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Antiviral escin derivatives from the seeds of *Aesculus turbinata* Blume (Japanese horse chestnut)

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**Abstract**

Porcine epidemic diarrhea virus (PEDV) causes severe diarrhea and high fatality of piglets, influencing the swine industry. Japanese horse chestnut (seed of *Aesculus turbinata*) contains many saponin mixtures, called escins, and has been used for a long time as a traditional medicinal plant. Structure-activity relationship (SAR) studies on escins have revealed that acylations at C-21 and C-22 with angeloyl or tigloyl groups were important for their cytotoxic effects. However, the strong cytotoxicity of escins makes them hard to utilize for other diseases and to develop as nutraceuticals. In this research, we investigated whether escin derivatives had no cytotoxicity at 20 μM on VERO cells, while compounds 2, 3, 5, and 6 showed strong cytotoxicity at similar concentrations on PEDV. Our results suggest that escin derivatives showed strong inhibitory activities on PEDV replication with lowered cytotoxicity. These studies propose a method to utilize Japanese horse chestnut for treating PEDV and to increase the diversity of its bioactive compounds.

**Keywords:**

Porcine epidemic diarrhea virus (PEDV), Horse chestnut, *Aesculus turbinata*, Escins

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**Coronaviruses** (CoVs) have been reported as the causes of different diseases at respiratory, enteric or central nervous system in many species including bats, pigs, horses and humans. These viruses are enveloped and single-stranded RNA viruses, containing the largest RNA genomes in mammalian viruses, ranging from 25.5 to nearly 32 kb in length. Until 2003, only two coronaviruses were identified as infecting humans. However, severe acute respiratory syndrome coronavirus (SARS-CoV), was newly identified in 2002 to 2003, causing 10–50% mortality in infected individuals, leading to genetic diversity, unlike other enveloped RNA viruses. PEDV of family *Coronaviridae* shares phyllogenetically common features with other coronaviruses. PEDV causes severe diarrhea, dehydration, vomiting in pigs of all ages, and high mortality of piglets, resulting in tremendous financial loss. Thus, these results imply the necessity of studying the characteristics of coronaviruses and discovering active drugs to prevent the fast and extensive spread of coronaviruses.

*Aesculus* L (Hippocastanaceae) contains 12 species of deciduous trees and has been cultivated as pharmaceutical crops for the production of Standardized Therapeutics Extracts of escins in China. The common name “horse chestnut” came from the uses of seeds for horses to treat overexertion or coughs, and it has been used as therapeutics purposes for anti-fever. Japanese horse chestnut...
(Aesculus turbinata) is a medicinal plant widely distributed in Japan and also has a small amount of cultivation in Korea and China. The seeds, which a large amount of escins were reported as its constituents, have been used for diverse biological activities including anti-inflammatory, anti-obesity, hypoglycemic, and anti-cancer effects.

Escins were also reported to possess strong antiviral effects against SARS-CoV with an EC50 of 6.0 µM (SI value of 2.5) and against anti-HIV-1 protease. However, the industrial utilization of escins for application to diseases and development as nutraceuticals has been limited to date due to their strong nonspecific cytotoxic effects. These reports prompted us towards the development of safer escin derivatives with anti-CoV activities. Previous studies on structure–activity relationship with escins suggested that acylation at C-21 and C-22 was necessary for the cytotoxic effects. The cytotoxicity can be enhanced with methylation at C-24 and a free hydroxyl at C-16 at oleanane-type structure and altered by the site of glycosides. Thus, alkaline and acid hydrolysis of escins was applied to detach acyl moieties at C-21 and C-22, and provide varieties of sugar moieties at C-3.

In this research, we reported ten compounds (1–10), including four new compounds 2, 3, 5, and 6, from the extract of A. turbinata after the two-step hydrolysis. We also measured their antiviral activities using the PEDV assay with isolated compounds and each fraction for safer utilization of Japanese horse chestnut.

The air-dried seeds of A. turbinata were extracted and separated through column chromatography using silica gel, RP-C18 and preparative HPLC to afford ten compounds, including four new (2, 3, 5, and 6) and six known (1, 4 and 7–10).

