (anise) family), is a key starting material for oseltamivir synthesis (Dewick, 2009; Kramer et al., 2003). Tea is a source of medicinal compounds that have not only beneficial physiological effects on human health but also inhibitory effects against a wide range of microorganisms, and it has been considered a healthy drink (Zhen et al., 2002). Black tea, which is popular in most of the Western world, and green tea, which is popular in the Orient, have been reported to act against influenza virus replication (Green, 1949; Imanishi et al., 2002; Kuzuhara et al., 2009; Nakayama et al., 1993; Song et al., 2005). By screening of different kinds of teas as influenza antivirals in cultured cells, we found that guava tea, which is mainly consumed in Oriental countries, has a stronger inhibitory activity than that of green tea against H1N1 clinical isolates, and we elucidated the mechanism of its anti-influenza virus action.

2. Materials and methods

2.1. Compounds and antibodies

Oseltamivir carboxylate ((3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate) was provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Acetylated trypsin, type V-S from bovine pancreas, for influenza growth inhibition assay and ethyl gallate (EGA) used as a standard for tannin content determination were purchased from Sigma Chemical Co. (St. Louis, MO). Two fluorogenic substrates, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNA) for sialidase activity assay and 4-methylumbelliferyl-β-D-galactoside (MU-Gal) for influenza growth inhibition assay, were purchased from Toronto Research Chemicals, Inc. (TRC, Ontario, Canada) and Calbiochem (San Diego, CA), respectively. Mouse IgG-conjugated anti-influenza nucleoprotein (NP) monoclonal (4E6) antibody was obtained using A/Memphis/1/71 (H3N2) as an antigen (Takahashi et al., 2008). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody and Alex 488-labeled goat anti-mouse IgG antibody were purchased from Jackson Immuno Research (West Grove, PA) and Molecular Probes (Eugene, Oregon), respectively. 4′,6-Diamidine-2-phenylindole dihydrochlroride (DAPI) was obtained from Boehringer Mannheim (Mannheim, Germany).

2.2. Cells

Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Tissue Culture Biological, Tulare, CA) and antibiotics (250 U/mL penicillin and 250 μg/mL streptomycin; GIBCO-BRL, Rockville, MD). β-Galactoside α2,6-sialyltransferase I (ST6Gal I)-expressing MDCK cells with the puromycin *N*-acetyltransferase gene (called AX4 cells), which have recently been developed to have a high expression level of sialyl-α2,6-galactose moieties (Hatakeyama et al., 2005), were grown in Dulbecco's modified essential medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated FBS and 7.5 μg/mL puromycin (Nacalai Tesque).

2.3. Viruses

Information on influenza virus isolates used in this study is shown in Fig. 1. The viruses were amplified in MDCK cells, and viral culture supernatants were harvested, concentrated, and stored at −80 °C as described previously (Sriwilaijaroen et al., 2011a).

2.4. Preparation of teas

Dried leaves of green tea (*Camellia sonensis*) and of guava tea (*Psidium guajava* Linn.) were purchased from a local market in Okinawa, Japan. To make tea, 20 g of each ground sample was steeped in 1000 mL of 85 °C water for 8 min, stirred gently for 10 s, filtered through filter paper, and left to cool down on ice. Tea extracts were filtered through a 3-μM polytetrafluoroethylene (PTFE) filter to remove remaining tea leaf particles prior to tannin analysis as described below. Each tea sample was diluted with cold water to obtain the same tannin value of 40 mg/100 mL and adjusted with sodium bicarbonate powder to obtain a final pH of 6.5.

2.5. Determination of tannin content

A colorimetric assay using the ferrous-tartrate method, the Japanese official analytical method for tea tannin, was used for quantitative analysis of tannin in tea samples (Iwasa and Torri, 1962). Each sample or water (background) was mixed with ferrous tartrate solution, containing 0.1% ferrous sulfate and 0.5% sodium potassium tartrate, and 1 M phosphate buffer, pH 7.5, at a 1:1:3 ratio. After incubation for 30 min at room temperature, the purple color that had developed in the mixture was measured at absorbance of 540 nm. The background subtracted absorbance was converted to tannin equivalent by the tannin standard curve using ethyl gallate as a standard. Tannin content was expressed as mg of tannin equivalents per 100 mL of sample.

2.6. Cytotoxicity assay

AX4 cell viability in the presence of various concentrations of an inhibitor was determined by using a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, the cells were incubated with an inhibitor for 24 h at 37 °C. After removal of inhibitor overlays, 10 μl of CCK-8 solution containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium-monosodium salt (WST-8) in 100 μl of medium was added to the cells. Following incubation at 37 °C for 45 min, absorbance of the formazan product directly proportional to the number of live cells was measured at 450 nm by a microplate reader (Bio-Rad Model 680, Hercules, CA).

