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Discovery of inhibitory materials against PEDV corona virus from medicinal plants

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Objective

Porcine epidemic diarrhea virus (PEDV) is an enveloped single-stranded RNA virus belonging to the family Coronaviridae. It is the causative agent of porcine epidemic diarrhea, dehydration, vomiting, and high mortality in the piglets. Most of newborn piglets infected by PEDV would be dying and pigs of all ages are also affected with a severe symptoms like massive diarrhea and dehydration.¹ Infection with this virus has been become a serious issue in the swine industry and outbreak resulted in serious economic losses in many swine producing countries, notably in Europe and Asia. Recently, new PEDV was noticed and its outbreak affected 23 US states by the end of January of 2014.²

As secondary substances from natural products are generally low-toxic's small molecules with high potential for chemical novelty and biological interests, extensive studies on medicinal plants may result in the investigation of new compounds with drug-like properties. It have been reported that about 50,000 species among total 500,000 plants in the world are used in traditional medicine. The recent outbreaks of

many virus-related new diseases like severe acute respiratory syndrome (SARS) in 2002-2003, novel influenza H1N1 virus in 2009-2010, continuous other influenza viruses in China, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012-present, and also MERS on May of 2015 in Korea suggest that bioactive compounds from natural products against infectious viruses should be continuously developed for the protection of human health.³

The main objective of our team using natural plants as research source is finding active compounds against zoonotic diseases by viruses at both human and animal. We have been screened thousands of plant extracts for antiviral activity against PEDV. During this process, *Camellia japonica*, *Saposhnikovia divaricata*, and *Dryoteris crassirhizoma* showed potential inhibitory effects on PEDV replication.⁴⁻⁶ Bioassay-guided fractionations of these active extracts afforded many compounds with inhibitory activities against PEDV replication. Herein, the antiviral activities, including action of mechanisms, against PEDV of all isolates were evaluated, and a brief structure-activity relationship was also discussed.

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Methods

General Experimental Procedures: Optical rotations were analyzed by a JASCO P-2000 polarimeter (JASCO International Co. Ltd., Tokyo, Japan). UV data were determined using an Optizen 3220 UV spectrophotometer (Mekasys Co. Ltd., Daejeon, Korea). IR spectra were recorded on a Nicolet 6700 FT-IR (Thermo Electron Corp., Waltham, MA, USA). NMR spectra were checked by Varian Unity Inova 500 and 600 MHz spectrometers, at the College of Pharmacy, Seoul National University, Korea. HRFABMS and HRESIMS were recorded on a JEOL JMS 700 (JEOL, Ltd., Tokyo, Japan) and Agilent 6530 Q-TOF (Agilent Technologies, Inc., Santa Clara, CA, USA) spectrometer, respectively. Column chromatography (CC) was performed with silica gel (63–200 μm particle size) and RP-C₁₈ (40–63 μm particle size) obtained from Merck (Darmstadt, Germany), as well as Sephadex LH-20 (Sigma-Aldrich Corp., St. Louis, Missouri, USA). TLC profiles were checked on RP-C₁₈ F₂₅₄ and silica gel 60 F₂₅₄ plates. HPLC was performed on a Gilson System with an Optima Pak C₁₈ column (10 μm particle size, 10 $\times$ 250 mm; RS Tech, Seoul, Korea) and a UV detector. All solvents used for extraction and isolation are of analytical grade.

Cell Culture and Virus Stock: Vero cells (African green monkey kidney cell line; ATCC CCR-81) were provided by American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 10% fetal bovine serum (FBS). PEDV was obtained from Choong Ang Vaccine Laboratory, Korea. Virus stock was kept at -80°C before use.⁷

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 grown in 96-well plates at 1×10^5 cells per well and adhered for 24 h before treatment. The cells were treated with different

concentrations of compounds and incubated for 48 h. Then, 20 μL of the 2 mg/mL MTT solution was added to each well and incubated for 4 h. Percentage cell viability is determined as the absorbance in the experiment well compared to that in the control wells, and toxicities of the compounds were defined as the percentage cell viability. Regression analysis was used to calculate the 50% cytotoxic concentration (CC_{50}).

Cytopathic Effect (CPE) Inhibition Assay: Vero cells were seeded onto 96-well plates at 1×10^5 cells per well. One day later, medium was removed and then washed with phosphate buffered saline (PBS). PEDV at 0.01 MOI was inoculated onto near confluent Vero cell monolayers for 2 h. The media was replaced by DMEM with several compounds at different concentrations. After incubation of 72 h at 37°C under 5% CO_2 atmosphere, cells were replaced with DMEM and 20 μL of 2 mg/mL MTT to each well and incubated for 4 h at 37°C . The 50% effective concentration (EC_{50}) was calculated using regression analysis, and the formula $\text{SI} = \text{CC}_{50}/\text{EC}_{50}$ was applied to determine the selective index (SI).⁸

