Antiviral Activity of *Corylus heterophylla* Fisch Against Porcine Epidemic Diarrhea Virus Infection

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The porcine epidemic diarrhea virus (PEDV) has recently been shown to cause huge economic losses in the global pork industry. Our results demonstrated that the extract dose-dependently inhibited the replication of PEDV and reduced the visible cytopathic effect (CPE). Treatment with *C. heterophylla* Fisch extract resulted in marked reduction of PEDV-induced cytokine and chemokine expression. The antiviral activity of *C. heterophylla* Fisch extract on PEDV replication was found to be primarily exerted at the early stages after infection. Taken together, our data indicate that *C. heterophylla* Fisch extract may be a good therapeutic agent for use against PEDV and also a potential candidate to be evaluated against other human and animal coronaviruses.

Key Words: *Corylus heterophylla* Fisch, Porcine epidemic diarrhea virus (PEDV), Antiviral activity

INTRODUCTION

Porcine epidemic diarrhea (PED), caused by the PED virus (PEDV), was first recognized in the United Kingdom in 1971 (1). PEDV is a devastating enteric disease that is characterized by vomiting, acute severe watery diarrhea, and dehydration (2). PEDV infections can occur in pigs of all ages, but infections are most serious in piglets, with morbidity and mortality often reaching 100% (3). PEDV infection has resulted in high economic losses in the Asian pig industry and the disease was also reported in 2013 in North America and in 2014 in South America. In addition, outbreaks were reported in Europe during 2015 (4, 5).

The optimum option is use commercial vaccine against PEDV infection. Recently, several PEDV vaccines have been developed (6, 7); however, the efficacy of the available commercial vaccines is limited and protective immunity is insufficient (8). Therefore, a highly effective, rapid-acting antiviral strategy against PEDV is needed.

Medicinal plants have been traditionally used for the treatment of various diseases or symptoms. The genus *Corylus*
among them consists of deciduous species that naturally occur in temperate forest areas in Europe, the Middle East, Asia, and North America (9). *Corylus heterophylla* Fisch is the most widely distributed Betulaceae plant in China and its yield accounts for more than 70% of total output in the domestic market (10). *C. heterophylla* Fisch plays a key role in water and soil conservation and in the ecological balance of certain types of forests (10). *Corylus* species are also an important source of taxol (paclitaxel), which is an effective medicine for the treatment of breast, ovarian, and lung cancer (11–13). In this study, we investigated whether *C. heterophylla* Fisch extracts exerted antiviral activity against PEDV in vitro. In addition, we elucidated the detailed action of *C. heterophylla* Fisch extract on PEDV replication.

**MATERIALS AND METHODS**

**Virus, cells, and reagents**

Vero (African green monkey kidney cell line; ATCC CCR-81) cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). PEDV CV 777 was obtained from the National Veterinary Research & Quarantine Service in Korea. To maintain the Vero cells, minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic for Streptomycin, Penicillin, Amphotericin B were used. Antibiotic-antimycotic, trypsin-EDTA, FBS, and MEM were acquired from Gibco BRL (Grand Island, NY, USA). Tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ, USA).

**Sample procurement**

*C. heterophylla* Fisch extract was supplied by the Ginseng Research Division, National Institute of Horticultural and Herbal Science, Eumseong-gun, Chungcheongbuk-do, Korea. Dried and ground the materials (100 g) from *C. heterophylla* Fisch were finely powdered with a blender. After powdered, which *C. heterophylla* Fisch powder were macerated in 1 liter of methanol at room temperature for 3 d. Then the macerate was filtered (Whatman No. 2) and the process repeated two times using 1 liter of methanol for maceration. The combined filtrates were evaporated using 40 °C water bath to afford 8 g of a dark-green residue on removal of solvent by rotary evaporator. The antiviral activity of *C. heterophylla* Fisch methanol extract was determined with a 5-fold diluted concentration ranging from 0.4 to 50 μg/ml in DMSO.

