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ORIGINAL PAPER

Anti-Influenza Virus Activity of Adlay Tea Components

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Abstract

Our previous study showed anti-influenza virus activity in adlay tea prepared from adlay seeds, naked barley seeds, soybean, and cassia seeds. In this study, we evaluated the anti-influenza virus activity of each component of this tea and analyzed their active ingredients. Each component was roasted and extracted in hot water; the extracts were tested for antiviral activity and their mechanisms of action were studied. All the tea components showed antiviral activity against the H1N1 and H3N2 influenza subtypes and against influenza B. The viral stages inhibited by the components were virus adsorption and replication in proliferative process, suggesting that the action mechanisms of the components might differ from those of oseltamivir acid. Of the tea components, soybean showed the strongest activity. Therefore, we analyzed its active ingredients by liquid chromatography quadruple time-of-flight mass spectrometry (LC/qTOF-MS) and daidzein and glycitein were detected as active ingredients. Here, anti-influenza virus action of glycitein was the first report.

Keywords Anti-influenza virus · Adlay · Naked barley · Soybean · Daidzein · Glycitein

Abbreviations

IFV	Influenza virus
CDC	Centers for Disease Control and Prevention
MDCK	Madin–Darby canine kidney
FBS	Fetal bovine serum
EMEM	Eagle's minimum essential medium
DMEM	Dulbecco's modified Eagle's medium
BSA	Bovine serum albumin
MOI	Multiplicity of infection
FFRA	Focus-forming reduction assay
SD	Standard deviation
IC_{50}	Half maximal inhibitory concentration
CC_{50}	50% cytotoxicity concentration
SIM	Selected ion monitoring

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Introduction

The influenza virus (IFV) is an RNA virus belonging to the family *Orthomyxoviridae*. There are three types in humans, influenza A, B, and C, with types A and B being particularly prevalent each winter. Infection with the virus can sometimes result in severe complications such as pneumonia and encephalitis [1]. Vaccines and antiviral drugs are used for the prevention and treatment of infection, respectively; however, the U.S. Centers for Disease Control and Prevention (CDC) has reported the adjusted vaccine effectiveness estimates for the influenza seasons from 2004 to 2019 to be 10 to 60% [2], indicating that the vaccines cannot reveal a stable preventive effect. In addition, the emergence of IFV strains resistant to amantadine and neuraminidase has become a serious problem in recent years [3–5]. Thus, a novel approach to protect against IFV infection is required.

Recently, functional food ingredients have received increased attention, with many reports of anti-IFV activity by various food ingredients, including tea polyphenols such as catechins, theaflavins, and procyanidins [6]. There have also been studies of the neuraminidase inhibitory activities of catechins in green tea [7–9], and of their inhibitory effect on IFV growth through acidification of the intercellular compartment [10, 11]. Besides, green tea has an inhibitory effect on nuclear factor-kappa B (NF-κB) which is a critical regulator of genes involved in inflammation, cell proliferation, and apoptosis [12]. It has also been suggested that cocoa polyphenols and anthocyanin pigments in hibiscus tea show anti-IFV activity [13, 14].

Our previous study [15] showed that adlay tea prepared from adlay seeds, naked barley seeds, soybean, and cassia seeds exhibited strong anti-IFV activity against the H1N1 and H3N2 subtypes and influenza B through inhibiting virus replication and virus adsorption to cells. The anti-IFV activity of the tea was effective even against oseltamivir-resistant viruses, suggesting that the mechanism of action of the tea differed from that of oseltamivir, a drug that inhibits viral neuraminidase [3]. In the present study, we conducted a detailed research of the anti-IFV activity of each component of the adlay tea, identifying the active ingredients.

Materials and Methods

Compounds

Daidzein, glycitein, biochanin A, and genistein were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Hot Water Extracts of the Four Adlay Tea Components

Adlay seeds (*Coix lacryma-jobi* L. var. *ma-yuen*; Akishizuku brand), naked barley seeds (*Hordeum vulgare* var. *nudum*; Ichibanboshi brand), soybeans (*Glycine max*; Sachiyutaka brand), and cassia seeds (*Cassia obtusifolia* L.) were provided by Sanyo, Co., Ltd., Kobe, Japan. These were roasted at 180 °C for 4 h and then powdered. Some of the roasted powder (2 g) was mixed with water (50 ml) and extracted in a hot water bath for 60 min at 80 °C, as described previously [15]. The extract was filtered through No. 2 filter paper followed by a Millex GV membrane (24 mm diameter and 0.22 μm pore size; Millipore, Billerica, MA) and freeze-dried. Each lyophilizate was further dissolved in same amount of water as that used before freeze-drying, filtered through a Millex GX membrane, and stored at −30 °C until further use.

