

Antiviral activity of CAVAC-1901, a combination of 3 standardized medicinal plants, against highly pathogenic influenza A virus in chickens

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ABSTRACT Three different medicinal plants that consisted of the formulated mixture (CAVAC-1901) have been traditionally used for distinct medicinal purposes in different areas. *Angelica dahurica* has been used as an important ingredient of a prescription, Gumiganghwaltang, for the common cold and influenza. *Curcuma longa* has been utilized for the treatment of asthma, and jaundice. *Pinus densiflora* (Korean red pine) has been used to improve memory and brain function for the treatment of vascular. Industrial livestock, which are characterized by dense breeding, are vulnerable to influenza infection, causing severe economic loss and social problems. However, there are no viable alternatives due to the risk of the occurrence of variants. Therefore, the aim of this study was to discover anti-influenza combinations of different medicinal plants with the concept of a multicomponent and multitarget (MCMT) strategy in traditional Chinese medicine (TCM). As part of a continuous project, 3 medicinal plants whose inhibitory activity against

influenza A was previously reported at the compound level, and the inhibition of cytopathic effects (CPEs) by these formulated mixtures was evaluated against influenza A virus H1N1. A selected combination with an optimal ratio exhibiting synergistic activity was assessed for its antiviral activity in chickens against the highly pathogenic avian influenza (HPAI) H5N6. The selected combination (CAVAC-1901) showed potent inhibitory effects on the expression of neuraminidase and nucleoprotein, by RT-qPCR, Western blot, and immunofluorescence assays. The antiviral activity was more evident in chickens infected with H5N6. The sample-treated group (50 mg/kg/d) decreased mortality and virus titers in various organs. Our results indirectly suggest synergistic inhibitory activity of the combination of 3 different medicinal plants with different modes of action. Taken together, an optimally formulated mixture (CAVAC-1901) could serve as an effective alternative to current measures to minimize damage caused by HPAs.

Key words: *Curcuma longa*, *Angelica dahurica*, *Pinus densiflora*, synergism, zoonosis, high pathogenic avian influenza

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INTRODUCTION

Influenza, a contagious respiratory disease caused mainly by influenza A viruses (IAVs), has been regarded by many people as an annual rite of passage. However, influenza, commonly called flu, is more dangerous than commonly known, especially to children younger than 5 yr old, pregnant women and elderly adults due to flu-related complications and the limitations of drugs

(Sullivan et al., 2019). Although modern medical technologies have shed light on the physiology and pathogenic biology of influenza and have produced a number of antiviral agents, the battle against the influenza virus is still ongoing. According to statistics from the Centers for Disease Control and Prevention (CDC), 151,700 to 575,400 people were killed during the 2009 H1N1 pandemic between April 12, 2009, and April 10, 2010, in the United States alone (CDC, 2019). Moreover, reports on human fatalities caused by zoonotic avian influenza viruses (AIVs), such as H5Nx and H7Nx, have been more frequent since the first documented zoonosis in 1997 (Short et al., 2015). Multilateral efforts have been made to minimize the damage caused by IAVs, such as antiviral medication, human and animal vaccination, disinfection, and mass culling, but these means have their own limitation or drawbacks. For instance, the continuous evolution of

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MATERIALS AND METHODS

Preparation of Plant Extracts and CAVAC-1901

IAVs through reassortments and gradual accumulation of mutations makes antivirals and vaccinations less effective and results in the inevitable necessity of the continuous development of antivirals and vaccines (Nelson and Worobey, 2018). For zoonotic AIVs, which have recently emerged as a new risk threatening human public health, mass culling, globally implemented to prevent further infection in livestock farms, has caused massive economic burdens, ethical problems, and soil pollution (Hseu and Chen, 2017). These challenges require us to develop a new approach for use in these multilateral efforts.

Many reports have demonstrated that the combination of antivirals with different mechanisms is effective. It has been shown that multicomponent therapeutics can act synergistically, a concept called multicomponent and multitarget (MCMT) in traditional Chinese medicine (TCM) (Keith et al., 2005; Dunning et al., 2014; Zhang et al., 2019). Compared to conventional antivirals that target a single protein, MCMT has advantages of lowering the probability of occurrence of variants that might make IAV less susceptible to antivirals during replication. The extensive application of MCMT even to animals would involve the use of a combination of traditional medicinal plants. Thus, for application of MCMT to animals, we adopted a strategy of combining 3 different traditional medicinal plants in which the inhibitory mechanism of the respective major constituents has been scientifically verified.

As part of an ongoing project to discover antiviral formulations, we chose 3 different medicinal plants, *Angelica dahurica*, *Pinus densiflora*, and *Curcuma longa*, based on scientifically reported evidence and also economical accessibility. *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. belonging to the Umbelliferae family has been used extensively for the treatment of colds, carbuncles, rheumatism, and ulcers in China, Japan, and Republic of Korea (Lee et al., 2015). Our previous studies on the plant clearly demonstrated that major constituents exert anti-influenza A activity via the inhibition of the early step of the viral propagation cycle (Lee et al., 2020). *Pinus densiflora* Siebold and Zucc. (Korean red pine) belonging to the Pinaceae family has been used to improve memory and brain function for the treatment of vascular and neuronal diseases such as strokes in Korea (Chun et al., 2008). Diterpenes, major constituents of especially resin, likely inhibit the expression of viral mRNA as well as nitric oxide production and inducible nitric oxide synthase activity (Ha et al., 2020). *Curcuma longa* L. (turmeric) belonging to the Zingiberaceae family has been utilized, primarily around India and China, for the treatment of various diseases such as asthma, jaundice, abdominal pain, and rheumatoid arthritis (Ayati et al., 2019). Curcuminoids that are predominantly present in *C. longa* play an important role in the inhibition of IAV by inhibiting NA (Dao et al., 2012). Therefore, we evaluated the inhibitory cytopathic effect (CPE) of the combination of these 3 medicinal plants at various ratios against IAV H1N1. Finally, with the selected combination reinforced by the addition of ascorbic acid and chitosan, anti-influenza activity was assessed in chickens infected by the highly pathogenic avian influenza (HPAI) A H5N6.

