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Identification of Soybean Components that Prevent the Entry of Influenza Virus

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Abstract

We have previously demonstrated the anti-influenza virus activity of a soybean extract, indicating the presence of compounds that prevent viral entry into cells. In this study, we sought to identify the compounds in hydrothermal extracts of soybean that blocked viral entrance and analyzed their mechanisms of action. Hot aqueous extracts of roasted soybeans were separated by reverse-phase chromatography on an octadecylsilyl C18 column, and the antiviral activity of individual fractions was measured during the viral entry and culture phases.

Fractions 2-5 were found to have a significantly high antiviral activity during the culture phase, whereas during the entry phase, fractions 2-4 showed strong antiviral activity, with fraction 4 having notably potent inhibitory effects. We accordingly analyzed fractions 3 and 4 using liquid chromatography quadrupole time-of-flight mass spectrometry and identified potential anti-viral compounds, including acacetin, 9,10,13-trihydroxy-11-octadencenoic acid, and soy saponin IV. Furthermore, the analysis of these active components revealed that acacetin and soyasaponin were active during the entry phase, and subsequent assessments of their inhibitory effects on influenza virus entry into cells revealed that acacetin inhibits the endocytosis process during viral uptake, whereas soyasaponin inhibits virus binding to its receptor. These findings thus indicate a novel mechanism for the anti-influenza activity of acacetin.

Keywords
Anti-influenza virus; attachment; entry; soybean; acacetin; soyasaponin

1. Introduction

The influenza virus (IFV) is an RNA virus within the family Orthomyxoviridae. Three types of influenza viruses infect humans, A, B, and C, among which, types A and B are responsible for seasonal epidemics of respiratory illness that occur almost annually during winter in temperate regions. Moreover, the occurrence of viral and bacterial co-infections can heighten the risk of mortality [1]. Although vaccines and antiviral drugs are used to prevent and treat infections, respectively, the fact that the US Centers for Disease Control and Prevention have reported adjusted vaccine efficacy estimates ranging from 10% to 60% for the 2004-2022 influenza seasons [2] would tend to indicate that. In contrast, vaccines can contribute to reducing the risk of influenza illness or its complications; however, their efficacy is variable. Furthermore, the emergence of IFV strains resistant to the drugs amantadine and oseltamivir has presented considerable challenges in recent years [3, 4]. Consequently, new approaches are needed for the prevention of IFV infection.

Functional food components and their potential anti-IFV activity have attracted growing attention in recent years, with several studies focusing on constituents such as the polyphenols found in tea (catechins, theaflavins, and procyanidins) [5]; the soybean polyphenol daidzein [6]; cocoa polyphenols [7]; and anthocyanin pigments in hibiscus tea [8]. Furthermore, catechins have been shown to inhibit neuraminidase, an enzyme essential for viral release [9], as well as IFV proliferation by acidifying the intercellular compartments [10]. In contrast, daidzein has been shown to activate 5-lipoxygenase via the MEK/ERK pathway, thereby producing 5-hydroperoxy eicosatetraenoic acid, which inhibits the intracellular replication of IFV [11, 12].

We have previously demonstrated that adlay tea, consisting of adlay, naked barley, cassia seeds, and soybeans, has anti-IFV activity against oseltamivir-resistant viruses [13]. However, although tea was found to inhibit the late stages of viral proliferation, our findings indicated that the mechanism differs from that of oseltamivir [3]. Furthermore, extensive investigations on the anti-IFV activity of components present in soybeans have revealed that a hot-aqueous extract of soybean suppresses the proliferation of influenza viruses and has a strong antiviral effect during the attachment or entry

stage of viral infection [14]. Daidzein has been identified as an antiviral component that occurs in substantial quantities in soybeans [6], of which the detailed mechanism of action and involvement in signal transduction have been studied [11, 12]. However, daidzein primarily inhibits the viral growth phase rather than the attachment or entry phase [11]. As the substances that inhibit the entry phase of the virus remain unclear, this study utilized a hydrothermal extract of soybeans (*Glycine max* L. Merr.) to perform constitutional analyses and identify the active components that contribute to inhibiting viral entry into host cells during the infection process.

2. Materials and Methods

2.1 Cell Lines and Viruses

Madin-Darby canine kidney (MDCK) cells (Research Institute for Microbial Diseases, Osaka University) used for influenza virus growth and virus-inhibitor assays were cultured in minimum essential medium (MEM: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), supplemented with 7% fetal bovine serum (FBS: Biowest Biotechnology Company, France). For the purposes of this study, we used the influenza A virus strain H1N1 (PR/8/34), with cell culture, viral infection, and recovery being performed as described by Sakata et al. [14].

2.2 Preparation and Partial Purification of Soybean Extracts

Yellow soybeans (Sachiyutaka 2018 harvest provided by Sanyo Co., Ltd., Kobe, Japan) were roasted, ground, and extracted with hot water based on the protocol described by Sakata et al. [14]. The extracts thus obtained were lyophilized, with 0.7 g of the dried product being suspended in 2 mL of water and subjected to reverse-phase flash column chromatography using an octadecylsilyl (ODS) column (10 g of ODS C_{18}, dissolved in methanol, packed into a 25 mm ϕ column). After the application of the sample, the column was washed six times [W fractions (Fr.)] with 50 mL of degassed ultrapure water and then eluted in a stepwise manner with a 50 mL volume of acetonitrile, in which the amount of acetonitrile was incrementally increased by 10% at each step (Frs. 2-11). The antiviral activity of each fraction was determined using an antiviral assay based on the protocol described by Nagai et al. [6]. Inhibition of viral replication by soybean extracts or partially purified samples in MDCK cells was evaluated using the focus-forming reduction assay (FFRA) method described by Sakata et al. [14]. Briefly, virus-infected MDCK cells in 96-well flat-bottom plates were subjected to indirect peroxidase staining to determine viral titers. Focus staining involves the sequential addition of murine monoclonal anti-HA antibodies and goat anti-mouse IgG antibodies conjugated to horseradish peroxidase. Visualization of the peroxidase reaction was facilitated using 3,3'diaminobenzidine and H_2O_2, and the number of foci in the immunostained infected cells was determined using an inverted light microscope. The active fractions were analyzed using liquid chromatography-mass spectrometry (LC-MS) described as follows.

