Jubanines F–J, cyclopeptide alkaloids from the roots of Ziziphus jujuba

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ABSTRACT
Five Ib-type cyclopeptide alkaloids, jubanines F–J (1–5), and three known compounds, nummularine B (6), daechuine-S3 (7), and mucronine K (8) were isolated from the roots of Ziziphus jujuba. Their structures were fully characterized by spectroscopic analyses in combination with chemical derivatization. Compounds 1–3, and 6 were evaluated for their antiviral activity against the porcine epidemic diarrhea virus (PEDV). Compounds 2, 3, and 6 showed potent inhibitory effects on PEDV replication.

1. Introduction
Cyclopeptide alkaloids are one of the chemotaxonomic specific constituents in Rhamnaceae plants, especially for Ziziphus species. They usually contain a p- or m-ansa 13-, 14-, or 15-membered ring structure that consists of a styrylamine moiety and two or three α-amino acid residues (Panseeta et al., 2011). In some cases, they possess one or two N-methyl or N,N-dimethyl amino acids. Cyclopeptide alkaloids of Rhamnaceae plants are divided into three types: Ia, Ib, or Ic, according to ring structure size. The Ib-type alkaloids have 13-membered macrocyclic structures composed of a monoxytrylamino moiety, a ring bond amino acid, and a β-oxypyrrolino moiety as an intermediate amino acid. Sedative, antibacterial, antifungal, antiplasmodial, antitumoral, and antimalarial effects have been reported for some cyclopeptide alkaloids (Gournelis et al., 1998; Panseeta et al., 2011; Tan and Zhou, 2006).

Ziziphus jujuba Mill. is a common species in the genus Ziziphus and is widely cultivated in Southern Europe and Asia, including Russia, India, the Middle East, and China (Outlaw et al., 2002). Although the fruit is the most widely used part of the plant for food, cyclopeptide alkaloids have been separated from other plant parts, especially from the stem bark. Four Ia-type (frangufoline, scutianine D, jubanine C, and mauritine A) and eight Ib-type cyclopeptide alkaloids (amphibine H, jubanine A, jubanine B, jubanine E, mucronine D, nummularine A, nummularine B, and zizycyclopeptide alkaloids (amphibine H, jubanine A, jubanine B, nummularine A, and nummularine B) and eight Ib-type parts, especially from the stem bark. Four Ia-type (frangufoline, food, cyclopeptide alkaloids have been isolated and reported from the Z. jujuba stem bark (Devi et al., 1987; Pandey et al., 2008; Tripathi et al., 2001; Tschesche et al., 1976). However, the chemical constituents of Z. jujuba roots have been rarely reported. To the best of our knowledge, only one publication has reported cyclopeptide alkaloids from the Z. jujuba root. These alkaloids are mauritine A and seven Ib-type cyclopeptide alkaloids: mucronine D, amphibine H, jubanine A, jubanine B, jubanine D, nummularine A, and nummularine B (Khokhar et al., 1994).

In this paper, the isolation and structural elucidation are described of five new Ib-type cyclopeptide alkaloids (1–5) with three known cyclopeptide alkaloids (6–8) (Fig. 1). Antiviral evaluations against PEDV are also reported.

2. Results and discussion
Air-dried pulverized roots of Z. jujuba were macerated with MeOH before preparing the alkaloid fraction with an acid–base method from the MeOH extract. This alkaloid fraction was subjected to silica gel column chromatography eluted with increasing polar mixtures of CHCl3/MeOH. Further purification was performed using Sephadex LH-20 and preparative HPLC. As a result, eight cyclopeptide alkaloids (1–8) were isolated and chemically characterized. The UV spectra of 1–8 showed absorption bands at around 270 and 320 nm, which were caused by the characteristic styrylamine chromophore (Panseeta et al., 2011). All eight compounds, therefore, could be 13-membered cyclopeptide
alkaloids. This is because these absorption bands are not observed with 14-membered rings due to the strain of the ring system, except when there is a tryptophan moiety (Gournelis et al., 1998). Three aromatic protons of the cyclopeptides exhibited an ABX spin system, e.g. \( \delta_H \) 6.70 (1H, d., \( J = 3.2 \) Hz, H-12), \( \delta_H \) 6.87 (1H, d., \( J = 9.2 \) Hz, H-15), and \( \delta_H \) 6.80 (1H, dd, \( J = 3.2, 9.2 \) Hz, H-16) for 1 (Table 2). This suggests that compounds 1–8 had a \( \beta \)-oxystyrylamino moiety, which then suggests that they are 13-membered cyclopeptide alkaloids (Lin et al., 2000). In addition, based on \( ^1H-^1H \) COSY and \( ^1H-^13C \) HMBC spectroscopic data analyses and comparison with previously reported chemical shift analogs, the presence of a \( \beta \)-oxystyrylamino moiety is possible for compounds 1–8 (Tan and Zhou, 2006). Thus, compounds 1–8 were confirmed as lb-type cyclopeptide alkaloids. The olefinic protons (H-1 and H-2) of 1–8 showed coupling constants (\( J \)) near 9.0 Hz, and a double bond geometry in styrylamino moieties was indicated to be in Z configurations.