The standardized crude extract from *A. dahurica* was prepared by extracting 9.6 kg of raw material with 70% EtOH by sonication (40°C, 1.5 h, 3 times). The roots of *Angelica dahurica* and the resin of *Pinus densiflora* were purchased from Duk-hyun dang herbal medicine market located in Seoul (Republic of Korea). Voucher specimens of *A. dahurica* (SNU2016-06) and *P. densiflora* (SNU2020-06) were stored at the Korea Bioactive Natural Material Bank, Research Institute of Pharmaceutical Science, College of Pharmacy, Seoul National University, Seoul, Republic of Korea. The 70% EtOH crude extract of roots of *C. longa* was purchased from Zhenjiang Koc Biotech Co., Ltd (Jiangsu, China). All extracts were lyophilized to make a dry powder of the extract. They were tested as single components or in combination at various ratios in DMSO for in vitro assays. A combination of 3 plant extracts with an optimal ratio reinforced by the addition of ascorbic acid and chitosan was named CAVAC-1901 (Choong Ang Vaccine Laboratories Co., Ltd.-1901, Daejeon, Republic of Korea) and administered for in vivo experiments in a solution [DMSO:PEG 400 (Sigma-Aldrich, St. Louis, MO):H₂O = 1:8:16 (v/v/v)]. For the sample preparation for quantitative analysis, 100 mg of lyophilized *C. longa* and *A. dahurica* and 10 mg of *P. densiflora* were scaled and mixed together with the ratio (1:1:0.1, w/w/w). After the addition of 3 mL of solvent, as used for standard compounds, they were extracted by using a sonicator at 35°C for 2 h. Then, they were centrifuged, and 10 μ L of clear supernatant was injected after filtration, which was repeated 3 times. Although the solubilities of ascorbic acid and chitosan were far different from those of the 3 marker compounds and they are included in small amounts, quantitative analysis was performed for the combined plant extract sample.

Preparation of the Stock Solution of Marker Compounds for Quantitative Analysis

To evaluate the contents of marker compounds of each constituting plant extract in the combined extracts, curcumin and imperatorin were purchased from Chengdu Must Bio-Technology Co., Ltd. (Zhejiang, China), and neoabietic acid was purified from the resin of *P. densiflora*. Standard stock solutions of these compounds with purities over 99.0% (HPLC) were prepared with a concentration of 10 mg/mL in a mixture of methanol and acetone (1:1, v/v). They were stored in a refrigerator after thorough sealing and diluted to make working solutions with proper concentrations.

Chromatographic Conditions for the Quantitative Analysis

HPLC-DAD analysis was carried out using a Thermo Fisher UltiMate 3000 HPLC system (Germering,

Germany) with a Phenomenex Kinetex C18 column (250 × 4.6 mm, 5.0 μm, 100 Å) (Torrance, CA) maintained at 25°C. The temperature of the sampler during analysis was maintained at 10°C to prevent evaporation of the solutions. A step gradient system using an aqueous acetonitrile (MeCN) system (A: H₂O containing 0.1% formic acid/B: MeCN) over 30 min (0.0–14.0 min, 40–55% B/14.0–24.0 min, 55–90% B/24.1–30.0 min, 40% B) at a flow rate of 1 mL/min was adopted. Ten microliters of solution were injected for every analysis, and a 250 nm wavelength was applied with consideration of the absorption maximum of the analytes.

Calibration Curve, Linearity, Limit of Detection, and Limit of Quantification

After HPLC analysis of the combined extract sample to obtain approximate peak areas of each compound, working solutions of marker compounds with 5 different concentrations (Table S1) were made, and their calibration curve was plotted by the quantity per injection (x) and the peak area (y) of a compound, which was expressed by the regression equation. Regression parameters of slope, intercept, and correlation factor (R) were calculated based on the linear regression data analysis tool in Microsoft Excel. The limit of detection (LOD) and limit of quantification (LOQ) were defined as concentrations corresponding to signal-to-noise ratios of 3 and 10, respectively. From linearity data, the LOD and LOQ were calculated by $3\sigma/S$ and $10\sigma/S$, where σ represents the standard deviation of the values and S represents the slope of the calibration curve.

Precision and Accuracy for the Validation of Linearity

For the precision study, working solutions with 3 different concentrations covering the concentrations of each marker compound in the combined extract were prepared in triplicate. Three samples of each marker compound were analyzed on the same day to determine the intraday precision. Three samples were also analyzed on 3 consecutive days to determine the interday precision. For the accuracy test, 3 standard working solutions were prepared with concentrations that were approximately 50, 100, and 150% of the analyte content in the combined extract. They were spiked into the combined extract at a 1:1 ratio (v/v). Accuracy values were calculated by $(a - b)/c \times 100$, where a , b , and c represent the measured amount, original amount, and spiked amount, respectively. Analyses were performed in triplicate at each concentration.

Cell Cultures

Madin-Darby canine kidney (MDCK) cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified

Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and with 5% CO₂. The cells were subcultured when cell confluence reached 70 to 80%.

