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Ethanolic Extract from *Echinacea purpurea* (L.) Moench Inhibits Influenza A/B and Respiratory Syncytial Virus Infection *in vitro*: Preventive Agent for Viral Respiratory Infections

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ABSTRACT: Among the most frequent causes of respiratory infections in humans are influenza A virus H1N1 (H1N1), influenza B virus (IVB), and respiratory syncytial virus (RSV). Echinacea is a perennial wildflower belonging to the *Asteraceae* family. *Echinacea purpurea* (L.) Moench is a species belonging to the Echinacea genus. Its characteristic compound, chicoric acid (CA), is known for its physiological activities, including antiviral effects and immune enhancement. Activities of *E. purpurea* 60% ethanol extract (EPE) and CA in inhibiting infections caused by H1N1, IVB, and RSV subtype A (RSV-A) were evaluated through plaque inhibition tests, quantification of viral gene expression, and analysis of transmission electron microscopy (TEM) images. Additionally, inhibitory activities of EPE and CA for hemagglutination and neuraminidase (NA) of H1N1 and IVB were determined. In the plaque reduction assays, both EPE and CA reduced infectivity against H1N1, IVB, and RSV-A. Furthermore, quantitative real-time polymerase chain reaction analysis revealed that EPE and CA reduced gene expression levels for H1N1, IVB, and RSV-A, whereas TEM image analysis confirmed their inhibitory effects on host cell infection by these viruses. Hemagglutination assays exhibited the ability of EPE and CA to hinder H1N1 and IVB attachment to host cell receptors. Furthermore, EPE and CA displayed inhibition activity against the NA of H1N1 and IVB. These findings suggest that EPE and CA can suppress the infection and propagation of H1N1, IVB, and RSV-A, demonstrating their potential as preventive and therapeutic agents for viral respiratory infections or as ingredients for health functional foods.

Keywords: chicoric acid, *Echinacea* extract, human influenza, respiratory syncytial virus, respiratory tract infections

INTRODUCTION

Respiratory virus infections are caused by various viruses, including influenza A and B viruses, severe acute respiratory syndrome virus, respiratory syncytial virus (RSV), and human rhinovirus. These viral respiratory infections tend to increase during the winter season (Uhteg et al., 2021), with influenza A/B and RSV being major contributors, especially during an influenza epidemic season (Borchers et al., 2013; Gaitonde et al., 2019; Centers for Disease Control and Prevention, 2023).

Among negative-sense RNA viruses in the *Orthomyxoviridae* family, influenza viruses can be classified into three types: A, B, and C. Among these, types A and B frequently cause widespread respiratory infections and diseases in humans (Paules and Subbarao, 2017; Keilman, 2019). Of the three, influenza A viruses pose the greatest threat as they can easily undergo antigenic variation, thereby leading to increased infectivity and the potential for epidemics (Paules and Subbarao, 2017) Influenza A viruses are categorized into different subtypes on the basis of antigenic properties of their viral surface proteins hemagglutinin (HA) and neuraminidase (NA). Subtypes such as influenza A virus H1N1 (H1N1) and H3N2 are responsible for seasonal influenza epidemics in humans (Kosik and Yewdell, 2019). In contrast, influenza B viruses (IVBs) exhibit a slower rate of antigenic variation than influenza A viruses. Moreover, they do not undergo antigenic shift (Valesano et al., 2020). Consequently, IVBs generally cause milder symptoms than influenza A viruses. However, they can still lead to severe morbidity and mortality, especially in young children and older

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adults (Peltola et al., 2003; Heikkinen et al., 2004; Mosnier et al., 2015; Otto et al., 2017; Kini et al., 2018; Narayan et al., 2020).

A major contributor to acute lower respiratory infections is RSV, which typically causes pneumonia and bronchiolitis (Checchia et al., 2011). RSV exhibits a high infection rate in children under the age of 5 and in individuals aged >65 years (Falsey and Walsh, 2000). Specifically, RSV plays a significant role in morbidity and mortality among the elderly and immunocompromised patients, potentially resulting in a disease burden similar to a nonpandemic influenza A (Falsey et al., 2005; Collins et al., 2013). RSV belongs to the *Pneumoviridae* family. As an enveloped negative-sense RNA virus, RSV is primarily classified into two major subtypes, A and B (Rima et al., 2017). These two subtypes can be distinguished using monoclonal antibodies against RSV surface proteins, including attachment glycoprotein, fusion protein, matrix protein, nucleoprotein, and phosphoprotein (Ramaekers et al., 2020).

Antiviral agents used for treating influenza and RSV primarily target the HA and NA of influenza and the fusion protein of RSV. However, frequent mutations in these target proteins have led to the emergence of drug-resistant variants, posing challenges in treatment (Douglas et al., 2005; Adams et al., 2010; Zhu et al., 2012; Yan et al., 2014; Bedford et al., 2015; Hussain et al., 2017; Goldhill et al., 2018; Lina et al., 2018; Dunning et al., 2020; Imai et al., 2020).

For these reasons, increasing efforts are undertaken to develop safer and novel antiviral therapies. Particularly, researchers in Asia, Europe, and the United States have shown a growing interest in exploring antiviral compounds from traditional medicinal plants to develop antiviral agents. For several years, traditional medicinal plants have been effectively used in the treatment of infectious diseases in humans. They offer the advantage of having a well-established safety profile in clinical settings (Lin et al., 2014; Liu et al., 2016; Duncan et al., 2020; Mani et al., 2020; Maurya et al., 2020).

Echinacea is a perennial wildflower plant belonging to the *Asteraceae* family and is primarily native to North America. Currently, it is also cultivated in various regions including Europe (Burlou-Nagy et al., 2022). The Echinacea genus comprises nine species and several varieties. Its three species [*Echinacea angustifolia*, *E. purpurea* (L.) Moench, and *E. pallida*] have been utilized as medicinal herbs (Burlou-Nagy et al., 2022; Yang et al., 2022). Echinacea plants have been traditionally utilized in North America for preventing and treating cold and flu symptoms. They are one of the most commonly used medicinal plants in both North America and Europe (Barrett, 2003). Although various species of Echinacea have demonstrated effectiveness, *E. purpurea* is the most frequently utilized species for treating colds, flu, and upper respiratory infections (Melchart et al., 1995; Burger et al., 1997).

According to previous research studies, *E. pupurea*-derived compounds including caffeic acid derivatives, alkylamides, polysaccharides, and glycoproteins, exhibit various physiological activities, such as antiviral and immune-stimulating properties (Mrozikiewicz et al., 2010; Manayi et al., 2015). Among caffeic acid derivatives, chicoric acid (CA) possesses a wide range of physiological activities, including inhibiting hyaluronidase, protecting collagen, exhibiting antiviral effects, inhibiting human immunodeficiency virus (HIV) type 1 integrase and replication, enhancing phagocytic and natural killer cell activities, and scavenging free radicals (Tsai et al., 2012).

Although previous studies have confirmed antiviral activities of *E. purpurea* extracts against human respiratory viruses (Hudson et al., 2005; Vimalanathan et al., 2005; Pleschka et al., 2009; Selvarani et al., 2013; Signer et al., 2020), the antiviral activity and mechanism associated with CA, the characteristic compound of *E. purpurea* extract, have not yet been reported. Thus, this study aimed to evaluate antiviral activity of a 60% ethanol extract of *E. purpurea* (EPE) and its characteristic compound, CA, against H1N1, IVB, and RSV subtype A (RSV-A), the most common respiratory infection viruses in humans. Additionally, this study aimed to explore antiviral mechanisms of EPE and CA against influenza H1N1, IVB, and RSV-A.