Cells and Viruses

Madin–Darby canine kidney (MDCK) cells were cultured in Eagle's minimum essential medium (MEM; Sigma-Aldrich, Inc., St. Louis, MO) containing 7% fetal bovine serum (FBS). CV-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% FBS. The following IFVs were used: influenza A H1N1 virus strains Puerto Rico/8/34 (PR/8/34), New Caledonia/20/99, and Beijing/262/95; oseltamivir-resistant influenza A H1N1 virus strains Osaka/2024/2009 and Osaka/71/2011 (provided by Osaka Prefectural Institute of Public Health); influenza A H3N2 virus strains Aichi/2/68 and Sydney/5/97); and influenza B virus strains Nagasaki/1/87 and Shanghai/261/2002. These were propagated in MDCK cells and cultured in DMEM containing 0.4% bovine serum albumin (BSA) and 2 μg/ml of acetyl trypsin (Sigma-Aldrich).

Antiviral Assays of the Hot Water Extracts

An antiviral assay was performed following the method of Kamei et al. [14] with slight modifications. IFV solution at a multiplicity of infection (MOI) of 0.0001 was added to MDCK cells in a 24-well plate (Thermo Fisher Scientific Inc., MA) and incubated for 1 h at 37 °C in 5% CO_2. The cells were washed with serum-free MEM and cultured in DMEM containing 0.4% BSA and 2 μg/ml acetyl trypsin plus hot water extract for 24–48 h at 37 °C in 5% CO_2. Virus titers were assessed in the culture supernatants by focus-forming reduction assay (FFRA), as described previously [15]. The anti-influenza effect was evaluated by determining the Selectivity index = CC_{50}/IC_{50} (IC_{50}, half maximal inhibitory concentration; CC_{50}, 50% cytotoxicity concentration).

Time-of-Addition Assay

A time-of-addition assay was performed following the method of Furuta et al. [16] with slight modifications.

Cytotoxic Testing and Hemagglutination Inhibition and Cell Fusion Inhibition Assays

Cytotoxic testing of the hot water extracts was performed by using a Cell proliferation kit (Roche Diagnostics Co. Ltd., Basel, Switzerland) following the manufacturer's protocol. Hemagglutination inhibition assays were performed by using a standard microassay [17]. The cell fusion inhibition assays followed the method of our previous study [15]. The influenza virus solution (PR/8/34: MOI of 0.001) was infected into CV-1 cells and cultured for 24 h. The infected cells were washed twice and incubated for 15 min in DMEM containing 10 μl/ml trypsin. The cells were washed twice and incubated for 30 min in DMEM plus hot water extract. The cells were washed and

treated with fusion medium to adjust to pH 5.0 and incubated for 2 min. The cells were washed twice and incubated for 3 h with DMEM containing 2% FBS. The cells were stained with Giemsa and the number of fused cells were counted.

Purification and Identification of Antiviral Compounds in the Hot Water Extract of Soybean

Roasted powder from the soybeans (2 g) was mixed with water (50 ml), and extracted in hot water bath at 80 °C for 60 min. The hot water extract was filtered through No. 2 filter paper followed by a Millex GV membrane and freeze-dried, and the lyophilizate (1 g) was then suspended in 2 ml of water and subjected to reversed-phase flash column chromatography using an Isolera Spektra (Biotage, Sweden, Uppsala). Antiviral activity of each fraction was determined with antiviral assay described earlier. The active fractions were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC/qTOF-MS).

Analysis of the fractions containing antiviral activity was performed according to the method described by Kammerer et al. [18] with some modifications.

