Supplementary Methods

Time-of-addition assay

The virus was diluted in MEM plus 0.4% BSA and the infected cells were cultured in DMEM containing 0.4% BSA and 2 μg/ml acetyltrypsin in five patterns (Figure S1): pretreatment, virus adsorption, and three stages of virus replication. Pattern 1, the pretreatment before virus adsorption, was performed as follows. MDCK cells were seeded into a 24-well plate. Hot water extract was added to the culture medium and the cells were cultured for 12 h at 37°C in 5% CO_2. The cells were then washed twice with serum-free MEM, IFV solution (PR/8/34: MOI of 0.01) was added, and the cells were incubated for 1 h at 37°C in 5% CO_2. The infected cells were washed twice with serum-free MEM and cultured for 8 h at 37°C in 5% CO_2. Pattern 2, virus adsorption, was performed by mixing the hot water extract and virus solution, and the mixture was adsorbed to MDCK cells for 1 h at 37°C in 5% CO_2. The infected cells were washed twice with serum-free MEM and then cultured in the same way as with Pattern 1. Pattern 3, virus replication was achieved by adding the virus solution as with Pattern 1, washing the infected cells twice with serum-free MEM, and culturing the infected cells in medium containing the hot water extract for 8 h at 37°C in 5% CO_2. Patterns 4 and 5 (virus replication at an early and late stage, respectively) were achieved as follows. The influenza virus solution was added as with Pattern 1. For Pattern 4, the infected cells were cultured in medium containing the hot water extract for 4 h; the culture medium was then replaced with new medium that did not contain the extract, and the cells were cultured for a further 4 h. For Pattern 5 (late stage replication), these two steps were performed in the reverse order, first culturing the infected cells in normal medium and

then with new medium containing the hot water extract. After all the patterns, the infected cells were frozen at −80°C and subjected to two freeze–thaw cycles prior to assessment by virus titer by focus-forming reduction assay.

Reversed-phase flash column chromatography

The extract fraction was developed on a pre-packed C18 cartridge (SNAP Ultra C18; 16 mm × 100 mm) and eluted in series with 264 ml of water, 132 ml each of mixtures of water and acetonitrile in various proportions, and 198 ml of 100% acetonitrile, with a flow rate of 12 ml/min. This yielded 50 fractions. Fractions 1–8 eluted with 100% water. Fractions 9–44 were eluted with a mixture of water and acetonitrile in the following v/v ratios: fractions 9–12, 9:1; fractions 13–16, 8:2; fractions 17–20, 7:3; fractions 21–24, 6:4; fractions 25–28, 5:5; fractions 29–32, 4:6; fractions 33–36, 3:7; fractions 37–40, 2:8; and fractions 41–44, 1:9. Fractions 45–50 were eluted with 100% acetonitrile. The eluent was detected with a Photodiode Array Detector (PDA) monitor at wavelengths of 200 nm and 250 nm and collected fractions were freeze-dried. Each lyophilizate was dissolved again in dimethyl sulfoxide and filtered through a Millex GX membrane for using to antiviral assay.

Liquid chromatography quadrupole time-of-flight mass spectrometry (LC/qTOF-MS)

The active fractions were analyzed in an LC20ADXR high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with a microTOF-QII quadrupole time-of-flight tandem mass spectrometer (Bruker Daltonics, Billerica, MA).

The ingredients were separated using a Synergi Hydro RP column (particle size = 2.5 μm; 100 mm × 3 mm internal diameter; Phenomenex, Torrance, CA). The mobile phase was (A) 2% acetic acid and (B) 0.5% acetic acid/acetonitrile = 1:1. The gradient began with 10% B and was changed to 24% B at 8 min, 30% B at 16 min, 55% B at 24 min, 100% B at 30 min, 100% B (isocratic) from 30 to 33.2 min, and 10% B from 34 to 36 min. The flow rate of the mobile phase was 0.4 ml/min. The temperature of the column oven was 40℃, and the sample injection volume was 5 μl. The mass spectrometry used the following conditions: mass range 50–3,000; spectra rate, 2 Hz; nebulizing gas, nitrogen (1.6 bar); drying gas, nitrogen (200℃, 8 l/min); capillary voltage, −4500 V for positive ions and +2800 V for negative ions; hexapole radio frequency, 200 Vpp; quadrupole ion energy, 5 eV; collision gas nitrogen (1.6 bar); collision energy, 10 eV; and collision radio frequency, 200 Vpp.

