







RESEARCH ARTICLE

# Antiviral Effects of Novel Herbal Medicine KIOM-C, on Diverse Viruses

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## Abstract

In order to identify new potential antiviral agents, recent studies have advocated thorough testing of herbal medicines or natural substances that are traditionally used to prevent viral infections. Antiviral activities and the mechanism of action of the total aqueous extract preparation of KIOM-C, a novel herbal medicine, against diverse types of viruses were investigated. *In vitro* antiviral activity against A/Puerto Rico/8/34 (H1N1) (PR8), vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) through the induction of type-I interferon related protein phosphorylation and up-regulation of pro-inflammatory cytokines in murine macrophage cells (RAW264.7) were determined. *In vivo*, KIOM-C-treated BALB/c mice showed higher survivability and lower lung viral titers when challenged with A/Aquatic bird/Korea/W81/2005 (H5N2), A/PR/8/34(H1N1), A/Aquatic bird/Korea/W44/2005(H7N3) or A/Chicken/Korea/116/2004(H9N2) influenza subtypes in contrast with the non-treated group. The present study revealed that total aqueous extract preparation of KIOM-C stimulates an antiviral state in murine macrophage cells and in mice leading to inhibition of viral infection and protection against lethal challenges.

## Introduction

Several viral pathogens cause malignant disease in humans (pandemic influenza virus) and animals (Newcastle disease virus) and animals worldwide, resulting in significant mortality and economic losses. Although various therapeutic drugs and vaccines have been developed to prevent and treat these diseases, the emergence of novel mutants and resistant strains reduces their efficacy. However, the lack of availability and high cost of effective drugs and vaccines necessitates further research on alternative approaches. In addition, because of the concerns

about the side effects of conventional medicine, the use of standardized herbal preparations as an alternative to conventional treatment for the prevention and treatment of various diseases has been on the rise [1]. For centuries, herbal medicines have been traditionally utilized for the treatment of various ailments, including viral diseases [2–4]. The discoveries of effective Western medicines, such as aspirin, which can be obtained from white Willow bark, or quinine, an antimalarial drug derived from the bark of *Cinchona officinalis*, have increased the interest for more elaborate research on herbal medicines and paved the way toward the acceptance of the efficacy of using standardized herbal preparations [5, 6].

KIOM-C, a compound mixture of herbal medicine, was found during a screening of over 100 herbal medicine formulations. Previous studies have shown that pigs supplemented with KIOM-C improved their overall growth performance and restored their viability from porcine circovirus-associated disease (PCVAD) [7]. Kim et al. revealed an increased production of anti-viral cytokines and a decreased level of pro-inflammatory cytokines and chemokines following to clearance of the Influenza virus in the respiratory tracts of mice due to the oral administration of KIOMC [8]. Recently, it has been reported that daily oral administrations of KIOM-C at doses of 170 and 510 mg/kg can efficiently block lung metastasis in C57BL/6J mice following injection of B16F10 cells in the tail vein [9]. Although these traditional herbal medicines from various herbal plants have been tested and proven to have potential against diseases, the breadth of their protection against a wide range of viruses and mechanisms of action has remained largely unknown.

The innate immune system is the first line of defense against microbial infection and consists of type I interferons (IFNs) and pro-inflammatory cytokines [10]. Type I IFNs,  $\alpha$  and  $\beta$ , are regulated by IFN regulatory factor 7 (IRF-7), IRF-3, NF- $\kappa$ B and several intracellular signaling molecules, which are activated by germline-encoded pattern recognition receptors that recognize the molecular pattern specific to microorganisms [11, 12]. During viral infection, a rapid production of IFNs is needed to prevent the spread of viruses in the host. In this study, we investigated the KIOM-C-induced signaling molecules that activate such antiviral mediators as type I interferons, pro-inflammatory cytokines and interferon-stimulatory genes, which may be responsible for the antiviral state in murine macrophage cells. In addition, we evaluated the efficacy of a total aqueous extract preparation of KIOM-C against A/Puerto Rico/8/34 (H1N1) (PR8), vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) *in vitro*. Furthermore, we tested the potential of KIOM-C against divergent subtypes of influenza A/Aquatic bird/Korea/W81/2005(H5N2), A/PR/8/34(H1N1), A/Aquatic bird/Korea/W44/2005 (H7N3) and A/Chicken/Korea/116/2004(H9N2) *in vivo*.

## Materials and Methods

### Ethical Statements

Treatment and challenge experiments for this study were conducted in BSL-2 and BSL-3+ laboratory facilities, respectively, with the approval of the Institutional Animal Care and Use Committee of Bioleaders Corporation, Daejeon, South Korea, protocol number: BSL-ABLS-13-

002. The survival rate was determined by death or a body weight loss cut-off of 25%, at which point the animals were killed. Out of 116 (total mice), 80 mice were assigned for the survival test against four different influenza subtypes. Among them 18 mice were directly died of viral infection and the rest were killed after final monitoring. All efforts were made to minimize the suffering of the animals, and all surviving mice were humanely killed by CO<sub>2</sub> inhalation for 5 min.

## Preparation of Total Aqueous Extract KIOM-C

KIOM-C (PCT Patent Application PCT/KR2010/000107) was provided by the KM-based Herbal Drug Development Group, Korea Institute of Oriental Medicine. KIOM-C consists of *Scutellariae Radix*, *Glycyrrhizae Radix*, *Paeoniae Radix Alba*, *Platycodongrandiflorum*, and *Zingiberofficinaletc* [7]. Fifty grams (g) of KIOM-C formula was placed in 1 liter of distilled water and extracted by heating for 3 hours (h) at 115°C using a medical heating plate (Gyeongseo Extractor Cosmos-600, Incheon, Korea). After extraction, KIOM-C was filtered out using testing sieves (150 µm) (Millex, Darmstadt, Germany), lyophilized, and stored in desiccators at 4°C. The extract was prepared by combining 10 g KIOM-C with 100 ml phosphate-buffered saline (PBS), vortexing for 30 min, and centrifuging the mixture at 2000 X g for 15 min. The supernatant was collected, and the pH was adjusted to 7.0. The total aqueous extract was then subjected to membrane syringe filtration (0.45 and 0.22 µm) (Millex, Darmstadt, Germany) and stored at 4°C until administration.

## Determination of Effective Concentration (EC<sub>50</sub>) of KIOM-C in Vitro

RAW264.7 cells were grown in 96-well plates ( $2.5 \times 10^4$  cells/well) and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. After 12 hours, the medium was replaced with two-fold serially diluted KIOM-C (50 µL/well) (1–25 µL/mL or 0.1–2.5 µg/mL). At 12 hour post treatment (hpt), cells were washed with PBS once and infected using DMEM containing 1% FBS. Cells were infected with PR8-GFP (MOI = 0.1), VSV-GFP (MOI = 1.0) or NDV-GFP (MOI = 1.0) viruses. At 2 hours post infection (hpi), the inocula were removed, washed with PBS once and replaced with DMEM containing 10% FBS. The experiments were performed in duplicate. GFP expression was measured 12 hpi with the Glomax multi-detection system (Promega, WI, USA), according to the manufacturer's instructions. Graphs were developed for the individual viruses based on the dilutions and the GFP expression values. The EC<sub>50</sub> values were then calculated as the extract concentration yielding 50% GFP expression.

