

Original Article

Investigation of Anti-inflammatory and Anti-oxidative Activities of *Lonicerae* Flos, *Citri* Pericarpium and *Violae* Herba Complex (LCVC)

Hong Kyoung Kim

Dept. of Acupuncture & Moxibustion Medicine, College of Korean Medicine, Daejeon University

Objectives: The anti-inflammatory and anti-oxidative activities of LCVC (*Lonicerae* Flos, *Citri* Pericarpium and *Violae* Herba Complex) have not been fully elucidated. The purpose of this study was to investigate the mechanisms underlying these effects in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages.

Methods: The evaluation of the anti-oxidative activity of LCVC was completed via DPPH and ABTS radical scavenging capacity, FRAP assay, measurement of polyphenol and flavonoid, assessment of ROS and NO levels in LPS-induced RAW 264.7 cells. The anti-inflammatory activity was defined by measuring the production of biomarkers (PGE₂, IL-1 β , IL-6 and TNF- α), proteins (ERK, JNK, P38, Nrf2, Keap1, HO-1 and NQO1) and expressions of genes (iNOS, COX-2, IL-1 β , IL-6, TNF- α , Nrf2, Keap1, HO-1 and NQO1) in LPS-induced RAW 264.7 cells.

Results: LCVC have polyphenol and flavonoid contents. The results of DPPH and ABTS free radical scavenging capacity and FRAP assay showed that the anti-oxidative activity was increased. Production of ROS, NO, IL-6, TNF- α , mRNA expressions of IL-1 β , IL-6, TNF- α , Keap1, iNOS and COX-2 were decreased, and NQO1, Nrf2, and HO-1 were increased. In protein expression, JNK and Keap1 were decreased, NQO1, Nrf2 and HO-1 were increased, and no relationships were observed with the ERK and P38 by LCVC.

Conclusions: These results suggest that LCVC may offer protective effects against LPS-induced inflammatory and oxidative responses through attenuating Nrf2/HO-1 pathway and MAPKs pathway. Therefore, we propose that LCVC has anti-inflammatory and anti-oxidative activities that have therapeutic potential in the treatment of inflammatory and oxidative disorders caused by the over-activation of macrophages.

Key Words : *Lonicerae* Flos, *Citri* Pericarpium, *Violae* Herba, LCVC, Anti-oxidative, Anti-inflammatory

Introduction

Inflammation is one of the mechanisms which immune cells, blood vessels, and molecular mediators are activated to defend the body due to infection or trauma¹⁾. However, if the inflammatory response is continuous or uncontrolled, excessive production of inflammatory factors can cause

various diseases such as rheumatoid arthritis (RA), alzheimer's disease (AD), hepatitis, pneumonia, tumor, and nephritis^{2,3)}.

Reactive oxygen species (ROS) generated in the human body can be removed through mechanisms of biological metabolism. However, if the ROS is over produced or oxidative response is uncontrolled, oxidative stress could

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• Correspondence to : Hong Kyoung Kim
Daedeok-daero 176beon-gil, Seo-gu, Daejeon, Republic of Korea
Tel : +82-42-470-9000, Fax : +82-42-477-9007, E-mail : goheekoo@naver.com

induce loss of cell function and apoptosis. It could cause variety of systemic problems to the kidney, liver, heart, blood vessels, skin, etc⁴).

Macrophages are one of the factors that activate the innate immune responses. They defend the body and repair cells by delicately regulating the signaling pathways that mediate the immune response⁵. Macrophages manage primary defense role at the body and if it react with Lipopolysaccharide (LPS) which is one of the main components of the outer membrane surrounding the peptide glycan of the surface layer of Gram-negative bacteria, they activate to cause inflammation by producing interleukin-6 (IL-6), interleukin-1 β (IL-1 β), cyclooxygenase-2 (COX-2), prostaglandin e2 (PGE2), tumor necrosis factor- α (TNF- α), iNOS (inducible nitric oxide synthase), nitric oxide (NO), and other various inflammatory mediators⁶⁻⁸).

NO, one of the major inflammatory mediators causing inflammatory diseases, plays an important role in cell signal transduction and is involved in various biopathological processes. It can cause DNA damage and mutations⁹. COX-2 over-produces PGE2, which can cause pain, fever, excessive inflammation, and cancer¹⁰. Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a major role in inhibiting inflammatory processes, and Nrf2 activators are utilized in the treatment of various inflammatory diseases¹¹. Heme oxygenase-1 (HO-1), a target of Nrf2, is an enzyme that promotes the decomposition of carbon monoxide (CO), biliverdin, and free iron. It reduces the production of inflammatory mediators and promotes the production of anti-inflammatory cytokines¹². It is known that

HO-1 plays a very important role in the mechanism of protecting cells from inflammatory and oxidative stress, and also regulates NO production of activated macrophages¹³. The mitogen-activated protein kinase (MAPK) pathway plays an important role in the inflammatory response. That process is activated by extracellular signal-regulated kinase (ERK), stress-activated protein kinase (JNK), and p38 mitogen-activated protein kinase (p38MAPK)⁸. LPS stimulation activate phosphorylation of ERK, JNK, and p38 to cause inflammation.