Supplementary Figure Legends

Figure S1. Outline of culture pattern in time-of-addition assay. MDCK cells were infected with A/PR/8/34 (multiplicity of infection 0.0001) and cultured in one of five patterns in a time-of-addition assay. Pattern 1 (-12h); Pattern 2 (-1–0h); Pattern 3 (0–8h); Pattern 4 (0–4h); Pattern 5 (4–8h). The following was added: adlay seeds (400 μg/ml), soybeans (300 μg/ml), naked barley seed (1500 μg/ml), cassia seeds (700 μg/ml). The virus titers were measured by focus-forming assays.

Figure S2. Fractionation and virus-inactivating ability of the hot water extract from roasted soybeans. (A) Elution profile of the flash column chromatography. (B) Anti-influenza virus activity of the collected fractions. The soybean extract was fractionated by preparative liquid chromatography using SNAP C18 column chromatography. The antiviral activity of fractions (Fr.) 1–50 was checked by antiviral assay. MDCK cells were infected with A/PR/8/34 (multiplicity of infection 0.0001) and the infected cells were incubated in medium containing 1% fraction solution for 24 h. The virus titers were measured by focus-forming assays. Statistical test vs. control (Cr): *** $P < 0.001$.

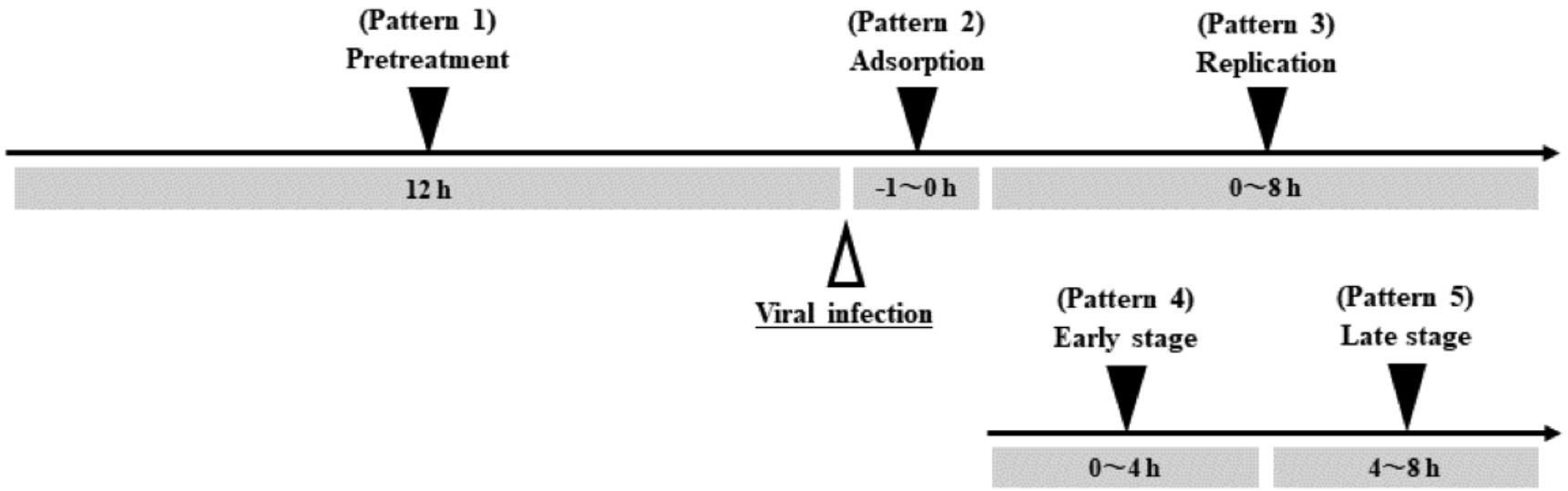

Fig. S1_Plant Foods for Human Nutrition_Nagai et al.