## Determination of Cytotoxic Concentration (CC<sub>50</sub>) of KIOM-C in Vitro

The CC<sub>50</sub> was evaluated in a cell viability assay through trypan blue exclusion test as described elsewhere [20]. The assay was performed using 48-well tissue culture plates. Increasing concentrations (1–160 µl/mL or 0.1–16 µg/ml) of KIOM-C extract was added to RAW264.7 (75–80% confluent) cell monolayers. After 12 h, the cell viability was determined by trypan blue exclusion test. Clarified cells from each treatment group were mixed with 0.4% trypan blue stain (Invitrogen, USA) at a 1:1 ratio. After staining, 10 µl of the mixture was applied to a hemocytometer to obtain the percentages of viable cells; the total number of viable/live cells per ml of aliquot was divided by the total number of cells/ml of aliquot multiplied by 100. Cell counting was done thrice. A graph of the concentrations of the extract as a function of cell viability was developed, and the CC<sub>50</sub> was calculated as the concentration of the extract resulting in 50% cell viability. The experiment was performed in duplicate.

## Cells and Viruses

RAW264.7 (ATCC TIB-71), MDCK (ATCC CCL-34, NBL-2) and Vero (ATCC CCL-81) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% 120 antibiotic/antimycotic solution (Gibco, Grand Island, NY, USA) at 37°C with 5% CO<sub>2</sub>. Green Fluorescent Protein (GFP)-tagged Influenza A {A/PuertoRico/8/34(H1N1) (PR8-GFP)}, Newcastle Disease Virus (NDV-GFP), and challenge Influenza viruses [{A/Aquaticbird/Korea/

W81/2005(H5N2)}, {A/PR/8/34(H1N1)}, {A/Aquaticbird/Korea/W44 /2005(H7N3)}, and {A/Chicken/Korea/116/2004(H9N2)}} were propagated in the allantoic fluid of 10-day-old chicken embryos. Vesicular Stomatitis Virus (VSV-GFP) was propagated on confluent Vero cells. The authors received the Green Fluorescence Protein (GFP)-tagged PR8, NDV and VSV viruses from Dr. Jae U. Jung, Department of Molecular Microbiology and Immunology, University of Southern California, USA.

### Virus Replication Inhibition Assay

A virus replication inhibition assay was performed using the GFP viruses described previously [13]. We decided to test viral pathogens previously used as challenged viruses in several studies such as Vesicular Stomatitis Virus [14–17], Newcastle Disease Virus [17–19] and Influenza A virus [13,17] to check the antiviral effect of KIOM-C against wide range of viruses. RAW264.7 cells were cultured in 12-well plates ( $8 \times 10^5$  cells/well) for 12 h. DMEM alone (untreated and virus-only groups), with 1000 U recombinant mouse interferon (IFN)- $\beta$  (positive control, Sigma, St. Louis, USA), or with 1  $\mu$ g/ml KIOM-C was added to the cells. After 12 h, the cells were infected with PR8-GFP (MOI = 0.1), NDV-GFP (MOI = 1) or VSV-GFP (MOI = 1). GFP expression was observed 12 hpi at 200X magnification. Cell viability was determined via trypan blue exclusion test [20]. Cell counts were performed in triplicate. GFP expression was measured 24 hpi with the Glomax multi-detection system (Promega, Wisconsin, USA) according to the manufacturer’s instructions [21].

### Virus Titration of Treated RAW264.7 Cells

Virus titers for PR8 and VSV-GFP were measured by plaque assays using Vero cells, as previously described. Briefly, cells ( $8 \times 10^5$  cells/well) were cultured on 12-well plates for 12 h. The medium was replaced with DMEM alone (untreated and virus-only groups) or DMEM with 1  $\mu$ g/ml of KIOM-C. After 12 h, the cells were infected with PR8-GFP (MOI = 0.1) or VSV-GFP (MOI = 1). Supernatants from each group were collected 24 hpi and serially diluted, and then 100  $\mu$ l of each dilution was added to the wells. Following 2–3 days of incubation, the cells were examined for GFP expression under the microscope. The titers were calculated using the number of GFP cells and the dilution factor [22].

For the titration of NDV-GFP, total mRNA from RAW264.7 cells was extracted and amplified to estimate the NDV-GFP RNA expression, as previously described, with some modifications. Cells ( $8 \times 10^5$  cells/well) were cultured in 12-well plates for 12 h. The medium was replaced with DMEM alone (untreated and virus-only groups) or DMEM with 1  $\mu$ g/ml KIOM-C. After 12 h, the cells were infected with NDV-GFP (MOI = 1) and collected at 0, 6, 12, and 24 hpi. Total mRNA was extracted using the RNeasy Mini Kit (Qiagen, Seoul, Korea), converted to cDNA, and PCR was performed using specific primers (Table 1). Equal amounts of PCR products were run on 1.5% ethidium bromide agarose gels and visualized using a Gel-Doc Imaging System (Bio-Rad, Seoul, Korea). Finally, the relative band intensity (RBI) of M

**Table 1. Primer sets used to quantify viral mRNA expression.**

Gene	Primers	
	Forward	Reverse
APMV-1 M Gene	5'-AGTGATGTGCTCGGACCTTC-3	5'-CCTGAGGAGAGGCATTGCTA-3'
GAPDH	5'-TGACCACAGTCCATGCCATC-3'	5'-GACGGACACATTGGGGGTAG-3'

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and GAPDH was determined using GelDoc Imaging System Band Quantification Software (Bio-Rad) [23, 24].

### Detection of IFN-β and Pro-Inflammatory Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The pro-inflammatory cytokine-inducing effects of KIOM-C on RAW264.7 cells were examined, as previously described. Briefly, RAW264.7 cells ( $2 \times 10^6$  cells/well) were cultured in a 6-well tissue culture (TC) plate. After 12 h, cells were treated with 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich), 1 μg/ml KIOM-C in DMEM containing 1% FBS, or medium alone and then incubated at 37°C with 5% CO<sub>2</sub>. The supernatant was harvested at 0, 12, and 24 h post-treatment (hpt), clarified by centrifugation at 2500 X g for 10 min at 4°C, and stored at -20°C until analysis. Clarified supernatant was dispensed into the murine IFN-β ELISA plate for measurement of the secreted murine IFN-β, and 10-fold diluted supernatant was dispensed into the mouse TNF-α, IL-6 and IL-12 capture antibody-coated ELISA plate (BD Bioscience, USA). The subsequent steps were performed according to the manufacturer's instructions. The entire test was performed in triplicate [25].

RAW 264.7 cells were cultured for 12 h in a 6-well TC plate. The cells were treated with either DMEM containing 1% FBS alone (negative control), 100 ng/ml LPS (positive control), or 1 μg/ml KIOM-C, before they were harvested at 0, 3, 6, 12, and 24 hpt. Total mRNA was prepared, and RT-PCR was performed as previously described [13, 24, 26]. The PCR primers are listed in Table 2.