Quantitative Real-Time PCR: Vero cells were grown to about 90% confluence in 6-well plates, infected with PEDV at 0.01 MOI and incubated for 2 h. Then, media was replaced by DMEM and cultured with compounds of interest at various concentrations. After 24 h, total RNA was isolated from the cells by following TRIzol method, and reverse transcribed using random primer (iNtRON Biotechnology, Inc, Seongnam, Korea) based on manufacturer's instruction. Real-time PCR was conducted using 2 μL of cDNA and Maxima SYBR Green qPCR master mix 2X (Thermo sci., Rockford, IL, USA). Cycling conditions for real-time PCR were follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Real-time PCR was conducted using the Step one Plus Real-time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). The data was analyzed with StepOne software v2.3.

Western Blot Analysis: The cultures were

prepared using similar methods to quantitative Real-time PCR. After 24 h, the cells were washed with cold PBS and kept at -80°C . For whole cell lysate, the cells were lysed on ice in 100 μL lysis buffer [50 mM NaF, 0.5% NP-40, 1 mM EDTA, 120 mM NaCl, 50 mM Tris-HCl (pH 7.6)] and centrifuged at 12,000 rpm for 20 min. Protein concentrations were calculated using protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After boiled for 5 min, aliquots of lysates were electrophoresed on 10% or 12% SDS-polyacrylamide gels. Protein in the gels were electrotransferred to nitrocellulose membranes (PVDF 0.45 μm , Immobilon-P, USA). Membranes were incubated with primary antibodies spike (S) protein, nucleocapsid (N) (AbFrontier Co., Ltd., Seoul, Korea) or mouse monoclonal actin antibody, and further incubated with secondary antibodies. Finally, they were detected using enhanced chemiluminescence Western blotting detection kit (Thermo Sci., Rockford, IL, USA).

Immunofluorescence Assay: Vero cells were grown on 8-well chamber slides (LAB-TEK, NUNC, Thermo Fisher Scientific, MA, USA) and PEDV at 0.01 MOI were injected to the cell monolayers for 2 h. After incubation at 37°C under 5% CO_2 atmosphere for 24 h, cells were washed with PBS (pH 7.4) three times and fixed with a 4% paraformaldehyde solution for 30 min at room temperature. After blocking with 1% BSA for 1 h, the cells were incubated overnight with monoclonal antibody against N protein of PEDV (AbFrontier Co., Ltd., Seoul, Korea) diluted 1:50 in PBS (pH 7.4). Then, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, Inc., West Grove, PA, USA) for 1 h after washing with PBS (pH 7.4). After washing three times with PBS (pH 7.4), the cells were stained with 500 nM DAPI solution for 10 min at room temperature and washed with PBS (pH 8.0) three times. Slides were mounted with mounting reagent for fluorescence (Vectashield, Vector Laboratories Inc., Burlingame, CA, USA) and observed by fluorescence microscopy (Olympus ix70

Fluorescence Microscope, Olympus Corporation, Tokyo, Japan).

Statistical Analysis: The results are expressed as the means $\pm$ SD of three independent experiments. Statistical analysis was conducted on Sigma Plot Statistical Analysis software, and differences between group mean values were determined by one-way analysis of variance followed by a two-tailed Student's *t*-test for unpaired samples, assuming equal variances. Statistical significance was accepted at $p < 0.05$.

Results and Discussion

1. Structure-Activity Relationships (SARs) of Oleanane Triterpenes from the Flowers of *Camellia japonica* against PEDV

The flowers of *C. japonica* were extracted with 70% EtOH using sonication. By means of diverse chromatographic methods, including silica gel, RP- C_{18} , LH-20, and HPLC, nine new (**1–4** and **10–14**) and six known (**5–9**, and **15**) oleanane triterpenes were purified and obtained (Fig. 1).

The purified fifteen oleanane-type triterpenes (**1–15**) were evaluated for their inhibitory effects on PEDV replication with azauridin as positive control. Vero cells were incubated with test compounds at different concentrations after inoculation with PEDV for 2 h. As shown in Table 1, compounds **6–9**, **11**, and **13** exhibited potent inhibitory effects on PEDV replication, with EC_{50} values at submicromolar range (0.06 ± 0.02 to $0.93 \pm 0.22 \mu\text{M}$), which was stronger than that of positive control (EC_{50} $3.37 \pm 0.71 \mu\text{M}$). In addition, compounds **6**, **9**, and **11** gave higher selective index (SI) values in this assay (44.54 ± 8.34 , 32.72 ± 6.22 , and 14.75 ± 1.62 , respectively), compared to azauridin (14.30 ± 1.24), while compounds **7** and **13** showed lower SI values (7.99 ± 0.28 and 6.68 ± 0.14 , respectively). Even though compound **8** gave considerable SI value (12.98 ± 2.34), it demonstrated strong cytotoxicity ($0.81 \pm 0.07 \mu\text{M}$) in this assay. Compounds **2**, **3**, **10**, and **15** also showed promising EC_{50} values at