Animals

All animal experiments in the study were approved by the Institutional Animal Care and Use Committee of Chungbuk National University (CBNUA-1554-21-02) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MD). Four-week-old male specific pathogen-free (SPF) chickens were purchased from Namduck SPF (Icheon, Republic of Korea). All animals were housed under SPF conditions and acclimatized for 2 d with free access to water and food.

Virus Stocks

Swine influenza H1N1 (A/Sw/Kor/CAN1/04, KCTC11165BP) was provided by Choong Ang Vaccine Laboratory (Daejeon, Republic of Korea) (Cho et al., 2019). Influenza A/AP/Korea/W612/2017 virus (H5N6) was kindly provided by Dr. Y. K. Choi at Chungbuk National University (Cheongju, Republic of Korea) and used for chicken experiments. All viral stocks were stored at -80°C prior to use (Song et al., 2009; Kim et al., 2018). Experiments employing H5N6, an HPAI, were performed in a biosafety level 3 (BSL-3) laboratory at Chungbuk National University. The 50% chicken lethal dose (CLD₅₀) of influenza A/AP/Korea/W612/2017 virus (H5N6) was calculated by referring to previously reported data (Choi et al., 2002).

Cell Viability

The cytotoxicity of the MDCK cells was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded on 96-well plates (1×10^5 cells per well) and incubated for 24 h prior to use. The cells were washed with phosphate-buffered saline (PBS) and treated with the test samples of various concentrations. To avoid the toxicity of the solvent, the final concentration of dimethyl sulfoxide (DMSO) in the culture medium was maintained at 0.05% (v/v). After 48 h of incubation, 20 μL of MTT reagent (2 mg/mL) was added to each well, followed by an additional incubation for 4 h. After the supernatant was carefully discarded, 100 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density was measured at 570 nm, and the statistical significance was calculated by comparison with the control group.

Cytopathic Effect Inhibition Assay

The CPE inhibition assay was carried out with a slight modification to a previously reported method (Ha et al., 2020). MDCK cells were seeded on 96-well culture plates at a density of 1×10^5 cells per well. After incubation for 24 h, the medium was removed, and the cells were washed twice with PBS. The influenza H1N1 viruses at a multiplicity of infection (MOI) of 0.01 were inoculated onto near confluent MDCK cell monolayers using DMEM containing 0.15 $\mu\text{g}/\text{mL}$ trypsin and 5 $\mu\text{g}/\text{mL}$ bovine serum albumin (BSA) for 2 h. The medium was removed, and the cells were washed with PBS, followed by replacement with new medium containing the various samples at different concentrations. After 3 d of incubation at 37°C under 5% CO₂ conditions, 20 μL of 2 mg/mL MTT solution was added to each well, and the plate was incubated for 4 h at 37°C. The statistical significance was calculated via comparison with the control group.

Quantitative Real-Time Polymerase Chain Reaction

MDCK cells were seeded into 6-well plates and the cells were washed twice with PBS and infected with the H1N1 A/PR/8/34 virus for 1 h. The medium was changed with a tested sample containing media. After 24 h of incubation, the TRIzol method was applied to isolate total RNA from the cells. Total RNA was reverse transcribed using random primers (Invitrogen, Eugene, OR) according to the manufacturer's instructions. Real-time PCR was performed using selective primers (Genotech, Daejeon, Republic of Korea) for neuraminidase using the AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Daejeon, Republic of Korea) (Table S2). The StepOnePlus real-time PCR system was applied with cycling conditions: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min. The data were extracted and calculated using StepOne software v2.3 (Applied Biosystems, Waltham, MA).

Immunofluorescence Assay

MDCK cells were maintained on sterilized glass coverslips (SPL Life Science, Daejeon, Republic of Korea) for 1 d. After infection with H1N1 (0.01 MOI) for 1 h, the cells were washed twice with PBS and transferred to DMEM containing the test compounds at different concentrations. Cells were incubated at 37°C and 5% CO₂ for 1 d and washed twice with cold PBS. The coverslip was fixed in 4% paraformaldehyde solution for 10 min, and the cells were permeabilized using 0.2% Triton X-100 solution on ice. The cells were blocked with 1% BSA solution for 1 h at room temperature. Subsequently, a monoclonal antibody against nucleoprotein (Novus Biologicals, Centennial, CO) was added, and the samples were incubated overnight. Antirabbit antibodies conjugated to fluorescein isothiocyanate (Abcam, Cambridge, UK) were added at the recommended

concentration and incubated for 2 h for binding of the antibody. The cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA) solution (500 nM) for 5 min at room temperature and washed again with cold PBS. The cells were mounted and observed under a confocal microscope (Confocal Scope TCS8, LEICA, Wetzlar, Germany).

Western Blot

The MDCK cell line was grown to approximately 90% confluency in a 6-well plate (1 d), and the cells were infected with H1N1 and incubated for an additional 1 h. After the medium was replaced with DMEM, samples were treated at different concentrations. After 24 h, the cells were washed with chilled PBS and lysed with EBC lysis buffer [1 mM EDTA, 0.5% NP-40, 50 mM NaF, 120 mM NaCl, and 50 mM Tris-HCl (pH 7.6)]. A protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) was used to measure the protein concentrations. SDS-polyacrylamide electrophoresis was performed using a gel electrophoresis kit. The PVDF membranes (0.45 μm Immobilon-P, Bio-Rad Laboratories, Inc.) to which the proteins were transferred were incubated with the antibodies overnight at 4°C and with the secondary antibodies at room temperature for 1 h in Tris-buffered saline. Finally, the samples were evaluated using an enhanced chemiluminescence Western blotting detection kit (Thermo Fisher Scientific).