2.7. Influenza growth inhibition assay

AX4 cells were washed and pretreated with DMEM alone or containing serial diluted inhibitors for 1 h at 37 °C. Influenza virus at a multiplicity of infection (MOI) of 0.03 was preincubated with a medium containing 2 μg/mL acetylated trypsin in the absence or presence of the inhibitor at the same concentrations as those used for pretreating cells for 1 h at 4 °C. Then the medium in the plate was replaced with the inhibitor-virus mixture and incubated for 19–20 h at 37 °C. Virus titer in infected cells was determined by a galactosidase-based fluorescent assay, and viral formation was observed by a peroxidase-based chromogenic assay. The infected cells were fixed and permealized with methanol and were then

(A)

Virus strain	M2 mutation (R/S)[a]	NA mutation (R/S)	OC anti-sialidase activity mean IC_{50} ± SD[c]
A/Narita/1/09 (H1N1pdm)	V28I; **S31N** (R)	V149I; Q430R[b] (S)	2.58 ± 0.09 nM
A/Yamaguchi/20/06 (huH1N1)	NA[d]	(S)	2.91 ± 0.40 nM
A/Kitakyushu/10/06 (huH1N1)	NA	**H274Y** (R)	1.42 ± 0.24 μM

[a] S, sensitive; R, resistant. Mutated amino acids that confer adamantane or NAI resistance are indicated in bold type (Sheu et al., 2008).
[b] Ile149 of loop-150 and Arg430 of loop-430 provide the 150 cavity-deficient active site in the pandemic 2009 N1 different from the group-1 NA but similar to the group-2 NA (Li et al., 2010).
[c] IC_{50} values shown are means ± SD of three independent experiments, each conducted in duplicate.
[d] NA, Not available.

(B)

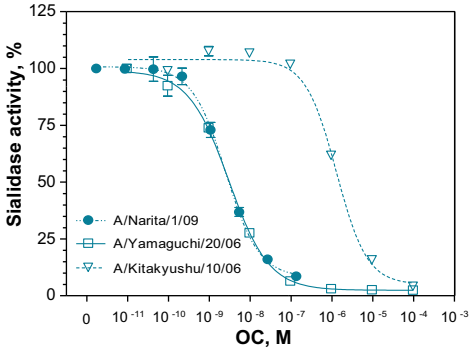


Fig. 1. Drug sensitivity of influenza viruses used in this study. (A) Information on molecular markers of resistance in M2 and NA genes of influenza viruses and summary of oseltamivir IC_{50} values obtained from non-linear curve fitting in Fig. 1B. (B) Curve comparison of oseltamivir susceptibility of different influenza clinical isolates tested by fluorometric-based NA inhibition assay. Each curve shows representative results of three independent experiments, each performed in duplicate. Each point is the mean ± standard errors of mean of duplicate samples. (See above-mentioned references for further information.)

stained with anti-viral NP mouse antibody (4E6) and β-galactosidase-conjugated anti-mouse IgG. The galactosidase reaction was initiated by adding a substrate solution containing 4-methylumbelliferyl-β-D-galactoside (MU-Gal) and $MgCl_2$. The fluorescent MU product was detected at Ex_{355}/Em_{460} by a Mithras LB940 microplate reader (Berthold Technologies, Pforzheim, Germany). For the peroxidase-based chromogenic assay, HRP-conjugated goat anti-mouse IgG antibody was added to the cells and virus in the infected cells was visualized as a blue color by incubation with H_2O_2, *N*,*N*-diethyl-*p*-phenylenediamine dihydrochloride and 4-chloro-1-naphthol.

2.8. Immunostaining and confocal microscopy

AX4 cells were grown overnight on coverslips. After washing, the cells were preincubated with or without an inhibitor for 1 h at 37 °C in a 5% CO_2 incubator. At the same time, influenza virus at an MOI of 0.13 was preincubated in DMEM medium with or without an inhibitor for 1 h at 4 °C. Following 1-h preincubation, the medium overlays on AX4 cells were replaced with influenza virus suspension and each coverslip was incubated for 4 h in the CO_2 incubator. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, at room temperature (RT) for 30 min, permeabilized with 0.5% Triton X-100 in PBS at RT for 5 min, and immunostained with mouse IgG-conjugated anti-influenza NP monoclonal (4E6) antibody in PBS with 0.1% Tween 20 (PBST) on ice for 40 min and then Alex 488-labeled goat anti-mouse IgG antibody (Molecular Probes, Eugene, Oregon) in PBST on ice for 40 min. Nuclei were visualized by DAPI (Boehringer Mannheim) directly under an Olympus IX71 fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at RT for 3–5 min. The coverslips were mounted and immunofluorescence images were processed and captured using a FluoView FV1000 laser scanning confocal microscope with a ×20 objective lens, zoom 1.6× and FV10-ASW 1.7 software (Olympus, Tokyo, Japan).