Statistical Analyses

Viral titers in the time-of-addition assay and antiviral assay for the soybean fractions were analyzed by Student's *t* test using Excel Toukei ver. 6.0 (Esumi, Tokyo, Japan). Values are presented as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results and Discussion

All four components of the adlay tea inhibited virus multiplication of all the laboratory-adapted and clinical isolates of influenza A and B viruses (Table 1), and effectiveness against oseltamivir resistant viruses such as Osaka/2024/2009 and Osaka/71/2011, implying that the mechanism of action of these components might differ from that of the inhibitor. Adlay seeds and soybeans were especially effective against type B viruses, suggesting a relationship between the antiviral activity of the components and the antigenic structure of the IFV. Soybeans contain many isoflavones [19], which have anti-IFV activity [20]. In the present study, soybeans exhibited the strongest activity. This was probably related to isoflavones contained. However, in our previous study, antiviral activity of adlay tea did not correlate with the polyphenol content [15], suggesting that adlay tea has many active ingredients other than polyphenols, and that polyphenols are not the principal active ingredients.

To investigate the stage of virus infection inhibited by the four components, we performed a time-of-addition assay in which the infection periods followed five patterns (Fig. S1): pretreatment before virus adsorption (Pattern 1), virus adsorption (Pattern 2), virus replication (Pattern 3), virus replication at an early stage (Pattern 4), and virus replication at a late stage (Pattern 5). The results are shown in Fig. 1. All the components blocked virus adsorption most strongly in the infection periods and the inhibition rate is more than 90%. Virus replication was also inhibited by all the components, although the naked barley seeds and cassia seeds were ineffective at the late stage of virus replication.

Because the components inhibited both virus adsorption and replication in the time-of-addition assay, we examined the mechanism of action in more detail by performing hemagglutination inhibition and cell fusion inhibition assays. However, viral neutralization and inhibition of cell fusion were not confirmed with any of the components (data not shown). During the growth process of the IFV, hemagglutinin binds to sialic acid on host cells and is taken up into the cells. Synthesis of the uncoated viral RNA (vRNA) and protein then occurs. The synthesized HA, NA, M1 and M2 protein are incorporated into the cell membrane of the host. New viral RNPs (vRNPs) composed of vRNA and viral protein PB1, PB2, PA and NP are transported to cell surface. Finally, the vRNPs acquire the cell membrane incorporating HA, NA, M1 and M2, and are released extracellular as a virion [21]. In the time-of-addition assay, all the components showed inhibitory effects during pretreatment, adsorption, and replication, indicating the possibility that binding between hemagglutinin and sialic acid is blocked, or of viricidal effects. However, none of the components showed any inhibitory effect in the hemagglutination inhibition assay. The fact strongly suggests that these components acted via other mechanism, such as inhibition similar to that by endocytosis inhibitors. In addition, the components showed no activity in the cell fusion inhibition assay, indicating that antiviral activity of the components was unrelated to hemagglutinin.

In this study, all four components showed inhibitory effects during pretreatment before adsorption of the virus, *i.e.*, the components might affect cell function, such as a signaling pathway. Indeed, a relationship between the IFV replication process and the signaling pathway such as P13K/AKT and Raf/Mek/ERK has been reported [22, 23]. These results suggest that the components of adlay tea exert a novel mechanism of action against the IFV, and that each component contains several active ingredients and exhibits antiviral activity at various stages.

Antiviral activity of the hot water extract of soybeans against PR/8/34 was measured for each collected fraction (Fig. S2). Fractions 1 and 2 and fractions 12–23 showed antiviral activity. The adsorbed active fractions (fractions 12–23) were analyzed by LC/qTOF-MS, with the mass detection using the target ions $[M + H]^+$ or $[M - H]^-$. With fractions 16 and 17, the ion peak was detected at *m/z* 253.0516 in the

Table 1 Effect of the components of adlay tea on the multiplication of various influenza virus strains