MATERIALS AND METHODS

Sample preparation

EPE was provided by NUON Co., Ltd. The manufacturing method and specifications were consistent with those detailed in a previous study (Kim et al., 2023). Aerial parts (leaves, stems, and flowers) of *Echinacea purpurea* (L.) Moench were dried and subsequently extracted using 60% ethanol (v/v) at 50°C for 2 h. The extracted solution was subsequently filtered, concentrated, and finally spray-dried to produce EPE with a yield ranging from 34.8% to 38.0% (w/w). EPE was standardized to contain approximately 2% CA and more than 4% polyphenols (data not shown). CA was purchased from TCI.

High-performance liquid chromatography (HPLC) analysis

HPLC analysis of EPE was performed using a LC-40D system (Shimadzu). The HPLC mobile phase comprised 0.1% phosphoric acid in water (solvent A) and acetonitrile (solvent B) at 1.5 mL/min flow through Waters SunFire C18 column (4.6×250 mm, 5 μm) maintained at 35°C. The ultra violet detection wavelength was 330 nm, and the injection amount was 10 μL. The gradient elution started with 90% solvent A and 10% solvent B at

0 min, adjusting to 78% A and 22% B at 13 min, followed by a shift to 60% A and 40% B at 14 min, which was maintained until 17 min. At 17.5 min, the system was swiftly returned to the initial conditions of 90% A and 10% B to allow for adequate column re-equilibration before the subsequent injection. The total runtime for each analysis was 22 min.

Cell lines and viruses

In this study, Madin-Darby canine kidney (MDCK) cells and human epidermoid carcinoma (HEp-2) cells used in this study were obtained from Korea Cell Line Bank. H1N1, IVB, and RSV-A were obtained from Korea Bank for Pathogenic Viruses. MDCK cells were employed as host cells for H1N1, and IVB. HEp-2 cells were utilized as host cells for RSV-A. MDCK cells were cultured and maintained in T75 cell culture flasks (Sarstedt) at 37°C with 5% CO_2 using Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antimycotics (Gibco). HEp-2 cells were cultured and maintained in T75 cell culture flasks at 37°C with 5% CO_2 using RPMI-1640 (Gibco) supplemented with 10% FBS and 1% antimycotics. When both cell types reached approximately 80% confluency, they were detached using Trypsin/EDTA (Sigma-Aldrich) and passaged in the same medium. The medium was refreshed every 48 h.

Cell viability tests

For cytotoxicity assay, cultured cells were trypsinized and seeded at approximately 80% confluency into 96-well cell culture plates (Sarstedt) and incubated for 24 h at 37°C with 5% CO_2. After removing the culture medium from cell culture plates, EPE was dissolved in dimethyl sulfoxide (DMSO) at 2, 4, 8, 16, 31, 63, 125, 250, 500, and 1,000 μg/mL concentrations and added to wells at 100 μL/well. CA was dissolved in DMSO at 0.37, 0.74, 1.48, 2.96, 5.93, 11.86, 23.72, 47.44, 94.88, and 189.75 μg/mL concentrations and added to wells at 100 μL/well. The negative control and solvent control groups were treated with the culture medium and DMSO, respectively. After treatment with test solutions, cells were incubated at 37°C with 5% CO_2 for 24 h. Test solutions were subsequently removed. WST-1 reagent (EZ-Cytox, DoGenBio) was added to each well and allowed to react in a CO_2 incubator. Following completion of the reaction, the absorbance was measured at 450 nm using a microplate reader (SpectraMAX ABS, Molecular Devices). The percentage viability was calculated using the following formula:

Viable cell number (%)
=OD450 test sample-treated cells/OD450 (vehicle-treated cells)×100

Each assay was performed in triplicate. Results were expressed as mean±standard deviation (SD).

Plaque reduction assay

For the plaque reduction assay, host cells were seeded into a 24-well cell culture plate (Sarstedt) 1 day in advance, reaching approximately 90% confluence. After 24 h, the culture medium was removed and cells were treated with EPE at a final concentration of 1,000 μg/mL or CA at a final concentration of 189.75 μg/mL. Frozen stocks of H1N1, IVB, and RSV-A were then rapidly thawed at 37°C in a water bath and diluted tenfold in their respective culture medium for infection. H1N1 and IVB were each treated to MDCK cells [multiplicity of infection (MOI)=0.0007 and 0.0004, respectively]. RSV-A was treated to HEp-2 cells (MOI=0.0007). To allow virus adsorption, all cells were subsequently incubated in a CO_2 incubator set at 35°C for 2 h. Subsequently, overlay media containing low melting agarose (Thermo Fisher Scientific) were added. Once the overlay media solidified, they were further cultured until cytopathic effects were observed under a microscope. After completing the incubation, cells were fixed with a formaldehyde solution for 30 min for cell staining. Following fixation, overlay media were removed and cells were stained with crystal violet solution.

The plaque number and infectivity titer [plaque forming unit (PFU)] for each well were calculated using the following formula:

Infectivity titer (PFU)=plaque number×dilution factor/added virus volume (mL)

The antiviral activity was calculated using the following formula:

Virus reduction rate (antiviral activity)
=(control PFU−sample-treated PFU)/control PFU

Hemagglutination assay

To measure hemagglutination activities of H1N1 and IVB in the presence of EPE or CA, each well of a round 96-well plate was filled with 100 μL of 0.15 M NaCl. H1N1 or IVB was serially diluted twofold in each well. EPE or CA was dissolved in 50% DMSO and added to each well to achieve a final concentration of 1,000 or 47.44 μg/mL, respectively. Subsequently, 50 μL 0.5% chicken red blood cell (RBC, Innovative Research) was added to each well and allowed to react at room temperature for 30 min. To measure the hemagglutination inhibition activity of each test sample, hemagglutination units (HAUs) were determined.

NA inhibition assay

For the NA inhibition assay, cells were seeded into six-well cell culture plates (Thermo Fisher Scientific) and grown until they reached approximately 90% confluence. After 24 h, the culture medium was removed and cells were treated with EPE at a final concentration of 1,000 μg/mL or CA at a final concentration of 189.75 μg/mL. Frozen stocks of H1N1 and IVB were rapidly thawed in a 37°C water bath and used to infect cells. H1N1 and IVB were applied to MDCK cells at a MOI of 0.6563 and 0.0375, respectively. After virus treatment, cells were allowed to absorb the virus for 2 h in a CO_2 incubator set at 35°C. Culture media were added and cells were cultured for 2 days. After harvesting the cells, the NA assay was performed according to the protocol provided by an NA activity assay kit (Abcam). The NA activity was calculated using the standard curve based on the galactose concentration and the following formula:

NA activity
=galactose amount from the standard curve/
(reaction time×reaction volume×dilution factor)
=nmol/min/mL=mU/mL

Viral gene expression analysis

Degrees of infection in host cells for H1N1, IVB, and RSV-A were analyzed by comparing the relative quantify of viral RNA using quantitative real-time polymerase chain reaction (qRT-PCR). For the viral gene expression analysis, MDCK and HEp-2 cells were seeded into six-well cell culture plates 1 day before the confluency reached approximately 90%. After 24 h, the culture medium was removed and cells were treated with EPE at a final concentration of 1,000 μg/mL or CA at a final concentration of 189.75 μg/mL. H1N1 and IVB were each applied to MDCK cells (MOI=0.6563 and 0.0375, respectively). RSV-A was applied to HEp-2 cells (MOI=0.0007). After virus treatment, cells were incubated for 2 h at 35°C in a CO_2 incubator to allow virus adsorption. Subsequently, culture media were added and cells were incubated for 2 days.