2.3 Analysis of Antiviral Components in a Hot-Aqueous Soybean Extract Using Liquid Chromatography-Mass Spectrometry

2.3.1 Liquid Chromatography-Spectrometry (LC-MS)

Active fractions were analyzed using an Agilent LCMS6430 high-performance liquid chromatography-mass spectrometry system (Agilent Technologies, Inc., CA, USA) equipped with a Senergi Hydro-RP 100 A column (3 mm i.d. × 100 mm, 2.5 µm; Phenomenex, Inc., CA, USA). The mobile phase was (A) 2% acetic acid and (B) 0.5% acetic acid/acetonitrile = 1:1. The separation gradient commenced with 10% B, with subsequent increases to 24% B at 8 min, 30% B at 16 min, 55% B at 24 min, and 100% B at 30 min, followed by 100% B (isocratic) from 30 to 33.2 min, and reduction to 10% B from 34 to 36 min. The flow rate of the mobile phase was 0.4 mL/min, the column oven temperature was 40°C, and the sample injection volume was 5 µL. Mass spectrometry was performed under the following conditions: mass range 50-3,000; spectra rate, 2 Hz; nebulizing gas, nitrogen (60 psi); capillary voltage, 2500 V; fragment voltage, 100 V.

2.3.2 Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC/qTOF-MS)

Detailed analysis of the active fractions was performed using 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 procedure used followed that described by Nagai et al. [6].

2.3.3 LC-MS Analysis (Triple Quadrupole, QQQ)

LC-MS analysis was performed according to the method previously described by Sakurai et al. [15]. Samples were injected into an LC-MS system comprising an Agilent 1260 Infinity binary LC and an Agilent 6430 triple-quadrupole LC-MS (Agilent Technologies Inc.) using a procedure described by Harada et al. [16].

2.4 Analysis of Antiviral Activity

2.4.1 Cell Viability Assay Following Treatment with Purified Compounds and Time-of-Addition Assay

Actual antiviral activity following treatment with purified compounds was examined based on assessments of cell viability using the procedure described by Sakata et al. [14] Briefly, to determine viral titers, virus-infected MDCK cells in 96-well flat-bottom plates were subjected to indirect peroxidase staining. Focus staining entailed the sequential addition of murine monoclonal anti-HA antibodies and goat anti-mouse IgG antibodies conjugated to horseradish peroxidase, with 3,3'diaminobenzidine and H_2O_2 being used for the development of the peroxidase reaction. The number of foci in the immunostained infected cells was determined using an inverted light microscope. To elucidate the timing of action, a time-of-addition assay was performed using the following procedure described by Sakata et al. [14]. MDCK cells were plated in 24-well plates and inoculated with A/PR/8/34 at a multiplicity of infection (MOI) of 0.01. Thereafter, serum-free DMEM (500 µL/well) containing 20 µg/mL acacetin or 150 µg/mL soyasapogenol B was introduced at different time points: between 1 h before infection and the time of infection (-1-0 h, corresponding

to adsorption and entry), and between 0-4, 4-8, or 0-8 h post-infection (replication). Following the respective incubation periods, the medium was replaced with a fresh medium, and the cells were cultured for a further 8 h post-infection. The infected cells were frozen at -80°C and subjected to two freeze-thaw cycles before determining viral yields using a focus-forming assay.

In general, in response to drug treatment, the curve of the viral titer corresponds to the following equation:

$$y = ae^{-bx} = Titer = ae^{-b \times concentration}$$

Given that the linear equation ln y = ln a - bx can be derived from this equation, we determined the coefficients of ln a and b and the goodness of fit by performing linear regression analysis using the experimental data. The slope of the coefficient of b was confirmed to be negative using a *t*-test.

2.4.2 Viral Entry Inhibition Assay

The inhibitory effects of soybean compounds on the adsorption of influenza virus onto MDCK cells were evaluated using the method described by Sakata et al. [14]. The influenza A/PR/8/34 virus (MOI = 10) was added to MDCK cells. After incubation for 1 h in a CO_2 incubator at 37°C or on ice, RNA was extracted from the cells and reverse transcribed to synthesize the corresponding cDNA [14]. RNA levels of the viral hemagglutinin (HA) protein adsorbed onto the cells were determined by quantitative polymerase chain reaction (qPCR) using a 7500 Fast Real-time PCR System (Applied Biosystems Inc., Norwalk, CT, USA).

2.5 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 24 for Windows. The Kruskal-Wallis test was used to assess differences between groups, and the Mann-Whitney U test was used to determine differences between treatment and control groups. Mean and confidence interval values were calculated following the logarithmic transformation and were subsequently retransformed to antilogarithmic values. The level of statistical significance was set at a two-tailed *p*-value of 0.05 or less.

3. Results

3.1 Antiviral Activity of a Hot-Aqueous Soybean Extract Fractionated Using Reverse-Phase Column Chromatography

Fractionation of the hot-aqueous extract of soybean was performed using a C_{18} reverse-phase column, with each fraction thus being assessed for antiviral activity. Washes 1-6 (W 1-6) represented non-adsorbed fractions, which were washed out using degassed ultrapure water. Having confirmed that the non-adsorbed antiviral components had been washed out, the adsorbed components were eluted using acetonitrile. Fr. 2-11 represented the adsorbed fractions, which were extracted using step-wise increases in acetonitrile solution. Antiviral activities were detected in W 1-5 and Fr. 2-9, with extreme viral inhibitory activities being observed in W1, W2, and Fr. 2-5 (Table 1). Although antiviral activity was also confirmed in the non-adsorbed fractions, we were unable to perform

analyses of these using LC-MS; therefore, no further fractionation was performed. Consequently, we focused primarily on the adsorbed fractions with confirmed antiviral activity.