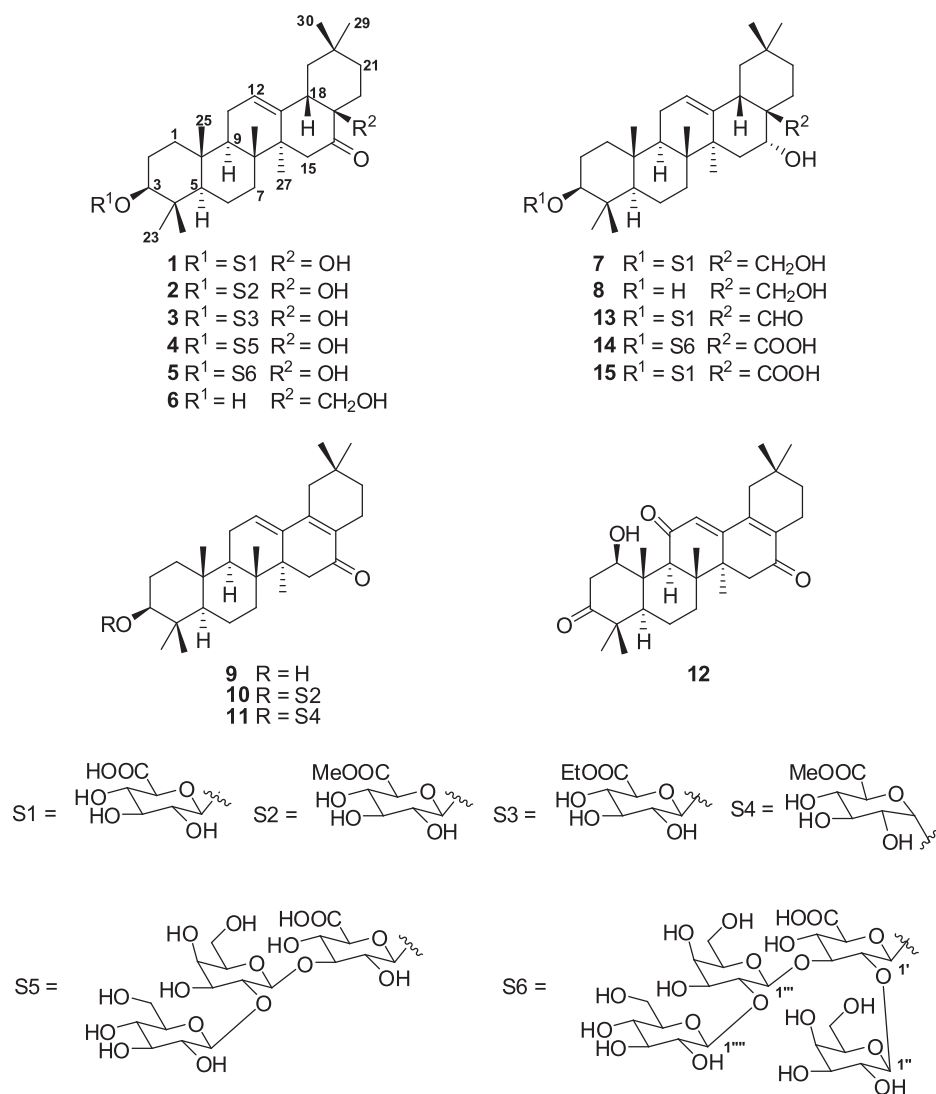


Fig. 1. Oleanane triterpenes 1-15 isolated from the flowers of *Camellia japonica*.

low micromolar range (1.09 ± 0.22 to $3.70 \pm 0.68 \mu M$).

However, there were no obvious antiviral activity observed for compounds **1**, **4**, **5**, **12**, and **14** in this assay. Moreover, the inhibition on viral replication of compound **9** was further supported by a time-course study, the infectivity of PEDV particles assay, and cell protection assay for PEDV infection (data not shown).

To discuss the SARs of *Camellia* triterpenes (table 1), the oleanane triterpenes (**1-15**) obtained in this investigation were divided into four groups based on the substitution pattern at the C-17 position: (1) substituted by OH (**1-5**); (2)

substituted by CH_2OH (**6-8**); (3) occurrence of an olefinic double bond between C-17 and C-18 (**9-12**); (4) substituted by CHO or COOH (**13-15**). Biological data of group 1 (**1-5**) indicated that the monoglycosylation by glucuronopyranosyl moiety with esterification at C-6' could improve the antiviral property (comparing **2** and **3** with **1**, **4**, and **5**). In group 2, the replacement of a hydroxy group by a ketone group at C-16 (comparing **6** with **8**) or monoglycosylation at C-3 (comparing **7** with **8**) induced lower cytotoxicity with keeping considerable antiviral effects. The antiviral profile of group 3 (**9-12**) indicated that monoglycosylation at C-3 could reduce cytotoxicity with keeping

