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Phloroglucinol as porcine epidemic diarrhea virus and canine corona virus inhibitors from *Dryoteris crassirhizoma*

Chosun University Graduate School

Department of Pharmacy

Ha Thi Kim Quy

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Porcine epidemic diarrhea virus (PEDV)와 canine corona virus (CCV) 바이러스의 저해물질로서 *Drysteris crassirhizoma*의 플로로글루시놀

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List of Abbreviations

aa	Amino acid
ATPase	Adenosine triphosphatase
BCoV	Bovine coronavirus
CCV	Canine coronavirus
CoV	Coronavirus
CMI	Cell-mediated immunity
CPV-2	Canine parvovirus type 2
DMEM	Dulbecco's modified Eagle's medium
EIMS	Electron impact mass spectrometry
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum
FCoV	Feline coronavirus
HPLC	High performance liquid chromatography
IBV	Infectious bronchitis virus
M	Membrane
MEM	Minimal essential medium
MeOH	Methanol
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Nucleocapsid
ORF	Open reading frame
PBS	Phosphate buffered saline
PED	Porcine epidemic diarrhea
PEDV	Porcine epidemic diarrhea virus
RNA	Ribonucleic acid

S	Spike
SARS-CoV	Severe acute respiratory syndrome – Coronavirus
SD	Standard deviation
TGEV	Transmissible gastroenteritis virus
TLC	Thin layer chromatography
TMS	Tetra methyl silane
TRSs	Transcription regulating sequences
UTR	Untranslated region

(국문 초록)

Porcine epidemic diarrhea virus (PEDV)와 canine corona virus (CCV) 바이러스의 저해물질로서 *Drysteris crassirhizoma*의 플로로글루시놀

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Porcine epidemic diarrhea virus (PEDV)와 canine corona virus (CCV)는 동물들에 심각한 질병을 일으키는 coronavirus 종류이며, 오랫동안 가축산업에 큰 위험요소로 여겨져 왔다. 바이러스 침투에 대한 효과적이고 효율적인 치료방법의 부족은 이들 PEDV 및 CCV 등의 코로나 바이러스에 대한 새로운 항 바이러스제 연구의 필요성을 더욱 부각시키고 있다.

In vitro상의 virus yield reduction assay를 사용하여, 바이러스 저해활성을 측정하는 방법을 사용하여 양치류 식물인 *Dryopteris crassirhizoma* (관중, Dryopteridaceae)의 뿌리의 에탄올 추출물로부터 항 바이러스 저해활성 물질을 분리하였다. 관중의 에탄올 추출물을 칼럼 크로마토그래피법을 사용하여 4개의 화합물을 얻고 이들 화합물의 ^{1}H, ^{13}C-NMR, HMBC 및 HMQC등의 분광학적인 방법을 이용하

여 구조결정한 결과 활성물질은 phloroglucinol 계열의 화합물 (1-4)로 결정하였다.

분리한 화합물 중 norflavaspidic acid-AB (3)와 methylene-bis-methylphlorobutyrophenone (4)은 낮은 농도에서 효과적인 PEDV와 CCV바이러스 량 감소와 세포변성효과를 보이는 등 얻어진 화합물 중 가장 강한 항바이러스 효과를 보였다. 또한, 화합물 (4)는 Vero 세포에 주입된 바이러스 PEDV의 구성부분에 있어서 중요한 spike (S), nucleocapsid (N), 및 membrane (M) 전사와 번역과정을 저해한다는 것을 발견하였다. 이러한 결과는 관중으로부터 얻어진 화합물 들이 PEDV의 감염에 의하여 발생하는 돼지 등의 가축질환에 대하여 효과적으로 사용할 수 있음을 보여준다.

ABSTRACT

Phloroglucinol as porcine epidemic diarrhea virus and canine corona virus inhibitors from *Dryoteris crassirhizoma*

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Porcine epidemic diarrhea virus (PEDV) and canine corona virus (CCV) are the types of coronaviruses that cause severe disease in animals and they are grave threat to the livestock industry for a long time. The lack of effective and efficient therapeutical treatment for that virus emphasizes the importance of research for new antivirals. Following an *in-vitro* virus yields reduction assay, the bioactivity guided successive chromatographic fractionation of EtOH extract of *Dryopteris crassirhizoma* (*Dryopteridaceae*) rhizomes led to the isolation of four phloroglucinol type compounds (**1-4**). Among those, compounds *norflavaspidic acid–AB* (**3**) and *methylene-bis-methylphlorobutyrophenone* (**4**) were the strongest antivirals that significantly reduced virus titers and cytopathic effect at low concentrations. Moreover, Compound (**4**) inhibited the spike (S), nucleocapsid (N) and membrane (M) gene transcription and the process of translation in PEDV infected to the Vero cell.

1. Introduction

1.1. The Coronavirus (CoVs)

Coronaviruses (CoVs) were first determined in the 1960s by using electron microscopy to visualize the characteristic spike glycoprotein projections on the surface of enveloped virus particles.[1] CoVs are generally spherically shaped pleomorphic particles ranging in diameter from 60 to 220 nm. They contain the viruses as deadly as SARS-CoV in addition to PEDV, TGEV, FCoV, CCV, BCoV, IBV etc. and responsible for seasonal or local epidemics of gastrointestinal and respiratory diseases in a variety of animals.

Coronaviridae family was previously believed to be monogeneric comprising 11 viruses, but this number has already reached 26 recently, and the family of coronaviridae has been classified phylogenetically to three groups : group 1(or alpha-coronaviruses), 2 (or beta-coronaviruses) and 3(or gamma-coronaviruses) and subsequent subgroups (Figure 1).[2, 3] The first two groups contain nine mammalian Coronaviruses, while the third group contains avian Coronaviruses.[4]

The diversity of Coronavirus arises frightful because of three main reasons. Firstly, the infidelity of RNA-dependent RNA polymerase is replicated, which leads to one mutation in every 1000 to 10,000 nucleotides.[5] Secondly, the random template switching during replication is probably mediated by a “copy choice” mechanism as Coronaviruses have a large number of homologous RNA recombination. Finally, it is the ability to accommodate and modify genes as it possesses the largest known genome among all known RNA viruses. With high potential increase of diversity mutation may lead to the zoonotic disease outbreak of Coronavirus with disastrous results.

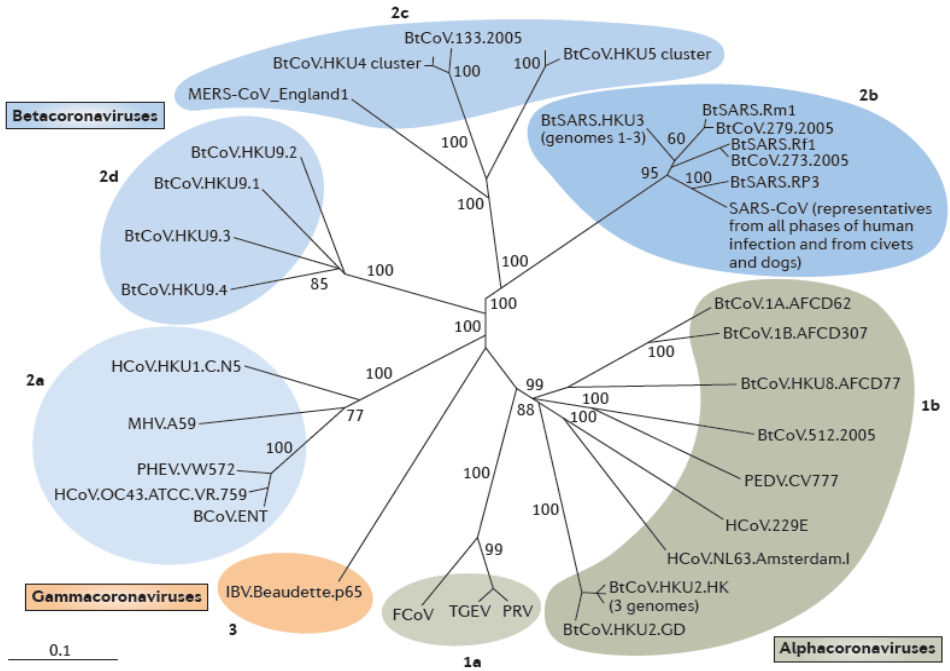


Figure 1. Classification of Coronavirus

Coronaviruses - enveloped viruses - with single stranded positive sense RNA genome about 30 kb in length that has a 5′ cap structure and 3′ polyadenylation tract. At First, the 5′ most open reading frame (ORF) of the viral genome in host cell is translated into a large polyprotein which is then cleaved by viral encoded proteases releasing different nonstructural proteins, including an RNA-dependent RNA polymerase, Adenosine Triphosphatase (ATPase) and helicase. Subsequently, these proteins replicate the viral genome and also generate nested transcripts used in the synthesis of the viral proteins. Transcription-regulating sequences (TRSs) located at the 5′ end of each gene represent signals which are used to regulate the discontinuous transcription of sub-genomic *m*RNAs. To illustrate those processing, the life cycle of coronaviruses was representative with SARS-CoV model (Figure 2).[6]