Viral RNA was extracted using a QIAamp MinElute Virus Spin kit (Qiagen). RNA concentration was measured using a NanoDrop ND-1000 (Thermo Fisher Scientific). cDNA synthesis was performed using a TOPscript cDNA synthesis kit (Enzynomics), with the amount of RNA sample being 2 μg. qRT-PCR was performed using a TOPscript SYBR Green qPCR premix (Enzynomics) and specific primers (Table 1) on a MIC qPCR Cycler (BMS). The qRT-PCR parameters for each virus sample are listed in Table 1. Cycle threshold values obtained from qRT-PCR were converted to $2^{-\Delta\Delta CT}$ values to compare relative expression levels with the control group (virus control) as a reference (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Transmission electron microscopy (TEM) analysis

MDCK and HEp-2 cells were seeded into six-well cell culture plates when they reached approximately 90% confluence the day before. After 24 h, the culture medium was removed. Subsequently, cells were treated with EPE at a final concentration of 1,000 μg/mL or CA at a final concentration of 189.75 μg/mL. H1N1 and IVB were each applied to MDCK cells (MOI=0.6563 and 0.0375, respectively). RSV-A was applied to HEp-2 cells (MOI=0.0007). Virus absorption was allowed to occur in a CO_2 incubator set at 35°C for 2 h. Subsequently, culture media were added and cells were cultured for 2 days. After harvesting 1×10^6 cells, they were fixed with 2% osmium tetroxide (OsO_4) at room temperature for 2 h. Cells were subsequently washed twice with 0.1 M phosphate buffer. After drying at room temperature, cells were immersed in propylene oxide for 10 min at room temperature. Next, cells were embedded at 60°C for 24 h. Subsequently, these cells were semi-thin sectioned to 150 nm in thickness and further sectioned into 70-nm-thin slices. Cells were then stained with 6% uranyl acetate and lead citrate and subsequently observed using TEM (JEM-1011, JEOL).

Statistical analysis

One-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons. Results were expressed as mean±SD. Differences between groups were considered statistically significant at $P<0.05$, $P<0.01$, and $P<0.0001$.

Table 1. Quantitative real-time polymerase chain reaction (qRT-PCR) information for viral gene expression analysis

Virus		Primer sequences (5′→3′)	Target gene	qRT-PCR condition
H1N1	F:	TCGCCGACTATGAGGAACTGAGGGA	Hemagglutinin	(95°C-15 s/61°C-20 s/72°C-60 s); 50 cycles
	R:	TGGTATCCCCGGGTTCCAGCAGAGT		
IVB	F:	AAAACAAATGCTCTGCGCCCCAAC	Nucleoprotein	(95°C-15 s/63°C-20 s/72°C-45 s); 50 cycles
	R:	CGTCTCCACCTACTTCATTCCCCCC		
RSV-A	F:	TGACCCATTAGTGTTCCCCTCTGATGAAT	Fusion protein	(95°C-15 s/64°C-20 s/72°C-30 s); 45 cycles
	R:	CTTCTGGCCTTACAGTATAGGAGCAGT		

IVB, influenza B virus; RSV-A, respiratory syncytial virus subtype A.

RESULTS

Analysis results of the marker compound in EPE

The marker compound in EPE was identified by HPLC analysis. As a result of the quantitative analysis, the CA content of approximately 2% in EPE was confirmed (Fig. 1). Optimized EPE was utilized for the following *in vitro* study.

Results of the cytotoxicity tests of EPE

When MDCK cells were treated with different EPE or CA concentrations, no significant cytotoxicity was observed at any tested concentrations (Fig. 2). The viability of MDCK cells treated with EPE at 1,000 μg/mL (the highest test concentration) or CA at 189.75 μg/mL (the highest test concentration) was 115.00%±3.30% or 91.26%±6.80%, respectively, showing no significant difference in viability compared with the control group (100% viability for the control group, both $P>0.05$). Similarly, viability of HEp-2 cells treated with 1,000 μg/mL of EPE or 189.75 μg/mL of CA was 108.00%±1.50% or 113.34%±7.00%, respectively, showing no significant difference in viability compared with the control group (both $P>0.05$).

The highest nontoxic EPE and CA concentrations and their viral infection inhibitory activities

Viral infection inhibitory activities of 1,000 μg/mL EPE or 189.75 μg/mL CA against H1N1, IVB, and RSV were analyzed using a plaque assay. EPE exhibited plaque formation inhibition activities against H1N1, IVB, and RSV-A (Fig. 3). Furthermore, CA showed plaque formation inhibition activities against H1N1, IVB, and RSV-A. Upon treatment with EPE or CA starting from a 10^{-5} dilution factor, H1N1 exhibited a reduced infection activity. Its infectivity titers were 26.25±4.57, 9.00±2.16, and 17.25±7.50 PFU/well for the solvent control, EPE treatment, and CA treatment groups, respectively. Antiviral activities of EPE and CA against H1N1 were 65.7% and 34.3%, respectively (Fig. 3A and Table 2). Upon treatment with EPE and CA starting from a 10^{-4} dilution factor, IVB showed a reduced infection activity. Its infectivity titers were 15.00±7.35, 5.00±3.56, and 14.00±0.82 PFU/well for the solvent control, EPE treatment, and CA treatment groups, respectively. Antiviral activities of EPE and CA against IVB were 66.7% and 6.7%, respectively (Fig. 3B and Table 2). Upon treatment with EPE and CA starting from a 10^{-1} dilution factor, RSV-A showed a reduced infection activity, with infectivity titers of 41.00±2.94, 6.00±3.92, and 9.75±5.38 PFU/well for the solvent control, EPE treatment, and CA treatment groups, respectively. Antiviral activities of EPE and CA against RSV-A were 85.4% and 76.2%, respectively (Fig. 3C and Table 2).

Evaluation of viral infection inhibitory activity of EPE and CA through analysis of viral gene expression levels

H1N1, IVB, and RSV-A gene expression levels in host cells were analyzed using qRT-PCR to assessed viral infection inhibitory activities of EPE and CA. The relative gene expression levels for each virus, comparing the solvent control, EPE treatment, and CA treatment groups to the group infected with the virus only (virus control), are depicted in Fig. 4. For H1N1, the relative viral gene expression levels in the solvent control, EPE treatment, and CA treatment groups were 1.43, 0.79, and 0.26, respectively (Fig. 4A). For IVB, the viral gene relative expression levels in the solvent control, EPE treatment, and CA treatment groups were 1.20, 0.32, and 0.31, respectively (Fig. 4B). For RSV-A, the viral gene relative expression levels in the solvent control, EPE treatment, and CA treatment groups were 1.08, 0.36, and 0.09, re-