### Evaluation of Expression of IFN Related Genes upon Viral Infection

For the evaluation of expression of IFN related genes upon the viral infection, RAW264.7 cells were cultured in 12-well plates ( $8 \times 10^5$  cells/well) for 12 h and DMEM alone (untreated group), with 100 ng/ml LPS (positive control, Sigma-Aldrich), or with 1.0 μg/ml KIOM-C was added to the cells. After 12 h, the cells were infected with PR8-GFP (MOI = 0.1) and harvested at 0, 12 and 24 hpi. Total mRNA was prepared, and RT-PCR was performed as mention previously. The PCR primers are listed in Table 2.

**Table 2. Primer sets used to confirm mRNA expression.**

Gene	Primers	
	Forward	Reverse
IFN-β	5' -TCCAAGAAAGGACGAACATTTCG-3'	5' -TGCGGACATCTCCCACGTCAA-3'
Mx1	5' -ACAAGCACAGGAAACCGTATCAG-3'	5' -AGGCAGTTTGGACCATCTTAGTG-3'
PKR	5' -GCCAGATGCACGGAGTAGCC-3'	5' -GAAAACCTGGCCAAATCCACC-3'
OAS	5' -GAGGCGGTTGGCTGAAGAGG-3'	5' -GAGGAAGGCTGGCTGTGATTGG-3'
ISG-15	5' -CAATGGCCTGGGACCTAAA-3'	5' -CTTCTTCAGTTCTGACACCGTCAT-3'
ISG-56	5' -AGAGAACAGCTACCACCTTT-3'	5' -TGGACCTGCTCTGAGATTCT-3'
IFN-α	5' -ATAACCTCAGGAACAACAG-3'	5' -TCATTGCAGAATGAGTCTAGGAG-3'
TNF-α	5' -AGCAAACCACCAAGTGGAGGA-3'	5' -GCTGGCACCAGTGTGGTTGT-3'
IL-6	5' -TCCATCCAGTTGCCCTTCTTGG-3'	5' -CCACGATTTCCAGAGAACATG-3'
GAPDH	5' -TGACCACAGTCCATGCCATC-3'	5' -GACGGACACATTGGGGGTAG-3'

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## KIOM-C Treatment and Western Blot Analysis

RAW264.7 cells were cultured in 6-well tissue culture (TC) plates ( $1 \times 10^6$  cells/well) and incubated at 37°C. After 12 h, the cells were treated with either DMEM containing 10% FBS alone (negative control), 100 ng/ml LPS (positive control) or 1 µg/ml of KIOM-C before being harvested at 0, 3, 6, 12, and 24 hpt. The cell pellets were washed with phosphate-buffered saline (PBS) and lysed in radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% IGEPAL, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 µg/ml each of aprotinin, leupeptin). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with lysed samples, followed by transfer onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). For immune detection, membranes were probed with phosphorylated and nonphosphorylated forms of rabbit anti-IRF3, anti-phospho-IRF3 (Abcam, Cambridge, England), anti-p65, anti-phospho-p65, anti-STAT1, anti-phospho-STAT1, anti-TBK1, anti-phospho-TBK1, anti-p38, anti-phospho-p38, mouse anti-ERK, and anti-phospho ERK (Cell Signaling Technology, Boston, MA, USA) or anti-β-actin (Santa Cruz Biotechnology, Texas, USA) antibodies at 4°C overnight. After washing with Tris-buffered saline containing 0.05% Tween 20, the membranes were reacted with anti-mouse or anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (IgG HRP) for 1 h at room temperature. Finally, the target proteins were detected using the WEST-ZOL plus Western blot detection system (iNtRON Biotechnology, Gyeonggi do, South Korea) and visualized by the enhanced chemiluminescence detection system (ECL-GE healthcare) with a Las-3000 mini lumino-image analyzer.

## Virus Challenge Experiment in BALB/c Mice

A total of 116 female BALB/c mice (5 weeks old) were purchased from Samtako (Seoul, Korea) and acclimated for 7 days at room temperature prior to use. Animals were separated into 4 experimental sets, containing 4 groups per set. Of the 4 sets, 1 had 4 groups of 14 mice each (6 for virus titration at 3 and 5 dpi and 3 for lung histopathology). The remaining sets had 4 groups containing 5 mice each. Mice were orally administered 10 mg/ml KIOM-C at a total volume of 200 µl at 1, 3, and 5 days before infection; at 1, 3, and 5 days after infection; or both pre- and post-infection.

Mice were infected intranasally (i.n.) with 5 times the 50% mouse lethal dose (MLD<sub>50</sub>) of H5N2, H1N1, H7N3 or H9N2 in 20 µl of PBS. Treatment and challenge experiments were conducted in BSL-2 and BSL-3<sup>+</sup> laboratory facilities, respectively, with the approval of the Institutional Animal Care and Use Committee of Bioleaders Corporation, Daejeon, South Korea, protocol number: BSL-ABLS-13-002. Body weights and survival were monitored for 13 dpi at fixed time points. At 3 and 5 dpi, 3 mice in each group of one set were randomly sacrificed to measure lung virus titers. At 5 dpi, another 3 mice were randomly sacrificed from each group of the same set for lung histopathology, and the remaining 5 mice per group were used for survival. The survival rate was determined by a death or cut-off of 25% in body weight loss, at which point the animals were killed. All efforts were made to minimize suffering, and all surviving mice were humanely killed using CO<sub>2</sub> inhalation for 5 min after final monitoring.

## Lung Virus Titer and Histopathology

Lung viral titers were measured by median tissue culture infectious doses (TCID<sub>50</sub>) using Madin-Darby canine kidney (MDCK) cells, as reported previously. Briefly, the mouse lungs were homogenized in 500 µl PBS containing antibiotic/antimycotic compounds. Confluent MDCK cells grown in 96-well microtiter plates were infected with 10-fold serial dilutions (in DMEM) of lung homogenate (50 µl/well). After 1 h at 37°C in a humid atmosphere with 5%

CO<sub>2</sub>, medium containing (TPCK) trypsin (Thermo Fisher Scientific, Rockford, USA) was added to the infected media and incubated for 72 h. Viral cytopathic effects were observed daily, and titers were determined by hemagglutination assay (HA) [27].

For histopathology, the lung tissues were immediately fixed in 10% formalin-containing neutral buffer, embedded in paraffin, sectioned at 4–6-µm thickness using a microtome machine, mounted on slides, and stained with eosin. Histopathological changes were examined by light microscopy, as previously described [28].

### Statistical Analysis

Differences between untreated and KIOM-C-treated groups were analyzed by Student’s *t*-test. *P* values less than 0.05 were regarded as significant, and those less than 0.01 were regarded as highly significant. Comparisons of survival were conducted by log-rank test using GraphPad Prism 6.0.