2.9. Agglutination and hemagglutination inhibition assay

An inhibitor was serially 2-fold diluted in PBS in a 96-well plate (50 μl/well). An equal volume of 0.5% (v/v) guinea pig erythrocyte suspension in PBS was added and mixed with the inhibitor. After incubation for 1 h at 4 °C, agglutination activity of the inhibitor was determined visually. For the hemagglutination inhibition assay, a 2-fold serial dilution of an inhibitor (25 μl/well) was mixed with an equal volume of influenza virus solution containing 4 HAU and incubated for 1 h at 4 °C before addition of 50 μl of 0.5% (v/v) guinea pig erythrocyte suspension. Viral hemagglutination inhibition activity of the inhibitor was visualized after a 2-h incubation at 4 °C.

2.10. Sialidase inhibition assay

A fluorometric assay utilizing MUNA as the sialidase substrate to produce a fluorescent product for quantitative measurement by a fluorometer was employed for evaluating sialidase inhibitory potential of an inhibitor according to the previously reported method (Sriwilaijaroen et al., 2009). In brief, 5 μl of influenza virus in 20 mM sodium acetate buffer, pH 6.0, equal to 1 enzyme unit (one unit being the quantity of neuraminidase that liberates 1 nM MU (equivalent to NA amount) per min under the assay conditions) was incubated with 5 μl of an inhibitor at various concentrations for 15 min at 37 °C. Five microliter of the MUNA substrate (final concentration equal to 1 K_m) was immediately added to initiate the enzymatic reaction at 37 °C and further incubated for 15 min. Fluorescence at 355 nm excitation and 460 nm emission of each reaction was detected after reaction termination with 100 mM sodium carbonate buffer, pH 10.6. The percent inhibition of sialidase activity was plotted versus inhibitor concentration to obtain the inhibition–dose response curve and the 50% inhibitory concentration (IC_{50}) value.

3. Results

3.1. Tea production and tannin content

Green tea and guava tea were prepared from infusion of *Camelia sinensis* and *P. guajava* Linn., respectively, with 85 °C hot water at a volume of 50 times the weight of tea leaves, followed by filtration, then tannin adjustment and finally pH adjustment. Tannins are water-soluble polyphenol compounds that contribute to the astringent taste and characteristic reddish-brown color of tea, and tannin content in tea is thus an important factor affecting tea quality (Chen et al., 2005). According to results obtained by using the ferrous-tartrate method, green tea contains 217 mg tannin/100 mL, whereas guava tea has 111 mg tannin/100 mL. In addition to being related to tea quality, tannins are responsible for several biological activities, such as anti-inflammatory and antioxidant activities (Chen et al., 2003; Hamauzu et al., 2005), and antimicrobial activities including antibacterial and anti-influenza activities (Akiyama et al., 2001; Hamauzu et al., 2005; Mantani

et al., 1999). Teas with the same tannin contents should be used for comparison of anti-influenza activities. Thus, tannin contents in both green tea and guava tea were adjusted to 40 mg/100 mL.