Compound 2 was obtained as a brownish amorphous powder. The positive HRESIMS showed [[M+H]+]− ion at m/z 649.3946 (calcd for 649.3946) and [M−H]+ ion at m/z 665.3901 (calcd for 665.3906), implying the molecular formula to be C38H58O11. The IR spectrum showed absorption due to hydroxyl

| No. | 2° | 3° | 5° | 6° |
|-----|-----|-----|-----|-----|
| 1   | 39.0 | 14.7 | 0.94 | 39.0 | 14.5 | 0.90 | 38.9 | 14.6 | 0.91 |
| 2   | 26.8 | 19.2 | 1.76 | 26.8 | 22.4 | (d-like; 11.1) | 27.2 | 2.23 | (d-like; 10.2) | 2.03 | (d-like; 11.0) |
| 3   | 89.1 | 3.42 | (dd-like) | 89.2 | 3.41 | (dd-like) | 89.2 | 3.63 | (dd-like; 11.8) |
| 4   | 39.7 |     |     |     |     |     | 44.7 |     |     |
| 5   | 56.0 | 0.80 |     | 55.9 | 0.82 |     | 56.4 | 0.94 | (d; 11.5) |
| 6   | 18.6 | 1.61 | 1.50 | 18.6 | 1.65 | 1.51 | 19.1 | 1.65 | 1.36 |
| 7   | 33.4 | 1.62 | 1.32 | 33.3 | 1.63 | 1.31 (overlap) | 33.7 | 1.59 | 1.31 |
| 8   | 40.2 |     |     | 40.2 |     |     | 40.3 |     |     |
| 9   | 27.2 | 1.77 |     | 47.2 | 1.77 | 47.2 | 1.76 | 47.2 | 1.74 (m) |
| 10  | 37.0 |     |     | 37.0 |     |     | 36.8 |     |     |
| 11  | 24.0 | 1.92 | 1.79 | 24.0 | 1.93 | 1.87 | 24.4 | 1.91 | 1.80 |
| 12  | 123.2 | 5.39 | (br s) | 123.1 | 5.42 | (br s) | 123.2 | 5.38 | (br s) |
| 13  | 144.1 |     |     | 144.1 |     |     | 144.2 |     |     |
| 14  | 42.2 |     |     | 42.2 |     |     | 42.4 |     |     |
| 15  | 34.5 | 2.10 | (d; 12.3) | 1.69 | 34.5 | 2.12 | (d; 11.8) | 1.71 | 2.08 | (d-like; 14.5) | 3.5 | 1.69 | (d; 13.5) |
| 16  | 68.0 | 5.03 | (overlap) | 68.0 | 5.04 | (overlap) | 68.2 | 5.02 | (br s) |
| 17  | 47.5 |     |     | 47.5 |     |     | 47.7 |     |     |
| 18  | 41.3 | 2.80 | (dd-like; 13.0) | 41.3 | 2.82 | (dd-like) | 41.3 | 2.80 | (dd; 14.0) |
| 19  | 48.4 | 3.06 | (t; 13.4) | 1.43 | 48.4 | 3.08 | (t; 13.5) | 48.5 | 3.02 | (t; 13.2) |
| 20  | 36.6 |     |     | 36.6 |     |     | 36.7 |     |     |
| 21  | 78.9 | 4.80 | (d; 8.6) | 78.8 | 4.82 | (d; 9.5) | 79.0 | 4.80 | (d; 9.6) |
| 22  | 77.5 | 4.63 | (d-like; overlap) | 77.4 | 4.65 | (d; 9.5) | 77.5 | 4.64 | (d; 9.5) |
| 23  | 28.3 | 1.30 |     | 28.3 | 1.31 |     | 28.6 | 1.31 |     |
| 24  | 17.2 | 1.00 |     | 17.1 | 1.02 |     | 63.6 | 4.40 | (d; 11.2) |
| 25  | 16.0 | 0.85 |     | 15.9 | 0.87 |     | 15.8 | 0.80 |     |
| 26  | 17.1 | 0.90 |     | 17.1 | 0.92 |     | 17.1 | 0.88 |     |
| 27  | 27.6 | 1.88 |     | 27.6 | 1.90 |     | 27.7 | 1.88 |     |
| 28  | 68.6 | 4.02 | (d; 10.3) | 3.72 | 68.5 | 4.04 | (d; 10.6) | 3.74 | 68.5 | 4.00 | (d; 10.5) |
| 29  | 30.8 | 1.32 |     | 30.7 | 1.35 |     | 30.9 | 1.33 |     |
| 30  | 19.6 | 1.38 |     | 19.6 | 1.43 |     | 19.8 | 1.40 |     |