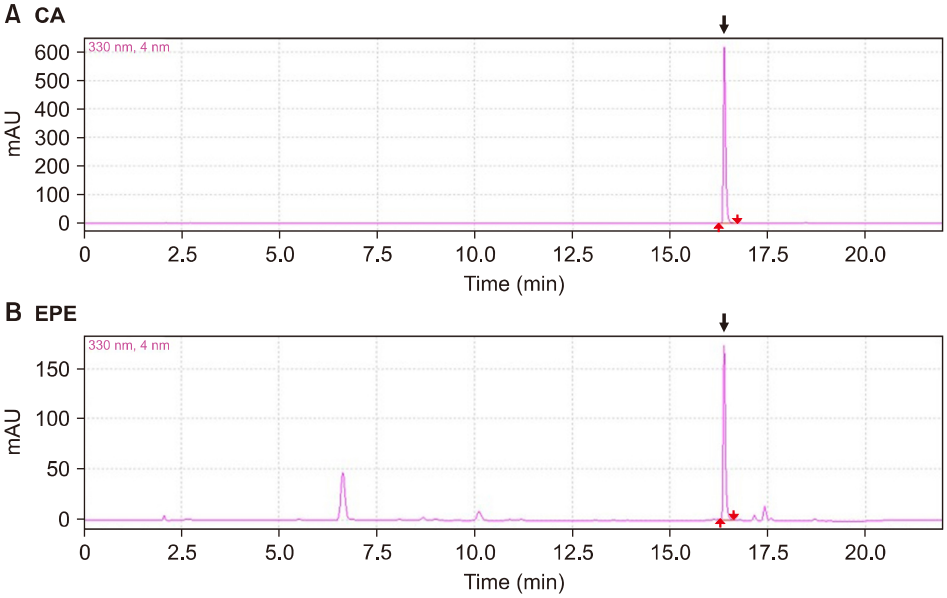

Fig. 1. High-performance liquid chromatography chromatogram of (A) chicoric acid (CA) reference material and (B) a 60% ethanol extract of *Echinacea purpurea* (EPE). The arrow indicates the peak for CA. The red arrows indicate the start and end of the CA peak.

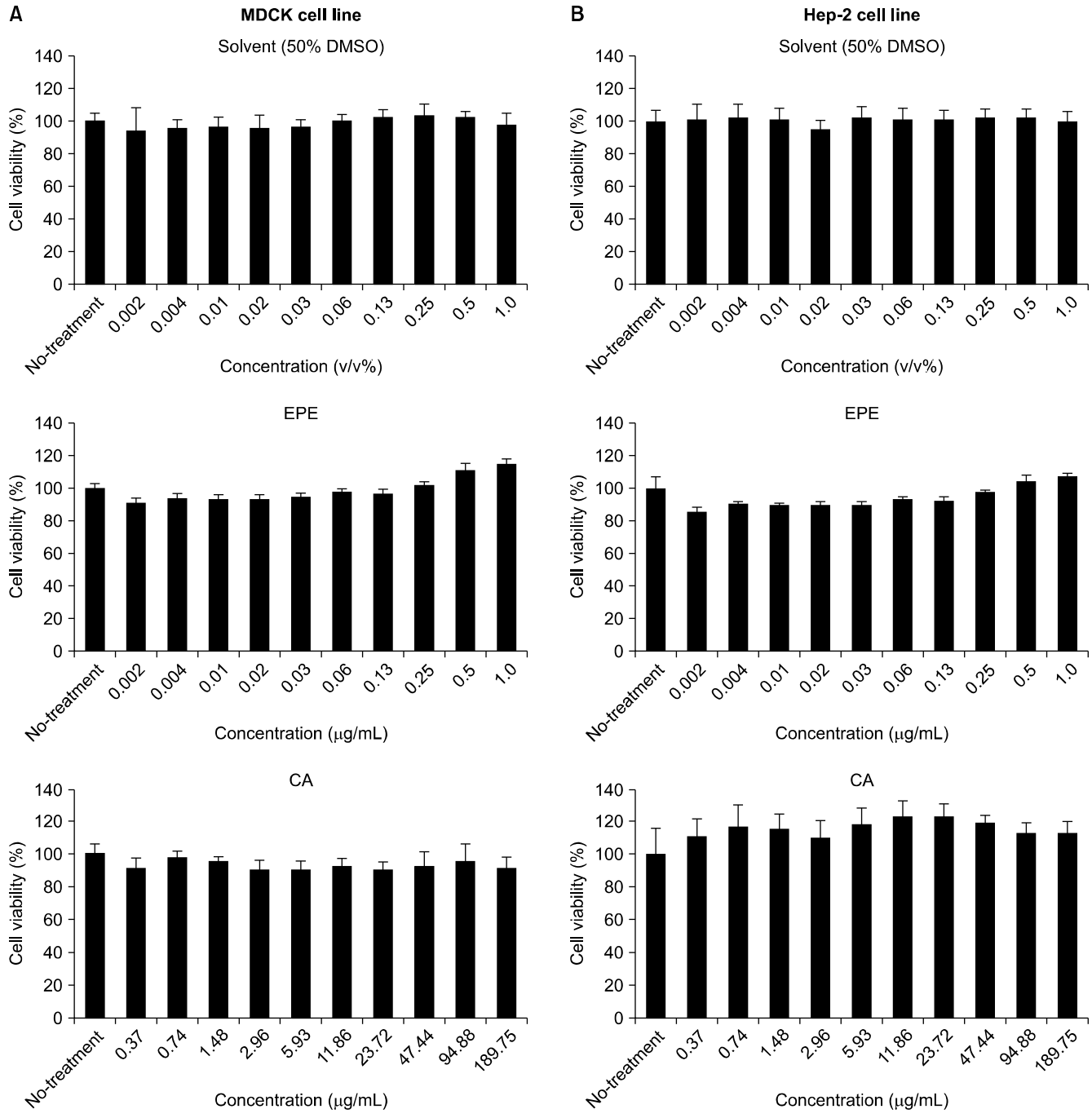

Fig. 2. In concentration-dependent cytotoxicity assessments, a 60% ethanol extract of *Echinacea purpurea* (EPE) and chicoric acid (CA) exhibit no toxicity to Madin-Darby canine kidney (MDCK) or HEp-2 cell lines. (A) Cell viabilities of each treatment group are evaluated compared with the untreated control group. Cell viabilities of the solvent control group (upper), EPE treatment group (middle), and CA treatment group (lower) using MDCK cell line are shown. (B) Cell viabilities of the solvent control group (upper), EPE treatment group (middle), and CA treatment group (lower) using HEp-2 cell line. Data are presented as mean±SD (n=8). DMSO, dimethyl sulfoxide.

spectively (Fig. 4C). These results indicate that EPE and CA can hinder the expression of viral genes for H1N1, IVB, and RSV-A, underscoring their ability to inhibit viral infection.