Table 1. Inhibition on PEDV replication by oleanane triterpenes 1–15

Compd	CC ₅₀ (μM)	EC ₅₀ (μM)	SI
1	4.95 ± 1.15	NA	—
2	26.02 ± 3.61	1.94 ± 0.39	13.39 ± 0.67
3	6.29 ± 2.23	1.09 ± 0.22	5.75 ± 0.75
4	>20	NA	—
5	5.91	NA	—
6	12.47 ± 0.97	0.28 ± 0.09	44.54 ± 8.34
7	7.23 ± 0.87	0.91 ± 0.07	7.99 ± 0.28
8	0.81 ± 0.07	0.06 ± 0.02	12.98 ± 2.34
9	9.32 ± 1.19	0.28 ± 0.11	32.72 ± 6.22
10	27.29 ± 5.63	2.90 ± 0.25	9.40 ± 1.04
11	13.72 ± 1.35	0.93 ± 0.22	14.75 ± 1.62
12	13.95	NA	—
13	2.25 ± 0.11	0.34 ± 0.01	6.68 ± 0.14
14	>20	NA	—
15	23.73 ± 1.80	3.70 ± 0.68	6.42 ± 0.58
Azauridin	48.17 ± 5.15	3.37 ± 0.71	14.30 ± 1.24

considerable antiviral activity (comparing **9** with **10** and **11**), and this trend depends on the type of attached sugar moieties. However, based on the activity data of compounds **2** and **10** and compounds **6** and **9**, the existence of an olefinic double bond between C-17 and C-18 slightly decreased the SI value. From the antiviral effects of compounds **13–15**, the substitution of a formyl group at C-17 increased anti-PEDV inhibition, and also increased the cytotoxicity (comparing **13** with **15**). However multiple glycosylation at C-3 would significantly decrease the antiviral activity (comparing **14** with **15**), and this observation was also supported by the data of compounds **4** and **5**.

Although chemical diversity of *Camellia* triterpenes, the above observations allowed the outline of SARs for this compound class: (1) the coexistence of a ketone group at C-16 and a hydroxymethyl group at C-17 positively contribute to the anti-PEDV property of *Camellia* triterpenes, which is supported by the comparison of biological data of compounds **6**, **8**, and **9**, as well as the highest SI value of compound **6**; (2) the multiple glycosylation at C-3 significantly decrease the anti-PEDV property of *Camellia* oleanane

saponins, which is supported by the biological data of compounds **4**, **5**, and **14**.

2. Inhibitory Effects of *Camellia* Triterpenes on Key Gene and Protein Synthesis during PEDV Replication.

During the PEDV life cycle, three key structural proteins, including spike, membrane, and nucleocapsid proteins, play a pivotal role. The spike protein functions as a key regulator in viral entry step. The membrane protein mainly regulates the viral assembly process, and the nucleocapsid protein, which binds to viral RNA, is a basic protein associated with the genome. Their biological functions place the three proteins as potential and promising targets for anti-PEDV research.⁹

Four structurally representative oleanane-triterpenes **6**, **9**, **11**, and **13** were evaluated for their biological effects on several key genes and proteins required for PEDV replication. First, we measured the levels of intracellular viral RNA, encoding PEDV nucleocapsid, spike, and membrane protein synthesis.^{10–12} After Vero cells, which were infected with PEDV, were treated

with test compounds at different concentrations for 24 h, total RNA was isolated from cells and analyzed by real-time PCR. Compounds **6**, **9**, **11**, and **13** were found to significantly reduce the RNA levels, associated with GP6 nucleocapsid, GP2 spike, and GP5 membrane protein. All of them (**6**, **9**, **11**, and **13**) demonstrated comparable or stronger inhibitory effects on RNA expression, compared with azauridin that was used as positive control in this assay. Compounds **6**, **9**, **11**, and **13** were also further evaluated for their inhibitory effects on GP6 nucleocapsid and GP2 spike protein synthesis during PEDV replication. Based on western blot analysis (Fig. 2A and 2B), compound **11** showed significant inhibitory effects on PEDV nucleocapsid and spike protein synthesis, which was even stronger than that of azauridin. Compound **13** demonstrated considerable inhibitory effects, at the corresponding concentration to that in RT-PCR analysis. Moreover, compound **11** was found to inhibit nucleocapsid protein synthesis in a dose-dependent manner at concentrations (Fig. 2C).

Based on promising EC_{50} , SI value, and the potent inhibition on PEDV RNA expression, compound **6** was further evaluated for its inhibition on PEDV replication, analyzed by an immunofluorescence assay. Compound **6** showed marked inhibition on PEDV replication in a dose-dependent manner at concentrations of 4.0, 2.0, and 1.0 μ M. It was found that compound **6** showed a comparable effect at a concentration of 4 μ M, compared with azauridin at 10 μ M (data not shown).