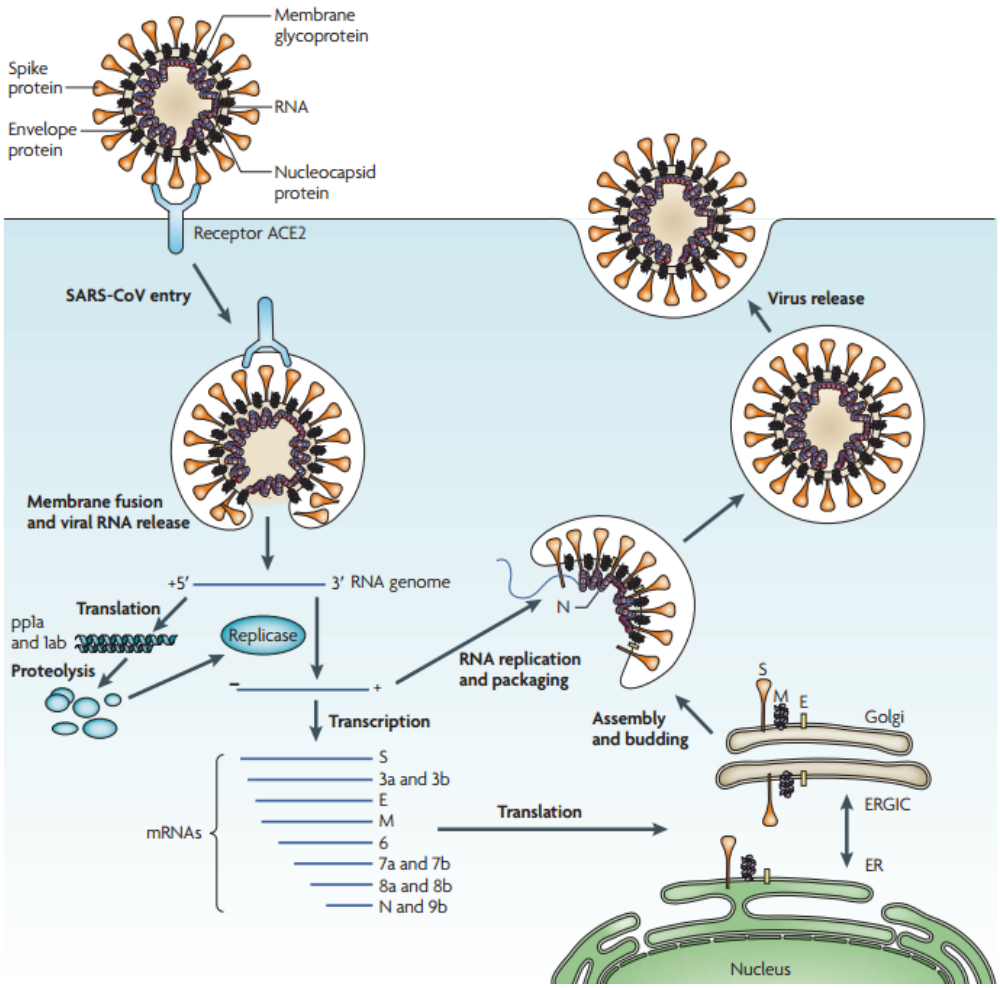


Figure 2. The life cycle of SARS-CoV in host cells

1.1.1. Porcrine epidemic diarrhea virus (PEDV)

In 1971, porcine epidemic diarrhoea (PED) was first observed among English feeder and fattening pigs.[7] During the 1980s and 1990s, PED was prevalent throughout Europe and is currently a source of concern in Asia, where outbreaks have been more acute and severe than those observed in Europe.[8]

PEDV, enveloped virus, owns an approximately 28 kb, positive-sense, single-stranded RNA genome with a 50 cap and a 30 polyadenylated tail.[9, 10] The genome consists a 50 untranslated region (UTR), a 30 UTR, and at least seven open reading frames (ORFs) that encode 4 structural proteins: spike (S), nucleocapsid (N), envelope (E) and membrane (M) and three non-structural proteins (Figure 3).[7, 11]

The PEDV S protein is a type I glycoprotein contains of 1,383 amino acids (aa). It comprises a signal peptide, neutralising epitopes, a transmembrane domain and a short cytoplasmic domain.[12-15] Similar with other coronavirus S proteins, the PEDV S protein is a glycoprotein peplomer (surface antigen) on the viral surface, where it has a pivotal role in regulating interactions with specific host cell receptor glycoproteins to mediate viral entry, and stimulating induction of neutralising antibodies in the natural host.[16, 17] Therefore, the S glycoprotein plays a primary target for the development of effective vaccines against PEDV.

The PEDV M protein is a triple-spanning structural membrane glycoprotein which is the most abundant envelope component. It has a short amino-terminal domain on the outside of the virus and a long carboxy-terminal domain on the inside.[18] The M protein plays an important role in the viral assembly process,[19, 20] in a-interferon (a-IFN) induction[21] and induces antibodies that neutralise the virus in the presence of its complement.[19, 20] Increasing the M glycoprotein research will help to understand about the genetic relationships, and the diversity of PEDV isolates and the epidemic situation of PEDV in the field.[22-25]

The N protein is a basic phosphoprotein associated with the genome. It binds to virion RNA and provides a structural basis for the helical nucleocapsid.[26] Interesting, it can be used as

the mark for the precise and early diagnosis of PEDV infection. It has also suggested that N protein epitopes may play importance in induction of cell-mediated immunity (CMI).[27]

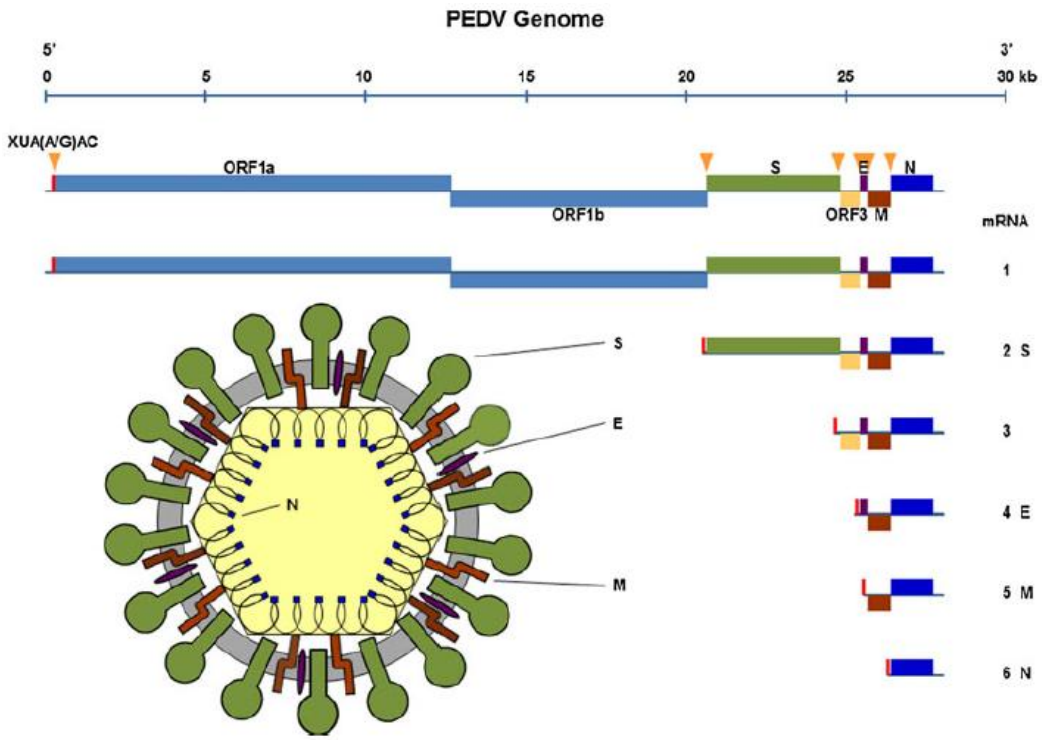


Figure 3. The structure of PEDV

1.1.2. Canine coronavirus (CCV)

Canine Coronavirus was first isolated from enteritis of dog in 1971.[28] When several CCVs have been outbreak in the world, they have become an important enteropathogen of the dog. The characteristics of CCV infection are high morbidity, low mortality and typical faecal-oral route of transmission.[29] Normally, systemic disease is not detected during CCV infection, although the virus has been isolated from various tissues of pups infected experimentally.[29] But, fatal disease commonly happens as a consequence of mingled infections with CCV together with canine parvovirus type 2 (CPV-2),[30, 31] canine distemper virus[32] or canine adenovirus type 1.[33]

Canine coronavirus is included in group 1 coronaviruses, which have type I and type II. Their evolution is related to feline coronavirus (FCoV) type I and type II. FCoV type II develops into heterologous recombination between CCV type II and FCoV type I, while FCoV type I is genetically similar to CCV type I. In addition, two FCoV biotypes that differ in pathogenicity have been observed in cats.[34]

1.1.3. Vaccines

The disease caused by PEDV was deficient in economic importance to start the vaccine development in Europe. Consequently, the trial of vaccine development was primarily constituted in Asian countries where the PEDV outbreaks have been so severe that the mortality of the new born piglets was risen.[35]

Similar to other groups of Coronavirus family, spike protein in PEDV is a glycoprotein peplomer- a surface antigen,[36] which contains 1,383 amino acids[37] and elicits a strong antibody response.[22] In addition, it is also responsible for mediating viral entry by interaction with the host cell's receptors.[38] Therefore, spike protein is used as a basal mark for the development of vaccines against PEDV.