Table 1 Inhibition of influenza virus replication by ODS C_{18} fractions of a hot-aqueous soybean extract.

	Virus titer mean † ($\times 10^4$ FFU/mL)	(95% CI)	Between-group comparison (1)	Differences between groups (2)	Difference from control (3)
Control	456243	(318170, 654234)			
Wash1	18626	(11496, 30179)		<W5,6**	**
Wash2	36529	(29124, 45816)		<W5,6**	**
Wash3	101731	(59864, 172879)			**
Wash4	102874	(44573, 237430)	$p = 0.007$		**
Wash5	226559	(134944, 380372)			**
Wash6	299800	(135306, 664271)			**
Fr2	5286	(1956, 14285)		<F7,8,9,10,11**	**
Fr3	23689	(10941, 51292)		<F8,9,10,11**	**
Fr4	50960	(42079, 61717)		<F10,11**	**
Fr5	35387	(27244, 45962)		<F10,11**	**
Fr6	114661	(65687, 200148)			**
Fr7	146226	(120740, 177091)	$p = 0.001$		**
Fr8	227430	(146911, 352079)			**
Fr9	204992	(104468, 402244)			**
Fr10	357266	(252800, 504899)			
Fr11	378603	(303506, 472280)			

MDCK cells were infected with influenza A/PR/8/34 virus at an MOI of 0.001 for 1 h. Following infection, 5% of each fraction was added to the medium, and 24 h later, the virus titer in the culture supernatant was determined using a focus-forming reduction assay.

† Values are presented as the means calculated after logarithmic transformation and the 95% confidence interval values were retransformed to antilogarithmic values.

(1) Kruskal-Wallis test.

(2) Dunn's test, **p < 0.05.

(3) Difference from control: Mann-Whitney U test, **p < 0.05.

To determine the components within the hot-aqueous soybean extract that inhibit the entry phase of the influenza virus, we added the C_{18} column fractions at the time of virus infection and measured the amount of virus adsorbed onto the cells by viral RNA quantification. A vigorous inhibitory activity against viral entry was observed in cells treated with W1, W2, and Frs. 2-4 (Table 2). Fr. 10, which had not shown antiviral activity earlier, also exhibited inhibition of viral entry (Table 2).

Table 2 Inhibition of viral entry by ODS C_{18} fractions of a hot-aqueous soybean extract.

	Virus titer mean † ($\times 10^4$ FFU/mL)	(95% CI)	Between-group comparison (1)	Differences between groups (2)	Difference from control (3)
Control	21589	(16631, 28025)			
Wash1	188	(2, 15535)		<W5,6**	**
Wash2	5370	(2696, 10694)		<W5,6**	0.05
Wash3	11093	(7484, 16443)	$p = 0.01$		**
Wash4	14530	(11834, 17840)			**
Wash5	18094	(15631, 20945)			
Wash6	19415	(13836, 27243)			
Fr2	4464	(2672, 7458)		<F6,7,8**	0.05
Fr3	5712	(2844, 11471)		<F7,8**	0.05
Fr4	2028	(1414, 2908)		<F5,6,7,8,11**	**
Fr5	11286	(5862, 21727)			0.05
Fr6	12952	(7608, 22048)	$p = 0.005$		0.05
Fr7	14414	(9238, 22489)			**
Fr8	13943	(7639, 25447)			0.05
Fr9	9416	(5312, 16691)			0.05
Fr10	6690	(3056, 14648)		<F7**	0.05
Fr11	12515	(6624, 23645)			0.05

MDCK cells were infected with influenza A/PR/8/34 virus at an MOI of 0.001 for 1 h. Following infection, 5% of each fraction was added to the medium. After culturing for 8 h, the cells were frozen overnight at -80°C, subjected to two freeze-thaw cycles, and the virus titers of the collected supernatants were determined using a focus-forming reduction assay.

† Values are presented as the means calculated after logarithmic transformation and the 95% confidence intervals were retransformed to antilogarithmic values.

(1) Kruskal-Wallis test.

(2) Dunn test, **p < 0.05.

(3) Difference from control: Mann-Whitney U test, **p < 0.05.

3.2 Analysis of Antiviral Components in the Hot-Aqueous Extract of Soybean

Fractions that were confirmed to strongly inhibit the entry phase of influenza virus infection (Frs. 2-4) and a fraction that showed weaker activity (Fr. 5) were analyzed, and candidate active components were identified by LC-MS and LC/qTOF-MS, as shown in Figure 1 and Table 3. Given that Fr. 9 and 10 were found to inhibit virus adsorption, we also analyzed the preceding (Fr. 8) and subsequent (Fr. 11) fractions, although we detected no evident activity. The 80%-90% acetonitrile fractions (Fr. 9 and 10), the components that we were unable to identify using LC-MS and LC/qTOF-MS, showed inhibitory effects only during the early stage of viral infection and had no detectable antiviral effect in activity assays in which the fraction aliquots were added after viral infection (Table 1 and Table 2). Polyphenols were detected in the initial half of the eluted C_{18} column adsorbed fractions, and components that are more lipophilic than polyphenols were detected in the second

half, thereby providing evidence to indicate that the soybean extract may have contained active components other than polyphenols that inhibit the entry phase of viral infection. We speculate that a possible factor precluding the detection of these components was the low sample weight obtained from the 80%-90% acetonitrile fractions (Fr. 9 and 10) following lyophilization (0.3 and 0.4 mg, respectively). In the future, identifying the active constituents of soybeans that inhibit the entry phase of viral infection will require examining the fractionation conditions, including the selection of appropriate column materials and elution solvents, with a particular focus on detecting liposoluble components.