**Antiviral activity assay**

Assays of antiviral activity were evaluated by the sulforhodamine B (SRB) method using cytopathic effect (CPE) reduction, as previously reported (14). Vero cells were seeded onto a 96-well culture plate at a concentration of 3 × 10⁴ cells per well. After 24 h, the cells were washed with phosphate buffered saline (PBS) in preparation for PEDV infection. A total of 90 μl of diluted PEDV suspension, containing the 50% tissue culture infective dose (TCID₅₀) of the virus stock to produce appropriate CPE within 2 d after infection and 10 μl of medium supplemented with trypsin-EDTA containing an appropriate concentration of the extract, was added. The antiviral activity of each extract was determined at five-fold diluted concentrations (0.4, 2, 10, and 50 μg/ml). Three wells were used as virus controls (virus-infected non-drug-treated cells) and three wells were used as cell controls (non-infected non-drug-treated cells). The culture plates were incubated at 37 °C in 5% CO₂ for 2 d. After washing one time with 1 × PBS, 100 μl of cold (-20 °C) 70% acetone was added to each well and the plates were left for 30 min at -20 °C. The 70% acetone was removed and the 96-well plates were left in a dry oven for 30 min. A total of 100 μl of 0.4% (w/v) SRB in 1% acetic acid solution was added to each well and left at room temperature for 30 min. Unbound SRB was removed and the plates were washed five times with 1% acetic acid. After drying for 24 h in a dry oven, the morphology of the cells was observed under a microscope at 0.4 × 10 magnification (Axiovert 10; Zeiss, Wetzlar, Germany) to identify the effect of the compounds on PEDV-induced CPE and images were recorded. Bound SRB was solubilized with 100 μl of 10 mM unbuffered tris-base solution and the plates were left on a table for 30 min. The absorbance was read at 540 nm using a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) with
a reference absorbance of 620 nm. To calculate the 50% inhibitory concentration (IC₅₀) value, the results were transformed to percentage of controls and the IC₅₀ value were graphically obtained from dose-response curves. The percent protection achieved by the extract in PEDV-infected cells was calculated using the following formula:

\[
\left( \frac{(OD_{t,\text{PEDV}} - OD_{c,\text{PEDV}})}{(OD_{c,\text{mock}} - OD_{c,\text{PEDV}})} \right) \times 100
\]

(expressed in %)

where \((OD_{t,\text{PEDV}})\) was the optical density measured with a given concentration of the test compound in PEDV-infected cells; \((OD_{c,\text{PEDV}})\) was the optical density measured for the control untreated PEDV infected cells; and \((OD_{c,\text{mock}})\) was the optical density measured for control untreated mock-infected cells.

The concentration achieving 50% protection according to the formula above was defined as the IC₅₀. The therapeutic index (TI) was defined as the 50% cytotoxicity concentration (CC₅₀)/IC₅₀ and was used to determine the effect of

\(C.\ heterophylla\) Fisch extract on the infectivity of PEDV particles.

**Cytotoxicity assay**

Cytotoxicity was conducted parrelly with antiviral assay, as above described (14). Vero cells were seeded onto a 96-well culture plate at a concentration of \(3 \times 10^4\) cells per well. After 24 h, the medium was removed and replaced with media containing the serially diluted extract. The cells were then further incubated for 48 h. The culture medium was removed and washed with PBS. An antiviral activity assay was conducted as described above. To calculate the CC₅₀ values, the results were transformed to percentage of controls and the CC₅₀ values were graphically obtained from the dose-response curves.

**Cytokine and chemokine measurement**

The levels of interleukin-6 (IL-6), interleukin-1 beta (IL-1β), chemokine (C-X-C motif) ligand 1 (CXCL1)/KC, and