Nowadays, the vaccines have been continually developed but satisfactory results have not come yet. Some reports have shown that the efficiency of such vaccines only reachs 50 % even at a very high dose.[39] In Korea, the vaccine against PEDV, DR13 strain, has been used but the results are not satisfactory.[4] However, only vaccine or another preventive measure is not sufficient to control the disease with consequences of the fatal magnitude. In addition to vaccines and preventive measures, the solutions to post infection condition still need to be explored further.

1.2. *Dryopteris crassirhizoma*

Dryopteris crassirhizoma from Dryopteridaceae family is a species growing in wet, shaded forests, open grassy areas, or along streams primarily in mountains (Figure 4).[40]

Classification:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Dryopteridales
Family	:	Dryopteridaceae
Genus	:	*Dryopteris*
Species	:	*D. crassirhizoma*

The rhizomes of this species have been used as a vulnerary, astringent, vermifuge, anti-inflammatory antibacterial and agent, and used internally in the treatment of haemorrhage, uterine bleeding and mumps.[40] Some phloroglucinol derivatives, such as albaspidins, norflavaspidic acids, flavaspidic acids, deaspidins, para-aspidins and filixic acids isolated from the genus *Dryopteris*[41] have been reported to possess the antioxidant, antibacterial and anti-tumor promoting activities.[42, 43] A previous study has demonstrated that acylphloroglucinols isolated from *D. crassirhizoma* inhibited fatty acid synthase, a potential therapeutic target to treat cancer and obesity.[44] Although a number of studies on the chemical constituents and biological activities of the genus *Dryopteris* was implemented, there have been still no researches with regard to its antiviral activities against coronavirus.

Figure 4. The plant, sliced dry rhizome and leaf of *Dryopteris crassirhizoma*

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

The rhizomes of *D. crassirhizoma* (1.0 kg) were purchased from a medicinal material market in Gwangju, South Korea and identified by Prof. Won Keun Oh, and a voucher specimen was deposited in College of Pharmacy, Seoul National University, Korea.

2.1.2. Chemicals reagents and chromatography

Silicagel (Merck, 63-200 μM particle size), Sephadex LH-20 and RP-18 (Merck, 40-63 μM particle size) were used for column chromatography. TLC was carried out with silica gel 60 F254 and RP-18 F254 plates. HPLC was carried out using a Gibson system with a UV detector and an Optima Pak C18 column (10 x 250 mM, 10 μM particle size, RS Tech, Korea). NMR spectra were obtained on a Varian Inova 500 MHz and 300 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). All solvents used for extraction and isolation were analytical grade. The EIMS data was measured on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer.

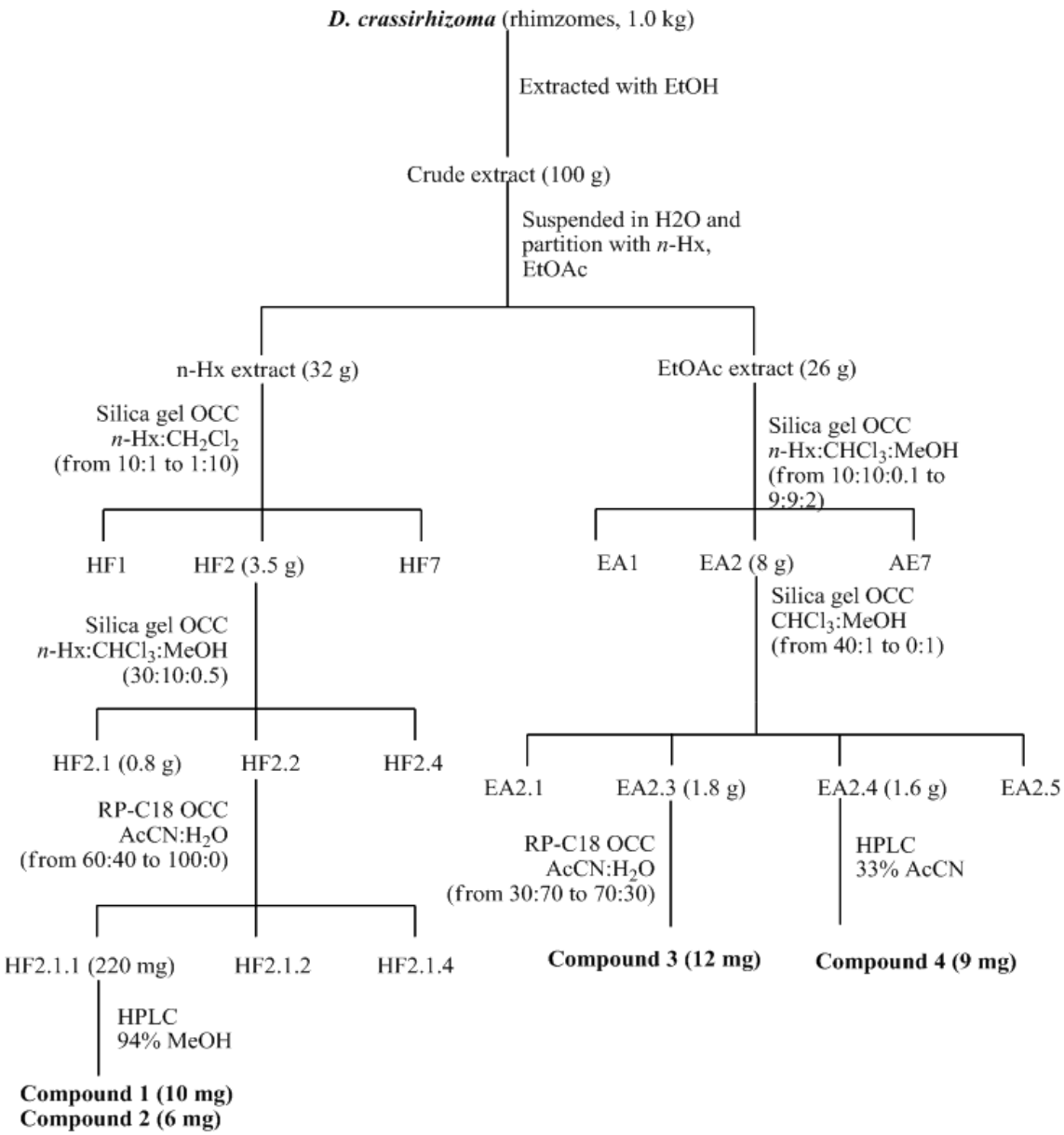
6-Azauridine was purchased from Sigma Chemical Company (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma, Korea.

2.2. Methods

2.2.1. Extraction and Isolation

All rhizomes were thoroughly dried and extracted with ethanol at room temperature for one week. As screening indicated *n*-hexane and EtOAc soluble parts inhibiting PEDV and CCV in a virus titer reduction assay, the obtained crude extract (100 g) was suspended in H_2O (2 L), and directly partitioned with hexane and EtOAc (2 L × 3, each solvent) sequentially. Further fractionation and separation was also guided by checking the inhibitory effect of each fraction on PEDV and CCV. The *n*-hexane part (32 g) was subject to a silica gel open column (4 × 20 cm; 63 – 200 μm particle size) eluting with *n*-hexane–CH_2Cl_2 (from 10:1 to 1:10) to give seven fractions (HF1 – HF7). The fraction HF2 (3.5 g) was further fractionated on a silica gel column (3 × 15 cm; 63 – 200 μm particle size) by *n*-hexane–$CHCl_3$–MeOH (30:10:0.5), to obtain four subfractions (HF2.1 – HF2.4). The fraction HF2.1 (0.8 g) was further subject to a reversed-phase column (1 × 20 cm; RP-C18, 40 – 63 μm particle size) eluted by AcCN–H_2O (from 60:40 to 100:0; 200 mL each) to yield four subfractions, HF2.1.1 to HF2.1.4. The fraction HF2.1.1 (220 mg) was further purified on a preparative HPLC system [YMC J-sphere ODS-H80® column (20 × 150 mm; 4 μm particle size); mobile phase AcCN in H_2O containing 0.1% HCO_2H (94% MeOH); flow rate 5 mL/min; UV detection at 254 nm] yielding compounds **1** (10 mg; t_R = 36.5 – 39.8 min) and **2** (6 mg; t_R = 42.0 – 46.0 min). The EtOAc part (26 g) was first chromatographed on a silica gel column (5 × 30 cm; 63 – 200 μm particle size) eluting with *n*-hexane–$CHCl_3$–MeOH (from 10:10:0.1 to 9:9:2) to provide seven fractions (EA1 – EA7). The fraction EA2 (8 g) was applied to a silica gel open column (4 × 20 cm; 63 – 200 μm particle size) eluting with $CHCl_3$–MeOH (from 40:1 to 0:1) to obtain five subfractions (EA2.1 – EA2.5). The fraction EA2.3 (1.8 g) was further purified using a reversed phase open column (2 × 20 cm; RP-C18, 40 – 63 μm particle size) eluting with AcCN–H_2O (from 30:70 to 70:30; 200 mL each) to afford compound **3** (12 mg). Part of EA2.4 (1.6 g) was purified by a

preparative HPLC [YMC J-sphere ODS-H80® column (20 × 150 mm; 4 μm particle size); mobile phase AcCN in H_2O containing 0.1% HCO_2H (33% AcCN); flow rate 5 mL/min; UV detection at 254 nm] giving compound **4** (9 mg; t_R = 25 – 30 min). By comparison of the spectroscopic data and optical rotation value with literature data, compounds **1 – 4** were elucidated as *albaspidin–PP* **(1)**, *albaspidin–PB* **(2)**, *norflavaspidic acid–AB* **(3)**, and *methylene-bis-methylphlorobutyrophenone* **(4)**, respectively (Figure 5).