3.2. Inhibition of clinical influenza isolate replication by guava tea in AX4 cells

Since human influenza isolates bind preferentially to sialic acids attached to galactose by an α2,6 linkage (Siaα2,6Gal) on the host cell membrane before invasion, AX4 cells, which highly express Siaα2,6Gal residues comparable to human airway epithelial cells, were used for evaluation of anti-influenza potency of inhibitors against clinical isolates. Cytotoxic effects of teas on AX4 cells determined after 24-h incubation using a cell counting kit 8 demonstrated that green tea and guava tea did not exhibit toxicity to AX4 cells at concentrations up to 25% but significantly decreased AX4 cell viability at 50% concentrations, with about 24% and 56% of the cells remaining alive, respectively (data not shown), and the inhibitory effects of teas on influenza virus replication in AX4 cells were thus determined at concentrations without cytotoxicity. Representative influenza clinical isolates used in this study were A/Narita/1/2009, A/Yamaguchi/20/06 and A/Kitakyushu/10/06, which are 2009 pandemic, NAI-sensitive seasonal and NAI-resistant seasonal viruses, respectively. A/Narita/1/2009 virus carries the most common M2 resistance-conferring S31N mutation, and A/Kitakyushu/10/06 isolate contains an H274Y mutation in the NA gene conferring oseltamivir resistance. Results obtained by using a fluorometric-based NA inhibition assay showed that oseltamivir carboxylate (OC) inhibited the sialidase activities of A/Narita/1/2009, A/Yamaguchi/20/06 and A/Kitakyushu/10/06 with IC_{50} values of 2.58 ± 0.09 nM, 2.91 ± 0.40 nM and 1.42 ± 0.24 μM, respectively (Fig. 1), confirming that A/Kitakyushu/10/06 strain is highly resistant to oseltamivir. This is consistent with previous reports showing that all of the circulating 2009 pandemic viruses are resistant to adamantanes but that most of them remain sensitive to both NAI drugs (Centers for Disease Control and Prevention, 2009; Lackenby et al., 2011). Comparable to the NAI results shown in Fig. 1, OC determined by a 19-h influenza growth inhibition assay with AX4 cells exhibited inhibitory activities against A/Narita/1/2009, A/Yamaguchi/20/06 and A/Kitakyushu/10/06 with IC_{50} values of 3.83 ± 1.64 nM, 11.57 ± 2.08 nM and 15.97 ± 7.76 μM, respectively (Fig. 2A), indicating that this simple method is accurate and reliable for evaluation of antiviral activity against influenza growth in cell culture. Of the influenza H1N1 viruses tested, the growth of A/Narita/1/2009 strain was the most susceptible to OC, possibly due to higher infectivity of 2009 pandemic viruses than that of seasonal influenza viruses (Mitchell et al., 2011). In parallel experiments, both green tea and guava tea inhibited growth of sensitive and resistant seasonal viruses with similar IC_{50} values (green tea: IC_{50} values of 1.44 ± 0.23% against A/Yamaguchi/20/06 and 1.33 ± 0.26% against A/Kitakyushu/10/06, guava tea: IC_{50} values of 0.58 ± 0.14% against A/Yamaguchi/20/06 and 0.23 ± 0.05% against A/Kitakyushu/10/06) (Fig. 2B). The amantadine-resistant A/Narita/1/09 pandemic virus was more sensitive to both green tea and guava tea than were the seasonal strains with IC_{50} values of 0.27 ± 0.06% and 0.05 ± 0.002%, respectively (Fig. 2B). These data demonstrated that the NA H274Y mutation and the M2 S31N mutation do not reduce influenza sensitivity to these teas. Of note, all clinical isolates were more sensitive to guava tea than to green tea, especially A/Narita/1/09 strain (Fig. 2B).

3.3. Effects of guava tea on the initial stage of influenza virus infection

To examine the effects of tea on the initial stage of virus infection, tea-treated AX4 cells were inoculated with tea-treated A/Narita/1/09 virus, a virus that is highly transmitted among humans and which was used as a representative H1N1 virus due to its highest susceptibility, for 4 h at 37 °C. Then virus in the cells was determined by immunostaining with anti-viral NP antibody and Alex secondary antibody, and the stained viruses were observed with a confocal laser scanning microscope. Fig. 3A shows that treatment of host cells and influenza A/Narita/1/09 virus with either green tea or guava tea before virus inoculation completely blocked virus infection in AX4 cells. To determine the mode of action of teas on prevention of influenza virus infection in host cells, the ability of tea to inhibit viral attachment to host cells by interaction either with cells or with viruses was determined simply by incubation of tea with erythrocytes in the absence or presence of the virus. It appeared that both green tea and guava tea can agglutinate guinea pig erythrocytes at concentrations of more than 1.56% and 0.39%, respectively (Fig. 3B). At tea concentrations without agglutination activity, they completely inhibited hemagglutination of influenza A/Narita/1/2009 virus at concentrations of 0.2% (green tea) and 0.1% (guava tea) as shown in Fig. 3C. Notably, both teas demonstrated hemagglutination inhibition activity with potency comparable to their infection inhibitory activity in cell culture studies. Again, guava tea showed about 4-fold stronger agglutination activity and 2-fold stronger influenza hemagglutination inhibition activity than those of green tea, suggesting that tea activity was associated with the source of tea.

3.4. Effects of guava tea on neuraminidase facilitating viral release and spread at the final stage of the influenza replication cycle

Neuraminidase is the other major glycoprotein protruding from the outer influenza surface. It cleaves sialic acid residues from the host cell membrane and influenza viral envelope, permitting efficient release and spread of progeny virions to new host cells. The ability of guava tea to inhibit the neuraminidase function of oseltamivir-sensitive and -resistant H1N1 viruses in comparison with green tea was tested by a standard fluorescent-based NAI assay. As a control, OC, an active form of oseltamivir, was evaluated in parallel and was shown to have IC_{50} values against A/Narita/1/2009 and A/Yamaguchi/20/06 about 1000-times lower than that against A/Kitakyushu/10/06 (Fig. 1). In contrast, both green tea and guava tea appeared to inhibit oseltamivir-sensitive (A/Yamaguchi/20/06) and -resistant (A/Kitakyushu/10/06) seasonal H1N1 viruses with similar IC_{50} values of 15.16 ± 1.09% and 19.45 ± 2.60% for green tea and 6.83 ± 0.80% and 7.50 ± 0.49% for guava tea, respectively (Fig. 4). These findings confirmed that oseltamivir-resistant mutation does not affect the inhibitory activity of either tea. Sialidase activity of the 2009 pandemic A/Narita/1/09 virus appeared to be approximately 8-fold and 16-fold more sensitive to green tea and guava tea, respectively, than that of seasonal viruses. NA amino acid variations between the pandemic 2009 virus and the seasonal viruses might account for the virus susceptibility to teas. In agreement with viral growth inhibition, agglutination and HAI results, guava tea showed sialidase inhibitory activity about 2–5-times higher than that of green tea Fig. 5.

