

Potential Anti-Influenza Virus Agents Based on Coffee Ingredients and Natural Flavonols

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Abstract

Natural phenolic compounds have been reported to directly inhibit influenza virus. Nonetheless, there have been no reports on the direct inhibitory effects of coffee ingredients. Here, we fractionated the hydrophobic and hydrophilic components of coffee. The hydrophobic fraction directly inhibited both a seasonal influenza A/Puerto Rico/8/24(H1N1) virus and a neuraminidase-resistant influenza A/Yokohama/77/2008(H1N1) virus infection in a dose-dependent manner, while the hydrophilic fraction did not show any inhibitory effects, even at concentrations above 100 µg/ml. The HPLC profile of the hydrophobic fraction indicated that caffeine is the major component. Indeed, caffeine alone showed comparable anti-influenza virus activity. Interestingly, caffeic acid also inhibited viral infection, while chlorogenic acid, which is an ester of caffeic acid and (-)-quinic acid, showed no obvious antiviral effect at less than 2 mg/ml. We previously reported a method for enhancing the anti-influenza virus activity of epigallocatechin gallate, a major tea catechin, by lipase-catalyzed acylation. Using this methodology, we synthesized fatty acid esters of caffeic acid and evaluated their influenza virus-inhibitory effects. It was found that dioctanoyl ester of caffeic acid exhibited approximately 38-fold higher direct antiviral activity. To understand the essential structure required for virus inhibition, we further examined the antiviral activity of natural flavonoids containing either the caffeic acid skeleton or its analogous structure. Flavonols (quercetin, myricetin and morin) and hexahydrobenzophenone containing extended planar pi-conjugated systems efficiently inhibited the virus infection. Flavonoids possessing both radical scavenging activity and cytotoxicity tended to show higher antiviral activity, probably due to their affinity with viral surface factors. On the other hand, there was no apparent correlation between their antiviral activity and antioxidative activity. These findings provide insight into the design of anti-influenza virus agents from natural polyphenols.

Keywords: Coffee extracts; Caffeic acid; Caffeine; Influenza virus; Flavonoids; Fatty acid esters; Antiviral activity; Antioxidative activity; Antiradical activity; Cytotoxicity

Abbreviations: PFU: Plaque Formation Unit; IC₅₀: 50% Inhibitory Concentration; CC₅₀: 50% Cytotoxic Concentration; DMSO: Dimethylsulfoxide; DPPH: 1,1-diphenyl-2-picrylhydrazyl radical; MDCK: Madin-Darby Canine Kidney; DMEM: Dulbecco's Modified Eagle's Medium; SOD: Superoxide dismutase-like; MTT: 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; EtOAc: Ethyl Acetate; Wat: Water; ND: Not Determined

Introduction

The threat of an influenza virus pandemic is increasing worldwide, highlighting the need for an antiviral agent that is able to inhibit a broad spectrum of viral infection. Current anti-influenza virus agents mainly target neuraminidase, an enzyme that plays an essential role in the release and spread of progeny virions, following the intracellular viral replication cycle. However, more than 90% of seasonal influenza viruses isolated in the northern hemisphere in 2008/2009 season were oseltamivir phosphate-resistant strains [1]. Although previously approved neuraminidase inhibitors can inhibit oseltamivir phosphate-resistant viruses [2,3], we must also be prepared for outbreaks of novel resistant strains.

On the other hand, green tea catechins have been reported to possess anti-influenza virus activity *in vitro* [4] and *in vivo* [5]. We reported that epigallocatechin gallate, a major green tea catechin, and its fatty acid derivatives directly inhibit various types of influenza virus including neuraminidase inhibitor resistant viruses [6,7]. Natural flavonoids in pomegranate [8], berries [9], and plant seeds [10] have also been reported to be effective for inhibition of virus infection. However, little is known about the antiviral activity of coffee extracts [10,11], despite its status as the most-consumed drink in the world.

The aim of this study is to obtain potent anti-influenza virus

compounds from coffee ingredients and to propose novel anti-influenza virus agents from natural food ingredients.

Materials and Methods

Coffee beans: Blend coffee (UCC Co. Ltd.; Produced in Colombia, 100% Arabica bean), Kona coffee (Hawaii Coffee Company; Produced in Hawaii, 100% Arabica bean). Coffee ingredients: caffeine, hesperetin, and ellagic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), caffeic acid and taxifolin were purchased from Enzo Life Sciences (Tokyo, Japan), (-)-quinic acid, naringenin, morin hydrate, hexahydroxybenzophenone, gallic acid and ethyl gallate were purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Chlorogenic acid was obtained from Wako. Luteolin, quercetin and myricetin were purchased from Cayman (Ann Arbor, MI), MDCK cells were obtained from Dainippon Sumitomo Seiyaku, DMEM was from Wako, fetal bovine serum was from MP Biomedicals, Inc., (Solon, OH), bovine serum albumin and agar were from Sigma-Aldrich (Tokyo, Japan), trypsin-EDTA was from Nacalai Tesque (Kyoto, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. unless otherwise noticed.