| GcA | 1°  | 107.4 | 5.03 | (overlap) | 107.0 | 5.03 | (overlap) | 106.7 | 5.18 | (d; 7.4) |
| 2°  | 75.7 | 4.13 | (t-like) | 75.1 | 4.15 | (t-like) | 75.7 | 4.12 | (t; 8.2) |
| 3°  | 78.4 | 4.32 | (t-like) | 76.1 | 4.37 | (t; 7.5) | 78.4 | 4.35 | (t; 8.8) |
| 4°  | 73.7 | 4.58 | (overlap) | 82.9 | 4.61 | (overlap) | 73.9 | 4.60 | (t-like) |
| 5°  | 77.9 | 4.67 | (overlap) | 76.6 | 4.75 | (d; 7.8) | 78.3 | 4.73 | (t-like) |
| 6°  | n.d. |     |     | n.d |     |     | n.d |     |     |

| Gc  | 1°  | 105.0 | 5.25 | (d; 6.4) |     | n.d |     |     |     |
| 2°  | 75.0 | 4.09 | (t; 8.0) |     | n.d |     |     |     |
| 3°  | 78.2 | 4.23 | (overlap) |     | n.d |     |     |     |
| 4°  | 71.7 | 4.15 | (overlap) |     | n.d |     |     |     |
| 5°  | 78.6 | 4.02 | (overlap) |     | n.d |     |     |     |
| 6°  | 62.7 | 4.53 | (d; 11.0) | 4.27 | (overlap) |     |     |     |

a Recorded in 1H (500 MHz) and 13C NMR (125 MHz).

b Recorded in 1H NMR (800 MHz) and 13C NMR (200 MHz).
The linkage position of the olean-12-ene triterpenoid glucopyranosiduronic acid was established by analyzing its 1H and 13C NMR data of 2 (Table 1). The HMBC correlation between H-3 (δH 1.40, δC 89.1) and C-12 (δC 123.2) and 144.2 (δC 139.2) confirmed the position of olean-12-ene triterpenoid glucopyranosiduronic acid. The linkage position of this β-D-glucopyranosiduronic acid moiety was identified by HMBC experiment from the correlation from H-1 (δH 5.03) to C-3 (δC 89.0). The linkage position of this β-D-glucopyranosiduronic acid was confirmed by the HMBC experiment from the correlation from H-1 (δH 5.03) to C-3 (δC 89.1) (Fig. 2A). Therefore, the structure of 2 was elucidated as (3β,16α,21β,22α)-16,21,22,28-tetrahydroxyolean-12-en-3-yl-β-D-glucopyranosiduronic acid, as an olean-12-ene triterpenoid glucopyranosiduronic acid, disclosing the presence of six tertiary methyl groups [δH 0.87 (CH3-25), 0.92 (CH3-26), 1.02 (CH3-24), 1.31 (CH3-23), 1.35 (CH3-29), 1.43 (CH3-30) and 1.90 (CH3-27)], an olefinic proton at δH 0.80 (br s) and two anomeric protons at δH 5.25 (1H, d, J = 6.4 Hz, H-1′) and 5.03 (1H, d, like, overlap, H-1′). The 13C NMR spectrum of 3 was very similar to that of 2, apart from the presence of one β-D-glucopyranosyl moiety in 3 (Table 1). The linkage position of the β-D-glucopyranosyl moiety was identified by HMBC experiment. The HMBC correlations between H-3 (δH 3.41) and C-1′ (δC 107.0) and also H-4′ (δH 4.61) and C-1′ (δC 105.0) were observed, indicating that β-D-glucopyranosiduronic acid was attached to the carbon C-3 as in 2, and the other β-D-glucopyranosyl was linked at the C-4′ of β-D-glucopyranosiduronic acid (Fig. 2A). Thus, the structure of 3 was established as (3β,16α,21β,22α)-16,21,22,28-tetrahydroxyolean-12-en-3-yl-O-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranosiduronic acid).