According to Herbology, the *Lonicerae* Flos, *Violae* Herba is classified as drugs for eliminating heat and relieving toxicity, and the *Citri* Pericarpium for Regulating Ki Flow. It is reported that these herbal medicines have pharmacological effects such as anti-oxidative, anti-inflammatory, and anti-tumor effect. *Lonicerae* Flos is effective for anti-fungal, and eliminating pathogenic heat from blood to cure dysentery¹⁴. Pharmacologically, it is reported to have antibacterial, anti-oxidative, anti-inflammatory, and anticancer effect¹⁵. As the *Violae* Herba is effective for clearing away heat from the Yeong and the blood systems, it has been prescribed as a drug to treat skin inflammation, enteritis, tuberculosis, and inflammatory diseases. Pharmacologically, it exhibits various physiological activities such as antioxidant, anti-inflammatory, pain suppression, brain cell protection, and antibacterial action against various pathogenic microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *dermatococcus*, and *pneumococcus*^{16,17}. As the *Citri* Pericarpium has the effects of controlling qi, eliminating dampness

and phlegm, it has been prescribed as a drug to treat bloating, anorexia, vomiting, nausea, cough¹⁸⁾. Pharmacologically, it is reported to have physiological activities such as antibacterial, antioxidant, anticancer, anti-inflammatory, and anti-obesity effects¹⁹⁾. According to previous studies, it has been demonstrated that *Lonicerae* Flos, *Citri* Pericarpium, and *Violae* Herba individually exhibit anti-inflammatory effects. As *Lonicerae* Flos and *Violae* Herba have similar medicinal properties, those are used in combination with each other in various inflammatory diseases, such as prostatitis, appendicitis, and mastitis¹⁴⁾. *Citri* Pericarpium is used clinically as a prescription in combination with *Lonicerae* Flos and *Violae* Herba²⁰⁾. As such combinations of prescription are widely used in clinical fields, effective verification of the anti-inflammatory effects of those would be of great help in the clinical evidence. However, research on complexes composed of these three herbs, *Lonicerae* Flos, *Citri* Pericarpium, and *Violae* Herba has not been implemented before. Therefore, the author administered LCVC to LPS-induced RAW 264.7 macrophages to measure changes of inflammatory mediators such as NO and PGE2, inflammatory cytokines such as TNF- α and IL-1 β , and inflammatory response mediators such as ROS. Furthermore, additional experiments were conducted to find out relationship with Nrf2/HO-1 pathway which is a strong antioxidant activity signaling pathway and MAPKs which is a major intracellular signaling pathway associated with antioxidant and anti-inflammatory effects. This study is reported as valid results were obtained by analyzing those factors.

Materials & methods

1. Materials

1) Sample

Lonicerae Flos, *Citri* Pericarpium, and *Violae* Herba Complex (LCVC) used in the experiment were purchased from Daejeon Korean medical pharmacy, Daejeon, Republic of Korea and used after removing unnecessary materials other than the medicinal part at the Department of Korean Pathology from Daejeon University.

2) Reagent

Gallic acid (Sigma, U.S.A.), ethanol (Merck, Germany), folin-Ciocalteu's phenol reagent (Merck, Germany), quercetin (Sigma, U.S.A.), aluminum nitrate nonahydrate (Sigma, U.S.A.), sodium carbonate (Sigma, U.S.A.), potassium acetate solution (Sigma, U.S.A.), 1,1-diphenyl-2-picryl-hydrazyl (DPPH : Sigma, U.S.A.), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS : Sigma, U.S.A.), Dulbecco's modified eagle's medium (DMEM : Gibco, U.S.A.), fetal bovine serum (FBS : Gibco, U.S.A.), penicillin-streptomycin (Sigma, U.S.A.), lipopolysaccharides from *Escherichia coli* O111:B4 (Sigma, U.S.A.), EZ-cytox (Daeilab, Korea), dulbecco's phosphate buffered saline (D-PBS : Welgene, Korea), trypan blue (Sigma, U.S.A.), accupower[®] cyclescript RT premix (dT20) (Bioneer, Korea), NO (Nitrix Oxide) plus detection kit (Intronbio, Korea), easy-spin[™] total RNA extraction kit (Intronbio, Korea), DEPC-DW (Bioneer, Korea), mouse IL-1 beta ELISA kit (Komabiotech, Korea), qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems, U.S.A.), mouse IL-6 ELISA kit (Komabiotech, Korea), mouse

TNF-alpha ELISA kit (Komabiotech, Korea), RIPA lysis and extraction buffer (Thermo Fisher, U.S.A.), protease inhibitor cocktail (Sigma, U.S.A.), phosphatase inhibitor cocktail 2 (Sigma, U.S.A.), phosphatase inhibitor cocktail 3 (Sigma, U.S.A.), sample buffer (Laemmli's 5x) (ELPISbiotech, Korea), Pierce™ BCA protein assay Kit (Thermo Fisher, U.S.A), 1.5M Tris-HCl, pH 8.8 w/SDS (Intronbio, Korea), 0.5M Tris-HCl, pH 6.8 w/SDS (Intronbio, Korea), Acrylamide-Bis Solution 30%, 29:1 (Intronbio, Korea), 10% ammonium persulfate (Thermo Fisher, U.S.A), TEMED (Bio-RAD, U.S.A.), 10X Tris-Glycine-SDS buffer (Intronbio, Korea), GangNam-STAIN™ prestained protein ladder (Intronbio, Korea), 10X transfer Buffer (Intronbio, Korea), methyl alcohol (Samchunchemicals, Korea), 10X TBS with Tween 20 (Intronbio, Korea), ultra pure bovine serum albumin (BSA : GenDEPOT, U.S.A.), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (Cell signaling, U.S.A.), p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (Cell signaling, U.S.A.), Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb (Cell signaling, U.S.A.), SAPK/JNK Antibody (Cell signaling, U.S.A.), Phospho-p38 MAPK (Thr180/ Tyr182) (D3F9) XP® Rabbit mAb (Cell signaling, U.S.A.), NRF2 (D1Z9C) Antibody (Cell signaling, U.S.A.), KEAP1 (D6B12) Antibody (Cell signaling, U.S.A.), p38 MAPK Antibody (Cell signaling, U.S.A.), NQO1 Antibody (Abcam, UK), HO-1 (E3F4S) Antibody (Cell signaling, U.S.A.), peroxidase-conjugated affinipure goat anti-mouse IgG (H+L) (Jackson immunoresearch, U.S.A.), miracle-star™, peroxidase-conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson immunoresearch,

U.S.A.), western blot detection system (Intronbio, Korea) were used in the experiment.