3. Coumarins from *Saposhnikovia divaricata* and Their PEDV inhibitory activities

A 70% ethanol extract of the radix of *S. divaricata* showed inhibitory effect against the PEDV. The 70% ethanol soluble extract was suspended in water and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. Bioactivity-guided fractionation of EtOAc-soluble fraction by successive chromatographic procedures (silica gel, RP-C₁₈, and HPLC) yielded three new

coumarin glycosides (**1–3**) together with ten known coumarins (**4–10**) (Fig. 3).

The structures of known coumarins were identified as praeruptorin F (**4**) and *cis*-3'-isovaleryl-4'-acetylkhellactone (**5**), praeruptorin B (**6**) and *cis*-3',4'-diseneciolykhellactone (**7**), *cis*-3'-isovaleryl-4'-seneciolykhellactone (**8**), (-)-*cis*-khellactone (**9**), scopoletin (**10**), oxypeucedanin hydrate (**11**), decurcinol (**12**), and umbelliferone (**13**), respectively, by comparing their spectroscopic data and optical rotations with the literature values.

All isolates were evaluated for their inhibitory activity against PEDV through CPE analysis in Vero cells. Of the tested compounds, compound **5** exhibited potent activity with EC_{50} values of 4.28 ± 0.64 μ M. Interestingly, the most potent compound **5** showed stronger activity as compared to positive control, azauridine (4.85 ± 0.43 μ M), with safety index more than 23.90. However, there was no obvious antiviral activity observed for compounds **10–13** in this assay. On the other hand, new coumarin glycosides **1–3** revealed moderate inhibitory activity against PEDV in a dose dependent manner without any cytotoxicity (Table 2).

To discuss the structure-activity relationships (SARs), coumarins **1–13** were divided into three groups based on the substitution patterns at C-7 and C-8: (1) 8-substituted coumarins **1–3** with *O*-sugar at C-7, (2) 8-substituted coumarins **4–9** with angular pyrano ring at C-8/C-1'/C-2'/C-3'/O/C-7, (3) non-8-substituted coumarins **10–13**. Among these compounds, group 3 (**10–13**) had no anti-PEDV activity, indicating the key role of functionalization at C-8 in the antiviral property of coumarins. Compared with group 1 (**1–3**), coumarins **4–8** showed stronger anti-PEDV affects based on EC_{50} values. However compound **9** in group 2 demonstrated similar EC_{50} values to those of coumarins **1–3**. The above observations implied that the existence of an angular pyrano ring at C-8/C-1'/C-2'/C-3'/O/C-7 bearing functionalization at C-1' and C-2' positively influenced the antiviral property of coumarins.