General procedures for organic synthesis: Reagents and solvents

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were obtained from commercial sources without further purification unless otherwise noted. The reaction process was monitored by Thin layer chromatography on Silicagel 60F254 (MerckGAA, Darmstadt, Germany) and a UV illuminator at 254 nm (Funakoshi Corporation, Tokyo, Japan). ¹H NMR spectra were recorded on a JEOL ECS 400 spectrometer (JOEL, Tokyo, Japan). Chemical shifts were reported in terms of δ (ppm) relative to tetramethylsilane in dimethylsulfoxide-d₆ (Acros Organics, Tokyo, Japan). All coupling constants are given in Hz. Flash column chromatography was performed on silica gel (40-63 μ m) under a pressure of about 4 psi. Organic solvents were evaporated using a rotary evaporator N-1000V (Shibata Scientific & Technology Ltd., Saitama, Japan), a SYS-20WR pump (Yamato Scientific Co., Ltd. Tokyo, Japan) and a coolant Coolman Pal C-330 (Shibata). Aqueous solutions were lyophilized using a DC 400 Freeze Dryer (Yamato) and a rotary vacuum pump (Hitachi Ltd., Tokyo, Japan). Samples were analyzed by reverse-phase HPLC using a JASCO PU-2080 plus pump system (Tokyo, Japan), a JASCO UV-2075 detector, a AS-2055 plus intelligent autosampler, a MX-2080-32 Mixer, a DG-2080-53 degasser, a GL Science Inertsil WP 300 C-18 column (150 mm \times 4.6 mm) and a 5 μ m C-18 column. Elution was performed with 30% methanol and 70% 5 mM potassium phosphate buffer (pH 2.5) at a flow rate of 0.4 ml/min for 22 min. Chromatographic peaks were detected with a UV detector at a wavelength of 280 nm. Mass spectra were obtained by a LTQ orbitrap XL (Thermo Scientific, Yokohama, Japan) and melting temperatures were obtained by a MP-S3 micro melting point apparatus (Yanaco New Science Inc., Kyoto, Japan).

Extraction of coffee bean ingredients

Commercially available ground coffee (Blend coffee or Kona coffee; 30 g) was subjected to extraction in 250 ml of hot water. Extracts were immediately cooled on an ice bath and hydrophobic compounds were extracted with ethyl acetate. Subsequently, the ethyl acetate phase was dried with magnesium sulphate and filtered, and the ethyl acetate was evaporated using a rotary evaporator. The remaining water phase was frozen in liquid nitrogen and subsequently freeze-dried using a lyophilizer.

HPLC analysis of coffee extracts and coffee ingredients

Dried powder of ethyl acetate extract and remaining water extract were individually dissolved in 1.0% methanol aqueous solution and the concentration was adjusted to 0.2 mg/ml. Samples were analyzed by octadecyl-modified silica gel column using eluents composed of 30% methanol and 70% 5 mM potassium phosphate buffer (pH 2.5). Chromatographic peaks were detected with a UV detector at a wavelength of 280 nm. Major coffee bean ingredients such as caffeine, caffeic acid and quinic acid shown in Figure 1 were analyzed by this method.

Influenza virus inhibitory assay for coffee extracts, coffee ingredients, octanoyl esters of caffeic acid, natural flavonoid and gallic acid derivatives

In order to evaluate the influenza virus-inhibitory effects of coffee extracts, coffee ingredients, natural flavonoids and their derivatives, we employed plaque formation inhibitory assay [12]. A monolayer of Madin-Darby Canine Kidney (MDCK) was prepared in a 6-well plate. Each sample was dissolved in DMEM containing 1.0% dimethyl sulfoxide (DMSO) and was directly incubated with influenza A/Puerto Rico/8/34/H1N1 or A/Yokohama/77/2008/H1N1 virus for 30 min prior to inoculation. Virus solution was then added to a monolayer of MDCK cells, and was incubated for 1 h at 37°C. Cell sheets were washed with Phosphate-Buffered Saline (PBS), and were incubated

with DMEM containing 0.8% agar for 2 days at 37°C. Cell sheets were fixed with 5% glutaraldehyde and the overlay agarose was removed, after which cell sheets were stained with 0.02% methylene blue solution. Plaques formed on each well were counted and the inhibitory effects of each sample on virus infection were evaluated. As a positive control, we employed quercetin, which is known to possess influenza virus inhibitory effects [13].