4. Discussion

As a result of the unpredictable ability of protective vaccines and the continued emergence of drug-resistant influenza viruses, new anti-influenza agents are being explored. Tea, a beverage that has great potential human health benefits with a long history, has received much attention for its anti-influenza effects. In 1949, black tea, which is derived from *Camellia sinensis* var. *sinensis* belonging to the family Theaceae, was reported to have anti-influenza activity (Green, 1949). Since the publication of that report, green tea, which is derived from the same plant but is different

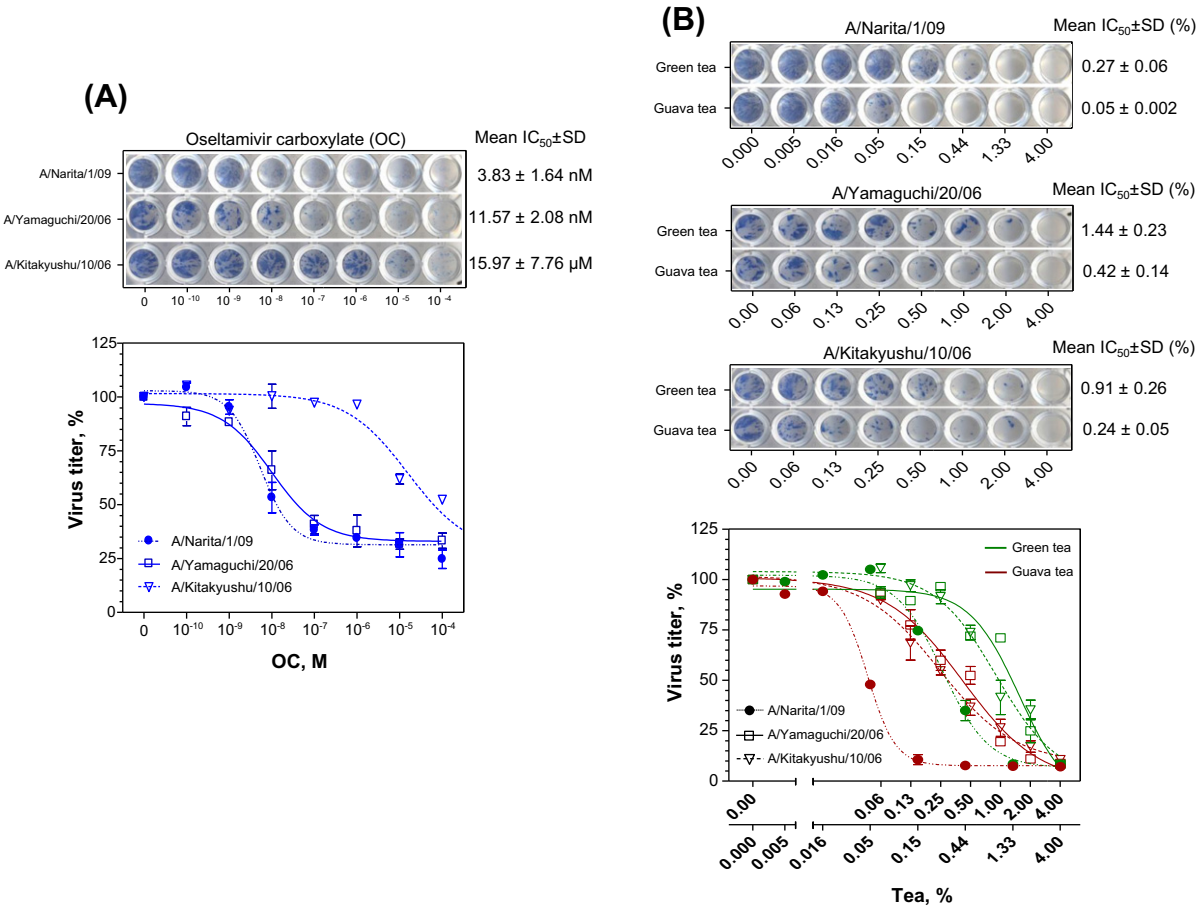


Fig. 2. Reduction of influenza virus yield in AX4 cells (upper panel) curvedly illustrated as a function of concentration (lower panel) of oseltamivir (A), green tea or guava tea (B).