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**Figure 1. The effects of \(C.\ heterophylla\) Fisch extract on PEDV-induced CPE.** Morphological assessment of PEDV-infected Vero cells following treatment with \(C.\ heterophylla\) Fisch extract. (A) Non-infected cells; (B) non-infected cells treated with \(C.\ heterophylla\) Fisch extract 10 µg/ml; (C) non-infected cells treated with \(C.\ heterophylla\) Fisch extract 50 µg/ml; (D) PEDV-infected cells; (E) PEDV-infected cells treated with \(C.\ heterophylla\) Fisch extract 10 µg/ml; (F) PEDV-infected cells treated with \(C.\ heterophylla\) Fisch extract 50 µg/ml.
chemokine (C-C motif) ligand 2 (CCL2) were determined using real time PCR. Vero cells with and without PEDV infection were treated with *C. heterophylla* Fisch extract (10 and 50 μg/ml). In addition, uninfected cells were treated with dimethyl sulfoxide (DMSO). After 6 h, total RNA was extracted using a QIAamp1 viral RNA mini kit (Qiagen, Limburg, Holland). cDNA was reverse-transcribed from 1 μg of total RNA using oligo (dT) primer and SuperScript™ II reverse transcriptase (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. For real-time PCR analysis, the cDNA was serially diluted 10-fold and amplified using a 7500 real time PCR system (Applied Biosystems, Foster City, CA, USA) with Power SYBR® Green PCR master mix (Applied Biosystems). The following primers were used: β-actin (sense 5'-CCA TCA TGA AGT GTG ACG TGG-3', antisense 5'-GTC CGC CTA GAA GCA TTT GCG-3'), CCL2 (sense 5'-TTA AAA ACC TGG A TC GGA ACC AA-3', antisense 5'-GTC CGC CTA GAA GCA TTT GCG-3'), CXCL1/KC (sense 5'-TGA GCT GCG GTG TTA CGG GT-3', antisense 5'-GTC CGC CTA GAA GCA TTT GCG-3'), IL-6 (sense 5'-CTG GAG TCA CAG AAG GAG TGG-3', antisense 5'-GTT TTG CCG AGT AGA TCT CAA-3'), and IL-1β (sense 5'-AAT CTG TAC CTG TCC GTG TT-3', antisense 5'-TGG GTA ATT TTT GGG ATC TAC ACT CT-3').

**Time-of-addition**

The time-of-addition effect of *C. heterophylla* Fisch extract was examined according to previously described procedures with minor modifications (15). Vero cells were seeded onto 96-well culture plates at a density of 3 × 10⁴ cells per well and incubated for 1 d. After washing with 1 × PBS, each 10 μg/ml of the *C. heterophylla* Fisch extract was then added to the cells either before (-1 h), during (0 h), or after (1, 2, 4, 6, 8, and 10 h) PEDV infection. After 48 h, antiviral activity was analyzed using the above-described SRB method.

**Statistical analysis**

One-way ANOVA followed by the Tukey post-hoc test using GraphPad Prism version 5 software (Graphpad, San Diego, CA, USA) was used to compare multiple groups. Values of *p* < 0.05 were considered significant at a 95% confidence level.
RESULTS

Antiviral activity of *Corylus heterophylla* Fisch extract against PEDV

After 2 d of PEDV infection, Vero cells treated with mock and *C. heterophylla* Fisch extract (10 and 50 μg/ml) did not display a difference in typical spread-out shape and showed normal morphology. PEDV-infected Vero cells treated with *C. heterophylla* Fisch extract (10 and 50 μg/ml) exhibited a reduced CPE compared with untreated PEDV-infected cells (Fig. 1). These results indicated that the CPE of PEDV infection is prevented by *C. heterophylla* Fisch extract at concentrations of 10 and 50 μg/ml.

To evaluate the antiviral activity of *C. heterophylla* Fisch extract, Vero cells were infected with PEDV and treated with *C. heterophylla* Fisch extract. *C. heterophylla* Fisch extract

<table>
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<tr>
<th>Test material</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; a</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; b</th>
<th>TI&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td><em>C. heterophylla</em> Fish</td>
<td>&gt; 50</td>
<td>2.87 ± 0.53</td>
<td>17.42</td>
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</table>

Results are presented as the mean IC<sub>50</sub> values obtained from three independent experiments carried out in triplicate ± S.D.

<sup>a</sup> Concentration required to inhibit virus-induced CPE by 50% (μg/ml).

<sup>b</sup> Concentration required to reduce cell growth by 50% (μg/ml).

<sup>c</sup> Therapeutic index = CC<sub>50</sub>/IC<sub>50</sub>.

Figure 3. Cytokine and chemokine gene expression levels reduced by *C. heterophylla* Fisch extract in Vero cells. Proinflammatory cytokines and chemokines measured in PEDV-infected Vero cells treated with *C. heterophylla* Fisch extract 6 h after infection.
displayed strong antiviral activity against PEDV, as indicated in Table 1. At concentrations of 10 and 50 μg/ml, cell viability was 80% or greater compared with control (Fig. 2A). In addition, the CC<sub>50</sub> value of <i>C. heterophylla</i> Fisch extract was greater than 50 μg/ml (Fig. 2B).