Synthesis of octanoyl esters of caffeic acid by lipase-catalyzed acylation

Synthesis of octanoyl esters of caffeic acid was accomplished using the previously reported lipase-catalyzed transesterification method [6]. Briefly, lipase PL from *Alcaligenes sp.* (100 mg) was added to 20 ml of N, N-dimethylformamide solution containing 4 (200 mg, 1.12 mmol) and vinyl palmitate (756 mg, 4.44 mmol). The mixture was stirred for 36 h at 57°C. After filtration of the reaction mixture, 100 ml of ethyl acetate was added to the solvent, which was washed three times with brine, three times with 5% NaHCO₃ aq and three times with 5% HCl aq. The organic phase was dried with Na₂SO₄, filtered, and the ethyl acetate was removed using a rotary evaporator vacuum system. The residue was purified by silica gel column chromatography using a mixture of 92% chloroform and 8% methanol, which resulted in 33% of a mixture of mono-octanoyl esters **6a** and **6b** (white solid, R_f value; 0.28 (chloroform:methanol=90:10 (v/v)) and 10% of dioctanoyl ester **7** (white solid, R_f value; 0.56 (chloroform:methanol=90:10 (v/v))).

¹H NMR of **6a** (400 MHz, CD₃OD) : 0.918 (3H, t, J=7.2 Hz, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 1.340 (6H, m, -COCH₂CH₂CH₂(CH₂)₃CH₃), 1.444 (2H, t, J=7.2 Hz, -COCH₂CH₂CH₂(CH₂)₃CH₃), 1.735 (2H, t, J=7.2 Hz, -COCH₂CH₂CH₂(CH₂)₃CH₃), 2.595 (2H, t, J=7.2 Hz, -COCH₂CH₂CH₂(CH₂)₃CH₃), 6.379 (1H, d, J=16 Hz, -CHCHCOOH), 6.988 (1H, d, J=8.4 Hz, 2-phenyl proton), 7.058 (1H, dd, J₁=1.6 Hz, J₂=8.0 Hz, 6-phenyl proton), 7.115 (1H, d, J₁=1.6 Hz, 5-phenyl proton), 7.535 (1H, d, J=16 Hz, -CHCHCOOH). Melting point: 125°C, ESI-MS calculated for C₁₇H₂₂O₅, 306.35; found, m/z 329.14[M +Na]⁺

¹H NMR of **6b** (400 MHz, CD₃OD) : 0.918 (3H, t, J=7.2 Hz, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 1.340 (6H, m, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 1.444 (2H, t, J=7.2 Hz, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 1.735 (2H, t, J=7.2 Hz, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 2.603 (2H, t, J=7.2 Hz, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 6.299 (1H, d, J=16 Hz, -CHCHCOOH), 6.913 (1H, d, J=8.4 Hz, 2-phenyl proton), 7.249 (1H, d, J=2.0 Hz,

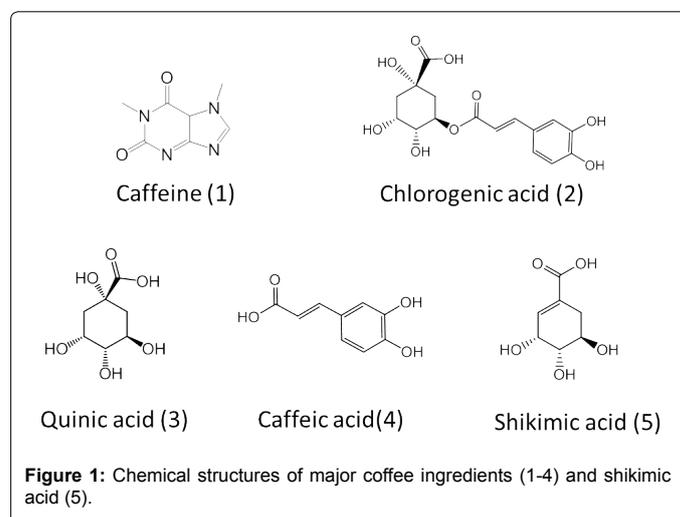


Figure 1: Chemical structures of major coffee ingredients (1-4) and shikimic acid (5).