### Results

#### Determination of the Effective Concentration (EC<sub>50</sub>) and Cytotoxic Concentration (CC<sub>50</sub>) of KIOM-C *in Vitro*

EC<sub>50</sub> values of KIOM-C were determined against divergent viruses *in vitro*. For this, we developed a modified GFP assay as previously described using RAW264.7 cell lines [29, 30]. GFP-tagged viruses were used and 50% reduction in GFP expression was considered as equivalent to the 50% reduction in virus titer. As shown in Table 3, KIOM-C inhibited the replication of PR8-GFP (MOI = 0.1), VSV-GFP (MOI = 1.0) and NDV-GFP (MOI = 1.0) by 50% at EC<sub>50</sub> values of 0.76 ± 0.03 µg/ml, 0.64 ± 0.02 µg/ml and 0.49 ± 0.01 µg/ml, respectively. Considering these EC<sub>50</sub> values, we selected 1.0 µg/ml as the optimum dosage of KIOM-C for further *in vitro* antiviral assays based on its effectiveness and convenience during the experiments.

The cytotoxicity of KIOM-C was assessed based on a cell viability test following treatment with various concentrations. KIOM-C had CC<sub>50</sub> values of 8.71 ± 0.07 µg/ml in RAW264.7 cells (Table 3). The selection indexes of KIOM-C (SI) for PR8, VSV and NDV on RAW264.7 cells were 11.4, 13.6 and 17.8, respectively, suggesting that the extract could be broadly useful as a prophylactic or therapeutic agent.

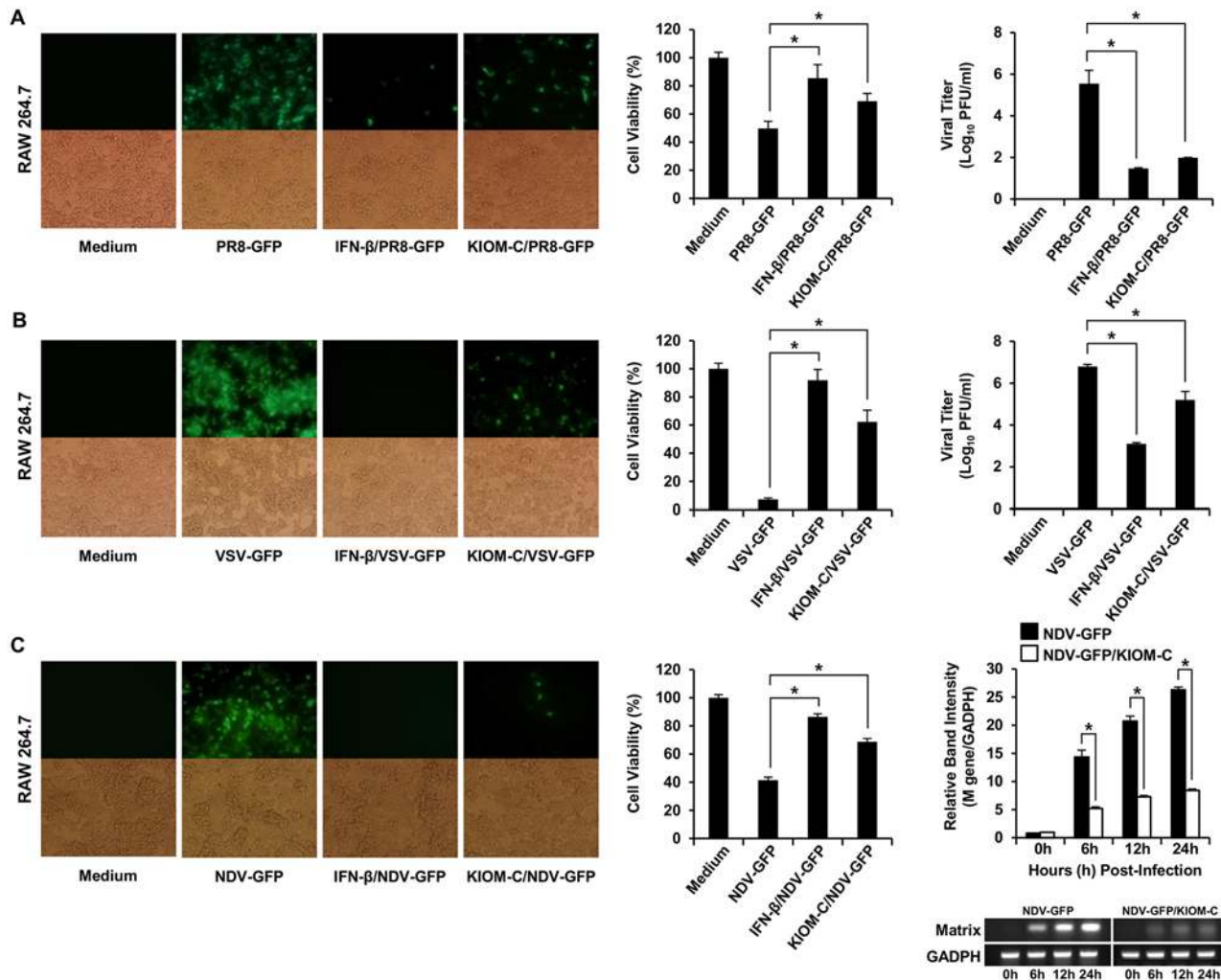
#### Inhibition of Virus Replications in KIOM-C-Treated RAW264.7 Cells

To determine the *in vitro* antiviral activity of KIOM-C, we checked the viral replication with GFP-expressing viruses treated with cytotoxic free KIOM-C (Fig 1). KIOM-C-treated RAW264.7 cells exhibited markedly reduced GFP expression, whereas the untreated group had high levels of GFP expression for PR8 (Fig 1A left panel), VSV (Fig 1B left panel), and NDV (Fig 1C left panel). When quantitated, the KIOM-C-treated cells showed a significant reduction in GFP expression compared to the untreated group (data not shown). These findings correlate with the viral supernatant titers of the VSV-GFP and PR8-GFP-infected cells in which

**Table 3. Determination of EC<sub>50</sub> and CC<sub>50</sub> of KIOM-C in RAW264.7 cells.**

	RAW264.7			CC <sub>50</sub> ± S.D. (µg/ml)
	PR8-GFP	VSV-GFP	NDV-GFP	
EC <sub>50</sub> ± S.D. (µg/ml)	0.76±0.03	0.64±0.02	0.49±0.01	8.71±0.07
SI (CC <sub>50</sub> /EC <sub>50</sub> )	11.4	13.6	17.8	

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**Fig 1. Anti-viral activities of the KIOM-C extracts in RAW264.7 cells.** Cells treated with media alone, 1 μg/ml KIOM-C extract, or 1000 U/ml recombinant mouse IFN-β 12 h prior to infection with (A) PR8-GFP, (B) VSV-GFP or (C) NDV-GFP at an MOI of 0.1, 1.0 and 1.0, respectively. Images of GFP expression (left panel) were obtained 12 hpi (200 X magnification). Cell viabilities (middle panel) were determined by trypan blue exclusion at 24 hpi, presented as a percentage of the controls (cells without treatment). Viruses were titrated from the supernatant as PFU (right panel). In the case of NDV-GFP, the expression of NDV M mRNA over time in each treatment group was confirmed by specific PCR primers, which are shown in Table 1. All samples were normalized using GAPDH. Equal amounts of PCR products were run on 1.5% ethidium bromide agarose gels and visualized using the GelDoc Imaging System. The relative band intensity (RBI) of M mRNA expression from the same experiment is shown. RBI was determined (gene/GAPDH) using GelDoc Imaging System Band Quantification Software. Error bars indicate the range of values obtained from three independent experiments. Virus titers in the cell supernatant are expressed as means ± SD. Error bars indicate the range of values obtained from counting in triplicate during three independent experiments (\*P<0.05 indicates a significant difference between groups compared by Student's *t*-test).