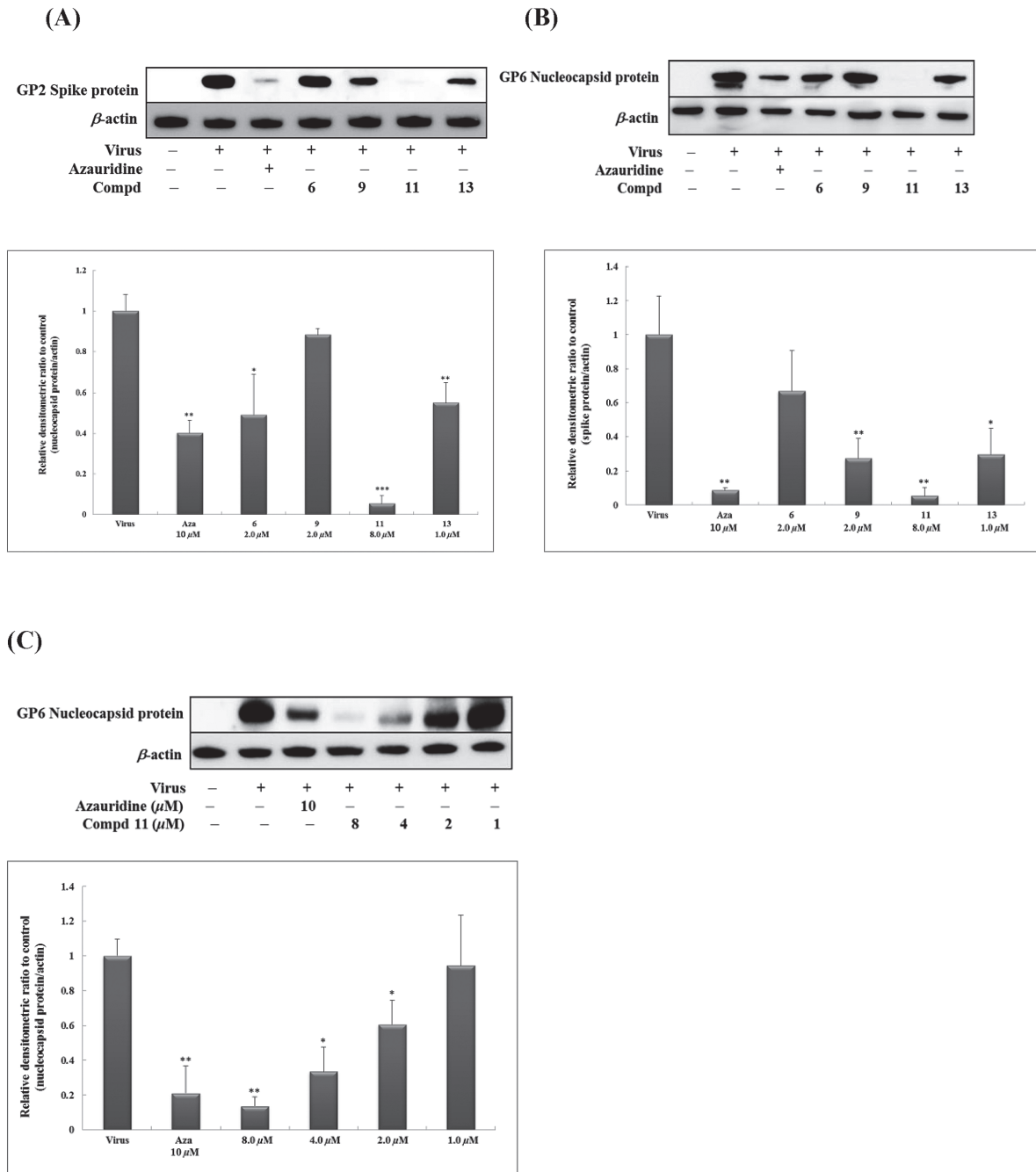


Fig. 2. Inhibitory effects of camellia triterpenes on PEDV GP6 nucleocapsid and GP2 spike protein synthesis, using western blot analysis. (A and B) Triterpenes **6**, **9**, **11**, and **13** inhibited PEDV nucleocapsid and spike protein synthesis at concentrations of 2 μ M, 2 μ M, 8 μ M, and 1 μ M, respectively. (C) Compound **11** inhibited the PEDV nucleocapsid protein synthesis in a dose-dependent manner.

The inhibitory effect of compound **5** on viral replication was determined by quantitative real-time PCR using specific primers for viral GP6 nucleocapsid, GP2 spike, and GP5 membrane encoding genes. Compound **5** exhibited inhibitory

effect on genes encoding PEDV GP6 nucleocapsid, GP2 spike, and GP5 membrane proteins in a dosedependent manner at concentrations of 10, 5, and 2.5 μ M. Compound **5** showed stronger inhibitory effects than positive control, azauridine,