Scheme 1. Isolation scheme of compounds from rhizomes of *D. crassirhizoma*

2.2.2. Viruses and cell

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) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Viruses were obtained from Choong Ang Vaccine Laboratory, Korea. Virus stock was stored at −80 °C until use.

2.2.3. Cytopathic effect (CPE) inhibition

2.2.3.1. Cytopathic effect assay

Vero cells were seeded onto 96-well culture plates at 1×10^5 cells per well. Next day, 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 hrs. The media was removed and replaced by DMEM with several compounds at different concentrations. Each concentration of compounds was determined in triplicate. The cultures were incubated for 3 days at 37 °C under 5% CO_2 atmosphere. Then, cells replaced with only DMEM and 20 µL of the 2 mg/mL MTT to each well and incubated at 37 °C for 4 hrs. After that, next steps were followed cytotoxicity assay and the 50% effective concentration (EC_{50}) was calculated by regression analysis. A selective index (SI) was determined using the formula SI = CC_{50}/EC_{50}.

2.2.3.2. Cytotoxicity assay

The cell viability was assessed using a MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide MTT methylthiazolyldiphenyl-tetrazolium bromide) based cytotoxicity assay. Vero cells were grown in 96-well plates at 1×10^5 cells per well and allowed to adhere for 24 hrs prior to treatment. The cells in 96-well plates were treated with various concentrations of compounds and incubated for 48 hrs. The final concentration of

DMSO in the culture medium was maintained at 0.05% (v/v) to avoid solvent toxicity. Subsequently, 20 µL of the 2 mg/mL MTT solution was added to each well of the plate and incubated 4 hrs. After removal of supernatant, 100 µL DMSO was added for solubilization of formazan crystals. Then the absorbance was measured at 550 nm. The percentage cell viability is expressed as toxicities of the compounds, where the higher the toxicity, the lower the cell viability. Percentage cell viability is defined as the absorbance in the experiment well compared to that in the control wells. Each experiment was carried out in triplicates. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis.

2.2.4. Virus titer reduction assay

In this assay, coronaviruses CCV, PEDV, TGEV, and IBV were respectively propagated into the appropriate host A-72 cell, Vero cell, STL cell, and SPF egg. All cell lines were maintained in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Antiviral activities of the compounds were determined through the virus yields reduction measured by the end-point titration technique. In short, quadruplicate confluent monolayers in 96-well microtitre plates were infected with 0.1 mL of serial tenfold dilutions of the virus suspension. The virus was allowed to absorb for 1 hr, then the dilutions of sample compounds in tissue culture medium were added. The cultures were incubated at 37 °C and examined microscopically after one day. The antiviral activity of each compound was determined by the difference between the virus titer in the absence and in the presence of the compound ($\log_{10}TCID_{50}$/mL).

2.2.5. 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 hrs. Then, media was removed and replaced by DMEM and cultured in the presence of various concentrations of compounds. After 24 hrs, total RNA was isolated from the cells following TRIzol method. The total RNA was reverse transcribed using random primer (iNtRON Biotechnology, INC, Korea) according to manufacturer's instruction. Real-time PCR was performed using selective primers for PEDV, and 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, 60 °C for 1 min. Real-time PCR was conducted using the Step one Plus Real-time PCR system. The data was analyzed with StepOne software v2.3 (Applied Biosystems).

Table 1. The selective primers for PEDV

Primer	Sequence (5'-3')
PEDVgp2 spike - forward	GCT AGT GGC GTT CAT GGT ATT TT
PEDVgp2 spike - reverse	ACG GCT CTT GCG AAA TGC
PEDVgp5 membrane - forward	TTT GAC GCA TGG GCT AGC T
PEDVgp5 membrane - reverse	GCA AGC CAT AAG GAT GCT GAA
PEDVgp6 Nucleocapsid - forward	GAA AAT CCT GAC AGG CAT AAG CA
PEDVgp6 Nucleocapsid - reverse	TTG CCG CTG TTG TCA GAC TT
Beta actin - forward	AGC GGG AA TCG TGC GTG ACA
Beta actin - reverse	GTG GAC TTG GGA GAG GAC TGG

2.2.6. Western blot analysis

The cultures were prepared similar methods with quantitative Real-time PCR. After 24 hrs, the cells were washed with cold PBS and stored at −80 °C. For whole cell lysate, the cells were lysed on ice in 100 µL lysis buffer [50 mM Tris-HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, 50 mM NaF] and centrifuged at 12,000 rpm for 20 min. Supernatants were collected from the lysates and protein concentrations were determined using protein assay kit (Bio-Rad Laboratories, Inc. USA). Aliquots of lysates were boiled for 5 min and 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 then incubated with primary antibodies spike (S) protein, Nucelocapsid (N) (Abfrontier, Korea) or mouse monoclonal actin antibody. The membranes were further incubated with secondary antibodies. Finally, they were detected using enhanced chemiluminescence Western blotting dectection kit (Thermo sci., Rockford, IL, USA).

2.2.7. Immunofluorescence assay

Vero cells were grown on 8-well chamber slides (LAB-TEK, NUNC, USA) and the cell monolayers were injected with PEDV at 0.01 MOI for 2 hrs. The solution was removed and replaced by DMEM, and treated with the compounds at the corresponding concentration. The cultures were incubated for 24 hrs at 37 °C under 5% CO_2 atmosphere. The cells were washed three times with PBS (pH 7.4) and fixed with a 4% paraformaldehyde solution for 30 min at room temperature. After blocking with 1% BSA for 1 hr, the cells were incubated overnight with monoclonal antibody against N protein of PEDV (Abfrontier, Korea) diluted 1:50 in PBS (pH 7.4). After washing with PBS (pH 7.4), the cells were incubated with FITC-conjugated goat anti-mouse lgG antibody (Jackson, ImmunoResearch, Inc.) for 1 hr. 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 3 times with PBS (pH 8.0). Slides were mounted with

mounting reagent for fluorescence (Vectashield, Vector Lab, Inc.) and observed by fluorescence microscopy (Olympus ix70 Fluorescence Microscope, USA).

2.2.8. Statistical analysis

The results are expressed as the means ± SD of three independent experiments. Statistical analysis was performed using Sigma Plot Statistical Analysis software. 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 either $p < 0.05$.

3. Results and Discussions

3.1. Isolation of compounds from rhizomes of *D. crassirhizoma*

Total 4 compounds (**1** to **4**) were isolated from the dried rhizomes of *D. crassirhizoma* by bioactivity guided fractionation using various successive chromatographic and other techniques like crystallization.

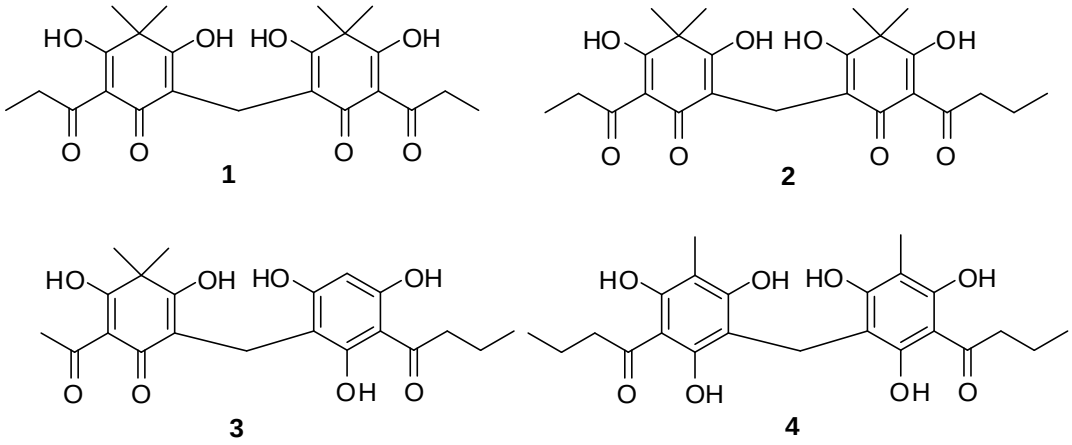


Figure 5. Structure of compounds (1 to 4) isolated from rhizomes of *D. crassirhizoma*

3.2. Structure determination of isolated compounds

Compound **1** (*albaspidin–PP*) was confirmed from the molecular ion peak at 431 [M - H]$^+$, 455 [M + Na]$^+$ in the ESI-MS. The ^{1}H-NMR in $CDCl_3$ spectra of compound **1** showed signals assignable to a phloroglucinol type [δ: 1.18 (6H, t, J = 7.2 Hz, H-10, -10′), 1.48 (6H, s, H-12, -12′), 1.55 (6H, s, H-13, -13′), 3.22 (4H, m, H-9, -9′), 3.32 (2H, br s, H-7)].