**Cytokine and chemokine levels in Vero cells treated with <i>C. heterophylla</i> Fisch extract**

Virus-induced chemokines and cytokines play a major role in recruiting leukocytes to the site of infection and activating innate immune responses to induce inflammation. Chemokine and cytokine production was evaluated at the gene expression level in PEDV-infected untreated Vero cells and in those treated with <i>C. heterophylla</i> Fisch extract, as well as uninfected Vero cells treated with DMSO and <i>C. heterophylla</i> Fisch extract. Compared with DMSO-treated Vero cells, PEDV-infected Vero cells displayed increased CCL2, IL-1β, IL-6, and CXCL1 gene expression levels 6 h after infection. There was no difference in cytokine and chemokine gene expression in uninfected Vero cells treated with <i>C. heterophylla</i> Fisch extract at concentrations of 10 and 50 μg/ml, compared with uninfected DMSO-treated Vero cells. Treatment of PEDV-infected Vero cells with <i>C. heterophylla</i> Fisch extract decreased the gene expression levels of CCL2, IL-1β, IL-6, and CXCL1 at concentrations of 10 and 50 μg/ml. These results suggested that <i>C. heterophylla</i> Fisch extract decreased gene expression of cytokine and chemokine at concentrations of 10 and 50 μg/ml in Vero cells (Fig. 3).

**Time-of-addition**

<i>C. heterophylla</i> Fisch extract was added to cells 1 h before PEDV infection and 0, 1, 2, 4, 6, 8, 10, and 12 h after infection. As indicated in Fig. 4, <i>C. heterophylla</i> Fisch extract displayed an inhibitory effect against PEDV infection after 0, 1, 2, 4, and 6 h with antiviral activity greater than 60%. However, at the other time points (-1, 8, 10, and 12 h), the antiviral activity of <i>C. heterophylla</i> Fisch extract was 40% or less.

**DISCUSSION**

PEDV is the causative agent of PED, dehydration, vomiting, and high mortality in piglets (12). To our knowledge, there have been no effective treatments or vaccines developed to prevent the economic loss associated with PEDV.

Traditionally, natural oriental herbal medicines have been used to relieve the symptoms of and cure viral infections, including cold, flu, and other viral diseases (16–21). In this study, <i>C. heterophylla</i> Fisch extract exhibited anti-PEDV activity with an IC<sub>50</sub> of 2.87 μg/ml, as determined by a CPE reduction assay (Table 1). In addition, it displayed no cytotoxicity to cells at the highest concentration tested (50 μg/ml). Therefore, <i>C. heterophylla</i> Fisch extract, with its potent inhibitory effect on the proliferation of PEDV, is a lead candidate for PEDV therapy.

A host’s innate immune system has the capacity to recognize a virus as foreign by pattern recognition receptors (PRRs), which leads to the expression of type I interferons (IFNα/β), pro-inflammatory cytokines, and chemokines (22). The secretion of pro-inflammatory cytokines and chemokines causes the recruitment of immune cells to combat the viral infection (23). <i>C. heterophylla</i> Fisch extract significantly reduced PEDV-induced expression of CCL2, IL-1β, IL-6,
and CXCL1 at concentrations of 10 and 50 μg/ml (Fig. 3).

To elucidate the action of *C. heterophylla* Fisch extract on PEDV multiplication in more detail, we investigated the effect of *C. heterophylla* Fisch extract on single steps of the infection cycle of PEDV. Based on the results of this time-course study, pre-incubation of the Vero cells with *C. heterophylla* Fisch extract did not protect the cells from PEDV infection (Fig. 4). Furthermore, *C. heterophylla* Fisch extract inhibited PEDV infection when it was added at the time of infection or during the early stages after viral inoculation (1, 2, 4, and 6 h), but not after 8 h or more (Fig. 4). This suggested that the mechanism of action involves inhibition of the early stages of viral replication after infection.

In conclusion, the present study indicated that *C. heterophylla* Fisch extract possessed anti-PEDV activity via inhibition of the early stages of viral replication. Treatment by *C. heterophylla* Fisch extract significantly changed the morphology of PEDV-infected Vero cells, inhibiting the formation of a visible CPE. *C. heterophylla* Fisch extract also effectively reduced PEDV-induced expression of CCL2, IL-1β, IL-6, and CXCL1. However, it isn't evident that the antiviral mechanism of *C. heterophylla* Fisch extract must be fully understood in order to develop a candidate for the treatment of PEDV infections. Therefore, we are planning to isolate pure compound showing antiviral activity from *C. heterophylla* Fisch extract and study detailed antiviral mechanism.

REFERENCES

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