3) Machine

Heating mantle with stirring for extraction apparatus (Misung scientific, Korea), rotary vacuum evaporator (EYELA FDU-540, Japan), freeze dryer (Ilshin biobase, Korea), autoclave (Sanyo, Japan), CO2 incubator (Sanyo, Japan), vortex mixer (Vision scientific, Korea), clean bench (Vision scientific, Korea), deep-freezer (Sanyo, Japan), centrifuge (Hanil, Korea), micro plate reader (Molecular Devices, U.S.A.), ice-maker (Brema, Australia), alpha cycler 1 PCRmax (PCRmax, U.K.), plate shaker (Lab-Line, U.S.A.), nanodrop (Thermo Fisher, U.S.A.), mini trans-Blot (Bio-RAD, U.S.A.), Rotor-Gene Q (Qiagen, Germany), fursion FX (Vilber, U.S.A.) were used in the experiment

2. Methods

1) Preparation of herbal complex extract

500 ml of distilled water was added to the amount of 1 tablet of the LCVC and heat reflux extracted for 3 hours. The extract was filtered with filter paper, concentrated under reduced pressure with a rotary vacuum evaporator, and freeze-dried using a freeze dryer to obtain 4.61 g (yield 15.38%) of powder. After that, it was stored in a cryogenic freezer (-80°C) and diluted in distilled water according to the concentration required for the experiment. The contents and dose of the prescription used in this experiment are as follows (Table 1).

Table 1. The prescription of LCVC

The name of herbal medicine	weight (g)
<i>Lonicerae</i> Flos	10
<i>Citri</i> Pericarpium	10
<i>Violae</i> Herba	10
Total amount	30

2) Evaluation of antioxidant efficacy

(1) Measurement of total polyphenol content

LCVC was set at concentration of 1 mg/ml. 0.5 ml of 50% FoilnCiocalteu's phenol reagent was added to 1 ml of LCVC and reacted at room temperature for 3 minutes. The reaction solution was sequentially mixed with 7.5 ml of distilled water and 1 ml of saturated Na₂CO₃ solution, standed for 30 minutes, centrifuged at an intensity of 14,000 g for 10 minutes, and absorbance at a wavelength of 760 nm was measured by taking the supernatant. The total phenol content was calculated according to the calibration curve prepared using gallic acid as a standard material.

(2) Measurement of total flavonoid content

The 0.5 ml mixture was made by mixing 0.1 ml of LCVC with a concentration of 1 mg/ml and 0.9 ml of 80% ethanol. 10% aluminum nitrate, 1 M potassium acetate 0.1 ml and 4.3 ml of 80% ethanol were added at the mixture. After those procedures, the mixture was left at room temperature for 40 minutes. Then, the absorbance at 415 nm wavelength was measured, and the content was obtained through a standard curve prepared using quercetin.

(3) Measurement of DPPH radical scavenging activity

The final concentrations of LCVC were diluted to 1, 10, 100, and 1000 µg/ml, respectively, and 150 µl of 0.2 mM DPPH solution dissolved in ethanol and 100 µl of LCVC were mixed and reacted at 37° C for 30 minutes. And the absorbance at a wavelength of 517 nm was measured. Distilled water was added as a control group of the sample, and ethanol was added as a control group for the DPPH solution to derive a correction value. The calculation of the DPPH radical scavenging ability was performed as follows.

$$\text{Scavenging activity (\%)} = \left(\frac{\text{absorbance (Control)} - \text{absorbance (Sample)}}{\text{absorbance (Control)}} \right) \times 100$$

(4) ABTS radical scavenging activity

The final concentrations of LCVC were diluted to 1, 10, 100, and 1000 µg/ml, respectively, and the ABTS solution was made by mixing 7.4 mM ABTS (2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) and 2.6 mM potassium persulphate. Thereafter, the prepared ABTS was left in a dark place for one day to form cations (ABTS⁺), the absorbance was measured at 732 nm, diluted until absorbance value was 1.5 or less, and 150 µl of the diluted ABTS⁺ solution and LCVC 5 µl of each was mixed and reacted at room temperature for 10 minutes, and absorbance was measured at 732 nm wavelength. ABTS radical scavenging ability was calculated as follows.

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance(Sample)}}{\text{Absorbance(Control)}} \right) \times 100$$

(5) Measurement of FRAP activity

FRAP reagent was prepared by mixing 10 mM TPTZ and 20 mM FeCl₃ dissolved in 300 mM sodium acetate buffer (pH 3.6) and 40 mM HCl in a ratio of 10:1:1, respectively. After that, 100 μ l of LCVC of 1, 5, 10, and 50 μ g/ml and 900 μ l of distilled water were added to a 15 mL tube, and 2 mL of FRAP reagent was added to the mixed solution, followed by reaction in the dark for 30 minutes. After the reaction, absorbance was measured at a wavelength of 593 nm, and antioxidant activity was obtained according to a calibration curve prepared using FeSO₄ as a standard material.