Compound 5 was obtained as a brown amorphous powder. HRESIMS of positive mode showed a [(M+H)+] ion at m/z 665.3898 (calcd for 665.3895) and [M−H]− ion at m/z 681.3852 (calcd for 681.3856), which indicated a molecular formula of C36H58O12. Absorption bands at 3385, 1657, and 1028 cm−1 in the IR spectrum demonstrated the presence of hydroxyl, olefinic bond, and glycosidic linkage, respectively. The 1H and 13C NMR data of 5 indicated the structure as an olean-12-ene triterpenoid glucopyranosiduronic acid, disclosing the presence of six tertiary methyl groups [δH 0.80 (CH3-25), 0.88 (CH3-26), 1.33 (CH3-29), 1.40 (CH3-30), 1.53 (CH3-23), 1.88 (CH3-27)], as well as an olefinic proton at δH 5.38 (br s, H-12) with two olefinic carbon signals at δC 123.2 (C-12) and 144.2 (C-13), and one β-D-glucopyranosiduronic acid moiety [one anomeric proton δH 5.18 (d, J = 7.2 Hz, H-1′), along with six carbon signals at δC 137.3 (C-6′), 106.7 (C-1′), 78.4 (C-3′), 78.3 (C-5′), 75.7 (C-2′), and 73.9 (C-4′)]. Compound 5 was similar to 2 except for the presence of a hydroxyl group at C-24 (δC 63.6) (Table 1). The HMBC correlation between H-3 (δH 3.63) and C-1′ (δC 106.7) confirmed the position of β-D-glucopyranosiduronic acid. The relative configuration of 5 was investigated by analysis of its ROESY spectrum (Fig. 2B).

Correlations between H2-24 (δH 4.40)/H3-25 (δH 0.80), H3-25 (δH 0.80)/H2-26 (δH 0.88), H2-26 (δH 0.88)/H2-28 (δH 4.00), H2-28 (δH 4.00)/H1-16 (δH 5.02), H-16 (δH 5.02)/H-22 (δH 4.64), H-22 (δH 4.64)/H2-30 (δH 1.40), H-30 (δH 1.40)/H-18 (δH 2.80), and H-18 (δH 2.80)/H2-22 (δH 4.64) were observed in the ROESY data, implying that all these protons were on the same side of the molecule. The relative configuration of compound 5 remained unaltered even...
after the two-step reaction except for the deacylation and the cleavage of the glucose linkage. Therefore, the structure of 5 was identified as \((3\beta,16\alpha,21\beta,22\alpha)-16,21,22,24,28\)-pentahydroxyolean-12-en-3-O-\(\beta\)-D-glucopyranosiduronic acid.\(^{23}\)

Compound 6 was isolated as a brown amorphous powder, with the molecular formula \(C_{42}H_{68}O_{17}\) by the HRESIMS at \(m/z\) 867.4345 [M+Na\(^+\)] (calcd for \(C_{42}H_{68}O_{17}Na^+, 867.4349\) and at \(m/z\) 843.4384 [M–H] (calcd for \(C_{42}H_{67}O_{17}, 843.4384\)). Its IR spectrum displayed strong absorptions for hydroxyl \((3405 \text{ cm}^{-1}/C_{0})\), olefinic bond \((1604 \text{ cm}^{-1}),\) and glycosidic linkage \((1033 \text{ cm}^{-1})\). The \(^1H\) and \(^{13}C\) NMR data of 6 suggested the structure as an olean-12-ene–D-glucopyranosyl (1\(\alpha\))–D-glycopyranosyl-(1\(\alpha\))–D-glucopyranosiduronic acid. \(^{23}\)

By analysing the ROEY data of compound 5, we also confirmed that the overall skeleton and relative configurations of new compounds 2, 3, 5, and 6 were identical with the escin series, after a two-step hydrolysis. Six known compounds 1, 4, and 7–10 were determined as protoaescigenin \((1), \^{24}\) escinidin \((4), \^{23}\) aesculaside B \((7),\) escin Ia \((8),\) escin Ib \((9), \^{13}\) and isoescin Ia \((10), \^{20}\) by comparison with literature data.

The cytotoxicity assay \(^{27}\) was done at a concentration of 10 \(\mu g/\) mL to compare the cytotoxic effects of the total extract and partitioned fractions before and after a two-step hydrolysis (Fig. 3A). The \(n\)-BuOH fraction, containing a large amount of escins, showed strong cytotoxicity compared to fractions obtained after the two-step hydrolysis. Interestingly, compounds 1–7 isolated from the fraction with the two-step hydrolysis were evaluated to have much lower cytotoxic effects than compounds 8–10 from the \(n\)-BuOH part at concentration of 20 \(\mu M\) (Fig. S22). Additionally, dose-dependent cytotoxic effects of compounds 8–10 were ascertained at concentrations of 2, 5 and 10 \(\mu M\) (Fig. S23).