Compound 1 was obtained as a white amorphous powder with molecular formula \( C_{29}H_{43}N_{5}O_{6} \) as indicated by ESI-qTOF-MS. From the analysis of \( ^1H, ^13C, ^1H-^1H \) COSY, and \( ^1H-^13C \) HMBC NMR spectroscopic data (Fig. 2), two valines and one terminal N-methylalano amino moiety were suggested in 1 (Table 1). The HMBC correlations between H-3 to C-4, H-6 to C-7, and H-9 to C-11 indicated linkages between a \( m \)-oxystyrylamino moiety and a valine, the valine and a \( \beta \)-oxoproline, and the \( \beta \)-oxoproline and the \( m \)-oxystyrylamino moiety, respectively. A NOE relationship between H-18 and H-21 was observed, which agreed with the \( \beta \)-oxoproline connection with another valine moiety. Thus, structure 1 was established as shown in Fig. 1. The CD spectrum of 1 exhibited two negative Cotton effects around 265 and 321 nm, suggesting 5,5'-S-configuration. This supported the 5,5'-S-configuration. A weak vicinal coupling between H-8 and H-9 (\( J = 3.2 \) Hz) suggests a trans orientation (Suksamarn et al., 2005). To confirm the configuration, acid hydrolysis of 1 was performed. The hydrolysate of 1 was treated with the \( \alpha \)-FDL (N\(^2\)-(5-fluoro-2,4-dinitrophenyl)-\( \alpha \)-leucinamide) and \( \beta \)-FDL (N\(^2\)-(5-fluoro-2,4-dinitrophenyl)-\( \beta \)-leucinamide) and the reaction products were analyzed by LC–MS (Fujii et al., 1997). \( \alpha \)-FDL derivatives are retained longer than the \( \beta \)-FDL derivatives on C-18 reverse phased HPLC, exhibiting amino acid residues of 1 as \( \alpha \)-Val and \( \alpha \)-N-Me-Ala, respectively. As a result, the configuration of compound 1 is shown in Fig. 1. Compound 1 was named jubanine F after its plant origin.

The molecular formula of compound 2 was determined to be \( C_{29}H_{45}N_{5}O_{6} \) by ESI-qTOF-MS. Compared to the \( ^1H \) and \( ^13C \) NMR spectra of 1, 2 differed in only one amino acid residue. Instead of a valine, two methyl (\( \delta_C \) 10.8 and 15.1), one methylene (\( \delta_C \) 24.5), and one methine (\( \delta_C \) 37.4) signals were observed. The \( ^1H-^1H \) COSY analysis indicated that these comprised an isoleucine residue.

### Table 1

\[
\begin{array}{cccccc}
1 & 2 & 3 & 4 & 5 \\
1 & 106.8 & 107.0 & 106.7 & 107.9 & 107.8 \\
2 & 121.5 & 121.5 & 121.5 & 122.0 & 116.9 \\
4 & 167.1 & 167.4 & 167.1 & 168.5 & 169.8 \\
5 & 60.7 & 60.7 & 60.3 & 53.8 & 53.4 \\
7 & 170.4 & 170.4 & 170.4 & 170.4 & 172.2 \\
8 & 64.5 & 64.7 & 64.5 & 64.8 & 64.8 \\
9 & 77.2 & 77.2 & 77.3 & 77.8 & 77.2 \\
11 & 151.1 & 150.9 & 151.1 & 150.6 & 150.5 \\
12 & 111.3 & 111.2 & 111.2 & 111.9 & 111.3 \\
13 & 124.3 & 124.2 & 124.3 & 124.1 & 124.1 \\
14 & 151.5 & 151.0 & 151.5 & 150.9 & 150.9 \\
15 & 113.8 & 113.7 & 113.8 & 113.6 & 113.7 \\
16 & 117.8 & 117.6 & 117.8 & 116.9 & 116.9 \\
17 & 32.6 & 32.6 & 32.6 & 32.6 & 32.3 \\
18 & 46.7 & 46.8 & 46.8 & 46.2 & 46.1 \\
20 & 171.6 & 171.5 & 171.7 & 168.5 & 170.3 \\
21 & 54.6 & 54.5 & 53.8 & 60.4 & 59.0 \\
23 & 175.0 & 172.6 & 175.0 & 169.8 & 172.5 \\
\end{array}
\]