3) Cell culture

RAW 264.7 cells were subcultured every 2-3 days in a cell incubator maintained at 37°C and 5% CO₂ environment using DMEM medium composed of 10% fetal bovine serum and 1% penicillin-streptomycin.

4) Measurement of cell viability

RAW 264.7 cells were aliquoted into a 24 well plate at 5×10^4 cells/well and cultured for 24 hours, then LCVC was treated at concentrations of 50, 100, and 200 μ g/ml, and cultured again for 24 hours. After 24 hours, 10 μ l of EZ-Cytox solution per 100 μ l of the cell culture medium was added and reacted in a cell incubator for 30 minutes. After the reaction, the change in absorbance seen at a wavelength of 450 nm was measured, and the cell viability relative to the control was expressed as a percentage.

5) Measurement of ROS production

RAW 264.7 cells were seeded in a 24 well plate at 5×10^4 cells/well and cultured for 24 hours. The normal group was not treated with LCVC and LPS, and the control group was treated with only LPS at a concentration of 100 ng/ml. The experimental group was treated with LCVC at a concentration of 50, 100, and 200 μ g/ml and LPS at a concentration of 100 ng/ml, and incubated for 24 hours again. Thereafter, the cells collected by centrifugation at 1200 rpm for 5 minutes were washed twice with cold PBS, DCF-DA and added to a concentration of 10 μ M, and placed at room temperature in a dark place for 30 minutes. After staining, cold PBS was added, centrifuged at 1200 rpm for 5 minutes, the supernatant was removed, and 400 μ l of PBS was suspended again. After that, the change of the fluorescence intensity of the remaining solution was measured with a flow cytometer and expressed as a percentage of the control.

6) Measurement of NO production

RAW 264.7 cells were seeded in a 24-well plate at 5×10^4 cells/well and cultured for 24 hours. The normal group was not treated with LCVC and LPS, and the control group was LPS at a concentration of 100 ng/ml. only processed. The experimental group was treated with LCVC at a concentration of 50, 100, and 200 μ g/ml and LPS at a concentration of 100 ng/ml and incubated for 24 hours again. After incubation, 100 μ l of the cell culture medium was added to a 96 well plate, 50 μ l of N1 buffer was added, and reacted at room temperature for 10 minutes. After the reaction, 50 μ l of N2 buffer was added and reacted at room temperature for 10 minutes.

After the reaction, the change in absorbance at 540 nm was measured, and the production amount relative to the control was expressed as a percentage.

7) Measurement of biomarker production

RAW 264.7 cells were seeded in a 6-well plate at 3×10^5 cells/well and cultured for 24 hours. After incubation, the normal group was not treated with LCVC and LPS, and the control group was treated with only LPS at a concentration of 100 ng/ml. The experimental group was treated with LCVC at a concentration of 50, 100, and 200 $\mu\text{g/ml}$ and LPS at a concentration of 100 ng/ml and incubated for 24 hours again. Then, 100 μl of the cell culture solution was added to a 96 well plate and reacted at room temperature for 2 hours. After the reaction, the reagents on the plate were discarded, and washing buffer was added and washed 4 times. After washing, 100 μl of detection antibody was added and reacted at room temperature for 2 hours. After the reaction, 100 μl of streptavidin-HRP was added to the plate and allowed to react at room temperature for 30 minutes. After the reaction, 100 μl of TMB or pink-ONE solution was put into each well, reacted for 15 minutes, 100 μl of stop solution was added, and absorbance was measured at 450 nm through a micro reader, and the absolute value was displayed based on a standard curve.

8) Measurement of gene expression

(1) Extraction of RNA

RAW264.7 cells were seeded in a 6-well plate at 3×10^5 cells/well and cultured for 24 hours.

After incubation, the normal group was not treated with LCVC and LPS, and the control group was treated with only LPS at a concentration of 100 ng/ml. The experimental group was additionally treated with LCVC at a concentration of 50, 100, and 200 $\mu\text{g/ml}$ and LPS at a concentration of 100 ng/ml, and incubated for 24 hours again. Thereafter, 1 ml of easy blue and 200 μl of chloroform were added to the cells, vortexed, and centrifuged at 13000 rpm and 4° C. for 10 minutes. After 400 μl of the supernatant and 400 μl of binding buffer were reacted at room temperature for 1 minute, 700 μl of the reaction solution was injected into the column and centrifuged at 13000 rpm for 30 seconds. 700 μl of washing buffer A was added to the column and centrifuged at 13000 rpm for 30 seconds. Then, 700 μl of washing buffer B was added and centrifuged in the same manner. After replacing the bottom of the column with a 1.5 ml tube, 30 μl of elution buffer was added to the column, reacted for 1 minute, and centrifuged at 13000 rpm for 1 minute to extract total RNA.

(2) cDNA synthesis

For the reverse transcription reaction, 1 μg of total RNA mixture (reaction buffer, dNTPs mixture, RNase inhibitor, stabilizer, oligo dT15 primer) of the RT premix kit was added, and DEPC-DW was added so that the final volume was 20 μl . After mixing this mixture well, react at 45°C for 60 minutes to synthesize first-strand cDNA, and leave at 95°C for 5 minutes to inactivate M-MLV RT and completed cDNA was used for polymerase chain reaction (PCR)

(3) Gene amplification

Real-time PCR was performed to amplify the synthesized cDNA, and put 1 μ l of cDNA, 2 μ l of each primer, 10 μ l of SYBR Green, and 5 μ l of DEPC-DW into a real time specialized tube and react at 95°C for 2 minutes. Then, the gene was amplified by repeating 40 times for 5 seconds at 95°C and 30 seconds at 62.5°C. The gene expression level was calculated compared to the control group, and the sequences of the primers used are shown in Table 2.