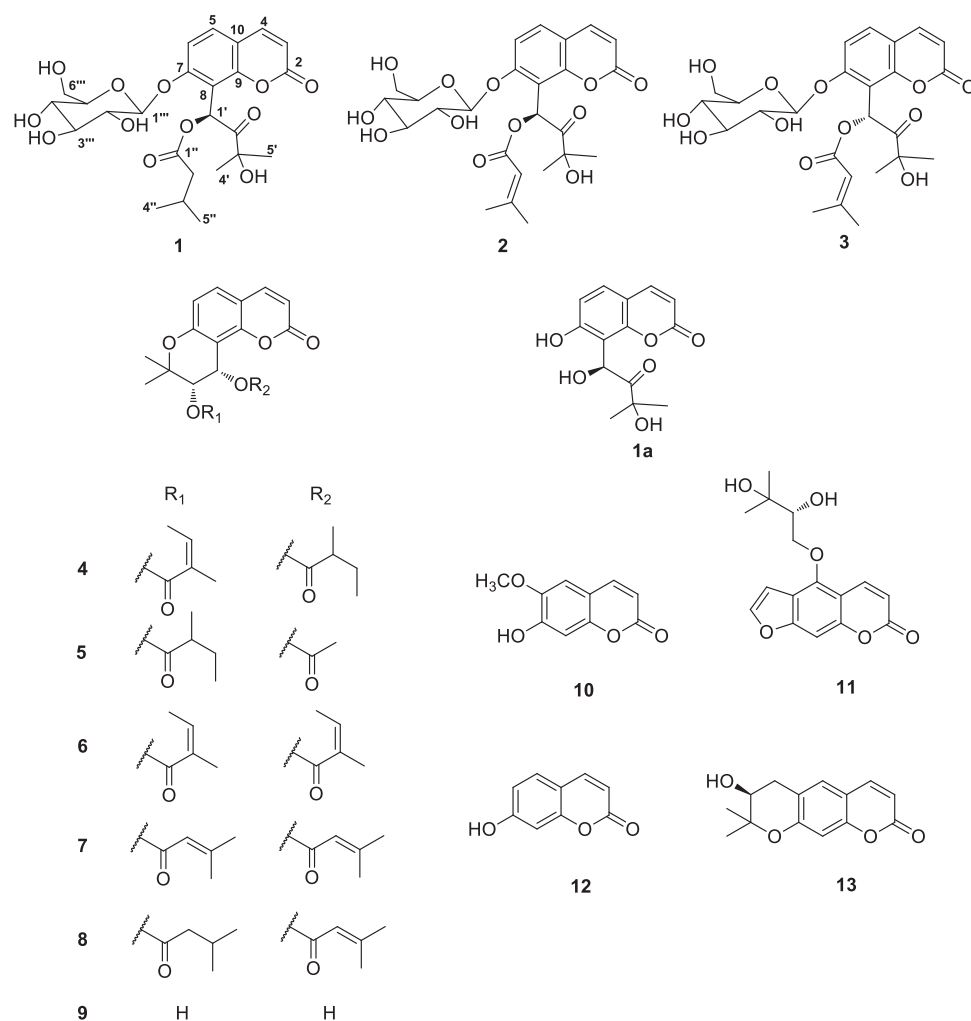


Fig. 3. Chemical structures of coumarins 1-13 from *Saposhnikovia divaricata* and the structure 1a.

for all three structural proteins encoding genes at the same test concentration of 10 μM (data not shown).

4. Phloroglucinols as PEDV Inhibitors from *Dryopteris crassirhizoma*

The successive bioactivity-guided chromatographic fractionations with an *in vitro* assay of virus yield reduction led to the isolation of four phloroglucinol-type compounds (1-4) from EtOH extract of *Dryopteris crassirhizoma* (Dryopteridaceae) rhizomes (Fig. 4).¹³

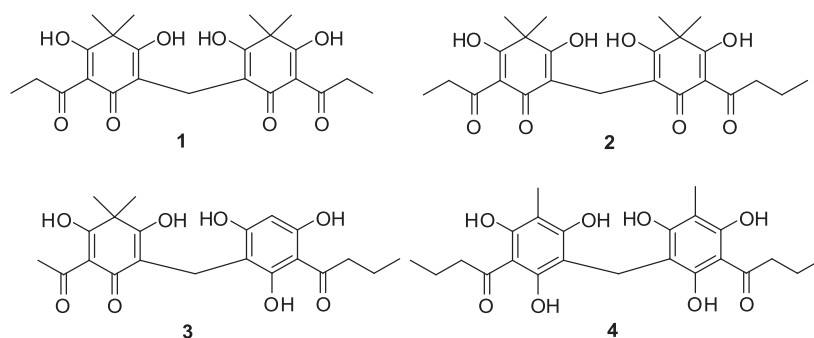
To confirm that phloroglucinols (1-4) inhibited PEDV, the purified four compounds (1-4) were evaluated whether they had inhibitory effects against PEDV replication with

6-azauridine as the positive control. Vero cells were incubated with test compounds at different concentrations after inoculation with PEDV for 2 hours. Compounds 3 and 4 were stronger inhibitory activities than the positive control against PEDV replication with EC_{50} values at low micromolar range (2.59 ± 0.14 to $1.58 \pm 0.24 \mu\text{M}$). Moreover, compound 4 displayed a considerably high selective index (SI) value (39.21 ± 0.27). Even though the relationships of structural activity of these compounds have not been illustrated thoroughly, we may suggest that carbonyl group at C-2 and C-2' could be related with their antiviral activity.

Considering their potent inhibition on PEDV replication, compound 4 was selected and

Table 2. Inhibitory effect of compounds 1-13 on PEDV induced CPE

Compound	EC ₅₀ (μM)	CC ₅₀ (μM)	SI
1	16.25 ± 1.97	>100	>6.25 ± 0.85
2	17.36 ± 2.12	>100	>5.85 ± 0.80
3	19.70 ± 1.66	>100	>5.11 ± 0.47
4	3.84 ± 0.45	15.51 ± 0.97	4.07 ± 0.25
5	4.28 ± 0.64	>100	>23.90 ± 4.11
6	1.09 ± 0.06	8.36 ± 0.42	7.67 ± 0.04
7	1.22 ± 0.09	9.98 ± 0.28	8.21 ± 0.40
8	0.60 ± 0.03	4.76 ± 0.79	7.89 ± 0.97
9	18.00 ± 1.25	>100	>5.58 ± 0.41
10	NA	—	—
11	NA	—	—
12	NA	—	—
13	NA	—	—
Azaauridine	4.85 ± 0.43	48.65 ± 7.95	9.94 ± 0.82

**Fig. 4. Structure of compounds (1-4) isolated from rhizomes of *D. crassirhizoma*.****Table 3. In vitro antiviral activity of compounds 1-4 from *D. crassirhizoma* against PEDV**