Compound **2** (*albaspidin–PB*): ESI-MS *m/z*: 445 [M – H]$^+$, 469 [M + Na]$^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.02 (3H, t, J = 7.2 Hz, H-11′), 1.18 (3H, t, J = 7.2 Hz, H-10), 1.48 and 1.49 (each 3H, s, H-12, -12′), 1.55 and 1.56 (each 3H, s, H-13, -13′), 1.70 (2H, m, H-10′), 3.17 (2H, m, H-9), 3.23 (2H, m, H-9′), 3.32 (2H, br s, H-7); 13C-NMR (400 MHz, CD_3OD) δ: 110.9 (C-1), 187.8 (C-2), 108.2 (C-3), 199.3 (C-4), 44.5 (C-5), 173.4 (C-6), 18.2 (C-7), 206.5 (C-8), 43.1 (C-9), 18.2 (C-10), 25.5 (C-12), 24.4 (C-13′), 110.9 (C-1′), 187.8 (C-2′), 108.1 (C-3′), 198.7 (C-4′), 44.4 (C-5′), 173.4 (C-6′), 207.4 (C-8′), 35.1 (C-9′), 8.6 (C-10′), 14.2 (C-11′), 25.5 (C-12′), 24.4 (C-13′).

Compound **3** (*norflavaspidic acid–AB*): ESI-MS *m/z*: 403 [M – H]$^+$, 427 [M + Na]$^+$; 1H-NMR (400 MHz, CD_3OD) δ: 0.99 (3H, t, J = 7.2 Hz, H-11′), 1.25 (6H, br s, H-12, -13), 1.68 (2H, m, H-10′), 2.43 (3H, s, H-9), 3.10 (2H, m, H-9′), 3.45 (2H, br s, H-7), 5.81 (1H, s, H-5′).

Compound **4** (*methylene-bis-methylphlorobutyrophenone*): ESI-MS *m/z*: 431 [M – H]$^+$, 455 [M + Na]$^+$; ^{1}H-NMR (400 MHz, acetone-d_6) δ: 0.95 (6H, t, J = 7.2 Hz, H-11, -11′), 1.67 (4H, m, H-10, -10′), 1.92 (6H, s, H-12, -12′), 3.10 (4H, m, H-9, -9′), 3.46 (2H, br s, H-7).

3.3. Anti-viral activity of isolated compounds against PEDV and CCV

All isolated compounds with purity more than 98 %, as assessed by HPLC and NMR spectroscopy, were tested for their inhibitory activity against CCV and PEDV by a virus titer reduction assay. The titer reduction assay was performed as previously described by the limit dilution method, using a 96-well microtiter plate with 4 wells per dilution.[20,21] The virus titer was estimated from cytopathogenicity of cells induced by viral infection and expressed as 50% tissue culture infectious doses/mL ($TCID_{50}$/mL). As shown in Table 2, the four compounds can significantly reduce the virus titer of CCV and PEDV. The titer of CCV control was $10^{5.5}$ $TCID_{50}$/mL whereas for those treated with compounds **1-4** at the final concentration of 25 μg/mL the titers were $10^{3.5}$, $10^{4.5}$, $10^{2.5}$ and 10^{2} $TCID_{50}$/mL respectively (reduced about 100, 10, 1000 and 3000 times respectively). The results were similar to PEDV, the virus titer of the control was 10^{6} $TCID_{50}$/mL whereas for those treated with compounds **1-4** the titers were $10^{3.5}$, $10^{3.5}$, 10^{4} and $10^{2.5}$ $TCID_{50}$/mL respectively (reduced about 300, 300, 100 and 3000 times respectively). These results showed that at the final concentration 25 μg/mL, these compounds can strongly inhibit virus replications and significantly increase cell survival after infected by the viruses. The results were more clearly when we performed this assay with different concentration of compound **4**, the strongest inhibitory compound. As shown in Figure 6, compound **4** express inhibitory activity against PEDV and CCV in a dose-dependent manner.

Table 2. Antiviral activities of phloroglucinols 1–4 from *D. crassirhizoma* against CCV and PEDV. Virus titers are presented as $\log_{10}$ 50% tissue culture infectious doses/mL ($TCID_{50}$/mL).

Compounds	Working concentration (μg/mL)	Virus titer*	
		PEDV	CCV
Control		6	5.5
Albaspidin-PP (**1**)	25	3.5	3.5
Albaspidin-PB (**2**)	25	3.5	4.5
Norflavaspidic acid-AB (**3**)	25	3	2.5
Methylene-bis-methylphlorobutyrophenone (**4**)	25	2.5	2

*Virus Titer : Log_{10} $TCID_{50}$/mL

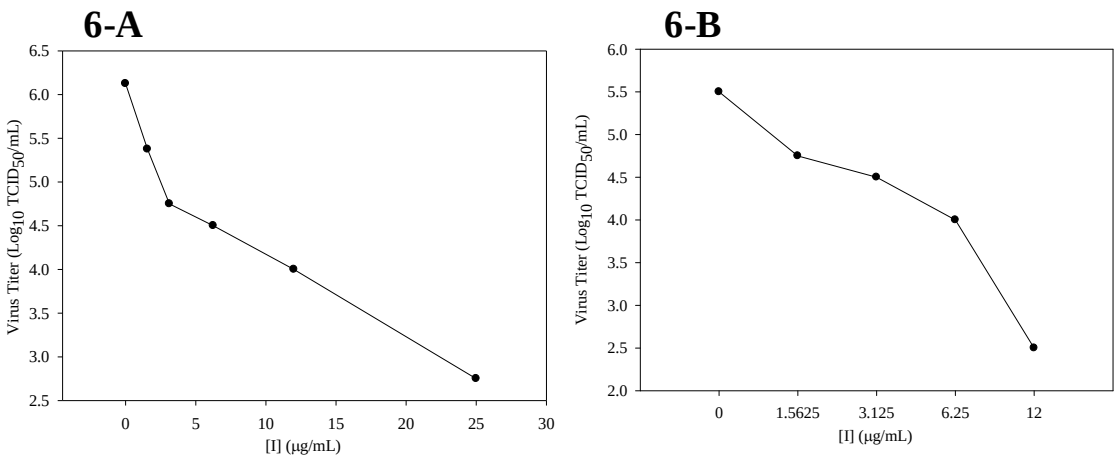


Figure 6. Antiviral activity of methylene-bis-methylphlorobutyrophenone (4) against PEDV (A) and CCV (B). Virus titers decreased when the concentration of the treatment compound increased. Virus titers are presented as $\log_{10}$ (50% tissue culture infectious doses/ml).

The purified four compounds (**1**–**4**) were evaluated against PEDV replication with azauridine as positive control. Vero cells were incubated with test compounds at different concentrations after inoculation with PEDV for 2 hrs. The data in Table 3 showed that, the inhibitory effects of compounds **3** and **4** exhibited stronger than positive control on PEDV replication with EC_{50} values at low micromolar range (2.5910 ± 0.1396 to 1.5761 ± 0.2386 μM). Moreover, compound **4** displayed highest the considerable selective index (SI) value (39.21 ± 0.27). Even though the structure activity relationships of these compounds have not been illustrated thoroughly, from data in Table 3 we may suggest that carbonyl group at C-2 and C-2′ could produce less antiviral activity. However, anti-PEDV inhibition increased when those positions were replaced by hydroxyl group.

Furthermore, to elucidate the action of compound **4** on against PEDV replication on the time-course study, we investigated the effect of compound **4** on virus replication after infection into Vero cells (1 hr to 20 hrs). The result suggests the compound **4** inhibited at an early stage of viral replication after infection (Figure 7).

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
Azauridine	4.73 ± 0.49	48.64 ± 2.76	10.28 ± 0.54