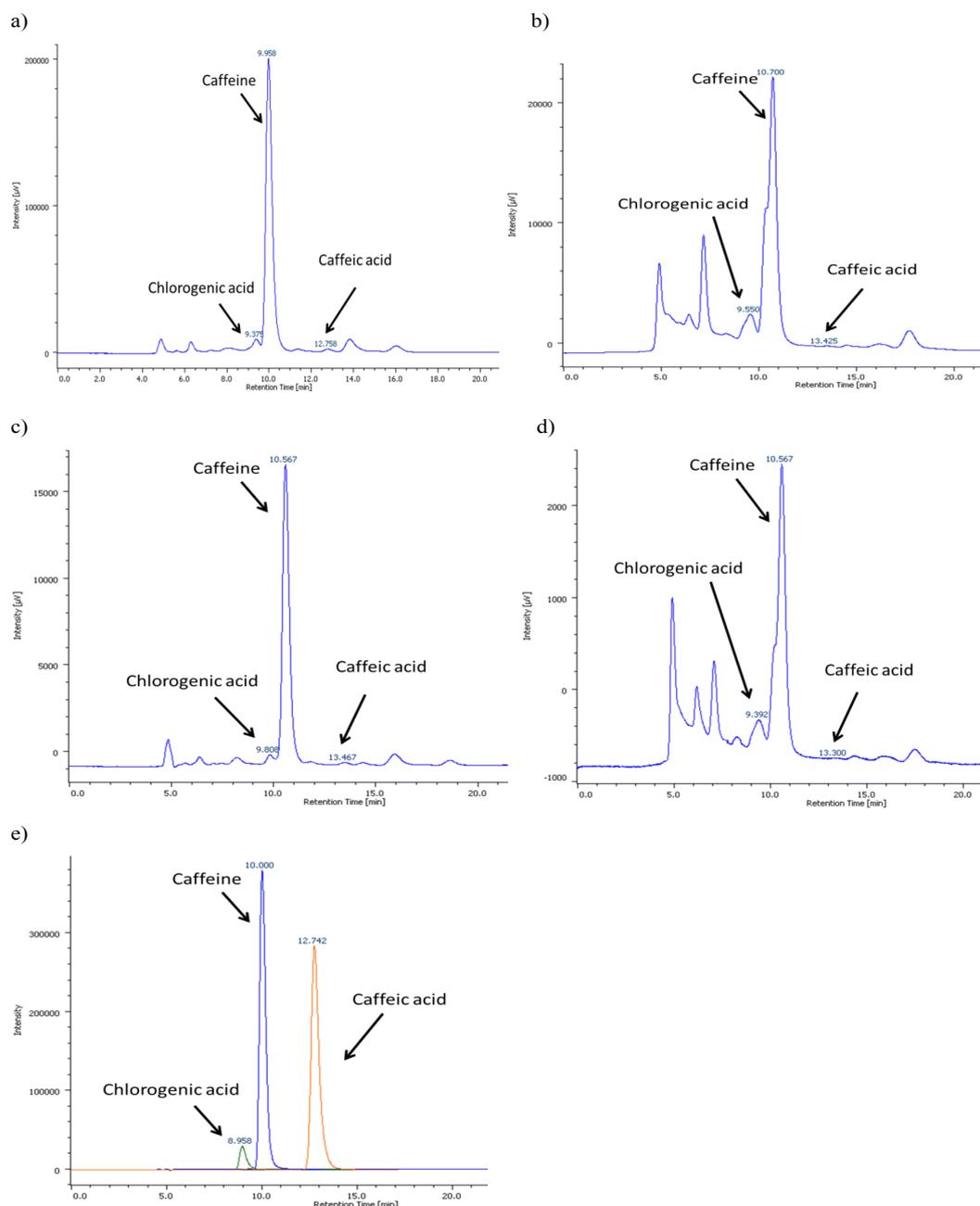


Figure 2: HPLC profiles of coffee bean extracts and the major coffee ingredients. Phenolic compounds in each fraction were detected by UV absorbance at 280 nm and their HPLC chromatograms are shown in Figures 2a-2d. a) ethyl acetate phase of Blend coffee, b) water phase of Blend coffee, c) ethyl acetate phase of Kona coffee, and d) water phase of Kona coffee. e) Retention time of chlorogenic acid, caffeine, and caffeic acid.

5-phenyl proton), 7.330(1H, dd, $J_1=2.0\text{Hz}$, $J_2=8.4\text{Hz}$, 6-phenyl proton), 7.547(1H, d, $J=16\text{ Hz}$, -CHCHCOOH). Melting point: 125°C, ESI-MS calculated for $C_{17}H_{22}O_5$, 306.35; found, m/z 329.14[M +Na]⁺

¹H NMR of 7 (400 MHz, CD₃OD) : 0.914 (6H, t, $J=7.2\text{ Hz}$, 2 x -COCH₂CH₂CH₂ (CH₂)₃CH₃), 1.331 (16H, m, 2 x -COCH₂CH₂CH₂ (CH₂)₃CH₃), 1.712 (4H, m, $J=7.2\text{ Hz}$, 2 x -COCH₂CH₂CH₂ (CH₂)₃CH₃), 2.561 (2H, t, $J=7.2\text{ Hz}$, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 2.575 (2H, t, $J=7.2\text{ Hz}$, -COCH₂CH₂CH₂ (CH₂)₃CH₃), 6.473(1H, d, $J=15.2\text{ Hz}$, -CHCHCOOH), 7.236 (1H, d, $J=8.0\text{ Hz}$, 2-phenyl proton), 7.457 (1H, d, $J=1.6\text{ Hz}$, 5-phenyl proton), 7.503(1H, dd, $J_1=2.0\text{Hz}$, $J_2=8.8\text{Hz}$, 6-phenyl

proton) 7.586 (1H, d, $J=16\text{ Hz}$, -CHCHCOOH). Melting point: 98°C, ESI-MS calculated for $C_{25}H_{36}O_6$, 432.55; found, m/z 455.24[M +Na]⁺