The \(n\)-BuOH and the other fractions from a two-step hydrolysis were evaluated for their PEDV inhibitory activities with 6-azauridine as positive control at 1, 2, 5, and 10 \(\mu g/\) mL (Fig. 3B). \(^{28}\) Up to 2 \(\mu g/\) mL, both fractions showed similar and mild inhibitory effects on PEDV replication, proving the original horse chestnut’s antiviral activities. The fraction after a two-step hydrolysis inhibited PEDV replication in a dose-dependent manner without cytotoxicity. The \(n\)-BuOH fractions above 5 \(\mu g/\) mL, which are expected to contain many escins, exhibited poor cell viability because of strong cytotoxic effects, even if it could show better PEDV inhibitory effects than the fraction from a two-step hydrolysis. Based on these data, the ten purified oleanane triterpenoids (1–10) were evaluated for their PEDV inhibitory effects with the same methods (Fig. S24). As compounds 8–10 shown...
strong cytotoxic effects on Vero cells at 20 μM, their PEDV inhibitory activities were evaluated at a concentration of 2 μM. Compounds 1–7 were tested at a concentration of 20 μM to compare their inhibitory effects on PEDV replication, providing less cytotoxicity in relatively high concentrations. Compound 4 showed the strongest inhibitory activity among the ten compounds 1–10.

Additionally, Compounds 4–6 exhibited concentration-dependent inhibition of PEDV replication at concentrations of 10, 20 and 40 μM, indicating improved cell viability from the two-step hydrolysis (Fig. S25). Based on cytotoxicity and CPE assays, structure-activity relationships (SARs) were studied. Isolated compounds 1–10 after the two-step hydrolysis suggested the presence of three important groups: (1) acylation at C-21, C-22 or C-28 (1–7 and 8–10), (2) methylation at C-24 (1–3 and 4–6), (3) existence of glycosidic linkages [(1–3) and (4 and 5–7)]. Group 1 (1–7 and 8–10) indicated that deacylation at C-21 could improve the cell viability (Figs. 3A and S22). The PEDV inhibitory effects of group 2 (1–3 and 4–6) demonstrated that methylation at C-24 could reduce antiviral activity. Group 3 [(1–3) and (4 and 5–7)] showed that the absence of glycosidic linkage also improved the antiviral effects (Fig. S24).

During the PEDV replication, two key structural proteins, spike and nucleocapsid proteins, take part in important roles.29 The spike protein regulates the entry stage of the virus30 and binding of nucleocapsid protein to viral RNA is crucial for viral transcription.31 Following the data of the cytotoxicity and CPE assays (Figs. S22–24), the five compounds 1 and 4–7 were selected for further evaluation. The inhibitory effects of compounds 1 and 4–7 on nucleocapsid protein synthesis at 20 μM were measured using Western blot (Fig. 4A).32 The five compounds showed moderate inhibitory effects on nucleocapsid protein synthesis, and compound 4 significantly inhibited nucleocapsid protein synthesis. Thus, compound 4 was further analyzed for its effects in nucleocapsid and spike protein synthesis with Western blot at concentrations of 10, 20 and 40 μM, and it was found to inhibit PEDV replication in a concentration-dependent manner (Fig. 4B).

On the basis of the above findings, compounds 4 and 6 were also measured with key genes and proteins crucial for PEDV replication by real time qPCR (qPCR).33 To measure the expression level of viral RNA encoding nucleocapsid and spike proteins, compounds 4 and 6 were treated in Vero cells at a concentration of 40 μM and total RNA was extracted for reverse transcription followed by polymerase chain reaction using the primers for PEDV (S-Table 1). Fig. 5A shows the RNA expression levels of two kinds of proteins with compounds 4, 6 and positive control. When the inhibitory effect of compound 4 was analyzed in detail at the concentrations

![Fig. 3.](A) Cytotoxicity assay of fractions at 10 μg/mL. The reaction fraction had no cytotoxicity at 10 μg/mL and the n-BuOH fraction showed significant cytotoxicity. (B) CPE inhibition assay of the n-BuOH fraction and the reaction fraction at concentrations of 1, 2, 5, and 10 μg/mL. Up to 2 μg/mL, the n-BuOH fraction and the reaction fraction from a two-step hydrolysis showed similar activities, but at high concentrations, the n-BuOH fraction showed cytotoxic effects and the reaction fraction had PEDV inhibitory effects in dose-dependent manner.