Verification of viral infection inhibitory activity of EPE and CA through TEM image analysis

To visually examine viral inhibitory activities of EPE and CA, degrees of infection in host cells for H1N1, IVB, and RSV-A were observed through TEM imaging. The TEM images of uninfected MDCK and HEp-2 cells and images of each virus infected host cell treated with solvent, 1,000 μg/mL EPE, or 189.75 μg/mL CA are displayed in Fig. 5. Virus particles of H1N1 were not observed upon EPE treatment. However, a decreasing trend of infection level was observed following CA treatment (Fig. 5B–5D). For

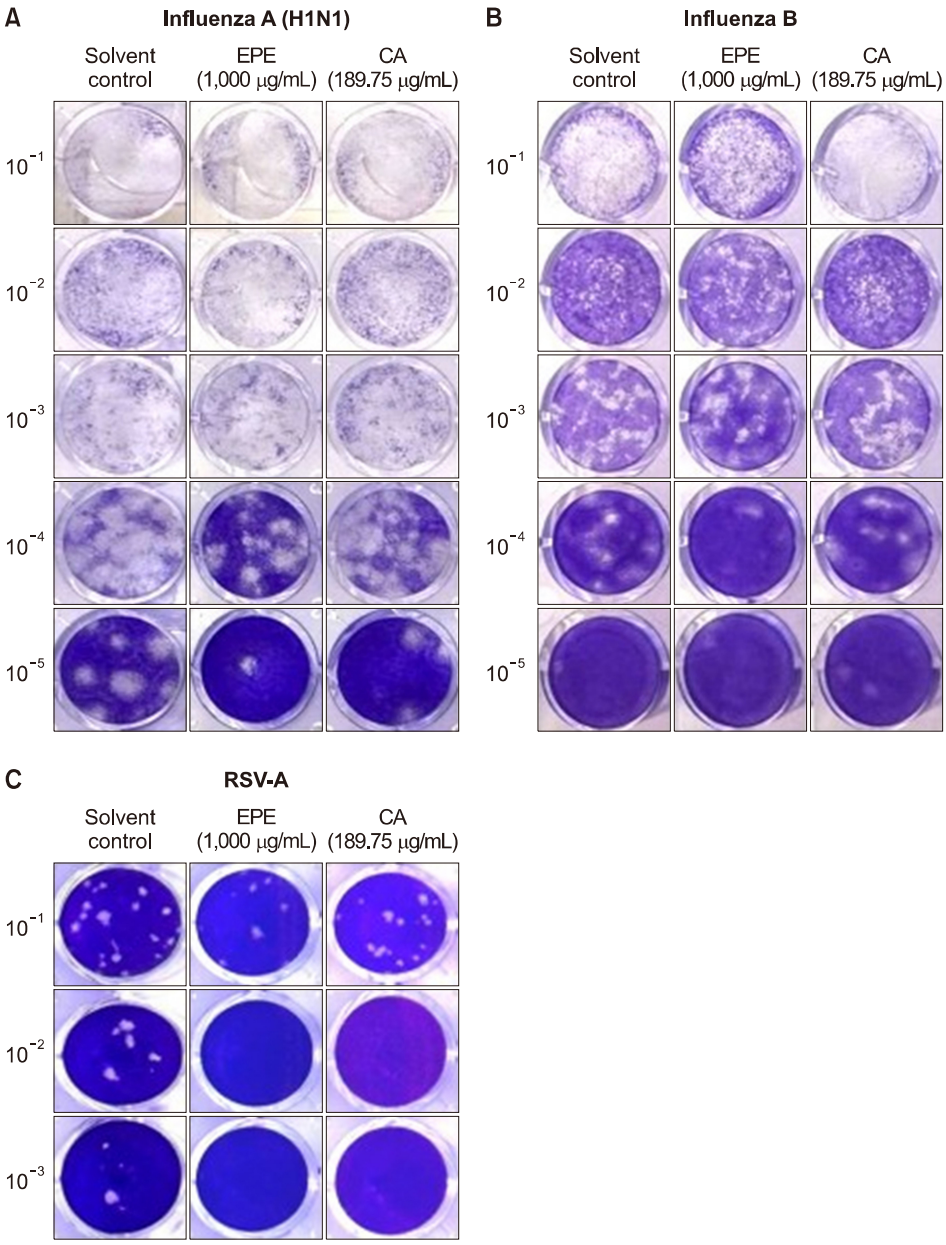

Fig. 3. A 60% ethanol extract of *Echinacea purpurea* (EPE) and chicoric acid (CA) exhibit inhibitory activities against viral infection in host cells. Viral infection inhibitory activities are evaluated using plaque reduction assays. Plaque reduction assay results for 1,000 μg/mL EPE and 189.75 μg/mL CA against (A) H1N1, (B) influenza B virus (IVB), and (C) respiratory syncytial virus subtype A (RSV-A) are shown. Madin-Darby canine kidney (MDCK) cells are used as host cells for H1N1, and IVB, whereas HEp-2 cells are used as host cells for RSV-A. The numbers denoted as powers of 10 with a negative exponent in each figure represent the serial dilution factor.

Table 2. Results of the analysis of infectivity titer and viral infection inhibitory activity for each group in the plaque reduction assay

Virus	Sample	Infectivity titer (PFU/well)	Viral infection inhibitory activity (%) (vs. solvent control)
H1N1	Solvent control	26.25±4.57	-
	EPE (1,000 μg/mL)	9.00±2.16	65.7
	CA (189.75 μg/mL)	17.25±7.50	34.3
IVB	Solvent control	15.00±7.35	-
	EPE (1,000 μg/mL)	5.00±3.56	66.7
	CA (189.75 μg/mL)	14.00±0.82	6.7
RSV-A	Solvent control	41.00±2.94	-
	EPE (1,000 μg/mL)	6.00±3.92	85.4
	CA (189.75 μg/mL)	9.75±5.38	76.2

Values are presented as mean±SD (n=4).
IVB, influenza B virus; RSV-A, respiratory syncytial virus subtype A; EPE, a 60% ethanol extract of *Echinacea purpurea*; CA, chicoric acid.

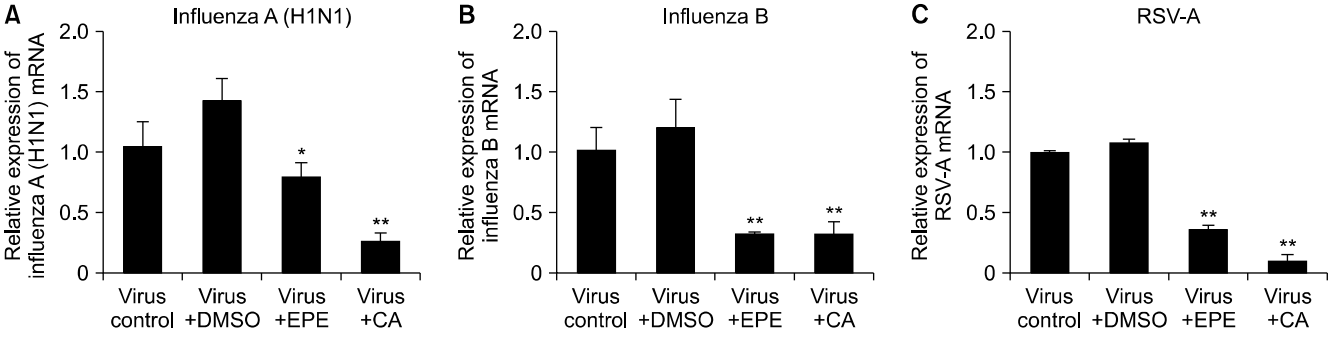

Fig. 4. Virus infection inhibitory activities of a 60% ethanol extract of *Echinacea purpurea* (EPE) and chicoric acid (CA) determined by viral gene expression quantification using quantitative real-time polymerase chain reaction (qRT-PCR). Comparison results of relative expression levels of viral genes for (A) H1N1, (B) influenza B virus (IVB), and (C) respiratory syncytial virus subtype A (RSV-A) following 1,000 μg/mL EPE or 189.75 μg/mL CA treatment are shown. Gene expression levels for each treatment group are analyzed relative to the viral control. Relative expression is calculated using the $2^{-\Delta\Delta CT}$ method. Data are presented as mean±SD (n=3). $^{*}P<0.05$ vs. the virus control group; $^{**}P<0.01$ vs. the virus control group. DMSO, dimethyl sulfoxide.