| R1 (ringbound a.a.) | Val | Val | ile | ile | ile |
|---------------------|-----|-----|-----|-----|-----|
| 1'                  | 28.3 | 28.6 | 35.2 | 35.6 | 36.5 |
| 2'                  | 19.7 | 19.8 | 24.5 | 24.3 | 24.1 |
| 3'                  | 17.3 | 17.4 | 11.7 | 11.1 | 10.7 |
| 4'                  | 16.2 | 15.6 | 14.8 |       |       |

- Recorded at 150 MHz.
- Recorded at 125 MHz.
- Recorded at 100 MHz.
- Recorded in CDCl_3.
- Recorded in DMSO-d_6.

Fig. 1. Chemical structures of compounds 1–8.

Fig. 2. Selected COSY, HMBC, and NOESY interactions for compound 1.
The HMBC cross-peaks of H-3 to C-4, H-6 to C-7, and H-22 to C-23 indicated connections between a m-oxystyrilamino moiety with a valine, the valine with a β-oxoproline, and an isoleucine with a N-methylalanine, respectively. The absolute configurations of C-5, C-8, C-9, C-21, and C-1" were all determined by chromatographic analysis on GITC (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate) derivatives (Hess et al., 2004). The GITC derivatives of l-ile and l-β-oxyproline were respectively confirmed through HMBC interactions and a valine with a N,N-dimethylalanine moiety was inferred from COSY and HMBC correlations. The absolute configurations of the two isoleucine residues and a N,N-dimethylalanine residue were confirmed by COSY and HMBC correlations. Two isoleucine residues and their spin systems with styrylamine, β-oxoproline, and terminal N,N-dimethylalanine moieties were inferred from COSY and HMBC correlations. The absolute configuration of the two isoleucine residues and a N,N-dimethylalanine residue in compound 5 were all determined by the chromatographic analysis on GITC and PGME derivatives. The absolute configurations of C-5, C-8, C-9, C-21, C-1', and C-1" of 5 were all identified as S. Compound 5 was named jubanin J.

After comparing NMR spectra with previously reports, known compounds 6 and 7 were identified as nummularine B (Panseeta et al., 2011) and daechuine-S3 (Lee et al., 2001), respectively. Compound 8 was identified as a compound previously isolated and reported, but not given a trivial name (Barboni et al., 1994). For
Marfrey’s reagent (o-FDLA and i-FDLA) and GITC were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The (S)-(+)-Phenylglycine methyl ester hydrochloride [(S)-(+)PGME] reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). The absolute configuration of amino acids was analyzed using Agilent 6120 quadrupole MSD consisting of the 1260 Infinity pump, 1260 Infinity autosampler, 1260 Infinity DAD detector (Agilent Technologies, Santa Clara, CA, USA), a Phenomenex column (Luna 5 µi C18 (2) 100 Å New Column, 4.6 mm × 100 mm, i.d.; 5 µm, Sungmoon Systech Co. Ltd., Seoul, Korea), and an Openlab ChemStation (Agilent) for data acquisition and processing.

4.2. Plant materials

*Z. jujuba* roots were collected in Jinju, Korea, in 2012. The plant was identified by Prof. Eun Ju Jeong (Gyeongnam National University of Science and Technology, Jinju, Republic of Korea). A voucher specimen (SUPH-1204-01) is deposited in the Herbarium in the Medicinal Plant Garden, College of Pharmacy at Seoul National University in Korea.