Evaluation of the Therapeutic Efficacy of CAVAC-1901 in Chickens Infected by H5N6

Chickens were assigned to 5 groups (sample-treated group, with a lower concentration; sample-treated group, with a higher concentration; positive control treated group; nontreated group; and uninfected group). CAVAC-1901 (at a dosage of 10 mg/kg or 50 mg/kg/d) or oseltamivir (1 mg/kg/d) as a positive control were administered 4 h prior to exposure to A/AP/Korea/W612/2017 virus (H5N6) (200 μL injection, $10^{3.5}$ EID₅₀/mL [5CLD₅₀, chicken lethal dose 50]). CAVAC-1901 and oseltamivir were provided daily for 5 d, and each group of 10 chickens was monitored daily for mortality up to 7 dpi.

Viral Titers From Different Organs of the Infected Chickens

Two chickens from each group were euthanized at 4 dpi and lung, spleen, kidney, intestine and brain tissues were extracted. After a series of processes, including homogenization and dilution, viral titers were determined by hemagglutination (HA) assay. Twofold serial dilution was carried out in 96-well U-bottom plates filled with 50 μL of 0.5% chicken and turkey red blood cells in PBS. After 30 min of incubation, the viral titer was measured as a unit of EID₅₀. For viral titration from the tracheas and cloaca of chickens,

swab specimens of surviving chickens from each group were subjected to determination of the viral titer using the HA assay for 5 d.

Statistical Analysis

Statistical analysis was conducted with SigmaPlot 9.0 using Student *t* test (statistical significance if $P < 0.05$). All results are calculated as the means \pm standard deviations.

RESULTS AND DISCUSSION

Quantitative Analysis of Marker Compounds from Combined Extract Samples

By using a proper validation method established by HPLC-DAD analysis, 3 marker compounds were well separated and quantified accurately in a combined extract sample (Figure 1). Linearity data, including the correlation regression equation, coefficient (*R*), LOD, and LOQ, were successfully obtained (Figure S1). The intra- and interday precision and accuracy values were also calculated and the % relative standard deviation (RSD) values of these assays were less than 10%, indicating that this method was well validated. The results showed that the contents of curcumin, imperatorin, and neoabietic acid in the combined extract samples were 3.923 ± 0.011 mg/g (of DW), 1.976 ± 0.016 mg/g, and 1.270 ± 0.001 mg/g, respectively (Tables 1 and S1).

Evaluation of the CPE-Inhibitory Activities of Individual Plant Extracts

As mentioned earlier, we already demonstrated the anti-influenza activities of the 3 plants and the mechanism of active constituents from each respective plant in previous reports (Dao et al., 2012; Ha et al., 2020; Lee et al., 2020). In consideration of reducing the possibility of the occurrence of resistant viruses, a combination of 3 different medicinal plants with various antiviral activities (MCMTs) is inevitably needed. The assay of CPE inhibition against influenza A virus H1N1 was carried

out for each individual plant extract in a dose-dependent manner at concentrations of 10, 1, 0.1, and 0.01 $\mu\text{g}/\text{mL}$. The results showed that all groups with concentrations over 0.1 $\mu\text{g}/\text{mL}$ exhibited inhibitory activity against H1N1 except for the group with *P. densiflora* (G3) at 10 $\mu\text{g}/\text{mL}$ in which cytotoxicity was observed (Table 2). This cytotoxicity seems to be attributed to high contents of diterpenes that are major components of resin acids (Zaidi et al., 2006; Kim et al., 2009). However, G3 with 1 $\mu\text{g}/\text{mL}$ ($59.3 \pm 1.5\%$) showed higher cell viability than the other 2 groups with same concentration ($38.1 \pm 1.3\%$ for G1 and $47.1 \pm 1.8\%$ for G2) and even higher concentrations ($38.8 \pm 3.0\%$ for G1 and $53.3 \pm 2.0\%$ for G2). Then, we selected the highest concentration exhibiting no cytotoxicity from each plant extract (Figure S2) and combined them together in various ratios, as shown in Table 2, to find any combination that showed synergistic activity.

Comparison of Activity of Combinations With Different Ratios

The results of the CPE inhibition assay for various combinatorial groups (G4–G10) demonstrated that G4, where *C. longa* (CL), *A. dahurica* (AD), and *P. densiflora* (PD) were mixed at a 1:1:0.1 ratio (w/w/w), exhibited slightly stronger inhibitory activity, with $62.7 \pm 0.9\%$ cell viability than any other combination group (Table 2). However, synergistic activity was more evident when comparing the activity of G4 with those of single plant groups (G1–G3). Among G5, G6, and G7, the tendency of activity was consistent with what was observed from the single plant groups (G1–G3), which means that G5, where the ratio of CL that showed the lowest activity ($38.8 \pm 3.0\%$ for G1) was higher than that of any other plants (3:1:0.1 = CL:AD:PD in G5), showed the lowest activity ($40.7 \pm 0.7\%$ for G5), and G7, in which the ratio of PD that showed strong activity even at a more diluted concentration ($59.3 \pm 1.5\%$ for G3) increased (1:1:0.3 = CL:AD:PD in G7), showed the same pattern ($60.6 \pm 3.0\%$ for G7). In contrast, G8 to G10 did not show a similar tendency. Moreover, we tested the CPE inhibition of G4 supplemented by the