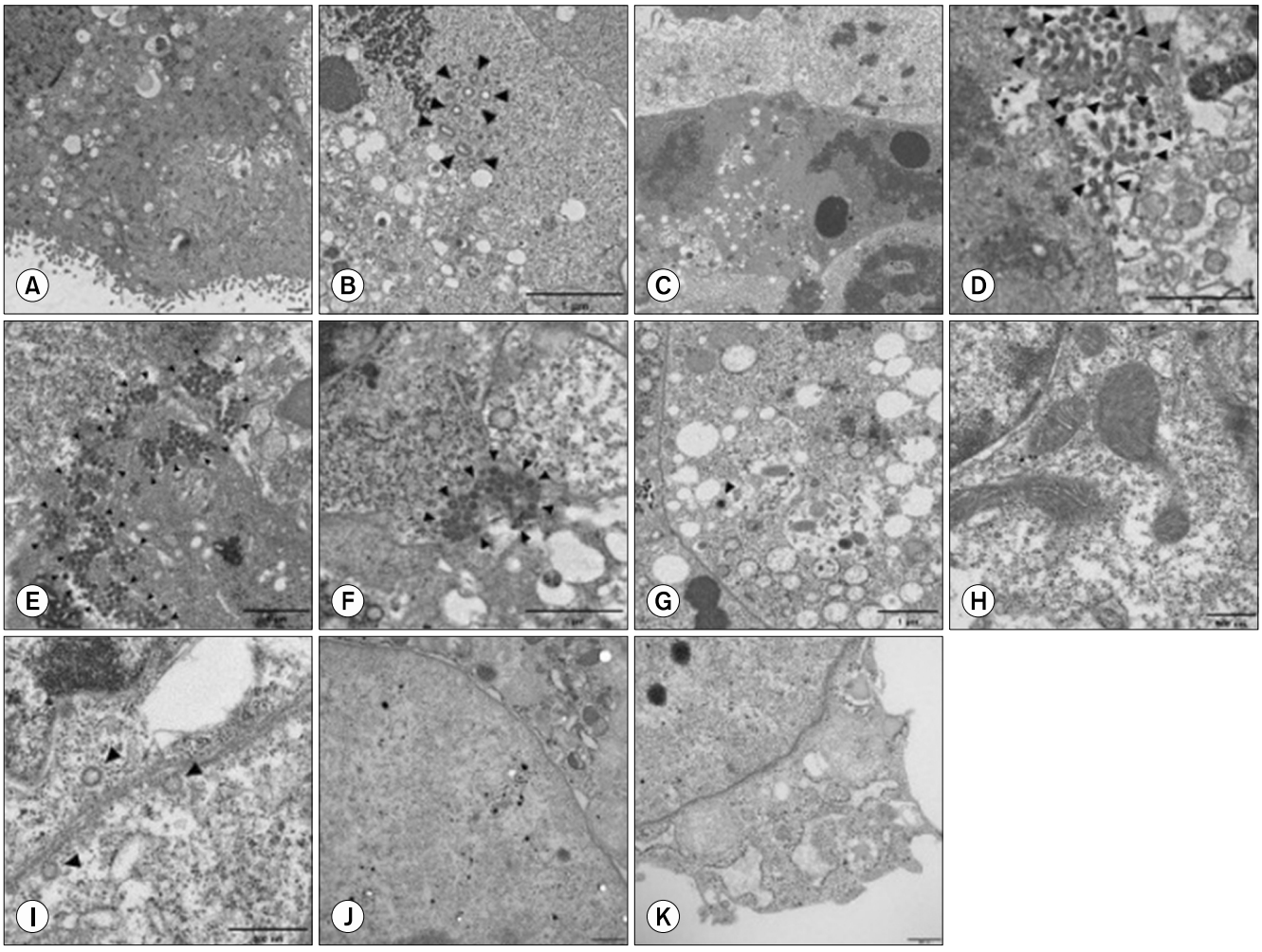

Fig. 5. Transmission electron microscopy (TEM) image analysis confirms that a 60% ethanol extract of *Echinacea purpurea* (EPE) and chicoric acid (CA) can inhibit virus infection in host cells. Madin-Darby canine kidney (MDCK) cells are infected with H3N2, H1N1, or influenza B virus (IVB), whereas HEp-2 cells are infected with respiratory syncytial virus subtype A (RSV-A). Following infection, they are treated with 1,000 μg/mL EPE or 189.75 μg/mL CA. Subsequently, TEM is then employed to assess the extent of viral infection. (A) Uninfected MDCK cell (×10 k magnification), (B) Solvent control of H1N1-infected MDCK (×10 k magnification), (C) H1N1-infected MDCK with EPE (×10 k magnification), (D) H1N1-infected MDCK with CA (×10 k magnification), (E) Solvent control of IVB-infected MDCK (×10 k magnification), (F) IVB-infected MDCK with EPE (×10 k magnification), (G) IVB-infected MDCK with CA (×10 k magnification), (H) Uninfected HEp-2 cell (×30 k magnification), (I) Solvent control of RSV-A-infected HEp-2 (×30 k magnification), (J) RSV-A-infected HEp-2 with EPE (×30 k magnification), (K) RSV-A-infected HEp-2 with CA (×30 k magnification). Virus particles are indicted by arrowhead. Scale bar: 1 μm (A, B, C, D, E, F, and G) or 500 nm (H, I, J, and K).