4.3. Extraction and isolation

Powdered dried roots of *Z. jujuba* (14.5 kg) were extracted through maceration with MeOH (2 × 60 L, for one week each) at rt. A crude extract (0.5 kg) resulted from extraction solvent removal. The extract was suspended in H2O and acidified with 1 N HCl to pH 3–4. The acidic solution was firstly extracted with EtOAc to yield the EtOAc fraction (186.2 g). The aqueous residue was basified with NaOH to pH 9 and extracted with CHCl3 to provide the alkaloid fraction (1.7 g), with the latter subjected to silica CC eluted with increasingly polar CHCl3–MeOH combinations (30:1, 10:1, 5:1, 3:1 and 1:1) to yield four subfractions, A1–4. Subfraction A3 (348.5 mg) was separated by Sephadex LH-20 CC eluted with CH2Cl2–MeOH (3:1) to give five subfractions, A3a–A3e. A3c was purified by a preparative HPLC (0.1% NH4Ac in H2O–MeCN, 7:3, 4 mL/min) to yield 1 (7.8 mg), 2 (53.1 mg), 3 (6.9 mg) and 6 (13.5 mg). A2 (207.8 mg) was subjected to Sephadex LH-20 CC eluted with CH2Cl2–MeOH (3:1) to give four subfractions, A2a–A2d. A2b was separated by preparative HPLC (0.1% NH4Ac in H2O–MeCN, 3:2 to 1:4, 4 mL/min) to yield 4 (2.7 mg), 5 (2.0 mg), 7 (1.6 mg), and 8 (2.0 mg).

4.3.1. Jubanine F (1)

White amorphous powder; UV (MeOH) $\lambda_{max}$ (log $e$) 276 (3.47), 323 (3.87) nm; CD (c 0.10, MeOH) $\lambda_{max}$ (Ae) 321 (–2.22), 294 (–0.9), 265 (–4.4), 234 (–1.8), 213 (–5.9) nm; $\delta^1$H 308.9 (MeOH); IR $\nu_{max}$ 2966, 2352, 2317, 1674, 1645, 1514, 1566, 1222 cm$^{-1}$; $\delta_{13}$C 558.3296 [M+H]$^+$ (calcd. for C24H24N2O6). For CD and NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) $m/z$ 544.3112 [M+H]$^+$.

4.3.2. Jubanine G (2)

White amorphous powder; UV (MeOH) $\lambda_{max}$ (log $e$) 273 (3.22), 323 (3.06) nm; CD (c 0.10, MeOH) $\lambda_{max}$ (Ae) 321 (–2.5), 294 (–0.9), 264 (–4.9), 231 (–0.5), 215 (–3.7) nm; $\delta^1$H 308.9 = 247.8 (MeOH); IR $\nu_{max}$ 3332, 2964, 1643, 1513, 1446, 1222, 1186, 1038, 1027 cm$^{-1}$; $\delta_{13}$C 558.3296 [M+H]$^+$ (calcd. for C24H24N2O6).

4.3.3. Jubanine H (3)

White amorphous powder; UV (MeOH) $\lambda_{max}$ (log $e$) 271 (3.65), 323 (3.50) nm; CD (c 0.10, MeOH) $\lambda_{max}$ (Ae) 321 (–4.2), 295 (–1.7), 262 (–8.9), 231 (–0.8), 215 (–6.2) nm; $\delta^1$H 308.9 = 280.9 (MeOH); IR $\nu_{max}$ 3337, 2966, 1678, 1643, 1513, 1432, 1222, 1031, 982 cm$^{-1}$; $\delta_{13}$C 558.3296 [M+H]$^+$ (calcd. for C24H24N2O6).
1006 cm⁻¹; See Tables 1 and 2 for 1H and 13C NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) m/z 572.3451 [M+H]^+ (calcld. for C₃₀H₄₈N₀₁₀S₀₄O₁₀, 572.3448).

4.3.4. Jubanine I (4)
White amorphous powder; UV (MeOH) λ max (log ε) 271 (3.15), 322 (3.27) nm; CD (c 0.02, MeOH) λ max (Δε) 322 (−0.8), 293 (0.0), 264 (−1.6), 236 (−0.7), 212 (−2.5) nm; [α]D²⁰ = −64.3 (MeOH); IR νmax 1506, 1430, 1392, 1362, 1303, 1031 cm⁻¹; See Tables 1 and 2 for 1H and 13C NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) m/z 572.3451 [M+H]^+ (calcld. for C₃₀H₄₈N₀₁₀S₀₄O₁₀, 572.3448).