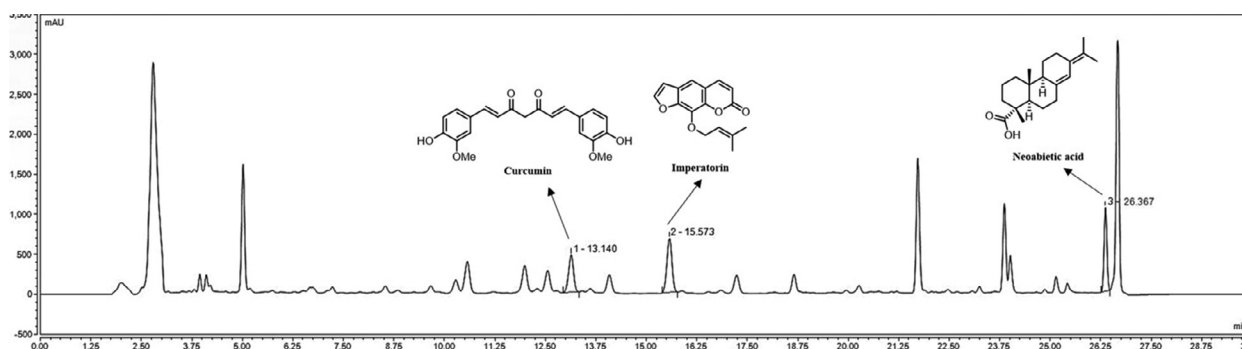


Figure 1. Optimized chromatogram of the combined extract sample for quantitative analysis. Curcumin, imperatorin, and neoabietic acid were identified as representative compounds of *C. longa*, *A. dahurica*, and *P. densiflora*, respectively, in the combined extract sample. A step gradient system using an aqueous acetonitrile (MeCN) system (A: H_2O containing 0.1% formic acid/B: MeCN) over 30 min (0.0–14.0 min, 40–55% B/14.0–24.0 min, 55–90% B/24.1–30.0 min, 40% B) at a flow rate of 1 mL/min was adopted. Ten microliters of solution were injected for every analysis, and a 250 nm wavelength was applied based on the absorption maximum of the analytes.

Table 1. Linearity data, LOD, and LOQ for 4 active furanocoumarins and their contents in combined extracts sample ($n = 3$).

Compound	Linearity range ($\mu\text{g/injection}$)	Regression equation	Correlation factor (R)	LOD ($\mu\text{g/injection}$)	LOQ ($\mu\text{g/injection}$)	Content (mg/g of DW)
Curcumin	0.02500–0.40000	$y = 24.269x + 0.8848$	1.0000	0.0732	0.2220	3.923 ± 0.011
Imperatorin	0.01250–0.20000	$y = 70.298x + 0.6160$	0.9998	0.0341	0.1034	1.976 ± 0.016
Neoabietic acid	0.00625–0.10000	$y = 95.195x + 0.3138$	0.9998	0.0235	0.0711	1.270 ± 0.001

addition of ascorbic acid and chitosan since there have been reports on anti-influenza A activity in animal models (Kim et al., 2013; Zheng et al., 2016). These results showed that the inhibitory activity of G13 was comparable to that of G4, whereas G11 and G12 exhibited lower efficacy at diluted concentrations (Table 2). Hence, we selected G13 as an optimally combined mixture (CAVAC-1901) and used it for the subsequent experiments.

Inhibitory Effect of CAVAC-1901 on the Expression of Viral Proteins

Based on the CPE assay data, the effects of CAVAC-1901 on the expression of neuraminidase (NA) and nucleoprotein (NP), which are essential for the replication of influenza virus in the viral life cycle, were tested at the transcriptional and translational levels. CAVAC-1901 was treated at concentrations of 10, 1 and 0.1 $\mu\text{g/mL}$, and incubated for 24 h with H1N1 virus-infected cells. The mRNA expression level of NA was evaluated using RT-qPCR. The CAVAC-1901-treated group showed that the expression level of NA was decreased in a dose-dependent manner compared to that in the virus-infected control group (Figure 2A). For further confirmation, the protein expression levels of NA and NP were evaluated at the translational level using a Western blot assay. The CAVAC-1901-treated groups exhibited a dose-dependent reduction in NA and NP expression upon H1N1 infection (Figure 2B). In the

immunofluorescence assay, the infected or noninfected cells (control group) were incubated with an anti-NP antibody and tagged with a green fluorescence secondary antibody. The CAVAC-1901 sample showed a dose-dependent decrease in production of nucleoprotein when compared to that of the virus-infected control, and the highest concentration (10 $\mu\text{g/mL}$) produced a strong reduction in NP production, which was comparable to the efficacy of ribavirin, the positive control (Figure 2C). Collectively, these results partially demonstrated the mechanism of CAVAC-1901 and proved that the mode of action verified by our previous studies at the single-plant level was retained in CAVAC-1901.

Protective Activities of CAVAC-1901 in Chickens Infected by Highly Pathogenic Influenza A Virus (H5N6)

CAVAC-1901 exhibited potent antiviral activity even in chickens infected with H5N6, a highly pathogenic strain. As shown in Figure 3, strong inhibitory activity in a dose-dependent manner was observed in CAVAC-1901-treated groups (33.3 and 83.3% survival rate in the 10 mg/kg and 50 mg/kg-treated group, respectively) and the protective activity observed in the CAVAC-1901-treated groups (50 mg/kg/d) surpassed that in the oseltamivir-treated group (66.7% of survival rate), whereas none of the nontreated groups survived at 7 dpi. On the other hand, since viral

Table 2. Cytopathic effects (CPE) inhibitory activities of plant extracts or their combinations with various ratios.