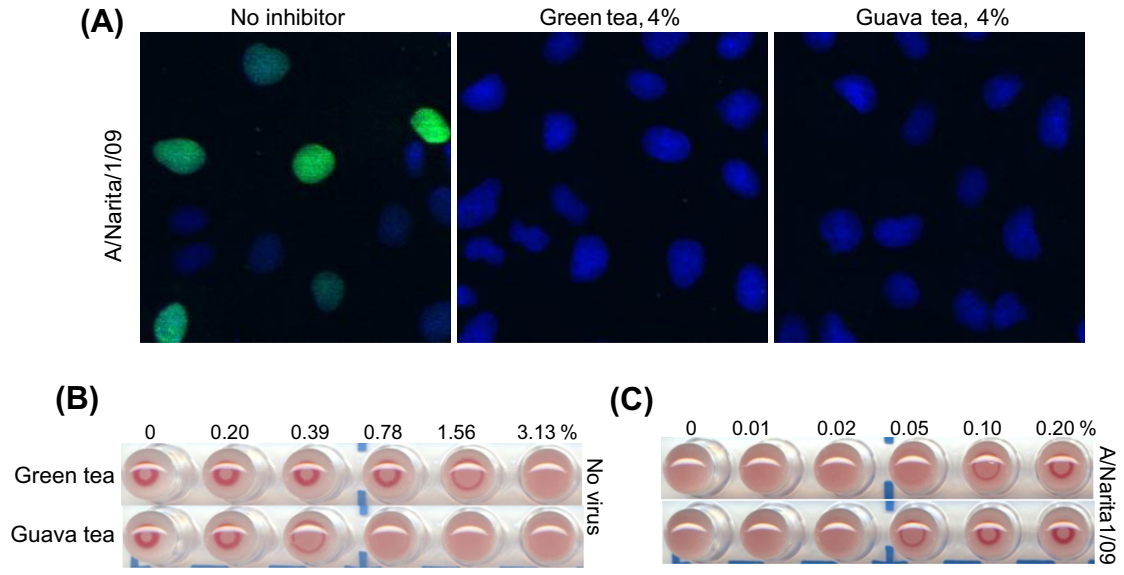
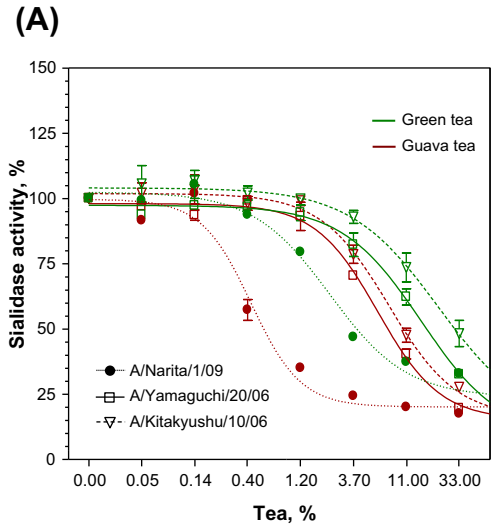


Fig. 3. Guava tea inhibits the early stage of influenza A/Narita/1/2009 virus infection. (A) Confocal fluorescent images of influenza virus NP protein (green) after 4-h addition of the virus to AX4 cells: the virus and cells were mock-treated (left) or treated with 4% v/v green tea (middle) or 4% v/v guava tea (right). (B) Both green tea and guava tea show agglutination activity with guinea pig erythrocytes in a dose-dependent manner. (C) Either green tea or guava tea can abolish influenza hemagglutination activity at low concentrations without a tea agglutination effect.

from black tea in that it is not fermented and, thus, polyphenols are less oxidized, has been intensively studied for its anti-influenza activity, bioactive components and biological mechanisms of action (Song et al., 2005). It is now accepted that catechin is a main



(B)

Virus strain	Anti-sialidase activity[a], mean IC_{50}±SD[b] (%)	
	Green tea	Guava tea
A/Narita/1/09 (H1N1pdm)	2.21±0.07	0.44±0.05
A/Yamaguchi/20/06 (huH1N1)	15.16±1.09	6.83±0.80
A/Kitakyushu/10/06 (oseltamivir-resistant huH1N1)	19.45±2.60	7.50±0.49

[a] Anti-sialidase activity of each sample was determined by neuraminidase inhibition assay as described in Materials and methods.
[b] IC_{50} values shown are means ± SD of three independent experiments, each conducted in duplicate.

Fig. 4. Guava tea shows inhibition of influenza sialidase activity. Dose–response curves of green tea (green) and guava tea (brown) were constructed against sialidase activity from different virus isolates (A), and non-linear regression fit yielded IC_{50} values that are summarized in the right panel (B).