9) Measurement of protein expression level

RAW264.7 cells were seeded in a 6-well plate at 3×10^5 cells/well and cultured for 24 hours. After incubation, the normal group was not

treated with LCVC and LPS, and the control group was treated with only LPS at a concentration of 100 ng/ml. The experimental group was treated with either LCVC at a concentration of 50, 100, or 200 μ g/ml and LCVC at a concentration of 100 ng/ml, or additionally treated with 100 ng/ml LPS and cultured again for 24 hours. Thereafter, RIPA buffer containing protease inhibitor cocktail I and phosphatase inhibitor II and III was added to the cells to extract proteins. The extracted protein was quantified using a pierce BCA protein assay kit, mixed with sample loading buffer, and reacted at 95°C for 5 minutes to prepare. The prepared proteins were separated by size by SDS-PAGE through 10% acrylamide gel, and

Table 2. The Sequences of Primers

Primer	F/R*	Sequences
iNOS	F	CGAAACGCTTCACTTCCAA
	R	TGAGCCTATATGCTGTGGCT
COX-2	F	AACCGCATTGCCTCTGAAT
	R	CATGTTCCAGGAGGATGGAG
IL-1 β	F	AAGAAGAGCCCATCCTCTGT
	R	GGAGCCTGTAGTGCACTTGT
IL-6	F	AGTCCTTCCTACCCCAATTTC
	R	GGTCTTGGTCCTTAGCCACT
TNF- α	F	ATGGCCTCCCTCTCATCAGT
	R	TTTGCTACGACGTGGGCTAC
Nrf2	F	AACAGAACGGCCCTAAAGCA
	R	TGGGATTCACGCATAGGAGC
Keap1	F	AACCTGTCACACTGCCAGC
	R	GGCAGTCGTATTGACCCAGT
HO-1	F	ATGGCGTCACCTCGTCAGAG
	R	GCTGATCTGGGGTTCCCTC
NQO1	F	TCGCCATTTTCTTCGGGCTA
	R	TGAGCAATTCCTTCTGCCC
β -actin	F	AGGGAAATCGTGCCTGACAT
	R	TCCAGGGAGGAAGAGGATGC

* F : forward, R : reverse

transferred to a PVDF membrane. The protein-transferred membrane was immersed in 5% BSA and reacted at room temperature for 2 hours. After washing 3 times with TBS-T buffer, p-ERK (1:2000), ERK (1:1000), p-JNK (1:2000), JNK (1:1000), p-P38 (1:1000), P38 (1:1000), Nrf2 (1:1000), Keap1 (1:1000), HO-1 (1:1000), NQO1 (1:1000), β -actin (1:5000) first antibody and reacted at 4°C for 14 hours. After washing again 5 times, the secondary antibody (1:10000) was added and reacted at room temperature for a hour, washed again 10 times, and the protein was developed through ECL solution. Then, the protein expression level was analyzed through chemidoc fusion FX.

3. Statistical processing

SPSS 21.0 was used to analyze experimental results which were expressed as mean±standard error of mean, multiple comparisons were performed using ANOVA, and significance was tested at $p<0.05$, $p<0.01$, and $p<0.001$ levels through Tukey's HSD test

Results

1. Total polyphenol and flavonoid content of LCVC

As a result of measuring the total polyphenol

content in LCVC using gallic acid as a standard material, it was found to be 61.47 ± 1.57 mg GAE/g and flavonoid content in LCVC using quercetin as a standard material, it was found to be 17.43 ± 1.11 mg QE/g (Table 3).

2. The antioxidant activity of LCVC

1) DPPH and ABTS radical scavenging activity

As a result of measuring the DPPH and ABTS radical scavenging ability according to the treatment of LCVC, the scavenging ability of LCVC was increased concentration-dependently (Table 4).

2) FRAP activation

As a result of measuring FRAP activity, FRAP activity of LCVC was increased concentration-dependently (Table 4).

3. Cytotoxicity

As a result of measuring cell viability, LCVC showed a concentration-dependent cell proliferation at a concentration of $50 \mu\text{g/ml}$ or higher, and showed a cell viability of less than 90% at a concentration of $400 \mu\text{g/ml}$ or higher (Table 5)

4. Effects on production of ROS

When the concentration of LCVC was $50 \mu\text{g/ml}$ or higher, the amount of ROS production

Table 3. Total Polyphenol and Flavonoid Contents of LCVC

	Total polyphenol (mg GAE [#])/g)	Total flavonoid (mg QE ^{##})/g)
LCVC	61.47 ± 1.57	17.43 ± 1.11

The results were described as mean \pm standard deviation.

[#]) Milligram of gallic acid equivalent (GAE) per gram of extract was used to express total phenol content

^{##}) Milligram of quercetin equivalent (QE) per gram of extract were used to express total flavonoid contents

decreased significantly (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$) compared to the control group (Table 6).