Sample	CL	AD	PD	Cell viability ¹ (%)			
				0.01 ($\mu\text{g/mL}$)	0.1 ($\mu\text{g/mL}$)	1 ($\mu\text{g/mL}$)	10 ($\mu\text{g/mL}$)
Control	-	-	-			100 \pm 9.8	
H1N1	-	-	-			23.8 \pm 6.2	
Ribavirin ²	-	-	-			69.8 \pm 1.2 ^{***}	
G1	1 ³	0	0	26.8 \pm 0.8	30.4 \pm 0.3	38.1 \pm 1.3*	38.8 \pm 3.0*
G2	0	1	0	29.1 \pm 0.3	46.4 \pm 2.4 ^{***}	47.1 \pm 1.8 ^{***}	53.3 \pm 2.0 ^{***}
G3	0	0	1	30.6 \pm 0.4	50.2 \pm 1.4 ^{***}	59.3 \pm 1.5 ^{***}	19.9 \pm 0.3
G4	1	1	0.1	32.4 \pm 0.4	50.6 \pm 1.1 ^{***}	56.6 \pm 0.7 ^{***}	62.7 \pm 0.9 ^{***}
G5	3	1	0.1	23.4 \pm 1.1	24.4 \pm 0.7	36.6 \pm 1.4 ^{***}	40.7 \pm 0.7 ^{***}
G6	1	3	0.1	34.1 \pm 1.5*	38.0 \pm 2.1 ^{***}	57.9 \pm 3.9 ^{***}	60.5 \pm 2.2 ^{***}
G7	1	1	0.3	42.4 \pm 2.2 ^{***}	47.8 \pm 3.3 ^{***}	55.2 \pm 1.8 ^{***}	60.6 \pm 3.0 ^{***}
G8	5	1	0.1	48.7 \pm 2.3 ^{***}	50.4 \pm 1.7 ^{***}	53.4 \pm 2.5 ^{***}	57.0 \pm 2.3 ^{***}
G9	1	5	0.1	36.2 \pm 1.3 ^{***}	41.3 \pm 1.8 ^{***}	43.3 \pm 1.2 ^{***}	57.2 \pm 2.0 ^{***}
G10	1	1	0.5	25.8 \pm 1.1	35.2 \pm 1.5 ^{**}	44.3 \pm 2.0 ^{***}	56.0 \pm 2.7 ^{***}
	G4	AS ⁴	CH ⁴				
G11	9	1	0	33.0 \pm 0.6	42.9 \pm 0.2*	52.2 \pm 1.1 ^{***}	60.9 \pm 0.7 ^{***}
G12	9	0	1	35.2 \pm 0.1 ^{***}	45.6 \pm 0.3 ^{**}	50.7 \pm 0.3 ^{***}	59.3 \pm 0.3 ^{***}
G13	9	0.5	0.5	34.4 \pm 0.3 ^{***}	50.6 \pm 0.4 ^{**}	58.5 \pm 0.1 ^{***}	59.8 \pm 0.2 ^{***}

¹Data are shown as the mean \pm SD ($n = 2$); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to the control group.

²The final concentration of ribavirin was 10 μM .

³The combinatorial ratio is presented as w/w/w.

⁴AS and CH stand for ascorbic acid and chitosan, respectively.