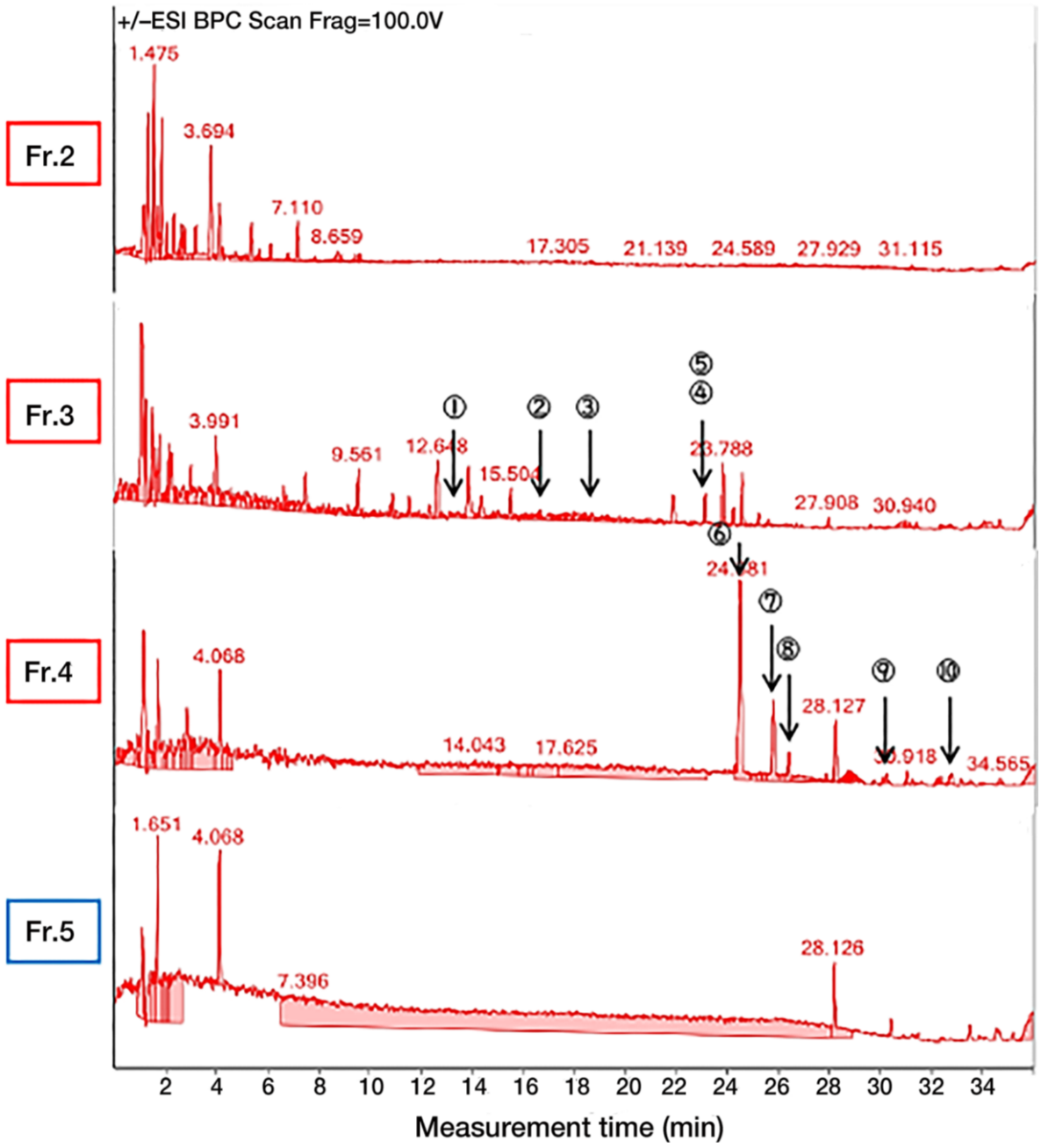

Figure 1 Base peak chromatograms obtained from LC/MS determinations of fractions of a hot-aqueous soybean extract**.** Arrows indicate fraction-specific peaks with confirmed antiviral activity.

Table 3 Candidate active constituents of a soybean extract detected based on LC/MS and LC/MS/MS analyses

No.	Fr.	Time (min)	MW	CF	Candidates
1.	3	13.4	479	$C_{23}H_{24}O_{11}$	Cirsimarin
2.	3	17.0	416	$C_{21}H_{20}O_9$	Daidzin
3.	3	19.1	432	$C_{21}H_{20}O_{10}$	Genistin
4.	3	23.1	458	$C_{23}H_{22}O_{10}$	6''-O-Acetyldaidzin
5.	3	23.1	518	$C_{25}H_{26}O_{12}$	Medicarpin 3-O-(6'-Malonylgluclside)
6.	4	24.4	254	$C_{15}H_{10}O_4$	Didzein
7.	4	25.6	284	$C_{16}H_{12}O_5$	Glycitein Acacetin
8.	4	26.0	474	$C_{23}H_{22}O_{11}$	6''-O-Acetylgenistin
9.	4	29.6	330	$C_{18}H_{34}O_5$	9,10,13-Trihydroxy-11-octadecenoic acid
10.	4	32.4	766	$C_{41}H_{66}O_{13}$	Soyasaponin IV

* No. Denotes the number of the corresponding peaks shown in Figure 1.