5. Effects on production of NO

When the concentration of LCVC was 50 μ g/ml or higher, the amount of NO production decreased significantly (*: $p < 0.05$, **: $p < 0.01$,

***: $p < 0.001$) compared to the control group (Table 7).

6. Effects on pro-inflammatory cytokine production

When the concentration of LCVC was 100 μ g/ml or higher, the amount of PGE2 and IL-1 β production decreased significantly (** : $p < 0.01$,

Table 4. DPPH, ABTS Radical Scavenging Activity and FRAP Value of LCVC

Concentrations (μ g/ml)	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	FRAP value (μ M)
Concentration I			
1	6.82 \pm 1.14	6.64 \pm 0.28	-
10	8.83 \pm 0.33	9.71 \pm 0.20	-
100	32.62 \pm 0.84	48.71 \pm 0.89	-
1000	80.59 \pm 0.23	89.47 \pm 1.11	-
Concentration II			
1	-	-	3.54 \pm 0.19
5	-	-	8.06 \pm 1.48
10	-	-	25.20 \pm 0.97
50	-	-	46.45 \pm 0.99

Table 5. Cell viability of LCVC in RAW264.7 Cell

Concentration (μ g/ml)	Cell viability (% of Control)
Control	100.00 \pm 1.69
50	100.11 \pm 2.87
100	102.03 \pm 3.76
200	104.32 \pm 2.91
400	88.56 \pm 1.79

Table 6. Effect of LCVC on ROS Level in RAW264.7 cell

Concentration (μ g/ml)	Reactive oxygen species (% of Control)
Normal	38.36 \pm 0.54
Control	100.00 \pm 1.89 ⁺⁺⁺
50	93.64 \pm 2.93 [*]
100	84.76 \pm 0.39 ^{**}
200	72.19 \pm 0.65 ^{***}

+++ : $p < 0.001$ compared to normal, * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$ compared to control

*** : $p < 0.001$) compared to the control group (Table 8). When the concentration of LCVC was 50 $\mu\text{g/ml}$ or higher, the amount of IL-6 and TNF- α production decreased significantly (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$) compared to the control (Table 8).

7. Effects on Gene expression

When the concentration of LCVC was 100 $\mu\text{g/ml}$ or higher, gene expression level of iNOS and COX-2 were significantly decreased (** : $p < 0.01$, *** : $p < 0.001$) compared to the control group (Table 9). When the LCVC concentration was 50 $\mu\text{g/ml}$ or higher, the gene expression level of IL-1 β , IL-6 and TNF- α were significantly decreased (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$) compared to the control group (Table 9). When the concentration of LCVC was 100 $\mu\text{g/ml}$ or higher, the gene expression level of

the Nrf2 was significantly increased (** : $p < 0.01$, *** : $p < 0.001$) compared to the normal group (Table 9). When the concentration of LCVC was 50 $\mu\text{g/ml}$ or higher, the Keap1 gene expression level was significantly reduced (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$) compared to the normal group (Table 9). When the concentration of LCVC was 100 $\mu\text{g/ml}$ or higher, the HO-1 gene expression level increased significantly (*** : $p < 0.001$) compared to the normal group (Table 9). When the LCVC concentration was 50 $\mu\text{g/ml}$ or higher, the expression level of the NQO1 gene was significantly increased (** : $p < 0.01$, *** : $p < 0.001$) compared to the normal group (Table 9).

8. Effects on protein expression

No change in protein expression level of ERK, P38 was found according to the concentration of LCVC, and no significance was observed (Table

Table 7. Effect of LCVC on NO Level in RAW264.7 cell

Concentration ($\mu\text{g/ml}$)	Nitric oxide (% of Control)
Normal	19.53 \pm 1.82
Control	100.00 \pm 2.94 ⁺⁺⁺
50	92.07 \pm 2.05*
100	75.19 \pm 0.27**
200	60.19 \pm 2.69 ⁺⁺⁺

⁺⁺⁺ : $p < 0.001$ compared to normal, * : $p < 0.05$, ** : $p < 0.01$, ⁺⁺⁺ : $p < 0.001$ compared to control

Table 8. Effect of LCVC on Level of pro-inflammatory cytokines in RAW264.7 cell

Concentration ($\mu\text{g/ml}$)	PGE2 (pg/ml)	IL-1 β (pg/ml)	IL-6 (pg/ml)	TNF- α (pg/ml)
Normal	82.67 \pm 12.56	33.64 \pm 2.25	101.19 \pm 8.82	218.42 \pm 48.14
Control	585.55 \pm 10.55 ⁺⁺⁺	261.84 \pm 8.26 ⁺⁺⁺	584.56 \pm 7.87 ⁺⁺⁺	1030.24 \pm 52.28 ⁺⁺⁺
50	567.24 \pm 35.86	254.17 \pm 7.78	479.44 \pm 26.04*	880.33 \pm 38.49*
100	470.27 \pm 17.46**	207.75 \pm 6.70*	379.65 \pm 29.29*	613.97 \pm 67.19**
200	433.72 \pm 11.62 ⁺⁺⁺	196.99 \pm 5.90 ⁺⁺⁺	338.65 \pm 4.96 ⁺⁺⁺	559.61 \pm 42.14 ⁺⁺⁺

⁺⁺⁺ : $p < 0.001$ compared to normal, * : $p < 0.05$, ** : $p < 0.01$, ⁺⁺⁺ : $p < 0.001$ compared to control

Table 9. Effect of LCVC on mRNA Expression Level of pro-inflammatory cytokines in RAW 264.7 cell