Virus type and strain	IC_{50} (μg/ml or ng/ml)[a]					SI[b]				
	Adlay seeds (Akishizuku)	Naked barley seeds (Ichibanboshi)	Soybean (Sachiyutaka)	Cassia seeds (from India)	Oseltamivir acid [d]	Adlay seeds (Akishizuku)	Naked barley seeds (Ichibanboshi)	Soybean (Sachiyutaka)	Cassia seeds (from India)	Oseltamivir acid [d]
A (H1N1)										
PR/8/34	356.9 ± 51.9	571.8 ± 47.6	131.5 ± 7.2	335.4 ± 68.5	0.45 ± 0.01	26	25	56	9	>445,521
New Caledonia/20/99	688.7 ± 153.6	636.3 ± 1.2	329 ± 46.1	114.3 ± 32.4	0.38 ± 0.18	14	22	23	26	>526,327
Beijing/262/95	422.1 ± 54.9	1264.4 ± 426.3	71.5 ± 5.9	115.3 ± 41.1	ND	22	11	104	27	ND
Osaka/2024/2009[c]	499.4 ± 77.9	544.5 ± 12.2	78.6 ± 7.3	210.5 ± 88.5	180 ± 59	19	26	94	14	>1114
Osaka/71/2011[c]	333.5 ± 96.7	354.6 ± 124.3	83.6 ± 19.0	98.1 ± 22.9	259 ± 75	28	40	88	30	>771
A (H3N2)										
Sydney/5/97	158.1 ± 44.4	470.9 ± 88.8	90.5 ± 30.4	76.7 ± 15.4	ND	80	88	62	14	ND
Aichi/2/68	117.9 ± 58.7	161.3 ± 99.9	118.5 ± 13.4	215.0 ± 96.8	0.93 ± 0.78	60	30	81	39	>215,824
B										
Nagasaki/1/87	59.4 ± 15.1	724.1 ± 81.0	35.4 ± 18.6	837.6 ± 147.3	6.10 ± 1.19	137	20	212	4	>32,813
Shanghai/261/2002	69.5 ± 3.4	494.3 ± 79.6	32.1 ± 16.7	376.0 ± 181.3	ND	160	29	232	8	ND

[a] The values are means of the results of antiviral assays from two independent experiments, the units of μg/ml and ng/ml are for tea components and oseltamivir acid, respectively

[b] Selectivity index = CC_{50}/IC_{50} (IC_{50}, half maximal inhibitory concentration; CC_{50}, 50% cytotoxicity concentration)

[c] Oseltamivir-resistant virus

[d] The values of oseltamivir acid are cited from our previous study [15]