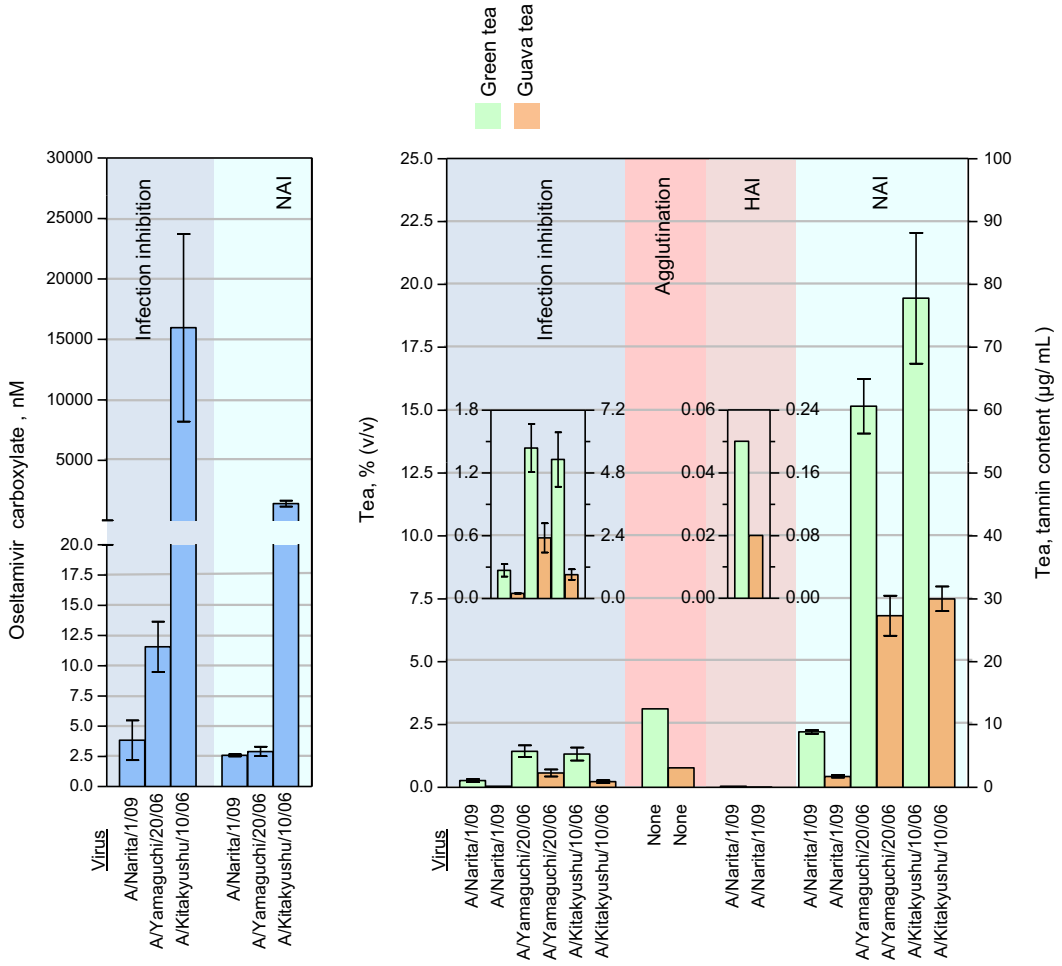


Fig. 5. Comparison of anti-influenza effects of green tea and guava tea on viral growth, cell protection, hemagglutination and sialidase activity. Values in the graphs are means ± standard deviations of three independent experiments.

active component in green tea possessing several properties responsible for anti-influenza activity including hemagglutination, neuraminidase and endonuclease inhibition (Kuzuhara et al., 2009; Nakayama et al., 1993; Song et al., 2005), and a variety of green tea catechin products, such as antiflu air purifier, antiflu mask and catechin candy, are now commercially available (Hara, 2001). In this

study, we showed that guava tea from *P. guajava* Linn. in the myrtle family (Myrtaceae) displayed a high level of anti-influenza activity.

P. guajava (guava) is a tropical and subtropical plant with a long history of traditionally medicinal uses. All of its parts, including shoots, leaves, bark, fruit, flower buds and roots, are used for treatment of various diseases, such as diarrhea and diabetes mellitus, and of several clinical symptoms, such as cough, fever and sore throat (Gutiérrez et al., 2008; Jaiarj et al., 1999). In Bolivia and Egypt, guava leaves are also used to treat pulmonary disease (Batick, 1984). In addition to possessing bactericidal activity (Jaiarj et al., 1999), guava leaves have been shown to possess activity against simian rotavirus (Gonçalves et al., 2005).

In this present study, guava tea was prepared in parallel with green tea by steeping the dried tea leaves in hot water. The ferrous-tartrate method revealed that tea from green tea leaves contains about 2-times higher total tannin content than that in tea from guava tea leaves. Tannins are polyphenolic secondary metabolites of high-order plants that play a role in defense against herbivores and pathogens (Beart et al., 1985). They are generally categorized into two groups: hydrolyzable tannins having molecular weights (MWs) of 500–3000 and condensed tannins, also known as proanthocyanidins, having MWs of 1000–20,000 (Cowan, 1999). Tannins provide bitter and astringent perception, and total tannin content is thus used for quality control of commercial tea products. Based on the same tannin contents, guava tea showed about 5-fold greater inhibitory effects than those of green tea on influenza replication in AX4 cells for all H1N1 viruses tested. These results indicated that guava tea contains a component(s) responsible for the anti-influenza property that may be different from green tea derived from leaves of different plant species.