4.3.5. Jubanine J (5)
White amorphous powder; UV (MeOH) λ max (log ε) 271 (3.13), 323 (2.82) nm; CD (c 0.10, MeOH) λ max (Δε) 321 (−2.0), 296 (−0.8), 262 (−4.3), 232 (−0.4), 215 (−3.2) nm; [α]D²⁰ = −172.8 (MeOH); IR νmax 3707, 2972, 2873, 2350, 1643, 1513, 1220, 1054, 1033, 1013 cm⁻¹; See Tables 1 and 2 for 1H and 13C NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) m/z 586.3597 [M+H]^+ (calcld. for C₃₉H₅₈N₁₀O₁₀S₀₄, 586.3605).

4.3.6. Nummaruline B (6)
White amorphous powder; UV (MeOH) λ max (log ε) 270 (3.84), 320 (3.68) nm; CD (c 0.10, MeOH) λ max (Δε) 321 (−6.6), 295 (−3.1), 264 (−12.5), 231 (+0.3), 217 (−9.6) nm; See Supplementary data for 1H and 13C NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) m/z 592.3107 [M+H]^+ (calcld. for C₃₂H₄₂N₁₀O₁₀S₀₄, 592.3125).

4.3.7. Daechuine-S3 (7)
White amorphous powder; UV (MeOH) λ max (log ε) 272 (3.95), 320 (3.79) nm; CD (c 0.40, MeOH) λ max (Δε) 321 (−22.7), 296 (−9.6), 263 (−49.1), 232 (−12.7), 216 (−45.0) nm; See Supplementary data for 1H and 13C NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) m/z 628.4074 [M+H]^+ (calcld. for C₃₈H₅₆N₁₀O₁₀S₀₄, 628.4059).

4.3.8. Mucronine K (8)
White amorphous powder; UV (MeOH) λ max (log ε) 270 (3.76), 323 (3.87) nm; CD (c 0.10, MeOH) λ max (Δε) 321 (−6.3), 295 (−2.6), 264 (−12.7), 232 (+0.9), 217 (−7.1) nm; See Supplementary data for 1H and 13C NMR spectroscopic data; ESI-qTOF-MS (positive ion mode) m/z 515.3245 [M+H]^+ (calcld. for C₂₈H₃₄N₁₀O₁₀S₀₄, 515.3245).

4.4. Acid hydrolysis of compounds
Approximately 0.2 mg of 1–3 and 0.1 mg of 4–5 were hydrolyzed with 6N HCl (100 μL) at 110 °C for 30 min with stirring. The hydrolysates were evaporated to dryness and then the dried hydrolysates were resuspended in H₂O (100 μL). The solutions were concd under reduced pressure.

4.4.1. Determining absolute configurations of the amino acids in 1–5 by the advanced Marfey’s method using LC–MS
Each hydrolysate (30 μg) was added to 1 M NaHCO₃ (200 μL) and 1% L- or L-FDLA in acetone (25 μL). The reaction vials were incubated and stirred for 30 min at 50 °C. The reactions were then quenched with 2 N HCl (100 μL). MeOH (100 μL) was added to prepare LC-MS samples. The reaction products were analyzed by HPLC–MS with a positive ion detection mode. H₂O–MeCN containing 0.05% HCO₂H was used as eluents with MeCN containing 0.05% HCO₂H increasing from 5% to 100% over 19 min at a flow rate of 0.7 mL/min. Authentic standards (200 μg) were also prepared and analyzed using the same procedure. The retention times of the hydrolysates and amino acid standards and L-FDLA-derivatives were as follows: L-Val/L-FDLA (tR 13.51 min, m/z 412 [M+H]^+), L-Ne-Ala-L-FDLA (tR 11.92 min, m/z 398 [M+H]^+) at 2, L-Ne-Ala-L-FDLA (tR 12.05 min, m/z 398 [M+H]^+) at 3, 1-Ile/1-alle-L-FDLA (tR 12.62 min, m/z 426 [M+H]^+) at 2, and L-Ile/L-alle-L-FDLA (tR 14.04 min, m/z 426 [M+H]^+).