doi:10.1371/journal.pone.0125357.g001

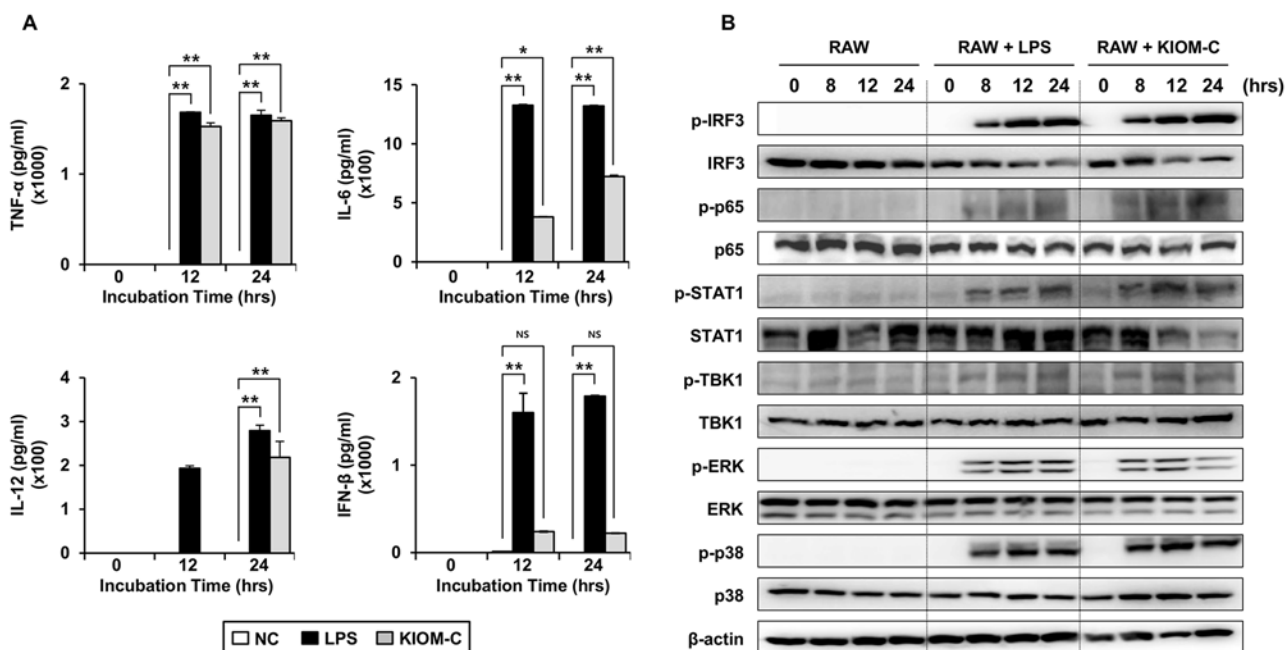
KIOM-C treatment significantly reduced the viral titer in comparison with the virus-only group (Fig 1A and 1B right panel). Similarly, KIOM-C-treated cells had a significant reduction in cell death compared with untreated cells, which had ≤50% cell viability within 24 hpi for all viruses (Fig 1A, 1B and 1C middle panel). We measured the mRNA expression of the NDV M gene via RT-PCR to estimate the replication (Fig 1C right panels) of NDV-GFP. As expected, the expression of the M-gene in the KIOM-C-treated cells was significantly lower than in the untreated group at 6–24 hpi. These results showed evidence that the herbal extract of KIOM-C could significantly reduce viral replication in RAW264.7 cells.



### KIOM-C Activates the Type I IFN Signal Pathway in RAW264.7 Cells

To elucidate the possible mechanism of the KIOM-C-induced inhibition of viral replication, we first measured the levels of secreted cytokines involved in the antiviral state of the treated and untreated RAW264.7 cells. KIOM-C was able to induce high levels of secreted TNF- $\alpha$  and IL-6 (Fig 2A upper panel) and a moderate secretion of IL-12 (Fig 2A lower panel) at 24 hpt, compared with LPS-treated cells. Likewise, although not at levels as high as in LPS-treated cells, KIOM-C-treated cells showed detectable amounts of IFN- $\beta$  compared to the media-treated cells (Fig 2A lower panel). The results show that TNF- $\alpha$ , IL-6, IL-12 and IFN- $\beta$  can be induced by KIOM-C, which may mediate the antiviral state in murine macrophage cells. And also, we confirmed the secretion of IL-6 and IFN- $\beta$  from KIOM-C-treated RAW264.7 cells in the presence of PR8-GFP infection. As shown in S1A Fig, after viral infection, KIOM-C-treated RAW264.7 cells induce levels of secreted IL-6 and IFN- $\beta$  like LPS-treated cells. These results suggest that KIOM-C mediate the antiviral state in murine macrophage cells by secretion of type-I IFN and proinflammatory cytokines.

To correlate these above observations with the IFN signal pathway, we examined the effects of KIOM-C on type I IFN-related protein phosphorylation. For this, whole cell lysates of KIOM-C extract-treated RAW264.7 cells were subjected to immunoblotting to analyze the expression of the phosphorylated and nonphosphorylated forms of IRF3, p65, STAT1, TBK1, p38 and ERK. As shown in Fig 2B, KIOM-C treatment in RAW264.7 cells up-regulated the phosphorylation of IRF-3, STAT1, and TBK1, which are important molecules in the type I IFN and NF-kb signaling pathway. The phosphorylation of IRF3 indicates the translocation of the



**Fig 2. Stimulation of an antiviral state by KIOM-C extract in RAW264.7 cells.** Cells were treated with DMEM + 1% FBS alone, with 100 ng/ml LPS derived from *E. coli* or with 1  $\mu$ g/ml KIOM-C extract and then incubated at 37°C with 5% CO<sub>2</sub>. Cells treated with DMEM + 1% FBS served as the negative controls. The supernatant from each group was harvested at 0, 12, and 24 hpt and clarified by centrifugation at 2500 X g for 10 min at 4°C. Ten-fold diluted supernatant was dispensed to the mouse. (A) The cytokines (TNF- $\alpha$ , IL-6, IL-12 or IFN- $\beta$ ) secreted in treated RAW264.7 cells were measured by cytokine ELISA using antibody-coated ELISA plates. The test was performed in triplicate. The data show representative means  $\pm$  SD of each murine cytokine measured over time from three independent assays. The asterisk indicates a significant difference between groups (\*\* $P$ <0.01; \* $P$ <0.05). N.S., not significant. (B) Immunoblot analysis was performed on the cell lysates of RAW264.7 cells treated with or without KIOM-C to assess the expression of the phosphorylated and nonphosphorylated forms of IRF3, p65, STAT1, TBK1, p38, ERK and  $\beta$ -actin time dependently (h).