Concentration ($\mu\text{g/ml}$)	mRNA fold change of factors											
	iNOS/ β -actin	COX-2/ β -actin	IL-1/ β -actin	IL-6/ β -actin	TNF- α / β -actin	Nrf2/ β -actin	Keap1/ β -actin	HO-1/ β -actin	NQO1/ β -actin	HO-1/ β -actin	Keap1/ β -actin	NQO1/ β -actin
Normal	0.22 \pm 0.05	0.08 \pm 0.02	0.32 \pm 0.02	0.18 \pm 0.01	0.14 \pm 0.01	1.00 \pm 0.02	1.00 \pm 0.03	1.00 \pm 0.03	1.00 \pm 0.03	1.00 \pm 0.03	1.00 \pm 0.03	1.00 \pm 0.03
Control	1.00 \pm 0.00 ⁺⁺⁺	1.00 \pm 0.00 ⁺⁺⁺	1.00 \pm 0.00 ⁺⁺⁺	1.00 \pm 0.00 ⁺⁺⁺	1.00 \pm 0.00 ⁺⁺⁺	-	-	-	-	-	-	-
50	0.97 \pm 0.03	0.98 \pm 0.01	0.93 \pm 0.02*	0.82 \pm 0.04*	0.84 \pm 0.02**	1.05 \pm 0.03	0.92 \pm 0.02*	1.03 \pm 0.02	1.15 \pm 0.03**	1.03 \pm 0.02	0.92 \pm 0.02*	1.15 \pm 0.03**
100	0.77 \pm 0.02**	0.89 \pm 0.01*	0.87 \pm 0.01**	0.58 \pm 0.03**	0.54 \pm 0.02***	1.11 \pm 0.01**	0.83 \pm 0.01**	1.32 \pm 0.03***	1.29 \pm 0.02***	1.32 \pm 0.03***	0.83 \pm 0.01**	1.29 \pm 0.02***
200	0.63 \pm 0.02***	0.72 \pm 0.03**	0.76 \pm 0.01 ^{xx}	0.49 \pm 0.01***	0.47 \pm 0.02***	1.23 \pm 0.02**	0.58 \pm 0.02***	1.57 \pm 0.04***	1.45 \pm 0.02***	1.57 \pm 0.04***	0.58 \pm 0.02***	1.45 \pm 0.02***

+++ : p<0.001 compared to normal, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to control (if there was no control, then normal was the reference)

Table 10. Effect of LCVC on Protein Expression Level of pro-inflammatory cytokines in RAW 264.7 cell

Concentration ($\mu\text{g/ml}$)	Protein fold change									
	p-ERK/t-ERK	p-JNK/t-JNK	p-P38/t-P38	Nrf2/ β -actin	Keap1/ β -actin	HO-1/ β -actin	Keap1/ β -actin	HO-1/ β -actin	Keap1/ β -actin	NQO1/ β -actin
Normal	0.27 \pm 0.01	0.39 \pm 0.02	0.12 \pm 0.00	1.00 \pm 0.02	1.00 \pm 0.03	1.00 \pm 0.06	1.00 \pm 0.03	1.00 \pm 0.06	1.00 \pm 0.03	1.00 \pm 0.08
Control	1.00 \pm 0.01 ⁺⁺⁺	1.00 \pm 0.02 ⁺⁺⁺	1.00 \pm 0.01 ⁺⁺⁺	-	-	-	-	-	-	-
50	1.02 \pm 0.02	0.91 \pm 0.02**	1.00 \pm 0.01	0.99 \pm 0.02	0.97 \pm 0.04	1.01 \pm 0.06	0.97 \pm 0.04	1.01 \pm 0.06	0.97 \pm 0.04	1.43 \pm 0.09***
100	1.00 \pm 0.03	0.75 \pm 0.02***	1.01 \pm 0.01	1.15 \pm 0.09*	0.89 \pm 0.03**	1.87 \pm 0.14***	0.89 \pm 0.03**	1.87 \pm 0.14***	0.89 \pm 0.03**	1.86 \pm 0.11***
200	1.04 \pm 0.02	0.44 \pm 0.01***	1.03 \pm 0.04	1.72 \pm 0.07***	0.57 \pm 0.02***	2.76 \pm 0.20***	0.57 \pm 0.02***	2.76 \pm 0.20***	0.57 \pm 0.02***	2.38 \pm 0.13***

+++ : p<0.001 compared to normal, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to control (if there was no control, then normal was the reference)

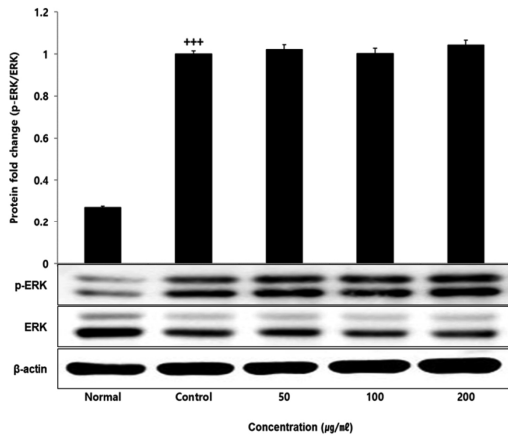


Fig. 1. Effect of LCVC on ERK protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 µg/mL of LCVC with 100 ng/mL LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, +++ : p<0.001 compared to normal)

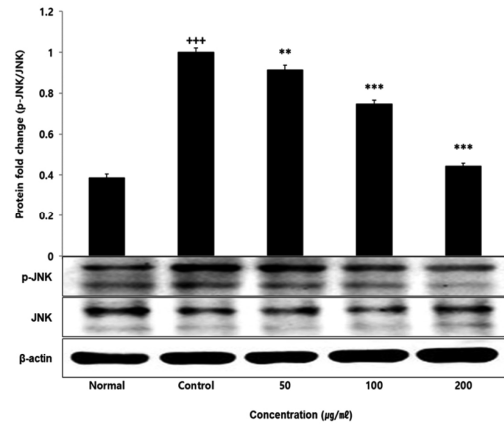


Fig. 2. Effect of LCVC on JNK protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 µg/mL of LCVC with 100 ng/mL LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, +++ : p<0.001 compared to normal, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to control).