To confirm the antiviral activity of the components that inhibited the entry phase of viral infection (shown in Figure 2 and Table 3), we performed activity assays using commercially available compounds. Among the identified active components, those found to lack inhibitory activity at the early stage of viral infection, along with glycosides, were excluded. The findings of these activity assays revealed that aglycones of acacetin (5,7-dihydroxy-4'-methoxyflavone) and soyasapogenol B (derived from soyasaponin IV) exhibited anti-influenza virus activity against the H1N1 A/PR/8/34 strain. The following equation was obtained based on linear regression analysis for the viral titer after acacetin treatment:

$$y = 16.718e^{-0.171x}$$

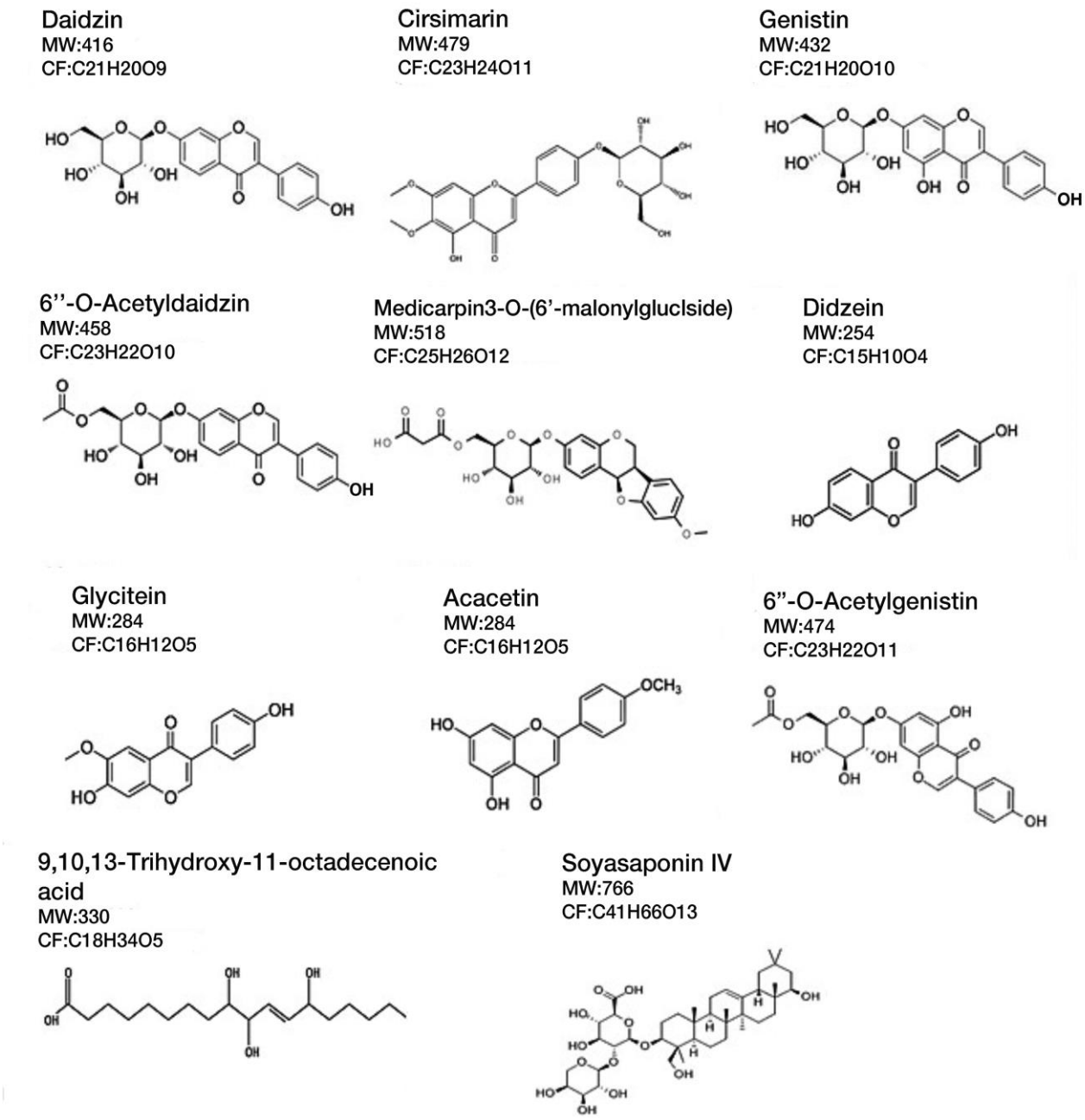

Figure 2 Structural formulae of candidate components detected in a hot-aqueous soybean extract by LC-MS and LC/qTOF-MS. Figure 1 and Table 3 show the candidate components' structural formulae.

The coefficient of determination R^2, which indicates the goodness of fit, was 0.937. This model indicated that the expected value of the viral titer for control samples was 16.718×10^6 FFU/mL. Substituting the 50% value of the control (16.718×10^6) into this equation, the respective half maximal inhibitory concentration (IC_{50}) value was calculated to be 4.05 μg/mL. Similarly, the following equation was obtained by linear regression analysis for the viral titer following treatment with soyasapogenol B:

$$y = 16.152e^{-0.052x}$$

The coefficient of determination was 0.976, and by substituting the 50% control viral titer of 16.152×10^6 into this equation, we obtained an IC_{50} value of 13.33 µg/mL (Figure 3). At all assessed concentrations of acacetin and soyasapogenol B, we detected no evidence of cytotoxicity. However, although qualitative and quantitative analyses of acacetin in the hot-aqueous soybean extract using LC-triple quadrupole MS (QQQ) duly revealed the presence of acacetin in this extract (Figure 4), the content was too low to enable quantification.