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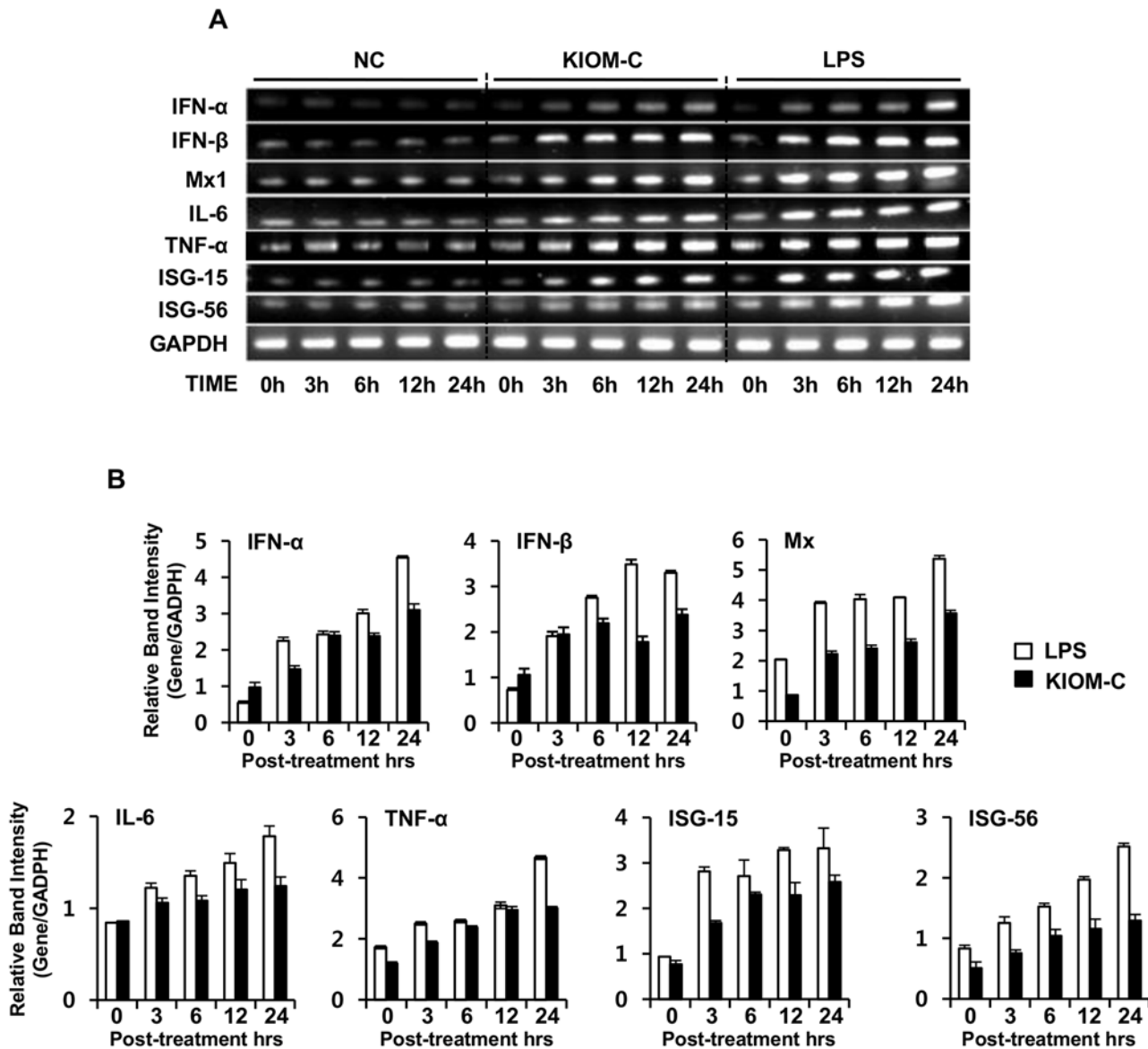
IRF3 molecules into the nucleus and the initiation of the transcription of type I IFNs. Consequently, the produced type I IFNs binds to the JAK-STAT pathway, leading to the phosphorylation of STAT1 and the transcriptional activation of interferon-stimulating genes (ISGs). Furthermore, the increased activation of p38 and ERK, which are required for the serine phosphorylation of STAT-1, indicated the active functions of ISGs [33]. Besides the activation of type I IFNs, extract-treated raw cells were able to generate an obvious activation of NF- $\kappa$ B (P65), leading to a strong secretion of pro-inflammatory cytokines. Our results clearly demonstrate that KIOM-C extract treatment can induce IRF3, p65, STAT1, TBK1, p38 and ERK phosphorylation at 8 hpt at levels that subsequently increased compared with untreated cells (Fig 2B right panel). Interestingly, the phosphorylation of these molecules by the induction of the extract is comparable to that obtained by LPS treatment, a known potent stimulator of TLR-4 (Fig 2B middle panel).

We further confirmed the presence of an interaction between KIOM-C and induction of IFN-stimulated genes in RAW264.7 cells. A time-dependent increase in the mRNA expression regarding IFN- $\alpha$ , IFN- $\beta$ , IL-6, TNF- $\alpha$ , ISG56, ISG15, and Mx1 was observed in KIOM-C-treated RAW264.7 cells, compared with untreated cells (Fig 3A and 3B). And also, the expression of IFN related genes upon viral infection was further confirmed in PR8-GFP infected RAW264.7 cells to ensure the specificity of KIOM-C in inducing strong antiviral response. As shown in S1B Fig, after viral infection, KIOM-C-treated RAW264.7 cells induced the antiviral (PKR, OAS, Mx-1), IFN- $\beta$  and IFN-stimulated genes (ISG-15 and ISG-56) both at 12 and 24 hpi. Importantly, the observed pattern was similar but greater compared with the LPS treated positive control. The overall results suggest that KIOM-C can induce the antiviral state by modulating the IFN signal pathway as well as the ISGs in RAW264.7 cells, which may result in the inhibition of virus replication.

## Protection Efficacy of KIOM-C against Lethal Influenza Infection in BALB/c Mice

To confirm the KIOM-C-induced *in vivo* protection efficiency and its breadth against the influenza virus, groups of BALB/c mice were treated with KIOM-C, before or after or before and after infection with 5MLD<sub>50</sub> of divergent subtypes of influenza A H5N2, H1N1, H7N3, or H9N2. The untreated (0.85% saline) group suffered severe illness and significant body weight loss by 3 to 7 dpi, succumbing by day 9 post-infection to all of the viruses tested. In contrast, the KIOM-C-treated mice showed a  $\leq 15\%$  body weight loss between 3 and 7 dpi and had begun to recover their lost weight by 8 dpi, returning to their normal states by 13 dpi. Furthermore, we observed that survival was different between the groups that had received KIOM-C pre- or post-infection. Interestingly, compared to the other two groups (pre- or post-treatment), mice treated with KIOM-C at both pre- and post-infection showed higher protection levels: 100%, 100%, 100%, and 80% for H5N2 (Fig 4A), H1N1 (Fig 4B), H7N3 (Fig 4C), and H9N2 (Fig 4D), respectively. These results indicate that KIOM-C has potential preventive and therapeutic activities against influenza A viruses and that a combined treatment (preventive and therapeutic) can increase the protection efficacy compared to single strategy (preventive or therapeutic) *in vivo*.