SOD-like activities of natural flavonoids and gallic acid derivatives

SOD-like activity of polyphenolic compounds was determined by SOD assay kit-WST (Dojindo Molecular Technologies, Inc., Tokyo, Japan) as specified by the manufacturer. Briefly, test compounds were dissolved in DMSO and were then serially diluted in dilution solution. Each test compound was mixed with xanthine oxidase solution followed

by addition of xanthine solution. The mixture was incubated for 30 min at 37°C, and the reduction in WST-1 formazan was monitored by absorbance at 450 nm.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity natural flavonoids and gallic acid derivatives

The radical scavenging activity of each sample on DPPH radicals was assessed using the method described by Gažák et al. [14] with some modification. A stock solution (1 mM) of each sample was prepared and diluted with methanol to various concentrations (0, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100 µM). An aliquot of 50 µL of each dilution was transferred to a 96-well microplate (Corning, Tokyo, Japan). A working solution of DPPH (100 µM) in methanol was prepared and then an aliquot of 150 µl was added to each well. After incubation for 30 min at ambient temperature, quenching at an absorbance of 490 nm was measured on a microplate reader (Biorad, Tokyo, Japan). Each dilution was performed in triplicate. Free radical-scavenging activities of test samples are expressed in terms of IC₅₀ values, which is the concentration of a sample required to decrease the absorbance at 490 nm by 50% when compared to the control response.

Cell culture

MDCK cells were grown in DMEM supplemented with 10% foetal bovine serum and maintained in a humidified incubator at 37°C with 5% CO₂ upon reaching confluence. Cells were trypsinized and passaged.

Cytotoxicity of coffee ingredients, natural flavonoids and their derivatives

The cytotoxicity of coffee extracts, coffee ingredients, natural flavonoids and their derivatives were examined by cell growth proliferation (MTT) assay. MDCK cells were seeded onto a 96-well plate and each sample was added to cells in a serial dilution from 2 mM. At 1 h post-incubation, cells were washed twice with PBS and subsequently incubated with DMEM for 24 h at 37°C. Cells were then washed twice with PBS and cell proliferation activity was monitored by MTT assay in accordance with the manufacturer's protocol.

Results and Discussion

HPLC analysis of coffee bean extracts

Coffee ingredients in the ethyl acetate phase of blend coffee (Blend-EtOAc) and Kona coffee (Kona-EtOAc) were monitored by absorbance at UV 280 nm and are shown in Figure 2a and 2c. Coffee ingredients in the water phase of Blend coffee (Blend-Wat) and Kona coffee (Kona-Wat) were also monitored under the same conditions and are shown in Figure 2b and 2d. From their peak profiles on HPLC chromatograms, phenolic compounds in each coffee extract were almost the same, regardless of the production region. The total peak intensity in the ethyl acetate phase was 5 to 8-fold higher than that of the water phase. To identify some of peaks in the chromatograms, we analyzed caffeine (1), chlorogenic acid (2) and caffeic acid (4) under the same analytical conditions, and found that their peaks appeared around 9, 10, and 13 min, respectively (Figure 2e). According to the HPLC profile in Figure 2a-2d, chlorogenic acid, caffeine and caffeic acid appeared at 9.375-9.808 min, 9.958-10.700 min and 12.759-13.467 min, respectively. Caffeine seemed to be the major aromatic ingredient in coffee extract, although the molar extinction coefficient of each compound differed at 280 nm.

Influenza virus inhibition activity of coffee extracts

The ethyl acetate phase of blend coffee (Blend-EtOAc) and Kona coffee (Kona-EtOAc) inhibited influenza A/Puerto Rico/8/34/H1N1(PR8) in a dose-dependent manner, regardless of the production region (Figure 3). The 50% virus inhibitory concentration (IC₅₀) values for Blend-EtOAc and Kona-EtOAc were 17.5 µg/ml and 7.2 µg/ml. On the other hand, the water phase of blend coffee (Blend-Wat) did not show virus-inhibitory effects, even at 100 µg/ml (Figure 3). These results indicate that the antiviral components are mainly present in the ethyl acetate extracted phase. Blend-EtOAc also inhibited infection by oseltamivir-resistant influenza A/Yokohama/77/2008/H1N1 and the IC₅₀ was 3.9 µg/ml (Figure 3). The antiviral effects of coffee extracts varied depending on target viral strain. Although further study is required in order to understand the mode of action, these data indicate that coffee ingredients bind differently to each viral surface component.

Influenza virus inhibitory assay of caffeine and caffeic acid

In order to understand the antiviral components in coffee extract, we examined the virus-inhibitory effects of 1, 2 and 4. Both 1 and 4 inhibited influenza A/Puerto Rico/8/34/H1N1 (PR8) in a dose-dependent manner and the IC₅₀ values were 19.1 µg/ml (98.0 µM) and 53.9 µg/ml (299 µM) (Figure 4). It has been reported that 1 is able to inhibit the expression of hemagglutinin protein in influenza A/Aichi-infected MDCK cells [10]. However, there have been no reports on whether 1 and 4 are able to directly inactivate influenza virus infectivity. In contrast to our expectations, 2 did not show virus-inhibitory effects even at concentrations of 100 µg/ml (282 µM). This indicates that the caffeic acid moiety of 2 is important for the virus inhibitory effects and the quinic acid moiety in 2 hinders the interaction between its caffeic acid moiety and viral surface components.