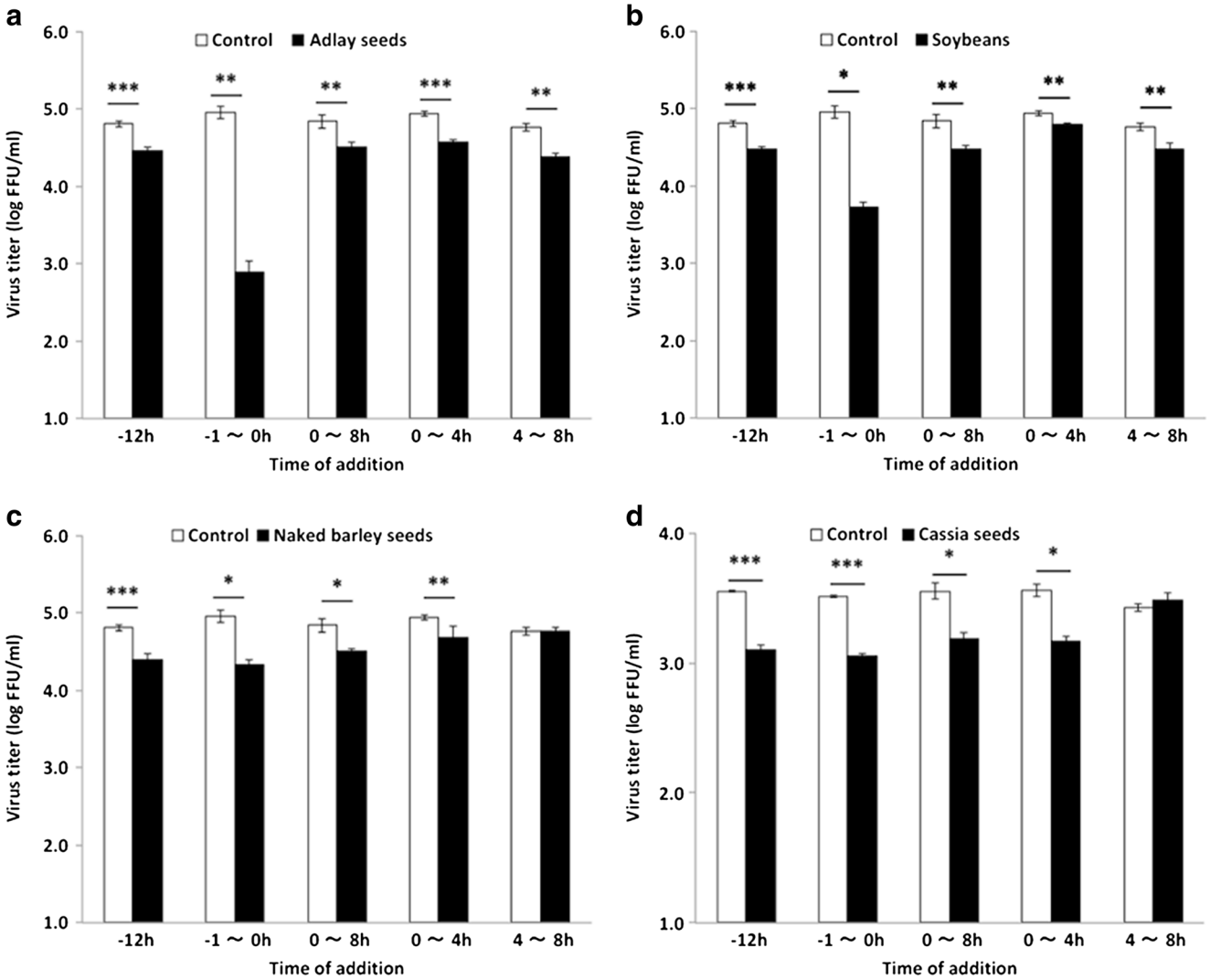

Fig. 1 The inhibition stages of the four components of adlay tea in virus multiplication. **a** Adlay seeds (Akishizuku); **b** soybeans (Sachiyutaka); **c** naked barley seeds (Ichibanboshi); and **d** cassia seeds. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

negative ion mode; this was thought to be daidzein, which has a molecular weight (MW) of 254. Considering fractions 18–20, the ion peak was detected at *m/z* 285.0765 in the positive ion mode for glycitein or biochanin A, as both have an MW of 284. The result obtained by SIM measurement of LC/MS for the retention time revealed that the peak of 18–20 fractions using LC/MS was completely overlapped by glycitein and not by biochanin A. With fractions 19–22, the ion peak was detected at *m/z* 271.0606 in the positive ion mode by using genistein, with an MW of 270. Here, we checked the antiviral activity of these components against PR/8/34 using the standard substances, which were isolated from soybean. As a result, it is suggested that daidzein and glycitein, as presented in Table 2, are involved as active ingredients in fraction 16–20 of the chromatography. Glycitein has not previously been reported to exhibit anti-IFV activity; this was a novel finding of the present study. However, other fractions also showed antiviral activity, suggesting that there are many active ingredients other than daidzein and glycitein. Thus, antiviral effect of soybeans is probably the combined effect of several ingredients. Further study is needed to identify other active ingredients and establish the mechanism of action.

Table 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	>17,182

[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} (IC_{50}, half maximal inhibitory concentration; CC_{50}, 50% cytotoxicity concentration)

Conclusions

This study showed antiviral activity by the four components of adlay tea (adlay seeds, naked barley seeds, soybeans, and cassia seeds). In the four components, soybean showed the strongest activity. When trying to purify the active ingredient from soybean, daidzein and glycitein were identified. Anti-IFV action of glycitein was the first report.

The intake of these components may be effective in prevention and treatment of influenza, as these components inhibited the virus adsorption and replication in this study. In order to clarify the effectiveness as a functional food, we need to conduct animal experiments and to check their effect on human population through a questionnaire in future studies.

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Compliance with Ethical Standards

Conflict of Interest The authors whose names are listed immediately below certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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References