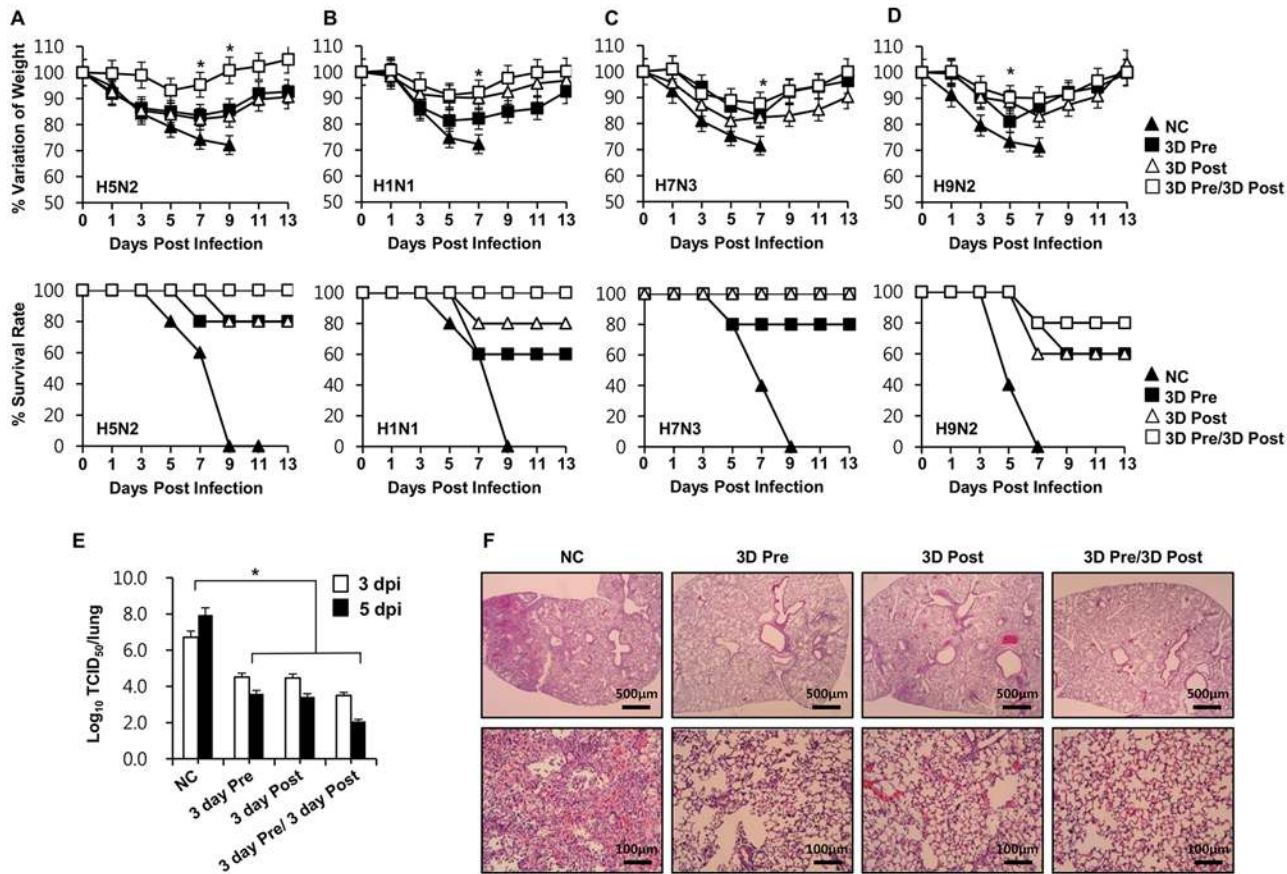
To determine the ability of KIOM-C to inhibit viral growth in the lungs of the infected mice, at 3 and 5 dpi of H5N2 infection, 3 mice from each group were sacrificed. Their lungs were collected for virus titration. These mice received KIOM-C before or after the infection and had lower viral titers at 3 and 5 dpi than the untreated group, which had titers of 6.7 and 7.9 log<sub>10</sub> TCID<sub>50</sub>, respectively (Fig 4E). The lungs from the KIOM-C-treated mice (both pre- and post-infection) also showed a significant reduction in the virus levels at 3 and 5 dpi



**Fig 3. Stimulation of interferon-stimulating genes (ISGs) in RAW264.7 cells.** The time-dependent changes in mRNA expression after treatment with KIOM-C extract were confirmed by RT-PCR using the primers shown in Table 2. All samples were normalized using GAPDH, and (A) equal amounts of PCR products were run on 1.5% ethidium bromide agarose gels and visualized using the GelDoc Imaging System. (B) The relative band intensities of IFN-β, IFN-α, TNF-α, IL-6, and IFN-related genes (Mx1, ISG15, and ISG56) were determined using the Image J analysis program and are shown as a bar graph. Error bars indicate the range of values obtained from three independent experiments.

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(Fig 4E). In addition, the untreated group had severe lung tissue damage with disrupted epithelial cells, and the perivascular and alveolar spaces were infiltrated with mononuclear cells. However, the KIOM-C-treated mice showed no remarkable pulmonary inflammation (Fig 4F). These results demonstrate that the KIOM-C-induced antiviral states are strong enough to inhibit virus replication, which promotes the survival of mice against lethal infections with influenza A.



**Fig 4. KIOM-C extract protects BALB/c mice against lethal infection with divergent influenza subtypes.** BALB/c mice were treated orally with 200  $\mu$ l/mouse of 100  $\mu$ g/ml KIOM-C at 1, 3, and 5 days pre-; 1, 3, and 5 days post-; or both pre- and post-infection, along with (A) A/Aquatic bird/Korea/W81/2005 (H5N2) (lower panel, log-rank test,  $P = 0.0016$ ), (B) A/PR/8/34(H1N1) (lower panel, log-rank test,  $P = 0.0019$ ), (C) A/Aquatic bird/Korea/W44/2005 (H7N3), (lower panel, log-rank test,  $P = 0.0006$ ) or (D) A/Chicken/Korea/116/2004(H9N2), (lower panel, log-rank test,  $P = 0.0048$ ) influenza subtypes. Mice treated with 0.85% normal saline (200  $\mu$ l/mouse) prior to virus challenge were considered the negative controls (NC). Weight loss (upper panel) and percent survival (lower panel) were recorded until 13 dpi. (E) The virus titers in the lung tissues were measured by TCID<sub>50</sub> at 3 and 5 dpi. (F) A representative image of the histopathological damage in eosin-stained lung tissue from mice treated with KIOM-C (pre-, post-, or pre- and post-infection) or 0.85% saline. Lungs of the KIOM-C-treated mice show clear alveoli without inflammatory cell infiltration, as opposed to the lungs of NC mice, which revealed features of severe pneumonitis. Tissues were observed under a light microscope at 200 X magnification. Bars denote means  $\pm$  SD. Comparisons of groups were analyzed by Student's t-tests and ANOVA. The asterisk indicates a significant difference between groups (\* $P < 0.05$ ).