Influenza virus inhibitory assay of fatty acid esters of caffeic acid

We prepared monoctanoyl ester (6a+6b) and dioctanoyl ester (7) of caffeic acid by lipase-catalyzed acylation (Figure 5). They showed enhanced anti-influenza virus activity against A/Puerto Rico/8/34/

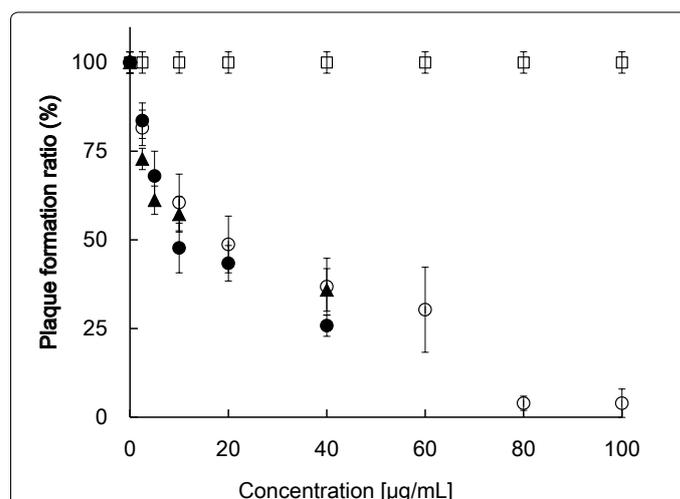


Figure 3: Influenza virus inhibitory effects of ethyl acetate extracts and remaining water extracts of coffee. Open circles: antiviral effects of ethyl acetate extract of blend coffee on influenza A/PR8; Closed triangles: antiviral effects of ethyl acetate extract of blend coffee on influenza A/Yokohama; Closed circles: antiviral effects of ethyl acetate extract of Kona coffee on influenza A/PR8; Open squares: antiviral effects of water phase after ethyl acetate extract on influenza A/PR8.

H1N1. The IC_{50} values of the mono-octanoyl esters (**6a+6b**) and dioctanoyl ester (**7**) were 20.9 $\mu\text{g/ml}$ (68.3 μM , 4.38-fold) and 3.4 $\mu\text{g/ml}$ (7.86 μM , 38.0-fold). It is interesting to note that **7** completely inhibited viral infection at 40 $\mu\text{g/ml}$ (Figure 6). This was probably because the two neighbouring octanoyl groups in **7** increased lipophilicity with the viral membrane, and enhanced the direction of virus-inhibitory effects.

Influenza virus inhibitory assay of natural flavonoids and gallic acid derivatives

Among the flavonoids in Figure 7, quercetin (**11**), myricetin (**12**), morin (**13**) and hexahydroxybenzophenone (**16**) showed influenza virus-inhibitory effects (Table 1). Other flavonoids that did not show any inhibitory effects at 2,000 ng/ml were eliminated from further study. Although epigallocatechin (**15**) has a similar structure to **13**, the lack of a planar pi-conjugated system caused the loss of antiviral effects (Table 1). From a structural viewpoint, the above compounds (**11**, **12**, **13** and **16**) possess an extended pi-conjugated system and more than two hydroxyl groups in the phenyl group at the C-2 position. Although luteolin (**8**) meets these criteria, the absence of a hydroxyl group at the C-3 position lowered its antiviral activity.

SOD-like activity of natural flavonoids and gallic acid derivatives

In order to understand the effects of antioxidative activity on antiviral activity, we evaluated the SOD-like activity of natural flavonoids that contain caffeic acid skeleton or analogous structures (Figure 7). It was found that **8**, taxifoline (**14**), **15** and gallic acid (**17**) showed SOD-like activity (Figure 7 and Table 1). Nevertheless, they did not show apparent antiviral activity at 2,000 nM. This indicates that there is no clear correlation between antioxidative and antiviral activities.

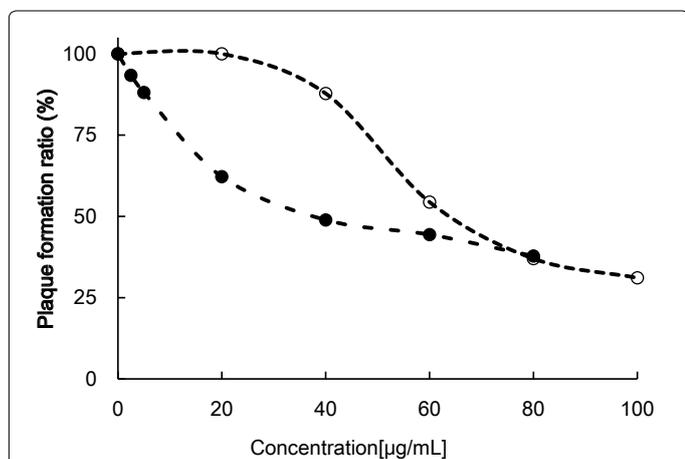


Figure 4: Influenza virus inhibitory effects of caffeine and caffeic acid. Closed circles: caffeine (**1**); Open circles: caffeic acid (**4**).