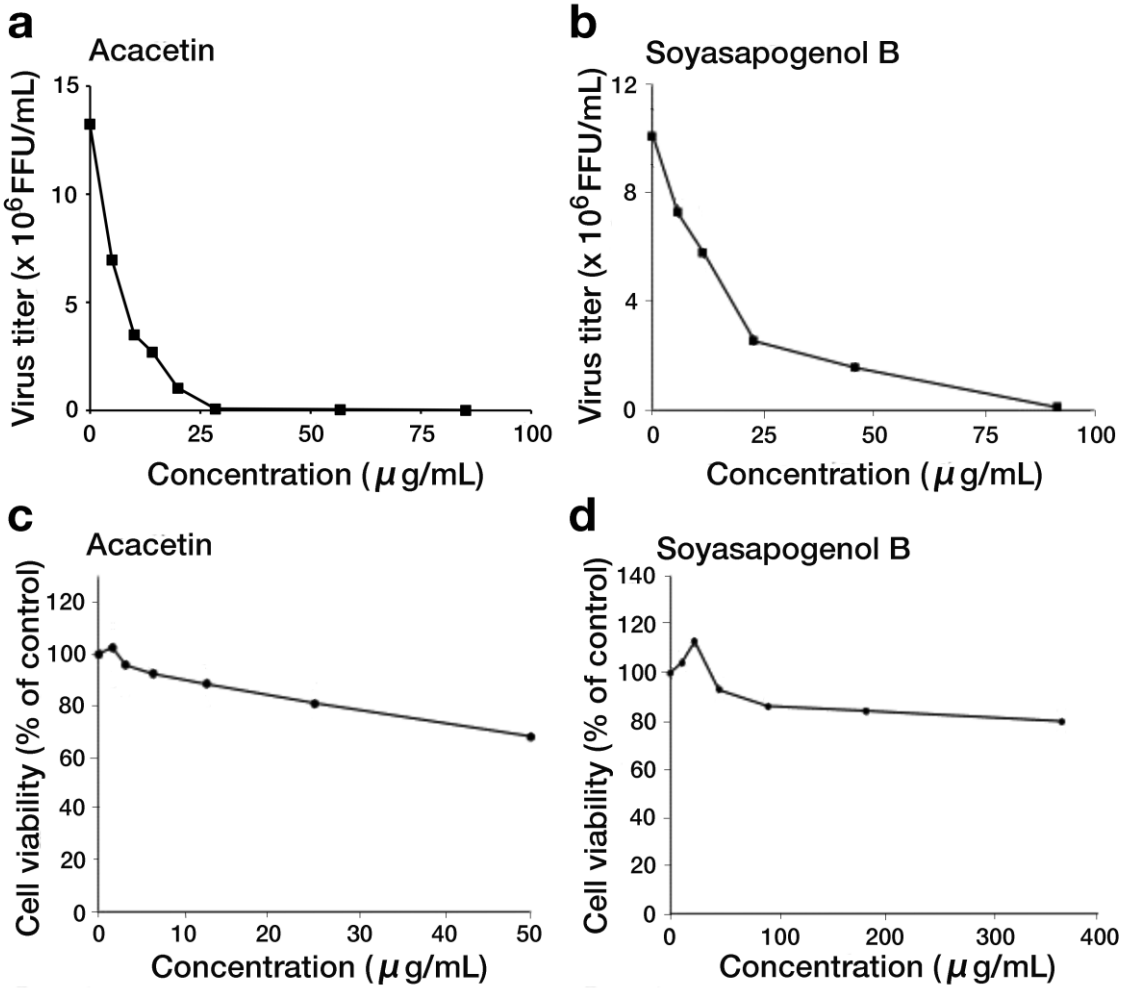

Figure 3 Effects of acacetin and soyasapogenol B on influenza virus proliferation and MDCK cell viability. (a, b) Inhibitory effects of soybean constituents on virus proliferation. MDCK cells were infected with influenza A/PR/8/34 virus at an MOI of 0.001 for 1 h. Following infection, the virus titer in the culture supernatant was determined using a focus-forming reduction assay 24 h after adding the sample-containing medium. (c, d) Cytotoxicity of the constituents of a hot-water extract of soybean. The viability of MDCK cells was determined using an MTT assay 24 h after the addition of acacetin (a and c) or soyasapogenol B (b and d). Data are presented as the means (n = 3).

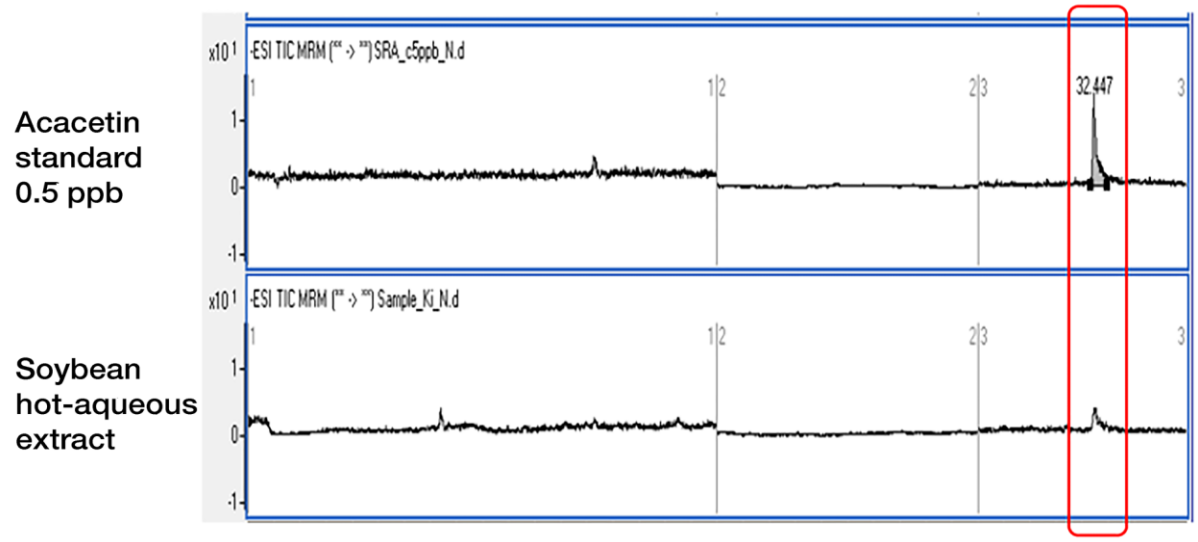

Figure 4 Qualitative measurement of acacetin in a hot-aqueous soybean extract using QQQ. Samples were injected into an LC-MS system comprising an Agilent 1260 Infinity binary LC and an Agilent 6430 triple-quadrupole LC-MS (Agilent Technologies Inc.). The peaks corresponding to acacetin are shown in the red box.