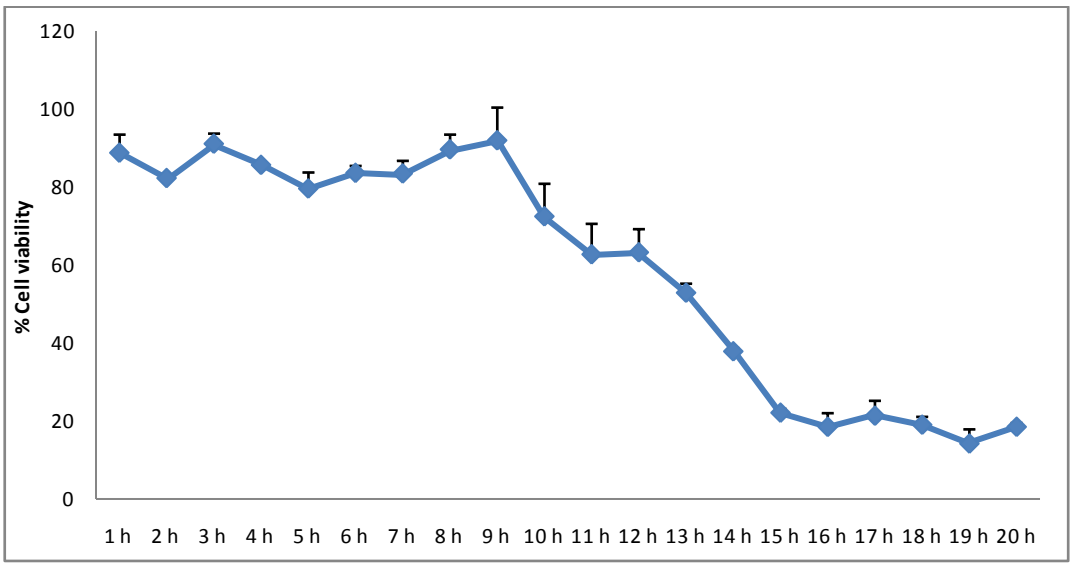
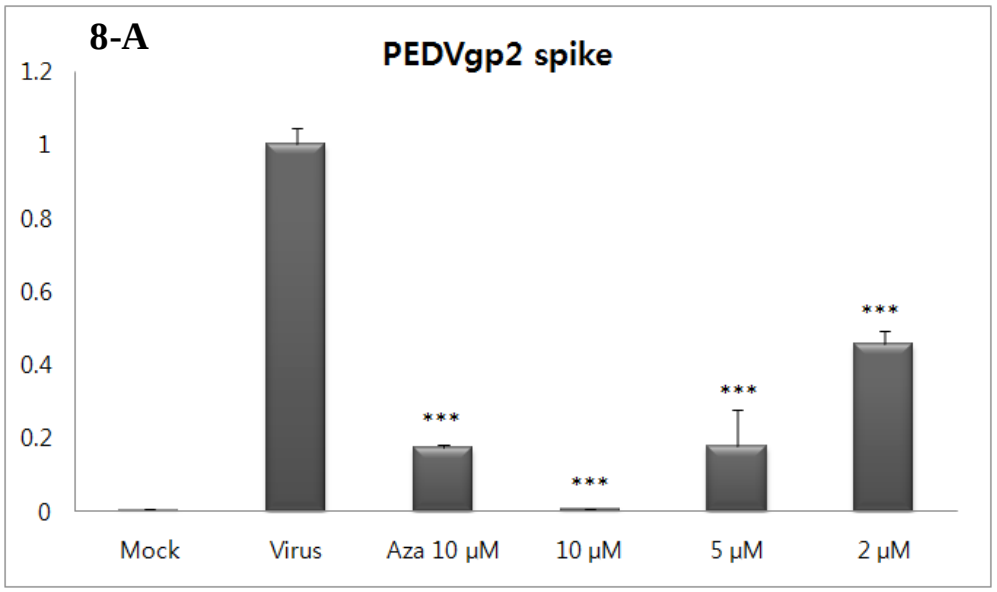
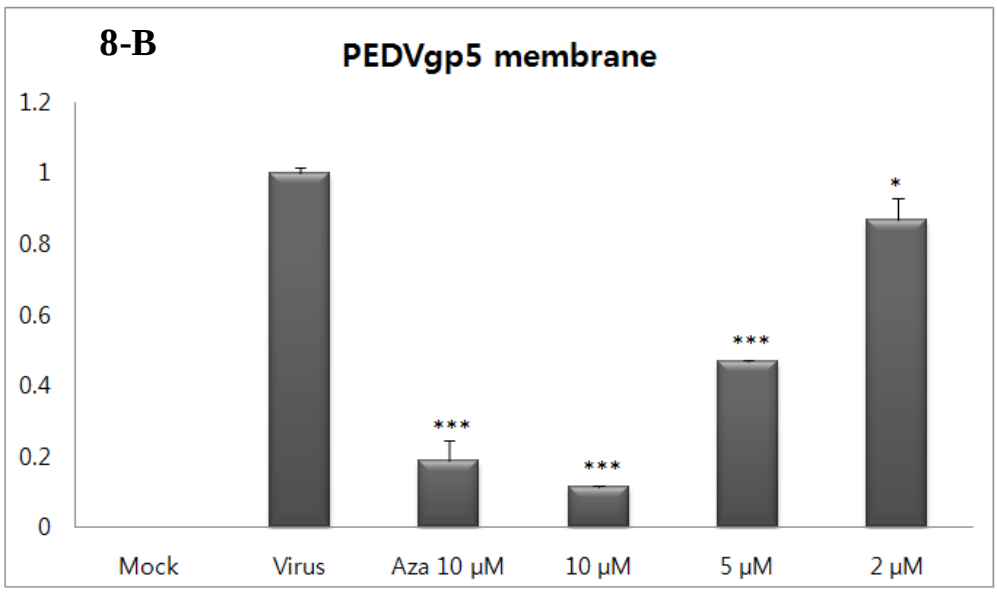


Figure 7. In vitro antiviral activity of compound 4 against PEDV replication on the time-course study

Considering their potent inhibition on PEDV replication, compound **4** was selected and evaluated for inhibitory effects on viral RNA synthesis. The levels of intracellular viral RNA (encoding PEDVgp2 spike, PEDVgp5 membrane and PEDVgp6 nucleocapsid) in viral-infected cells after treatment at different concentrations were measured by real-time PCR analysis. Total RNA was isolated from the cells after 24 hrs, and real-time PCR was performed using selective primers for PEDV. As shown in Figure 8 compounds **4** significantly reduced the RNA levels in dose-dependent manner, associated with PEDVgp2 spike (Figure 8A) PEDVgp5 membrane (Figure 8B) and PEDVgp6 nucleocapsid (Figure 8C), at concentrations of 10 μM, 5 μM and 2 μM, respectively. Moreover, to clarify compound **4** inhibited on PEDV replication dependent concentration and time, immunoblot analysis also confirmed that compound **4** decreased the level of spike protein and nucleocapsid protein after 24 hrs treatment with viral-injected cells and inhibited at an early stage of viral replication after infection (Figure 9). We then examined the emission of green fluorescence of nucleocapsid

production. As shown in Figure 10, compound **4** treatment reduced green fluorescence in a concentration-dependent manner.





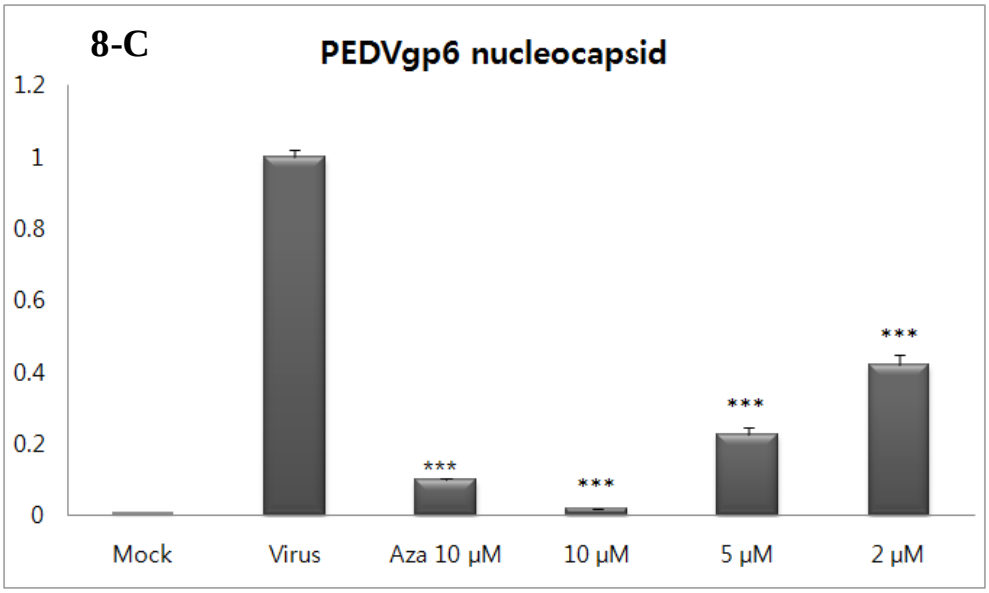
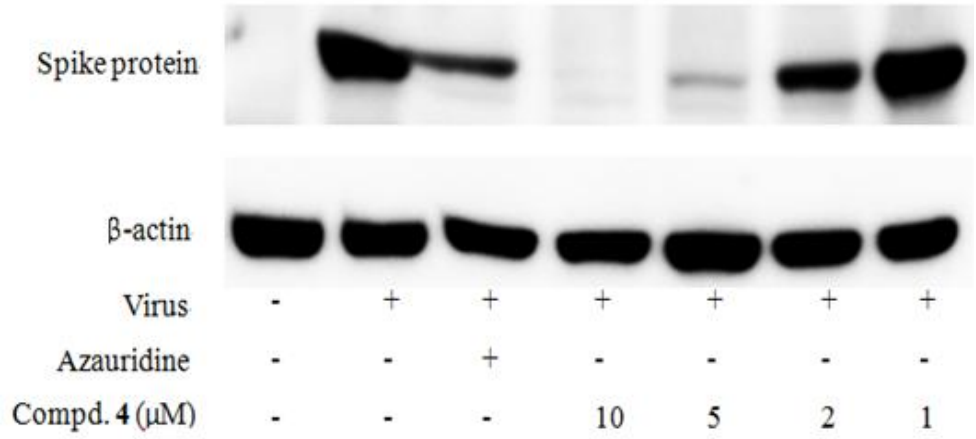
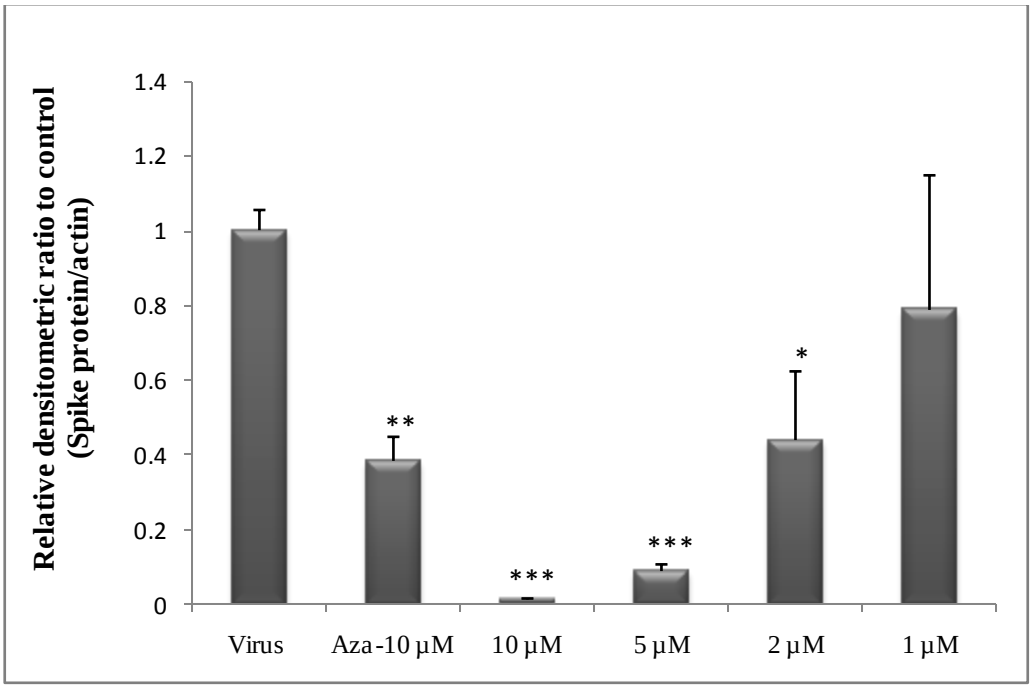