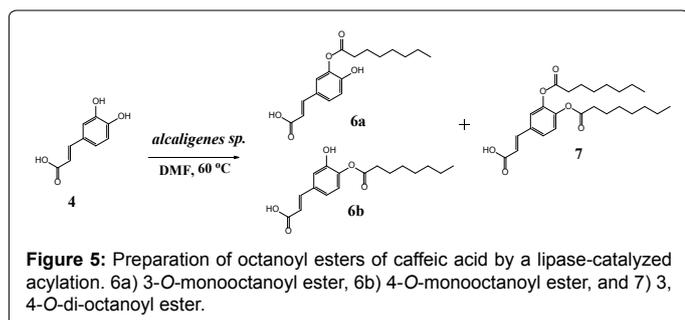


Figure 5: Preparation of octanoyl esters of caffeic acid by a lipase-catalyzed acylation. **6a**) 3-O-mono-octanoyl ester, **6b**) 4-O-mono-octanoyl ester, and **7**) 3,4-O-di-octanoyl ester.

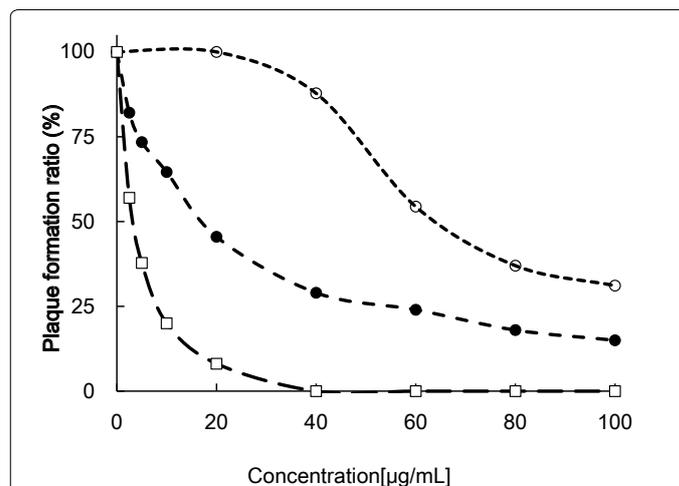


Figure 6: Influenza virus inhibitory effects of caffeic acid and octanoyl esters. Open circles: caffeic acid (**4**), Closed circles: mono-octanoyl esters (**6a+6b**), Open squares: dioctanoyl ester (**7**).

DPPH free radical scavenging activities of natural flavonoids and gallic acid derivatives

Influenza is an envelope virus. The outer layer is composed of a lipid membrane taken from the host cell. Lipid peroxidation of viral membranes by free radicals may cause serious damage to virus infectivity. Hence, we evaluated DPPH free radical scavenging activity of **8-19** to understand the possible relationship between radical scavenging activity and antiviral activity. Flavone (**8**), flavonols (**11-13**) and gallic acid derivatives (**16-19**) showed radical scavenging activities, and although there may be some correlation, the radical scavenging activities were not sufficient to explain the antiviral activity.

Cytotoxicities of natural flavonoids and gallic acid derivatives

The cytotoxicity of each compound (**8-19**) was evaluated by cell proliferation assay. Flavones (**8-9**), flavonols (**11-13**) and **16** exhibited relatively high cytotoxic effects when compared with other flavonoids, although their selectivity index (CC_{50}/IC_{50}) was larger than 500. Interestingly, they all have planar pi-conjugated systems. This may have increased cell membrane permeability. As influenza virus membrane is composed of the host cell membrane, cell membrane affinity and radical scavenging activity may be important for inactivating virus infectivity.

Conclusion

In conclusion, the coffee ingredients in ethyl acetate extract directly inhibited influenza virus infection. The antiviral compounds in the extract were identified as caffeine and caffeic acid. We introduced octanoyl groups in 3-phenyl group of caffeic acid by a lipase-catalyzed acylation. The dioctanoyl ester showed 38-fold increased anti-influenza virus activity, probably due to its increased lipophilicity to the virus membrane. This simple method can be useful for improving anti-influenza virus activity of various types of natural phenolic compounds. Meanwhile, we evaluated natural flavonoids that contain a caffeic acid skeleton or analogous structure. From a structural viewpoint, flavonols (**11-13**) that have both extended pi-conjugated systems and more than two hydroxyl groups in the phenyl group exerted higher antiviral activities (Table 1). Among these three compounds, **13** showed the highest antiviral activity. The neighboring three hydroxyl groups at the B-ring increased the antiradical activity and cell membrane permeability, resulting in higher antiviral activity. Flavanone **10**,