3.3 Analysis of the Antiviral Activities of the Soybean Components Acacetin and Soyasapogenol B

Acacetin and soyasapogenol B were added to infected cells at designated time intervals to determine the growth stage at which IFV inhibition occurred. On the basis of virus titer levels, we found that acacetin inhibited all stages of the entry phase and early and late cultivation stages (Figure 5a), whereas soyasapogenol B had more potent inhibitory effects at the entry and late cultivation stages (Figure 5b). These findings accordingly revealed that acacetin and soyasapogenol B are active components that contribute to inhibiting the entry phase during viral infection.

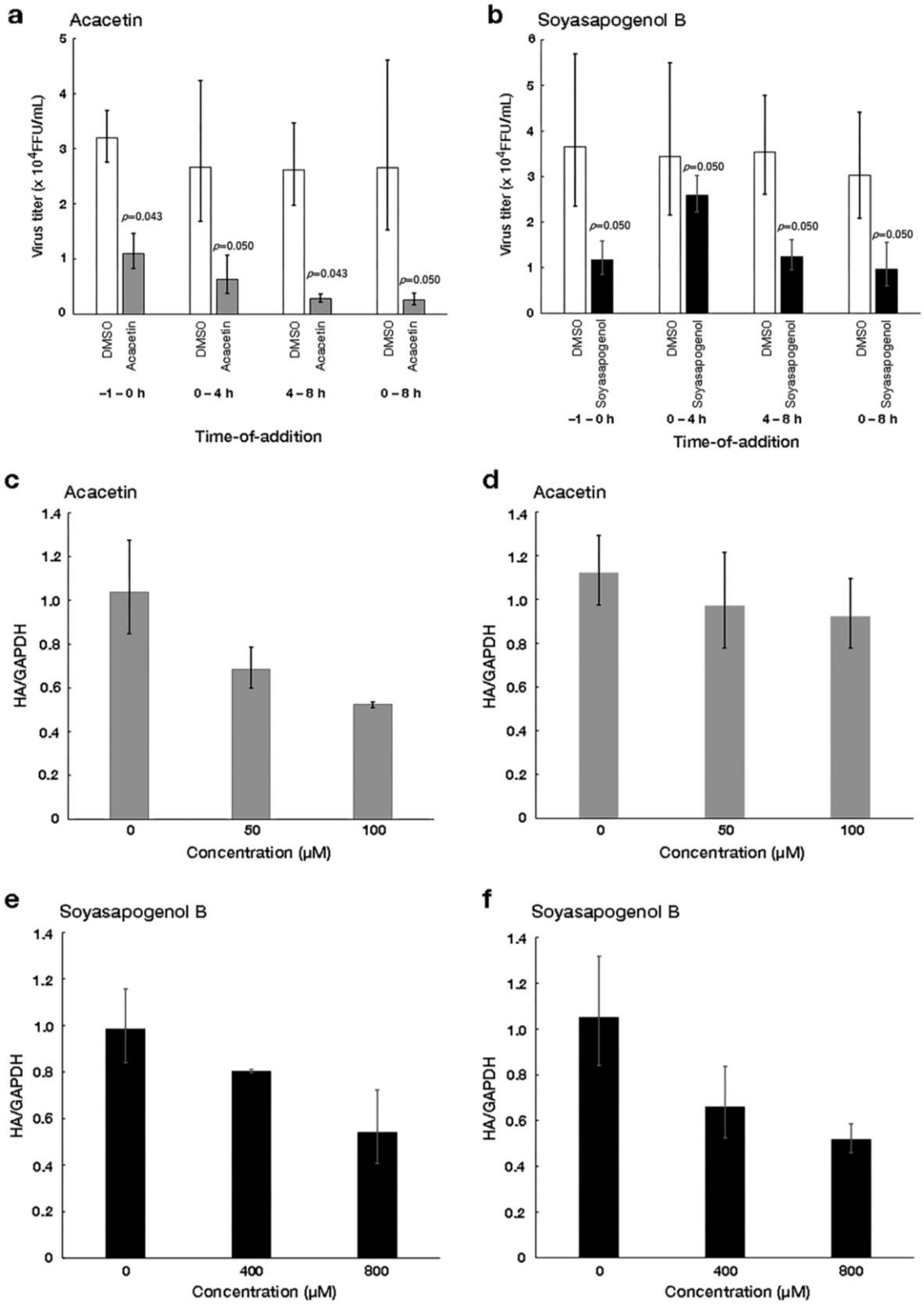

Figure 5 Virus growth inhibition stage and evaluation of the inhibition of virus adsorption by acacetin and soyasapogenol B. (a, b) In the inhibition phase assay, MDCK cells were infected with influenza A/PR/8/34 virus at an MOI of 0.01 for 1 h, and a time-of-addition assay was performed according to the method described by Sakata et al. [14]. Data expressed as the means ± 95% CI are representative of three independent experiments.

The viral titer of the collected supernatant was determined using a focus-forming reduction assay. For adsorption inhibition experiments, influenza A/PR/8/34 virus was added to MDCK cells at an MOI of 10, and following incubation for 1 h at 37°C (c, e) and 4°C (d, f), the amount of viral RNA adsorbed or incorporated into the cells was evaluated by qPCR. Acacetin (a, c, d) and soyasapogenol B (b, e, f). Data are expressed as the mean ± 95% CI (n = 3).