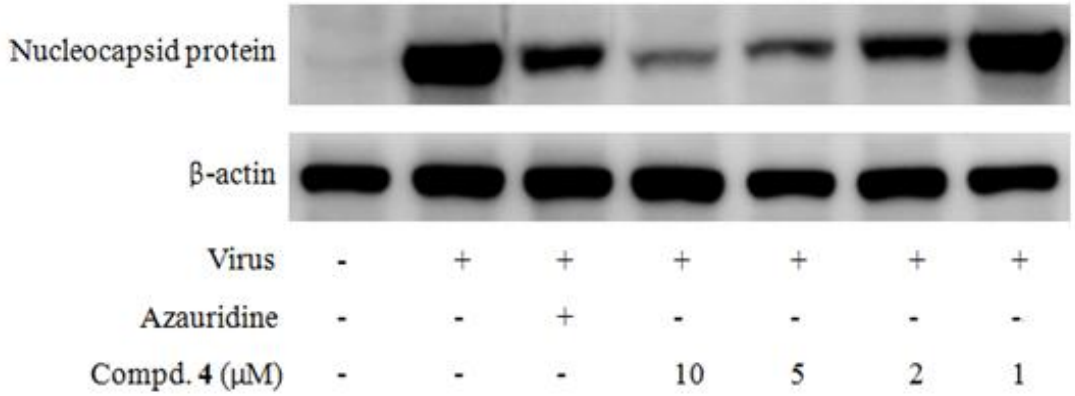
Figure 8. Quantitative RT-PCR analysis demonstrating the inhibition of PEDV replication by compound 4 via suppressing genes encoding viral structural protein synthesis. Dose-dependent inhibition of gene encoding PEDVgp2 spike protein (**A**); PEDVgp5 membrane protein (**B**) and PEDVgp6 nucleocapsid protein (**C**).

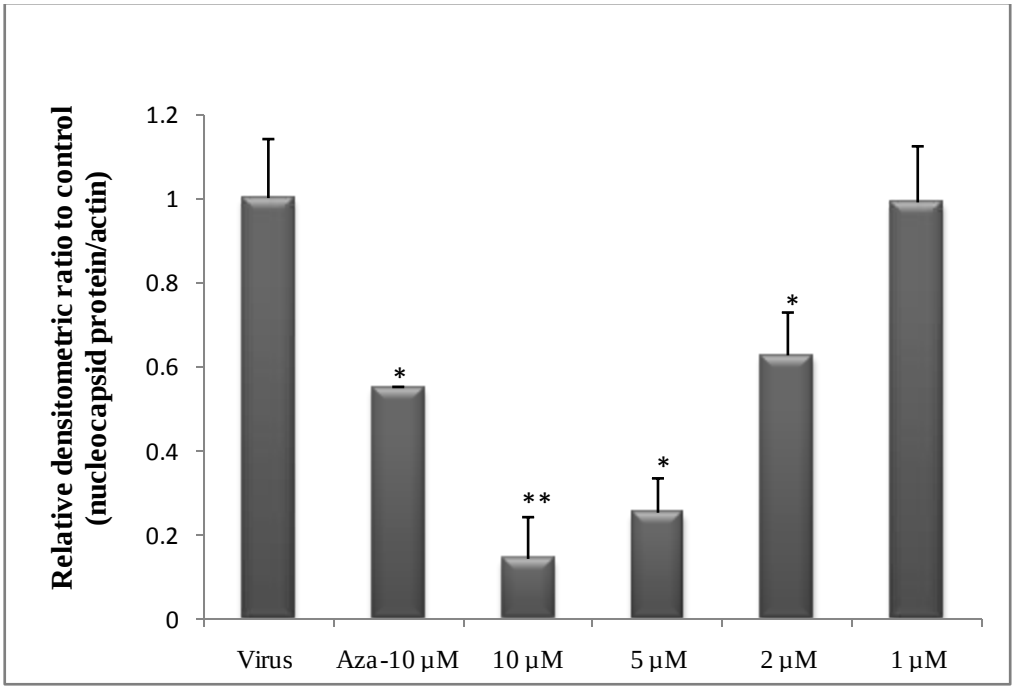
9-A





9-B





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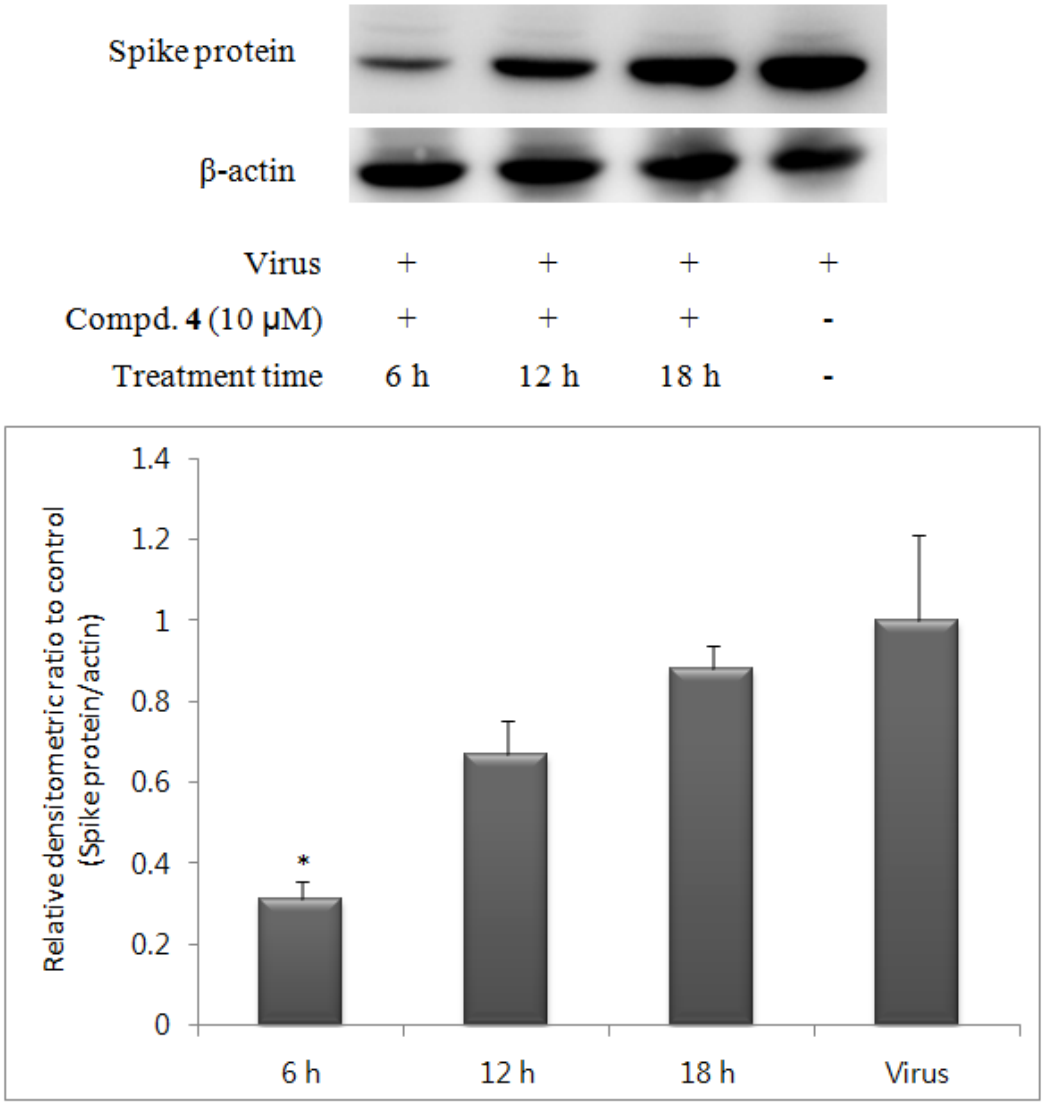