Compounds	EC ₅₀ (μM)	CC ₅₀ (μM)	SI
Albaspidin-PP (1)	5.53 ± 0.47	112.89 ± 6.38	20.40 ± 0.62
Albaspidin-PB (2)	5.69 ± 0.88	117.17 ± 8.85	20.58 ± 1.94
Norflavaspidic acid-AB (3)	2.59 ± 0.14	84.42 ± 3.79	32.58 ± 0.31
Methylene-bis-methylphlorobutyrophenone (4)	1.58 ± 0.24	61.81 ± 8.90	39.21 ± 0.27
6-Azaauridine	4.73 ± 0.49	48.64 ± 2.76	10.28 ± 0.54

evaluated for inhibitory effects on replication of viral RNA. After treatment of compounds at different concentrations, the levels of intracellular viral RNA (encoding PEDVgp2 spike, PEDVgp6 nucleocapsid and PEDVgp5 membrane) in viral-infected cells were measured by Real-time PCR analysis. After 24 hours incubation, total RNA

was isolated from the cells and the Real-time PCR was carried out using selective primers for PEDV. As shown in Fig. 5, compound **4** significantly reduced the RNA levels in a dose-dependent manner, associated with PEDVgp2 spike, PEDVgp6 nucleocapsid, and PEDVgp5 membrane at concentrations of 10 μM, 5 μM and

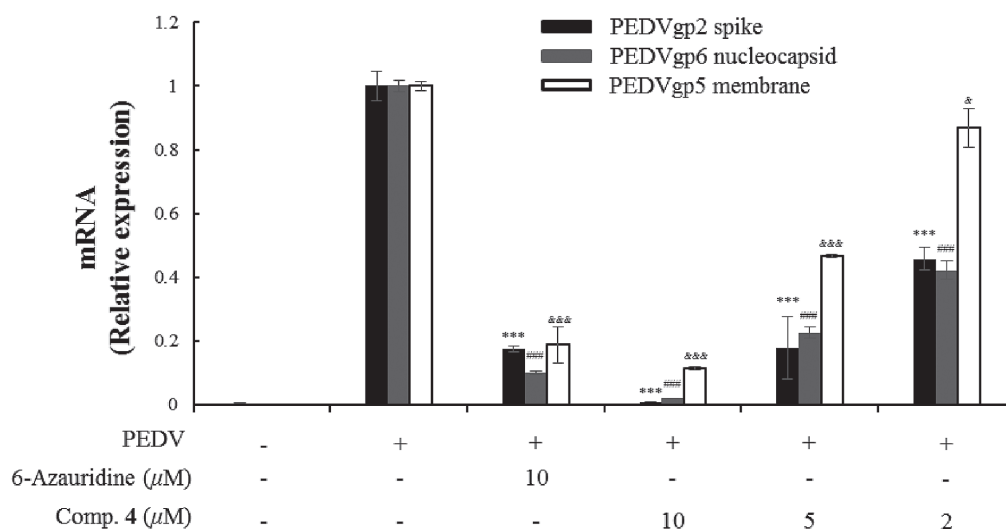


Fig. 5. Quantitative Real-time PCR analysis shows that the inhibition of PEDV replication by compound 4 is inhibited by the genes encoding viral structural protein synthesis. Confluent cells were incubated with PEDV for 2 hours and then treated with positive control 6-Azauridine and various concentrations of compound 4. After 24 hours of incubation, the cells were lysed to isolate the total RNA. Real-time PCR was performed with cellular RNA (β -actin) as an internal control and specific primers for viral RNA encoding PEDVgp2 spike, PEDVgp6 nucleocapsid, and PEDVgp5 membrane protein.

2 μ M, respectively. Furthermore, to clarify that compound 4 inhibited on PEDV replication depending on concentrations, immunoblot analysis also confirmed that compound 4 decreased the level of spike and nucleocapsid protein after 24 hours treatment with viral-infected cells (data not shown).

Conclusion

Bioassay-guided fractionations of *C. japonica*, *S. divaricata* and also *Dryopteris crassirhizoma* yielded many inhibitory materials against PEDV. A CPE assay, RT-PCR analysis, and western blot analysis, and immunofluorescence assay indicated that these compounds showed potent and promising inhibitory effects on PEDV replication by targeting key structural protein synthesis and relevant gene expression. Our studies provided a new class of natural scaffolds that may be able to be developed as potential anti-PEDV agents via inhibiting viral replication. The clear SARs outlined will facilitate the further structure optimization of this compound class for

developing novel antiviral agents. Furthermore, human corona viruses, like MERS, from the same Coronaviridae family shares some similar replication mechanism with PEDV, and the development of MERS vaccine and PEDV vaccine shared similar theory.^{14,15} Therefore, the anti-PEDV molecules obtained in this study could also be the noteworthy candidates for further investigation against the fatal human coronaviruses, including MERS.¹⁶

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