Having established that acacetin and soyasapogenol B inhibit the entry phase of viral infection, we subsequently examined the inhibitory effects of these compounds on viral adsorption to cell membranes. We accordingly observed that at 37°C, acacetin inhibited adhesion to HA cells in a concentration-dependent manner, with a significant difference being detected (Figure 5c). Compared with the mean virus copy number of 1.037 obtained for HA cell adhesion in the control cells (95% CI: 0.846, 1.273), the value for cells treated with 100 µM (28.4 µg/mL) acacetin was 0.525 (95% CI: 0.509, 0.536), the difference between which was significant. In contrast, at 4°C, no significant differences in virus copy number were observed regarding the inhibition of adhesion of acacetin-treated HA cells (Figure 5d). However, in response to treatment with soyasapogenol B, we detected a reduction in virus copy number at both 37 and 4°C (Figure 5e and 5f, respectively). At 37°C, inhibition of HA cell adhesion was found to be significantly concentration-dependent. Compared with the mean copy number of 0.986 (95% CI: 0.840, 1.157) obtained for the control HA cells, the value obtained for cells treated with 800 µM (367.0 µg/mL) soyasapogenol B was significantly lower at 0.543 (95% CI: 0.408, 0.723). Similarly, at 4°C, we found that soyasapogenol B had a significant concentration-dependent inhibitory effect on viral adhesion to HA cells. Compared with the mean value of 1.0352 (95% CI: 0.841, 1.318) obtained for the control HA cells, that received for cells treated with 800 µM soyasapogenol B was significantly lower at 0.519 (95% CI: 0.459, 0.586). These findings thus indicate that soyasapogenol B inhibited the adsorption of viruses to cells and that acacetin inhibited viral uptake in a temperature-dependent (energy-dependent) manner, although similarly to the hot-aqueous soybean extract, did not influence the adsorption of viruses to cells.

4. Discussion

In this study, we established that the soybean constituents acacetin and soyasapogenol B inhibit the entry phase of viral infection, and our findings indicate that acacetin and soyasapogenol B inhibit endocytosis and the binding of viruses to cells, respectively. However, we have yet to ascertain whether acacetin inhibits clathrin-dependent endocytosis, as has been demonstrated for soybean extracts [14]. Nevertheless, these findings provide evidence that acacetin is among the active constituents of a hot-aqueous soybean extract that inhibits the entry phase of infection. Similar to the synergistic enhancement of the anti-IFV effects observed with an extract of *Euglena* and quercetin [17], we speculate that given their overall low content in the extract, different soybean components may have a synergistic inhibitory effect.

Soyasapogenols are a type of saponin with surfactant properties that are used as emulsifiers and expectorants [18]; specific saponins can inhibit elevations in blood glucose levels and have anti-obesity effects [19]. In human breast cancer cells, soyasapogenols have been demonstrated to have estrogenic and antiproliferative properties [20], whereas soyasapogenol A has been observed to target CARF, thereby suppressing tumor growth [21], and ME3738, a derivative of soyasapogenol B, has been reported to enhance the anti-hepatitis C virus activity of interferons [22]. To the best of

our knowledge, however, the present study is the first report on soyasapogenol B as an inhibitor of influenza virus receptor binding.

Acacetin is a natural flavonoid compound that has been reported to have anticancer [23], antioxidant [24], and anti-inflammatory [25] properties, and the findings of recent studies have indicated that acacetin inhibits the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway [26]. Notably, in this regard, it has been established that the intracellular PI3K signaling pathway is activated in response to extracellular stimuli and IFV infection [27, 28], and it has accordingly been shown that inhibition of the PI3K signaling pathway inhibits viral infection [27]. Moreover, this signaling has been found to play regulatory roles in multiple endocytic pathways [29]. These findings suggest that acacetin may inhibit the cellular uptake of influenza virus by blocking the PI3K/Akt signaling pathway, which is involved in endocytosis. Given that an extract of soybean has also been demonstrated to inhibit clathrin-dependent endocytosis [14], it would be desirable to establish whether acacetin inhibits clathrin-dependent endocytosis in influenza virus uptake and to examine further the effects of acacetin on the intracellular signaling pathways and proteins required for the progression of this endocytic process. Further biochemical studies are also required to elucidate the mechanisms underlying the inhibitory activity of acacetin. Moreover, given the low concentrations of acacetin in soybeans, studies should aim to identify compounds that have potential synergistic or additive interactions with acacetin.

5. Conclusions

In this study, we aimed to identify and characterize components of a hot aqueous soybean extract that inhibit the entry phase of viral infection. On the basis of fractionating extract samples and conducting component analysis, we identified a number of putative active constituents with an inhibitory effect on viral cell entry. Among these, we established that acacetin has antiviral effects against IFV, which we assume to be associated with an inhibition of the energy-dependent uptake of the virus into cells.

Abbreviations

BSA	Bovine Serum Albumin
CC_{50}	50% Cytotoxicity Concentration
CF	Composition Formula
95% CI	95% Confidence Intervals
DMEM	Dulbecco's Modified Eagle's Medium
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
FFRA	Focus-Forming Reduction Assay
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HA	Hemagglutinin
IC_{50}	Half Maximal Inhibitory Concentration
IFV	Influenza Virus
LC-MS	Liquid Chromatography-Mass Spectrometry
LC/qTOF-MS	Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
MDCK	Madin-Darby Canine Kidney

MOI	Multiplicity of Infection
MW	Molecular Weight
ODS	Octadecylsilyl
qPCR	Quantitative Polymerase Chain Reaction
SD	Standard Deviation

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Author Contributions

NS: Investigation, Formal analysis and Writing-original draft preparation. YH: Investigation, Methodology and Formal analysis, KW: Formal analysis. RY: Supervision. HS: Methodology and Supervision. YI: Conceptualization, Writing-original draft preparation, Writing-review and editing, and Funding acquisition. All authors have read and approved the submitted version.

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Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

Datasets generated in this study are available from the corresponding author upon request.

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