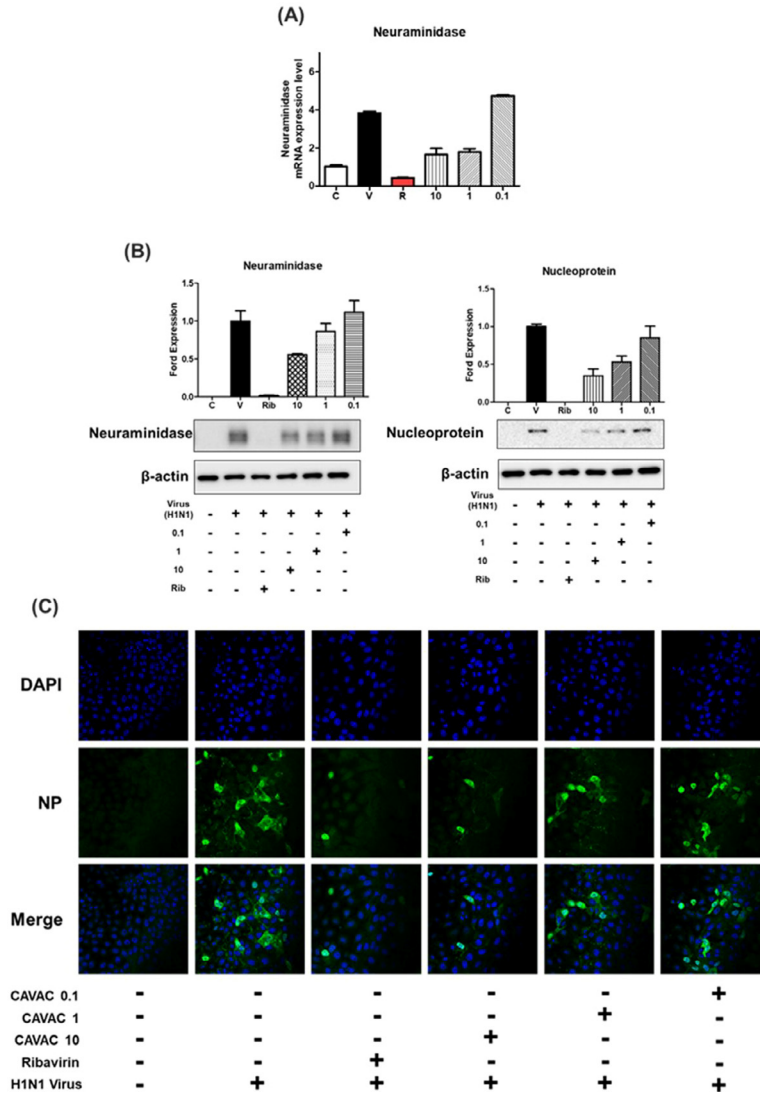


Figure 2. Effects of CAVAC-1901 on the gene expression levels of NA and NP. (A) MDCK (Madin-Darby canine kidney) cells were seeded onto 96-well plates, and the cells were infected with the H1N1 virus for 1 h and then treated with CAVAC-1901 at concentrations of 10, 1, and 0.1 $\mu\text{g}/\text{mL}$. Ribavirin at a concentration of 10 μM was used as a positive control in all experiments. The infected cells were incubated for 24 h and harvested for further analysis. The expression of viral NA was evaluated at the transcriptional level by using RT-qPCR. Data are presented as the mean \pm SD ($n = 3$) and compared to the expression of control groups. (B) The cells were treated in the same manner as in the RT-qPCR experiment, and the cells were collected for Western blot analysis. The protein levels of NA and NP were detected and normalized to the protein level of β -actin. (C) The effect of CAVAC-1901 on the expression of NP was visualized by immunofluorescence analysis. After the influenza virus H1N1 was inoculated for 1 h, MDCK cells were incubated with CAVAC-1901 or ribavirin as a positive control for 24 h. The cells were then fixed with formalin and stained with antibodies and DAPI, and image analysis was achieved by using a confocal microscope.

infection and replication occur through the fecal-oral route or respiratory tract, tracheal and cloacal swabbing is commonly implemented to estimate the severity of infection by determining the viral titer from swabs (Wille et al., 2014). As a result of viral titration from tracheal and cloacal swabs, clear differences among groups were observed from 2 dpi, in which CAVAC-1901-treated groups (50 mg/kg/d) showed a dramatically decreased viral titer (1.00 EID₅₀/0.2 mL) at 2 dpi when compared to that of the positive control group (1.33 EID₅₀/0.2 mL). Viral replication on the CAVAC-1901-treated groups (10 mg/kg/d) increased at 2 dpi compared to that at 1 dpi (Figure 4A). However, even the CAVAC-1901-treated groups (10 mg/kg/d) exhibited suppressed viral shedding to some extent when compared to that of the nontreated group.

Interestingly, the time-point of evident viral clearance differed in the control, CAVAC-1901-treated groups (10 mg/kg/d and 50 mg/kg/d) at 5 dpi, 4 dpi, and 2 dpi, respectively. The same patterns were observed from cloacal swabs except that viral titers started increasing from 2 dpi in all infected groups when compared to those at 1 dpi, indicating that transmission to the cloaca had not occurred until 1 dpi (Figure 4B). However, the extent of the increase in viral titer in the CAVAC-1901-treated groups (50 mg/kg/d) was much less than that in any other group, which reveals that CAVAC-1901 could effectively suppress viral replication.

HPAIs, such as H5N1 and H5N6, are characterized by severe pathogenicity in multiple organs due to their broad tissue tropism, especially in chickens (Proença-

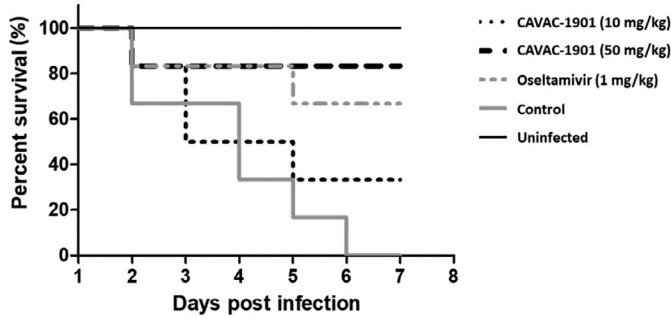


Figure 3. Anti-influenza activity of CAVAC-1901 in chickens infected by HPAI H5N6 ($n = 10/\text{group}$). Chickens, 4 wk of age, were intranasally inoculated with A/AP/Korea/W612/2017 virus (H5N6) at a dose of $10^{3.5}\text{EID}_{50}/\text{mL}$ ($200 \mu\text{L}$ injection per chicken, 5CLD_{50}). Samples were orally administered to chickens by a syringe once per day for 5 d. Changes in the survival rates of chickens were monitored daily for 8 d after infection in the CAVAC-1901-treated group (10 mg/kg; black dotted line, 50 mg/kg; black dashed line), oseltamivir-treated group (1 mg/kg; gray dashed line), control group (gray solid line), and uninfected group (black solid line).

Módena et al., 2007). More specifically, low-pathogenicity avian influenza (LPAI) brings about mild or subclinical respiratory or gastrointestinal infection in poultry with symptoms such as ruffled feathers and a drop in egg production in birds. In contrast, HPAI causes fatal systemic infection with high mortality rates (up to 100%) (Alexander, 2000). This difference lies in the types of cleavage sites in their HA precursor proteins and proteases required for cleavage of HA precursor proteins. LPAI uses trypsin-like proteases, which are predominantly present in the epithelial cells of the respiratory and gastrointestinal tracts to cleave its

monobasic site in its HA precursor protein, whereas HPAI utilizes convertases, which are ubiquitously found in a variety of cell types, to cleave its multibasic site (Wood et al., 1995; Swayne, 2007). Hence, we evaluated viral titers from various organs to assess the severity of infection. As shown in Figure 5, in all organs, high viral titers were observed in the nontreated group. Additionally, viral titers in the lung and intestine were greater than those in the spleen, kidney and brain in most groups. Notably, the CAVAC-1901-treated groups (50 mg/kg/d) showed significantly decreased viral titers, with values less than 0.6 ($\text{EID}_{50}/0.2 \text{ mL}$) in all organs, which were much less than those in the positive control group, which had values in the range of 1.0 to 2.3 ($\text{EID}_{50}/0.2 \text{ mL}$). Considering the pathophysiology of HPAI, this observation clearly demonstrated that CAVAC-1901 is effective against HPAI.