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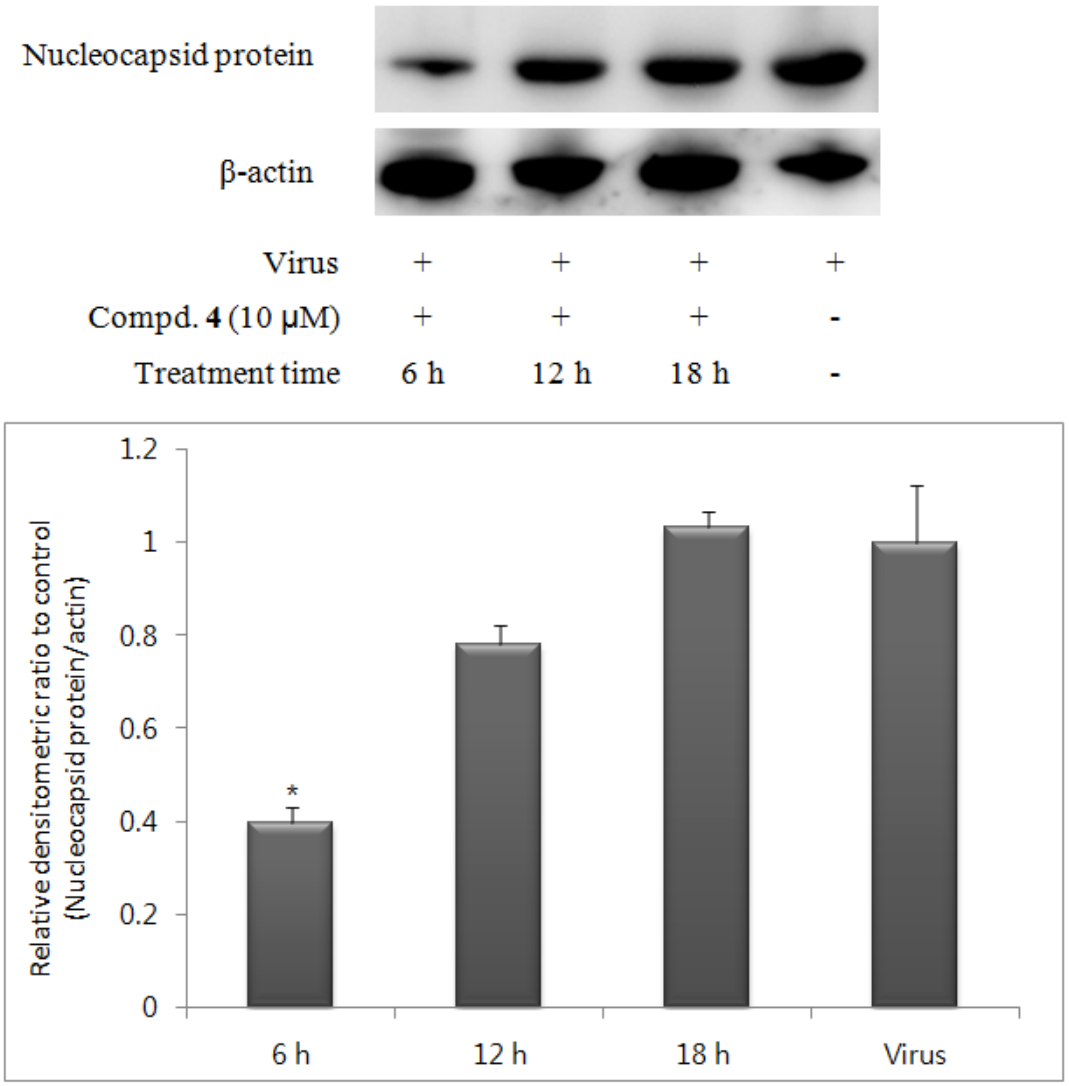

Figure 9. Western blot analysis revealing the inhibitory effects of compound 4 on viral structural protein synthesis. Dose-dependent inhibition of spike protein (**A**) and nucleocapsid protein (**B**); Time dependent inhibition of spike protein (**C**) and nucleocapsid protein (**D**) at concentrations 10 μM.

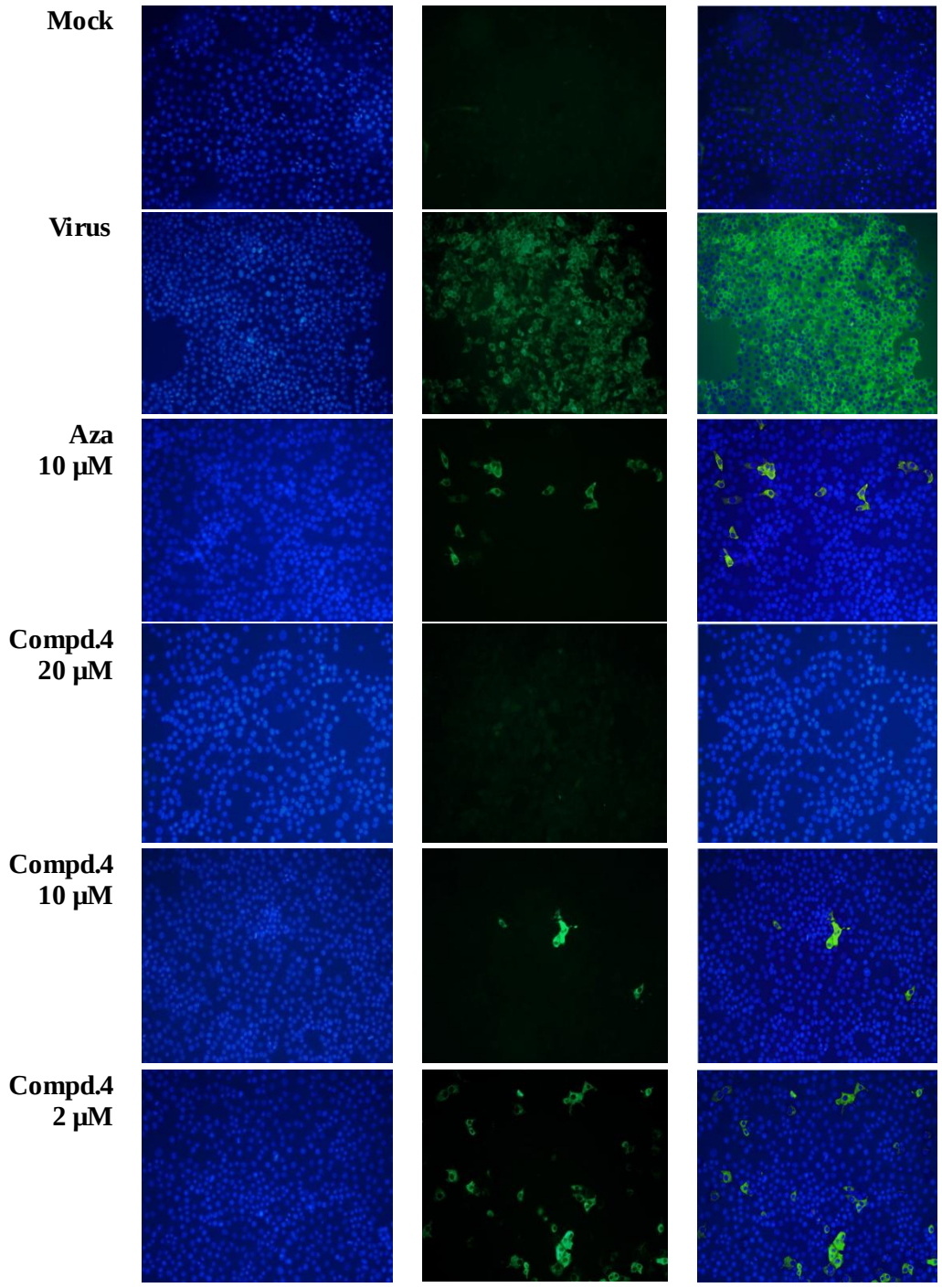

Figure 10. Immunofluorescence images showing compound 4 inhibited PEDV replication in dose-dependent manner.

4. Conclusions

Porcine and canine epidemic diarrhea becomes a serious illness in pigs and dogs with disastrous consequences in cattle based agro-economy. Several vaccines have been licensed in few countries, but their efficiency being undependable, the cattle industry is still in serious threat. Furthermore, dangerous viruses from coronavirus family such as SARS, have been a culprit to human epidemics and still posture a threat with recently new Coronavirus being found killing many people in the world. Because of the same family of viruses, they share some similar replication mechanism, the molecules against PEDV and CCV maybe have significant activities on human protection from the other Coronaviruses. Therefore, with an objective of finding efficient compounds against that virus, the plant *Dryopteris crassirhizoma* was studied by bioactivity guided fractionation leading to isolation of four phloroglucinol derivatives which significantly inhibited the PEDV and CCV. Especially, compound **4** (*methylene-bis-methylphlorobutyrophenone*) was found to have good antiviral property with viral replication inhibition at an early stage and in dose-dependent manner. Although the structure activity relationships of these phloroglucinol derivatives have not been investigated thoroughly, our results suggest that the hydroxyl group at C-2 and C-2′ is important for increasing the activity. Thus, this compound could be used as a marker component for quality control of antiviral botanical supplement. Further studies on the synthesis of its analogs and the mechanism of action may provide an opportunity for development of new therapeutic agents against not only PEDV and CCV but also other types of Coronaviruses.

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