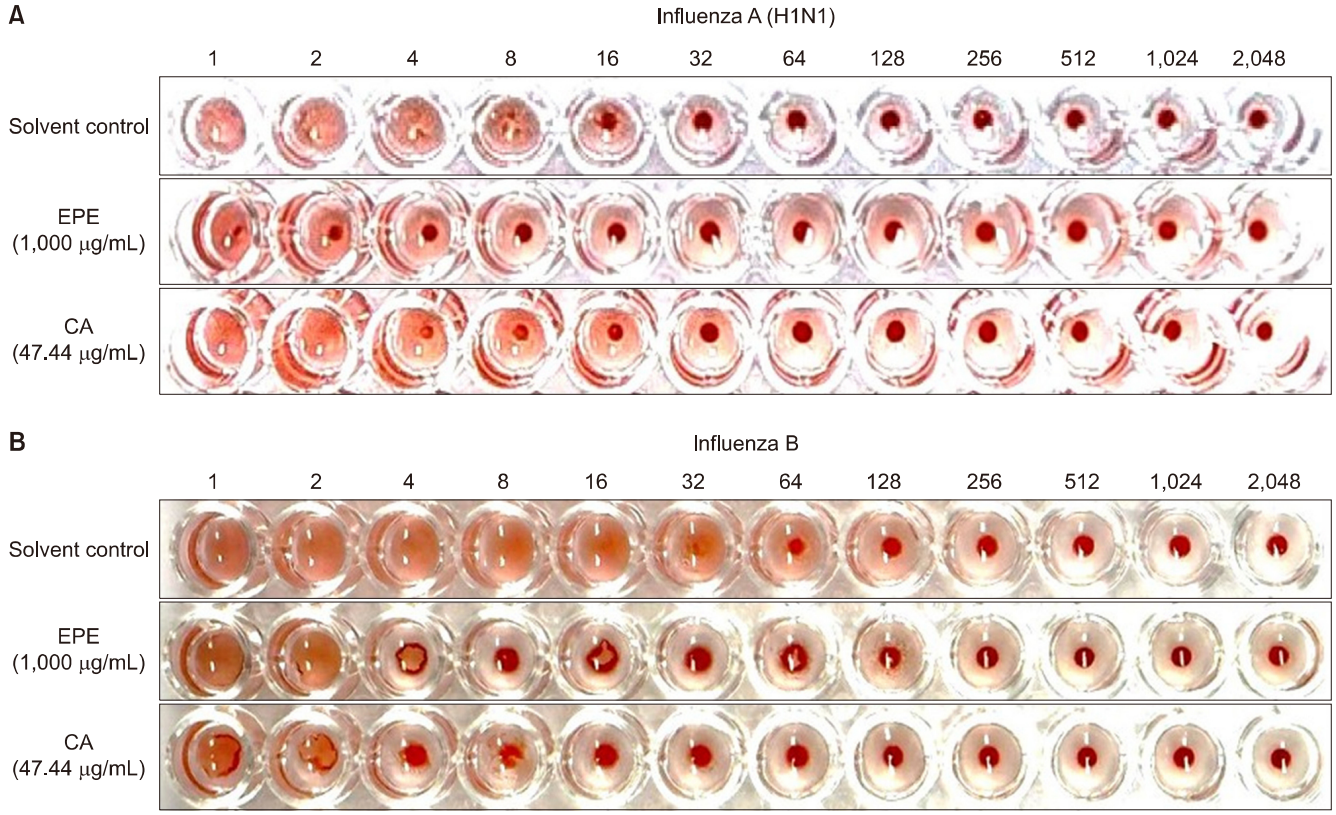

Fig. 6. Viral hemagglutinin (HA) inhibition activities of a 60% ethanol extract of *Echinacea purpurea* (EPE) and chicoric acid (CA) confirmed through a hemagglutination inhibition assay. Results of the hemagglutination inhibition assay using 1,000 μg/mL of EPE and 47.44 μg/mL of CA for (A) H1N1 and (B) influenza B virus (IVB) are shown. Numbers above each well in the image represent a twofold dilution factor.

IVB, a reduction in virus particles was observed upon EPE and CA treatment (Fig. 5E–5G). Moreover, virus particles of RSV-A were not observed in the EPE or CA-treated groups (Fig. 5I–5K). TEM image analysis results exhibited a similar trend to viral infection inhibition activities of EPE and CA, as confirmed by plaque reduction tests and viral gene expression analysis.

Interaction of EPE and CA with viral hemagglutination

Hemagglutination assays were performed for H1N1 and IVB to evaluate the inhibitory effect of EPE and CA on the interaction between the viral HA and the surface receptors on the host cell. We examined the direct interaction between the virus and EPE/CA by inspecting the viral hemagglutination activity in the presence and absence of both. CA at a 189.75 μg/mL concentration exhibited hemolytic activity against chicken RBC. Thus, we performed hemagglutination inhibition assays for CA at a decreased concentration of 47.44 μg/mL.

The results of the hemagglutination assay are demonstrated in Fig. 6. In the solvent control group, the HAU for H1N1 was 16; after treatment with EPE and CA, the HAUs were 2 and 0, respectively (Fig. 6A and Table 3). These results indicate that RBC agglutination is more pronounced at higher virus concentrations owing to sample treatment and that EPE and CA could effectively inhibit hemagglutination by H1N1. For IVB, the solvent control group had 32 HAUs, whereas it was 2 HAUs after EPE or CA treatment (Fig. 6B and Table 3). These results suggest that EPE and CA could effectively inhibit hemagglutination by IVB.

Viral NA inhibition activities of EPE and CA

We evaluated viral NA inhibition activities of EPE and CA against H1N1, and IVB, all of which had NA on their viral surfaces. NA can hydrolyze sialic acid at the end of polysaccharide chains. The resulting hydrogen peroxide generated from the enzymatic reaction with galactose can react with a probe, producing a detectable fluorescence

Table 3. Results of measuring hemagglutination unit (HAU) for each group in the hemagglutination inhibition assay

Virus	Sample	HAU
H1N1	Solvent control	16
	EPE (1,000 μg/mL)	0
	CA (47.44 μg/mL)	2
IVB	Solvent control	32
	EPE (1,000 μg/mL)	2
	CA (47.44 μg/mL)	2

Values are presented as mean±SD (n=3).
IVB, influenza B virus; EPE, a 60% ethanol extract of *Echinacea purpurea*; CA, chicoric acid.

signal, allowing for NA activity measurement (Hľasová et al., 2019).

The results of NA activity analyses for each group are presented in Fig. 7. NA activity analyses for each group, categorized by the virus, revealed that in the H1N1 infected control group and the solvent control group, NA activities were 13.67±0.81 and 14.08±1.08 mU/mL, respectively. In contrast, the 1,000 μg/mL EPE-treated and 189.75 μg/mL CA-treated groups exhibited NA activities of 10.32±0.60 and 10.36±1.71 mU/mL, respectively. For IVB, NA activities in the infected control group and the solvent control group were 13.76±0.14 and 13.51±0.11 mU/mL, respectively. The 1,000 μg/mL EPE-treated and 189.75 μg/mL CA-treated groups exhibited NA activities of 8.89±0.12 and 10.24±1.03 mU/mL, respectively. These analysis results indicate that EPE and CA could demonstrate NA inhibitory activities against H1N1 and IVB.

DISCUSSION

This study aimed to analyze the inhibitory activity and mechanisms of EPE and its characteristic compound, CA, against H1N1, IVB, and RSV-A, which are major causes of viral respiratory infections in humans. Evaluations of virus infection inhibition and propagation inhibition activities of EPE and CA were performed through plaque reduction tests, viral gene expression analysis, and TEM image analysis. Additionally, hemagglutination inhibition and NA activity tests were used analyzing virus inhibition mechanisms of EPE and CA.

Viral proteins HA and NA are crucial factors in virus host cell infection and propagation (Kosik and Yewdell, 2019). HA, primarily noted in influenza viruses and located on the viral envelopes, is responsible for attaching virus particles to host cells (Gamblin et al., 2021). When the virus initiates infection in host cells, HA can bind to sialic acid present on the termini of glycans on the surface of most host cells (Liu et al., 1995; Stray et al., 2000). This binding represents the first step wherein virus particles adhere to host cells, preparing the virus for penetration into the host cell. Therefore, receptor binding of HA and subsequent virus penetration play a crucial role in the initiation stage of viral infection (Gamblin et al., 2021). Conversely, NA is not required in the initial stages of infection (Liu et al., 1995). Moreover, NA is located on the surface of virus particles and plays a crucial role in completing the infection cycle by activating virus particle release and preventing virus particle aggregation by HA and other NA molecules through desialylation, thereby separating virus particles from host cells (Chockalingam et al., 2012; Romero-Beltran et al., 2016; Yamayoshi et al., 2017).