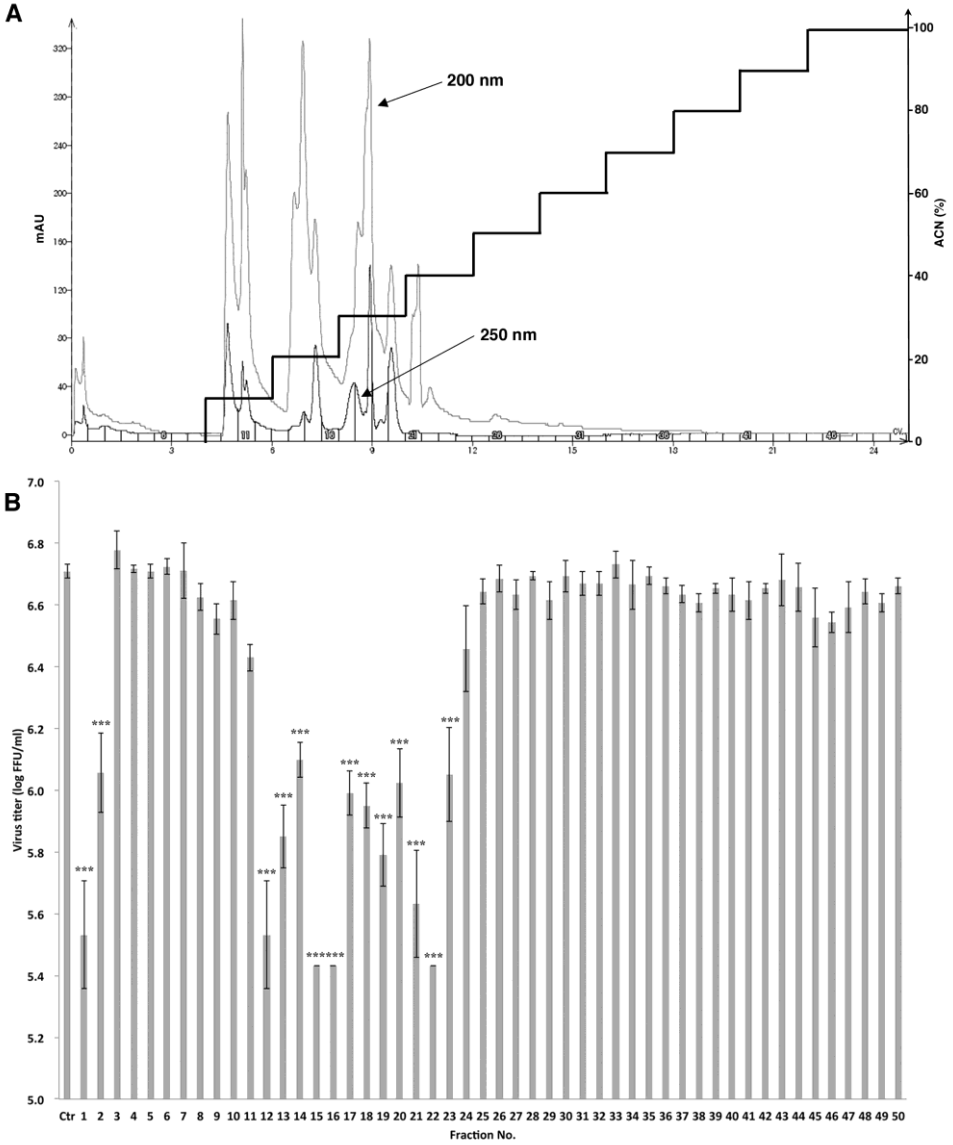

Fig. S2_Plant Foods for Human Nutrition_Nagai et al.