When 4% tea-treated AX4 cells were challenged with 4% tea-treated influenza 2009 pandemic strain, viral replication in the cellular nucleus at 4 h post infection could not be detected in either green tea or guava tea-treated cells, indicating that both teas inhibited the early stage of cell infection by the influenza virus. These results were supported by results of assays of agglutinating activity and tests of hemagglutination inhibition suggesting that at a high dose (green tea: $\geqslant$1.56%; guava tea: $\geqslant$0.39%), components present in the teas bind to guinea pig erythrocytes and connect them (agglutination), while at a lower dose (green tea: $\geqslant$0.1%; guava tea: $\geqslant$0.05%) without agglutination effects, components in the teas play a role in prevention of virus binding to erythrocytes. These properties were observed in a single compound, mumefural and its derivative, in a previous study (Sriwilaijaroen et al., 2011a), indicating that the component in teas that has agglutination and viral-hemagglutination inhibitory activities may be the same compound. A catechin (flavonol, a monomer of condensed tannin), (−)-epigallocatechin gallate (EGCG), from green tea and a polymerized catechin, theaflavin digallate (TF3), from black tea were shown by electron microscopy to agglutinate influenza A virus particles, inhibit hemagglutination and prevent infection of MDCK cells (Nakayama et al., 1993). These findings suggest that black tea, green tea and guava tea have the same mechanisms of prevention of virus adsorption to host cells.

Guava tea appeared to inhibit more than one target on the influenza virus particle: not only hemagglutinin but also neuraminidase. Guava tea showed sialidase inhibition with lower IC_{50} values than those of green tea. Two catechins, EGCG and (−)-epicatechin-3-gallate (ECG), isolated from green tea have been shown to inhibit influenza sialidase activity (Song et al., 2005). Comparison of the effectiveness of tea against 2009 pandemic (containing amantadine-resistant genes), seasonal-sensitive and seasonal-resistant (OC) influenza A H1N1 viruses revealed greater effectiveness against the pandemic virus and no significant difference in the effectiveness of teas against seasonal-sensitive and seasonal-resistant (OC) viruses, indicating that virus mutations in M2 and NA genes do not affect antiviral effectiveness of these teas.

Both teas are likely to act as viral entry blockers and as viral spread inhibitors of influenza virus. However, hemagglutination inhibitory activity is likely to primarily account for the anti-influenza activity of teas due to their hemagglutination inhibitory concentrations being near viral growth inhibitory concentration. Other mechanisms of influenza inhibitory action inside cells, including effects on acidification of the endosome by green tea and (−)epigallocatechin (EGC) (Imanishi et al., 2002) and inhibition of the endonuclease activity of viral RNA polymerase by catechin with a galloyl group (EGCG and ECG) (Kuzuhara et al., 2009), have been demonstrated and, thus, the possibility of these additional influenza targets being present in guava tea cannot be ruled out. Combination therapy has often been used for many infectious diseases, and combination therapy has been increasingly considered for influenza treatment to improve drug efficacy and delay drug-resistant development as well as to treat mixed infections. Tea is a mixture of components with multiple targets, which seem to act synergistically. Separated tea mixture provided lower anti-influenza activity than did unseparated tea; thereby, tea seems to have potential as a combination treatment. However, we are now making efforts to isolate, identify and characterize active ingredients of guava tea. Biological activity of each component will be explored to find a key component for use as a promising lead for anti-influenza development. Although catechin derivatives, low-molecular-weight polyphenols (MW of less than 500 g/mol), have been identified in guava leave extracts (Khan et al., 2011; Matsuo et al., 1994), screening of guava tea fractions performed by molecular weight-based fractionation using Vivaspin ultrafiltration devices (GE Healthcare) for anti-influenza activity has suggested that components with molecular weights of approximately 5000–10,000 (possibly being condensed tannins) and over 100,000 (possibly being polysaccharides) might be mainly responsible for anti-influenza activity.

In conclusion, we have shown that tea made from guava leaves has potent anti-influenza activity, most likely through prevention of viral entry into host cells. The tea inhibits all pandemic 2009, sensitive and resistant H1N1 strains that have shown an alarming spread worldwide. Consumption of this tea with numerous antimicrobial activities, especially during influenza season, might be useful for preventing and fighting influenza viruses and its complications.

Competing interests

S.F. and K.K. are employees of Pokka Corporation. The authors confirm that this does not alter their adherence to all Antiviral Research policies on sharing data and materials.

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