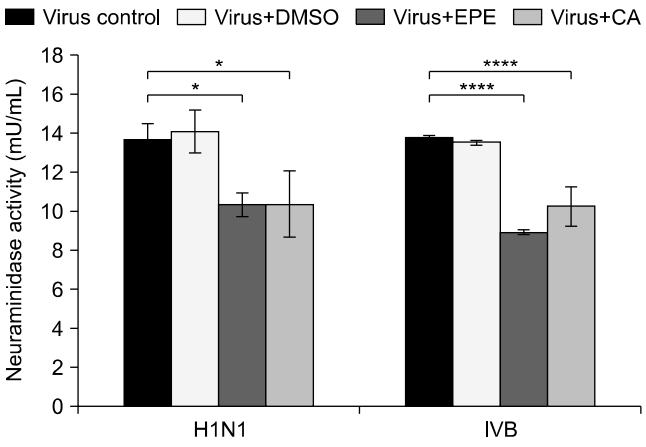

Fig. 7. A 60% ethanol extract of *Echinacea purpurea* (EPE) and chicoric acid (CA) exhibit inhibitory activities against viral neuraminidase (NA). The inhibition activity against viral NA is evaluated through an NA assay. Results of the NA assay for H1N1, and influenza B virus (IVB) using 1,000 μg/mL EPE or 189.75 μg/mL CA are shown. Data are presented as mean±SD (n=3). *P<0.05 vs. the virus control group; ****P<0.0001 vs. the virus control group. DMSO, dimethyl sulfoxide.

The efficacy of EPE and CA in interfering with the hemagglutination process of H1N1 and IVB is demonstrated by hemagglutination assays. This interference suggests a potential mechanism wherein EPE and CA disrupt the binding ability of these viruses to host cells, thereby hindering initial viral attachment. Additionally, viral NA inhibition activity assessment showed the NA inhibitory properties of EPE and CA against H1N1 and IVB. These collective findings imply a dual interference by EPE and CA, affecting both the initial attachment and final release stages of H1N1 and IVB viral infections, presenting a promising strategy in combating these respiratory pathogens. Furthermore, the results obtained from plaque reduction tests elucidated the antiviral properties of EPE and CA against the targeted viruses. EPE and CA exhibited plaque formation inhibition against H1N1, IVB, and RSV-A. Specifically, both EPE and CA displayed considerable antiviral activities against RSV-A. This finding was corroborated by qRT-PCR analysis, demonstrating the effective suppression of viral gene expression across all tested viruses upon EPE and CA treatment. TEM image analysis supported these observations, indicating reduced virus particle counts for H1N1, IVB, and RSV-A following EPE or CA treatment. These results demonstrate that EPE and CA possess inhibitory activities against infection and propagation of different types of viruses, such as H1N1, IVB, and RSV-A, suggesting their potential for preventing and alleviating symptoms of major seasonal respiratory virus infections.

Through this study, we confirmed the inhibitory activity of EPE and CA against respiratory infection viruses, including H1N1, IVB, and RSV-A, and provided a general mechanism of action. In a previous study, we have evaluated the immune-enhancing potential of EPE oral

administration (EP 60% ethanolic extract) in Bagg Albino laboratory breed (BALB/C) mice. This treatment resulted in NK cell cytotoxic activity augmentation and cytokine level modulation (Kim et al., 2022). NK cells and various cytokines play crucial roles in immune responses against not only respiratory infection viruses, but also various viral infections (Ma et al., 2021). Therefore, their increased activity and production can enhance immune responses against viral infections and potentially improve therapeutic outcomes (Rajaram et al., 2020; Razizadeh et al., 2023). Furthermore, Echinacea contains various compounds such as alkylamides, flavonoids, and polysaccharides. These bioactive substances possess inhibitory activities against influenza and various viruses (Barnes et al., 2005; Sharma et al., 2009; Fusco et al., 2010; Elufioye et al., 2020; Morimoto et al., 2023). Interestingly, although both EPE and CA demonstrated antiviral activities against all three viruses, CA had a lower overall activity than EPE, suggesting that the potent antiviral activity observed in EPE can be attributed to the synergistic interactions among various bioactive compounds present in EPE, which collectively exert a more pronounced effect than CA alone.

EP contains several bioactive components including polyphenols such as caffeic acid derivatives, alkamides, polysaccharides, and glycoproteins (Mrozikiewicz et al., 2010). Among these, caffeic acid derivatives and alkamides exhibit inhibitory effects against herpes simplex virus, HIV, and influenza viruses (Birt et al., 2008; Hudson and Vimalanathan, 2011). We here confirmed the inhibitory activity of CA, a caffeic acid derivative and a marker compound of EP, against H1N1, IVB, and RSV-A. However, other compounds present in EP may also possess antiviral activities, and interactions among these compounds could potentially enhance the overall antiviral effect. Therefore, in this study, the antiviral activity observed in EPE could be attributed not only to CA but also to the synergistic effects of multiple compounds. This finding represents a limitation of the study, as we did not fully explore the interactions between various compounds in EP and their collective impact on the antiviral activity. To develop a more comprehensive and deeper understanding of their effect, future research should focus on elucidating the antiviral mechanisms of these compounds in combination.

Additionally, this study employed the highest nontoxic concentration of EPE in cell-based assays to evaluate its antiviral effects and ensure cell viability. This approach, while revealing the maximum impact of EPE, limited the exploration of dose-dependent effects. To overcome this limitation, future research will include animal experiments to examine various doses and develop a comprehensive dose-response curve, which is essential for optimizing potential therapeutic applications.

In conclusion, the findings of this study highlighted the promising potential of EPE as both a therapeutic agent and a functional food ingredient for viral respiratory infection prevention and treatment, attributed to its antiviral and immunomodulatory properties. Specifically, the antiviral and viral multiplication inhibitory activities of EPE and CA against H1N1, IVB, and RSV-A were demonstrated. EPE and CA effectively inhibited hemagglutination caused by H1N1 and IVB, as well as hindered the NA activity, underscoring their potential utility in combating respiratory virus infections. However, notably, although the focus was on CA, other compounds within EPE may also contribute to its overall antiviral effect through potential synergistic interactions, a factor not fully explored in this study.

Despite these promising results, the current study was conducted using the highest nontoxic concentration of EPE in cell-based assays to ensure cell viability and establish a baseline of antiviral activity. This approach limited the investigation of dose-dependent effects and did not fully explore the interactions among various compounds in EPE. Consequently, our understanding of the collective impact of these compounds on antiviral activity remains incomplete. Specifically, for RSV-A, our investigation was limited to assessing only the inhibitory activity against its infection, highlighting the need for a more comprehensive analysis.

To address these limitations, future research will involve animal experiments to evaluate various EPE doses and develop a comprehensive dose-response curve for antiviral activity. This step is crucial for optimizing potential therapeutic applications and will allow for a deeper understanding of the antiviral mechanisms of EPE and its constituents in combination. Such investigations will be critical in validating the use of EPE as a therapeutic intervention or dietary supplement, enhancing resistance to viral respiratory infections, and providing a more robust scientific basis for its application in clinical and health food markets.

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AUTHOR DISCLOSURE STATEMENT

Author SKL, DRL, DEM, SHP, DGK, EJK, and BKC were employed by the company NUON Co., Ltd. The remaining authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: SKL, BKC, KBK. Analysis and interpretation: DRL, DEM, SHP, DGK, EJK. Data collection: SKL, DRL, DEM, EJK. Writing the article: SKL. Critical revision of the article: SKL, BKC, KBK. Final approval of the article: all authors. Statistical analysis: SKL, DEM, EJK. Obtained funding: BKC. Overall responsibility: KBK.

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