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## Discussion

Natural herbal medicines are gaining popularity as a means to control viral infections [33, 34, 35] due to their safety and low incidence of side effects. As a substitute for chemosynthesis drugs and vaccines, they show potential against a wide range of viruses, such as vaccinia, vesicular stomatitis virus, sendai [36–38], etc. In particular, the anti-influenza virus effects of several herbal extracts have been reported [39–41]. Here, we demonstrated that the oral administration of a novel herbal mixture (KIOM-C) protects against the divergent subtypes of influenza A virus in BALB/c mice. A previous study revealed that Baicalin, the most dominant compound (at 15.03–15.12%) in KIOM-C, had an antiviral activity against a wide range of viruses, inhibiting the entry of human immunodeficiency virus-1 into host cells [42] and suppressing sendai viral growth in mice [43]. Furthermore, Baicalin was also demonstrated to block Chlamydia trachomatis infection in vitro [44]. The immune-regulatory and immune-enhancing effects of these herbal plants used in the KIOM-C formulation have also been investigated [45–48]. Despite their potential

usage against a wide range of viruses, no detailed mechanism has yet been described. Although we were unable, in the present study, to demonstrate the mechanism of an individual compound and its antiviral effects, we were certainly able to show that a total compound of KIOM-C was sufficiently able to protect against viral replication through the induction of an antiviral state and the modulation of the innate immune response.

Aside from the non-immune cells, innate immune cells, such as macrophages and dendritic cells (DCs), initially recognize viral infections so that they can rapidly evoke the induction of type I interferons and pro-inflammatory cytokines, generating anti-viral immune responses [31, 32]. Likewise, we hypothesize that KIOM-C may induce an antiviral state in murine macrophage cells via the induction of antiviral cytokines and the modulation of the immune response and overall inhibition of virus replication. Thus, we determined that type-I IFN, pro-inflammatory cytokines, and IFN-stimulated genes, at both the mRNA (Fig 3A and 3B) and secreted-protein levels (Fig 2A), affected KIOM-C-treated RAW264.7 cells. Thus, cytokines possess functions that are both beneficial and detrimental to the host, in addition to their regulation of harmful functions. For example, a cytokine storm is crucial for effective protection against viral infection, particularly in influenza virus infections [49]. KIOM-C-treated RAW264.7 cells showed a notable pattern of cytokine regulation, correlating with the observations found in the cell viability assay in this study (Fig 1A, 1B and 1C middle panel).

For a clearer understanding of the effect of KIOM on the activation of type I IFN signaling molecules, a detailed investigation of IRF-3, P-65, TBK 1, STAT 1, ERK and p38 protein phosphorylation was performed on extract-treated RAW264.7 cells. These include the key signaling molecules of the type I interferon and NF-Kb pathway. Upon stimulation of the PRRs (Pattern Recognition Receptors) of the host cell by pathogen-associated molecular patterns (PAMPs), downstream signal transduction initiates the induction of type I interferons and pro-inflammatory cytokines to up-regulate the antiviral status of the host cell [50, 51]. However, the phosphorylation of IRF3 is a key indicator of signal transduction that initiates the transcription of type I interferons. A previous study has shown that cells lacking IRF3 are unable to produce type I IFN in viral infections and that TBK1 and IKK-I control both IRF3 and STAT1 [52]. In this study, we found that the herbal extract KIOM-C treatment can induce the phosphorylation of TBK1, IRF3 and STAT1, providing evidence of the downstream activation of the signaling molecules in the type I IFN pathway. In addition, the activation of NF-Kb (P65), which leads to a strong secretion of pro inflammatory cytokines, could also be observed in KIOM-C-treated RAW264.7 cells. These secreted inflammatory cytokines activate the immune cells and act to rapidly clear the viruses. Similarly, KIOM-C showed significant reductions in pulmonary virus titers and lung tissue damage after a lethal infection of the influenza A virus (Fig 4E and 4F). We also found that mice treated with KIOM-C, pre- or post-infection, showed protection against lethal infections of influenza; mice treated both pre- and post-infection had stronger protection than any of the other groups, potentially indicating that KIOM-C can induce an antiviral state independently.

In conclusion, our findings clearly demonstrate that KIOM-C can be a significant alternative antiviral therapeutic agent to disrupt viral infection through the activation of type I IFN signaling molecules and pro-inflammatory cytokines, providing an antiviral state in murine macrophage cells. *In vivo* results indicate that KIOM-C treatment can reduce influenza-induced mortality by disrupting viral replication or preventing viral infection by creating an antiviral state in the lungs. However, further study is needed to identify the specific substance/compound present in the herbal formulation that induces this antiviral response, both *in vivo* and *in vitro*. In addition, the specific signaling pathways, the best dosage for practical application, and the duration that the extract can be effective in the host should be examined. Finally, this study suggests that KIOM-C has anti-viral functions against a wide range of viruses, such as NDV, VSV, and PR8 *in vitro*, and divergent influenza A subtypes H5N2, H1N1, H7N3, and H9N2 *in vivo*. Moreover,

our findings describe a possible mechanism of action of KIOM-C in viral infection via the induction of the type-IIFN signaling pathway. Thus, the use of KIOM-C as a preventive or therapeutic medicine, in combination with the current vaccines or individually, constitutes a promising means to control viral infections in both prophylactic and therapeutic applications.

## Supporting Information

**S1 Fig. Determination of the level of type-I interferon, pro-inflammatory cytokine and IFN related gene induction by KIOM-C *in Vitro* by ELISA and real-time PCR Analysis upon viral infection in the presence of viral infection.** (A) RAW264.7 cells were treated with DMEM containing 10% FBS alone, with 100 ng/ml LPS, or with 1.0 µg/ml KIOM-C and incubated at 37°C with 5% CO<sub>2</sub>. 12 hpt, LPS or KIOM-C treated cells were infected with PR8-GFP (MOI = 1.0) and supernatant from each group was harvested at 0, 12 and 24 hpi and clarified by centrifugation at 2500 x g for 10 min at 4°C. Clarified supernatants were dispensed into the murine IFN-β and IL-6 capture antibody-coated ELISA plate to measure cytokine secretion. The test was performed in duplicate for IFN-β and in triplicate for IL-6. The data shows representative means ± SD of each murine cytokine measured over time. (B) RAW264.7 cells were treated with DMEM + 10% FBS alone, KIOM-C (1.0 µg/ml), or 100 ng/ml of LPS and infected with PR8-GFP (MOI = 1.0). The time-dependent changes in mRNA expression after treatment in RAW264.7 cells were confirmed by real-time PCR using the primers shown in Table 2. Real-time PCR was carried out with the use of a QuantiTect SYBR Green PCR kit (Qiagen) on a Mygenie96 thermal block (Bioneer). (TIF)

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## Author Contributions

Conceived and designed the experiments: CJK JSL WKC JYM. Performed the experiments: MRT MYEC MEP PW THK. Analyzed the data: MYEC JSL. Contributed reagents/materials/analysis tools: JSL JYM. Wrote the paper: MRT MYEC JSL.

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