1. Morishima T, Togashi T, Yokota S et al (2002) Encephalitis and encephalopathy associated with an influenza epidemic in Japan. Clin Infect Dis 35:512–517. https://doi.org/10.1086/341407
2. CDC Seasonal Flu Vaccine Effectiveness Studies. (2019) https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm
3. Kiso M, Mitamura K, Sakai-Tagawa Y et al (2004) Resistant influenza a viruses in children treated with oseltamivir: descriptive study. Lancet 364:759–765. https://doi.org/10.1016/S0140-6736(04)16934-1
4. Dapat IC, Dapat C, Baranovich T et al (2012) Genetic characterization of human influenza viruses in the pandemic (2009-2010) and post-pandemic (2010-2011) periods in Japan. PLoS One 7:e36455. https://doi.org/10.1371/journal.pone.0036455
5. Zaraket H, Kondo H, Hibino A et al (2016) Full genome characterization of human influenza a/H3N2 isolates from Asian countries reveals a rare amantadine resistance-conferring mutation and novel PB1-F2 polymorphisms. Front Microbiol 7:262. https://doi.org/10.3389/fmicb.2016.00262
6. Yang Z-F, Bai L-P, Huang W et al (2014) Comparison of *in vitro* antiviral activity of tea polyphenols against influenza a and B viruses and structure–activity relationship analysis. Fitoterapia 93:47–53. https://doi.org/10.1016/J.FITOTE.2013.12.011
7. Nakayama M, Suzuki K, Toda M et al (1993) Inhibition of the infectivity of influenza virus by tea polyphenols. Antivir Res 21:289–299. https://doi.org/10.1016/0166-3542(93)90008-7
8. Müller P, Downard KM (2015) Catechin inhibition of influenza neuraminidase and its molecular basis with mass spectrometry. J Pharm Biomed Anal 111:222–230. https://doi.org/10.1016/J.JPBA.2015.03.014
9. Song J-M, Lee K-H, Seong B-L (2005) Antiviral effect of catechins in green tea on influenza virus. Antivir Res 68:66–74. https://doi.org/10.1016/J.ANTIVIRAL.2005.06.010
10. Imanishi N, Tuji Y, Katada Y et al (2002) Additional inhibitory effect of tea extract on the growth of influenza a and b viruses in MDCK cells. Microbiol Immunol 46:491–494
11. Nakayama M, Toda M, Okubo S, Shimamura T (1990) Inhibition of influenza virus infection by tea. Lett Appl Microbiol 11:38–40. https://doi.org/10.1111/j.1472-765X.1990.tb00131.x
12. Di Lorenzo C, Dell'Agli M, Sangiovanni E et al (2013) Correlation between catechin content and NF-κB inhibition by infusions of green and black tea. Plant Foods Hum Nutr 68:149–154. https://doi.org/10.1007/s11130-013-0354-0
13. Baatartsogt T, Bui VN, Trinh DQ et al (2016) High antiviral effects of hibiscus tea extract on the H5 subtypes of low and highly pathogenic avian influenza viruses. J Vet Med Sci 78:1405–1411. https://doi.org/10.1292/jvms.16-0124
14. Kamei M, Nishimura H, Takahashi T et al (2016) Anti-influenza virus effects of cocoa. J Sci Food Agric 96:1150–1158. https://doi.org/10.1002/jsfa.7197
15. Nagai E, Iwai M, Koketsu R et al (2018) Inhibition of influenza virus replication by adlay tea. J Sci Food Agric 98:1899–1905. https://doi.org/10.1002/jsfa.8671
16. Furuta Y, Takahashi K, Kuno-Maekawa M et al (2005) Mechanism of action of T-705 against influenza virus. Antimicrob Agents Chemother 49:981–986. https://doi.org/10.1128/AAC.49.3.981-986.2005
17. Dowdle WR, Kendal AP, Noble GR (1979) Influenza viruses. In: Lennette EH, Schmidt NJ (eds) Diagnostic procedures for viral, rickettsial, and chlamydial infections, 5th edn. American Public Health Association, Washington, DC, pp 585–609
18. Kammerer D, Claus A, Carle R, Schieber A (2004) Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. J Agric Food Chem 52:4360–4367. https://doi.org/10.1021/jf049613b
19. Malenčić D, Cvejić J, Miladinović J (2012) Polyphenol content and antioxidant properties of colored soybean seeds from Central Europe. J Med Food 15:89–95. https://doi.org/10.1089/jmf.2010.0329
20. Liu A-L, Wang H-D, Lee SM et al (2008) Structure–activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their *in vitro* anti-viral activities. Bioorg Med Chem 16:7141–7147. https://doi.org/10.1016/J.BMC.2008.06.049
21. Matsuoka Y, Matsumae H, Katoh M et al (2013) A comprehensive map of the influenza a virus replication cycle. BMC Syst Biol 7:97. https://doi.org/10.1186/1752-0509-7-97
22. Seo Y-J, Pritzl CJ, Vijayan M et al (2013) Sphingosine kinase 1 serves as a pro-viral factor by regulating viral RNA synthesis and nuclear export of viral ribonucleoprotein complex upon influenza virus infection. PLoS One 8:e75005. https://doi.org/10.1371/journal.pone.0075005
23. Michael P, Brabant D, Bleiblo F et al (2013) Influenza a induced cellular signal transduction pathways. J Thorac Dis 5(Suppl 2):S132–S141. https://doi.org/10.3978/j.issn.2072-1439.2013.07.42