In light of our result and the report that not migratory wild birds but poultry and their trade are implicated in the geographic transmission of HPAI (van der Kolk, 2019), CAVAC-1901 could have a far-reaching impact and application with respect to the “one health” concept, which is an interdisciplinary collaborative effort to achieve welfare for humans, animals and the environment. The occurrence of HPAI not only threatens human society but also places a considerable burden on the environment (Short et al., 2015). There have been few reports on anti-influenza activity especially in livestock such as chicken and pig. Green tea by product extract was evaluated for its anti-influenza activity in chicken (Lee et al., 2012). However, majority of reports have shown the activity of single natural product extracts such as

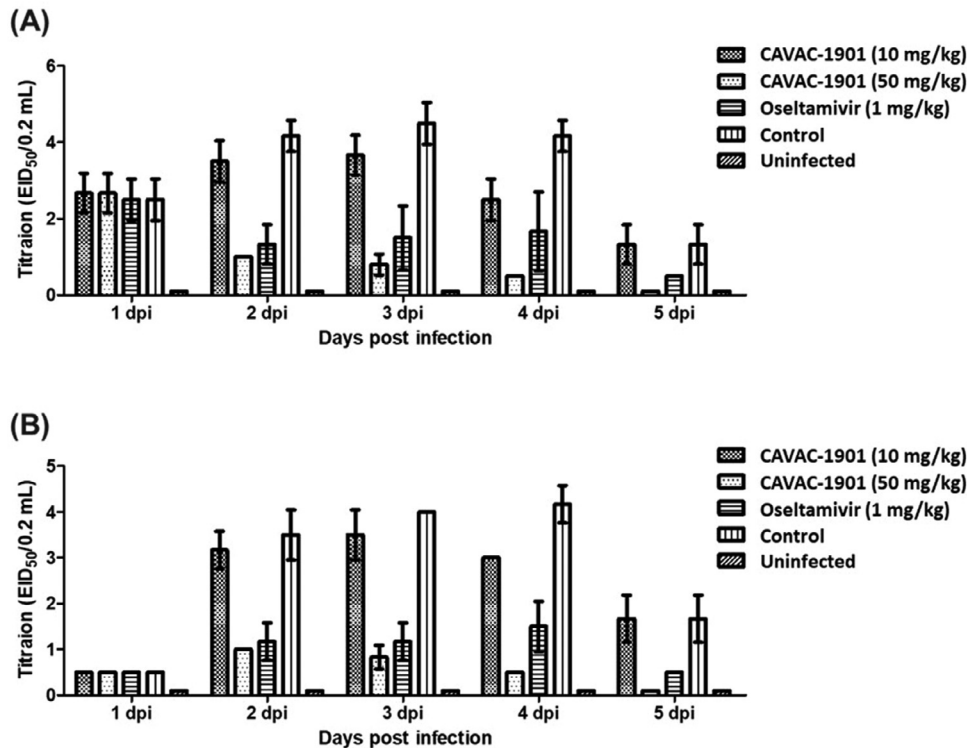


Figure 4. Viral titers of chickens after H5N6 infection in each group. Tracheal (A) or cloacal swabs (B) were obtained from surviving chickens in each group and they were subjected to HA assay to determine the viral titers daily until 5 dpi. Data are shown as the mean \pm SD; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to the control group.

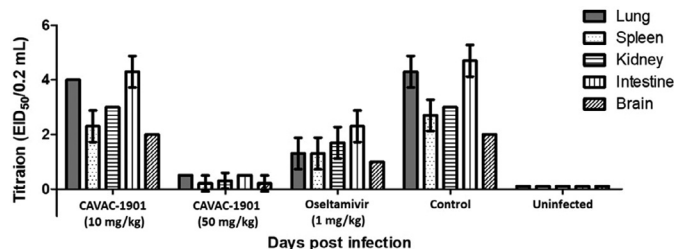


Figure 5. Viral titers of different organs of chickens after H5N6 infection in each group. Two chickens from each group were euthanized at 4 dpi and organs including the lung, spleen, kidney, intestine, and brain were extracted from each. After homogenization and dilution, an HA assay was implemented to determine viral titers from the aforementioned extracted organs. Data are shown as the mean \pm SD; * P < 0.05, ** P < 0.01, and *** P < 0.001 compared to the control group.

propolis, roots of *Pelargonium sidoides*, and leaves of wild black currant in mouse using mouse-adapted influenza virus (Shimizu et al., 2008; Theisen and Muller, 2012; Haasbach et al., 2014). The present study scientifically demonstrated for the first time that CAVAC-1901, which is composed of 3 plant extracts to be used for treating various human diseases, could be applied as a preventive tool against influenza virus. Multiple components in CAVAC-1901 with different modes of action could lower the probability of the occurrence of resistant variants. Thus, treatment with CAVAC-1901 would be supportive to current measures as part of multilateral efforts.

CONCLUSIONS

In our present study, as an application of MCMT, we combined 3 different plant extracts whose active constituents were evaluated for their mode of action and anti-influenza activity through our previous studies and evaluated the inhibition of CPE, in combination with different ratios. Our optimally formulated mixture of extracts, CAVAC-1901, exhibited strong inhibitory effects on the expression of viral proteins. More interestingly, it showed strong inhibitory activities against HPAI of H5N6 in animal experiments using chickens. Our observations represent an alternative solution for zoonotic influenza as well as human-circulating influenza in the respect that the medicinal plants composing CAVAC-1901 have been used in humans for a long time.

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DISCLOSURES

All authors declare that they have no conflict of interest regarding this study.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2022.102315](https://doi.org/10.1016/j.psj.2022.102315).

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