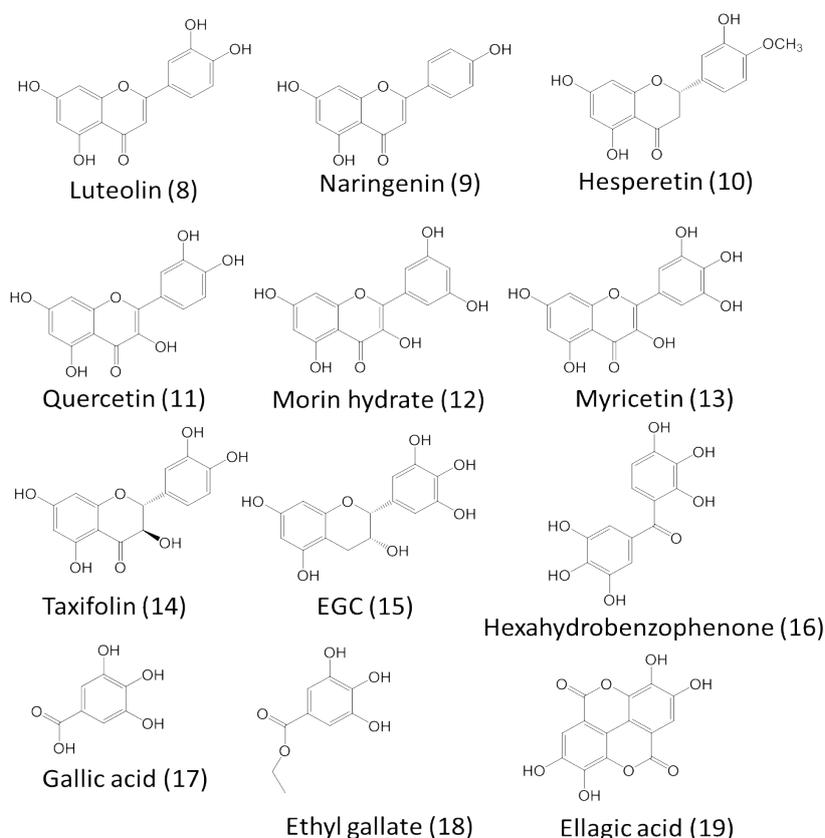


Figure 7: Chemical structures of natural flavonoids (8-15) and gallic acid derivatives (16-19).

	Compound	Antioxidative activity ¹ IC ₅₀ [nM]	Antiradical activity ² IC ₅₀ [nM]	Anti-influenza viral activity ³ IC ₅₀ [nM]	Cytotoxicity CC ₅₀ ⁴ [μM]
8	Luteolin	51.0	73.1	>2,000	225
9	Naringenin	>100	>100	>2,000	425
10	Hesperetin	ND	>100	>2,000	>1,000
11	Quercetin	>100	65.5	1,400	480
12	Morin Hydrate	ND	71.3	1,400	425
13	Myricetin	97.1	38.9	900	505
14	Taxifolin	60.9	>100	>2,000	>1,000
15	Epigallocatechin	26.0	>100	>2,000	>1,000
16	Hexahydroxy benzophenone	ND	25.8	900	475
17	Gallic acid	22.3	51.5	>2,000	>1,000
18	Ethyl gallate	ND	28.4	>2,000	>1,000
19	Ellagic acid	ND	22.6	>2,000	>1,000

¹Antioxidative activity was measured by superoxide dismutase-like assay

²Antiradical activity was measured by DPPH free radical scavenging assay

³Anti-influenza virus activity was measured by plaque formation inhibitory assay

⁴Cytotoxicity was measured by cell proliferation (MTT) assay

IC₅₀: the 50% inhibitory concentration

CC₅₀: the 50% cytotoxic concentration

ND: Not Determined

Table 1: Antioxidative, antiradical and anti-influenza virus activities, and cytotoxicity of natural flavonoids and their analogues.

flavanonol **14**, and flavan-3-ol **15**, which possess a non-planar structure did not show any virus inhibitory effects at 2,000 nM. This indicates that the double bond between C2 and C3 and the carbonyl at C4 on the center ring are important for retaining antiviral activity. Although flavones **8** and **9** have planar structures, the lack of a C3 hydroxyl group

lowered the antiviral activity, probably due to reduced water solubility. Gallic acid derivatives **16-19** showed relatively high anti-radical activities, but only **16** exhibited the antiviral activity. It is possible that **17-19** had insufficient cell membrane permeability. From these data, flavonoids and gallic acid derivatives that contain planar structure and possess both anti-radical activity and cell membrane permeable properties possess anti-influenza virus activity. There were no clear correlations between antioxidative activities and antiviral activities.

Based on the present study, we propose that novel octanoyl esters of caffeic acid and natural flavonoids, such as quercetin (in onion), myricetin (in walnut) and morin (in guava leave) be studied as potential influenza virus-inhibitory agents.

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