4.4.2. Determining absolute configurations of the amino acids in 2–5 by GITC analysis using LC–MS
Each hydrolysate (30 μg) was treated with 6% trimethylamine (40 μL) and 1% GITC reagent (40 μL). After 30 min of incubation at rt, 5% AcOH (40 μL) was added to a reaction vial to quench the reaction. MeOH (50 μL) was added to the reaction residues to prepare LC–MS samples. An authentic standard was also prepared from the same procedure. For the GITC product analysis of natural hydrolysates and standard amino acids, the following chromatographic method was used: solvent A was H₂O:MeCN with 0.1% HCO₂H (95:5, v/v), solvent B was MeCN with 0.1% HCO₂H. The LC–MS program for detecting the GITC-derivatives was set as 5% solvent B (0–7 min), 5–27% solvent B (7–12 min), 27–28.5% solvent B (12–45 min), 28.5–100% solvent B (45–46 min), and 100–100% solvent B (46–51 min) at a flow rate of 1 mL/min. The reaction products were analyzed on a positive mode with a LC–MS system. The co-injection experiments of the GITC-derivatized hydrolysates with authentic amino acid derivatives (L-Ile and L-alle-Ile) were established so that Lle residues in 2–5 are all Lle (Retention time: L-Ile-GITC (33.67 min)/L-alle-Ile-GITC (33.31 min)).

4.4.3. Preparing L- and d,N,N-dimethylalanine (Choi et al., 2012)
The L and D-alanine (8 mg) standards in H₂O were individually treated with HCHO (27 μL) and 10% Pd/C (10.4 mg). The mixtures were subjected to H₂ for 16 h and each reaction mixture was boiled and then dried under reduced pressure.

4.4.4. Determining absolute configurations of N,N-dimethylalanine in 4 and 5 by PGME derivatization and LC/MS analyses
Each dried L- and d,N,N-dimethylalanine was dissolved in tetrahydrofuran (THF, 500 μL). After adding 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 8.8 μL) to each vial, each mixture was stored at rt and stirred for 5 min. (S)-(+)PGME (10.5 mg) was added to each vial and each mixture was stirred for 6 h at rt. The reaction products were dried under N₂ gas and extracted with CH₂Cl₂. The CH₂Cl₂ soluble products were analyzed by LC–MS [same column but 150 mm length instead of 100 mm; solvent A was H₂O:MeCN with 0.05% HCO₂H (95:5, v/v), solvent B was MeCN with 0.05% HCO₂H with gradient solvent system as follows: 5% solvent B (0–2 min), 5–10% solvent B (2–12.5 min), 10–30% solvent B (12.5–15 min), flow rate 0.7 mL/min. The authentic amides, the (S)-(+)PGME product of L- and d,N,N-dimethylalanine (m/z 265 [M+H]^+), were eluted at 8.83 and 10.24 min, respectively. The (S)-(+)PGME product of N,N-dimethylalanine in 4 and 5 hydrolysates were observed at 8.79 and 8.73 min, respectively, retention time for LC–MS analyses. The absolute configurations of N,N-dimethylalanine in 4 and 5 were identified as both L forms (5 configuration).

4.5. Cell culture and virus stock
Vero cells (African green monkey kidney cell line; ATCC CCR-81) were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS). PEDV was obtained from Choong Ang Vaccine Laboratory, Korea. The virus stock was kept at −80°C before use.
4.5.1. Cytotoxicity assay

The cell viability was calculated using a MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Vero cells were adhered at 1 × 10^6 cells per well in 96-well plates and grown for 24 h before treatment. The cells were treated with various concentrations of compounds. To avoid solvent toxicity, the final DMSO concentration was maintained under 0.05% (v/v) in the culture medium. After incubating further for 48 h, MTT solution (2 mg/mL, 20 μL) was added to each well and kept for 4 h. After removing the supernatant, DMSO (100 μL) was added to solubilize formazan crystals. Consequently, the absorbance was measured at 550 nm. The percentage cell viability is the absorbance in the experiment well compared to that in the control wells and compound toxicity is the percentage cell viability. Regression analysis was used to calculate 50% cytotoxic concentration (CC50).

4.5.2. Cytopathic effect (CPE) inhibition assay

Vero cells were seeded onto 96-well plates at 1 × 10^5 cells per well. The medium was removed a day later and washed with phosphate buffered saline (PBS). PEDV at 0.01 MOI was inoculated onto near-confluent Vero cell monolayers for 2 h. The medium was replaced by DMEM with various concentrations of compounds. After incubating for 72 h at 37°C under 5% CO2 atmosphere, cells were replaced with DMEM and MTT (2 mg/mL, 20 μL) to each well and incubated for 4 h at 37°C. The 50% effective concentration (EC50) was calculated using regression analysis, and the formula SI = CC50/EC50 determined the selective index (SI).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.09.001.

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