![Fig. 4.](A) Inhibitory effects of compounds 1 and 4–7 on PEDV nucleocapsid synthesis, using Western blot assay. Compounds 1 and 4–7 inhibited PEDV nucleocapsid synthesis at a concentration of 2 μM. (B) Inhibitory effects of compound 4 on PEDV nucleocapsid and spike protein synthesis, using Western blot analysis.
of 10, 20 and 40 μM, compound 4 inhibited the RNA expression of nucleocapsid and spike proteins in a dose-dependent manner (Fig. 5B). On the basis of inhibition of PEDV RNA expression, compound 4 was further studied for its inhibitory effects on PEDV replication, by performing an immunocytochemistry assay (Fig. 5C).34 We observed green fluorescence in virus-infected cells but no signals in mock-treated cells. This result revealed that compound 4 had noticeable inhibitory effects on PEDV replication in a dose-dependent manner at concentrations of 10, 20 and 40 μM.

3C-Chymotrypsin-Like protease (3CL protease) is vital for proteolytic processing of viral replication in coronaviruses. As escin was reported as a SARS-CoV 3CL protease inhibitor,15 we performed docking modelling of compound 4 into the active site of SARS-CoV 3CL\textsuperscript{pro} (PDB ID code 3V3M).35 The binding site was predicted by the 2D program of DS 4.0. As shown in Fig. 5D, the hydroxyl group of C22 and C16 of 4 formed hydrogen bonds with the oxygen atom of the carbonyl group of Glu166. Additionally, the methyl group of C23 and the B ring of 4 showed hydrophobic interactions with Cys145 and Leu27 through their side chains. The CDOCKER interaction energy was calculated to be -38.63 kcal/mol. The 3CL\textsuperscript{pro} binding energy value of compound 4 was unstable and weaker than that of the reference ligand OEM. However, clear key amino acid interactions of compound 4 with 3CL\textsuperscript{pro}, proposed the mode of action as inhibition of 3CL protease and explained inhibitory possibility of the SARS-CoV of escin derivatives.

This research demonstrated that including the four new compounds (2, 3, 5, and 6), ten oleanane-type triterpenoids (1–10) were isolated from the seeds of Aesculus turbinata (Japanese horse chestnut). The cytotoxicity of the n-BuOH fraction was decreased with compounds 1–7 isolated from two-step hydrolysis. Especially, two compounds 4 and 6 showed strong inhibitory activities against PEDV in a dose-dependent manner. The present study proposed a way to utilize Japanese horse chestnut for treating PEDV with lowered cytotoxic effects and to increase the diversity of bioactive compounds.

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Supplementary data
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31. Whole-cell lysates were prepared by scraping adherent-cultured cells with 100 μl of lysis buffer (0.5% NP-40, 50 mM NaF, 1 mM EDTA, 120 mM NaCl, and 50 mM Tris-HCl (pH 7.6)) and centrifuged at 12,000 rpm for 20 min. Protein concentrations of the supernatant were calculated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of lysates were separated by 10–12% SDS-PAGE and electrophoretically transferred to PVDF membranes (PVDV 0.45 μm. Immobilon-P, USA). Membranes were incubated overnight with antibodies against spike (S) protein, nucleocapsid (N) (AbFrontier Co., Ltd, Seoul, Korea) or mouse monoclonal actin, and further incubated with secondary antibodies. Protein bands were detected using an enhanced chemiluminescence Western Blotting Detection System (ECL, Amersham Biosciences). Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Vero cells were seeded at 90% confluence in 6-well plates, infected with PEDV (0.01 MOI), and incubated for 2 h. The medium was removed and washed with phosphate-buffered saline (PBS). PEDV (0.01 MOI) was inoculated onto confluent monolayers of Vero cells for 2 h. The media were replaced by DMEM with different concentrations of compounds. After 24 h of incubation, the cells were detached with 0.05% trypsin-EDTA and washed twice with PBS (pH 7.4). Then, the cells were resuspended in DMEM, treated with compounds for 24 h, washed in PBS (pH 7.4) and fixed in 4% paraformaldehyde solution for 30 min at room temperature. The slides were blocked with 1% BSA for 1 h, incubated with monoclonal antibody against N protein of PEDV (AbFrontier Co., Ltd, Seoul, Korea) diluted 1:50 with PBS (pH 7.4) for 1 h. The slides were washed 3 times with PBS, incubated with FITC-conjugated goat anti-Rabbit IgG antibody (Abcam, Cambridge, UK) for 1 h. After washing three times with PBS, the slides were stained with 500 nM DAPI solution for 10 min at room temperature and washed with PBS (pH 8.0) three times. Mounting reagent (Vecashield, Vector Laboratories Inc., Burlingame, CA, USA) was used. The slides were observed under a fluorescence microscope (Olympus ix70 Fluorescence Microscope, Olympus Corporation, Tokyo, Japan).