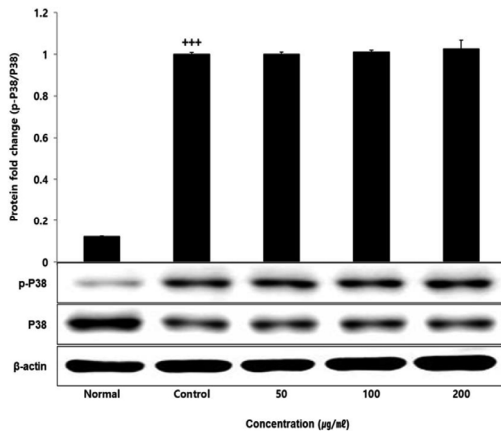


Fig. 3. Effect of LCVC on p38 protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 µg/mL of LCVC with 100 ng/mL LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, +++ : p<0.001 compared to control)

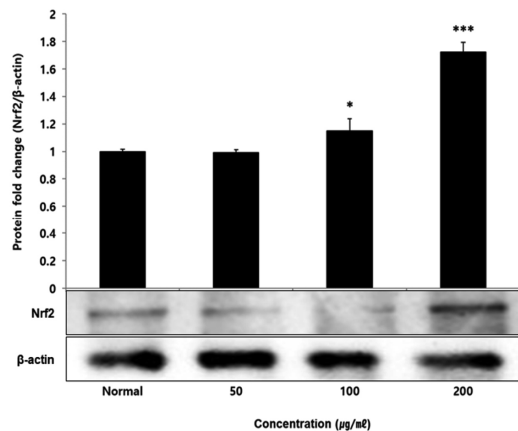


Fig. 4. Effect of LCVC on Nrf2 protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 µg/mL of LCVC with 100 ng/mL LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to normal).

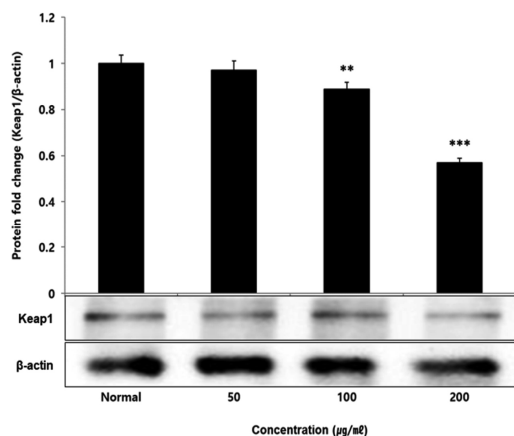


Fig. 5. Effect of LCVC on Keap1 protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 μg/ml of LCVC with 100 ng/ml LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to normal).

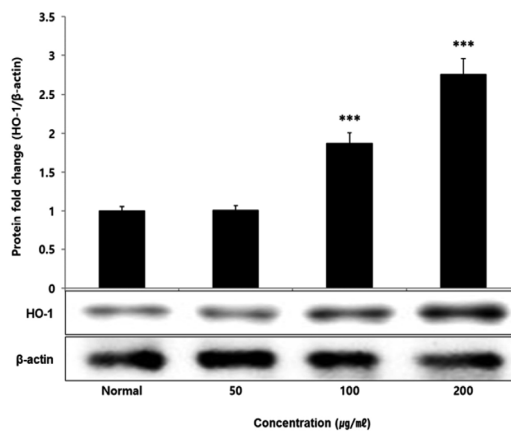


Fig. 6. Effect of LCVC on HO-1 protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 μg/ml of LCVC with 100 ng/ml LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to normal).

10)(Fig. 1,3). When the concentration of LCVC was 50 μg/ml or higher, the expression level of JNK protein decreased significantly (** : p<0.01, *** : p<0.001) compared to the control group (Table 10)(Fig. 2). When the concentration of LCVC was 100 μg/ml or higher, the protein expression level of Nrf2 and HO-1 increased significantly (* : p<0.05, *** : p<0.001) compared to the normal group (Table 10)(Fig. 4,6). When the LCVC concentration was 100 μg/ml or higher, the Keap1 protein expression level was significantly decreased (**p<0.01, ***p<0.001) compared to the normal group (Table 10)(Fig. 5). When the LCVC concentration was 50 μg/ml or higher, the expression level of NQO1 protein increased significantly (***p<0.001) compared to the normal group (Table 10)(Fig. 7).

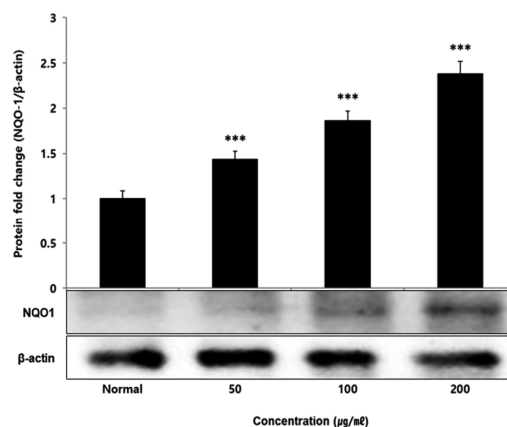


Fig. 7. Effect of LCVC on NQO1 protein expression level in RAW264.7 cell

RAW264.7 cell were treated 50, 100, and 200 μg/ml of LCVC with 100 ng/ml LPS for 24 h. The total cell extracts were analyzed via 10% SDS-PAGE and western blot analysis using respective primary and secondary antibodies. The results of the three independent experiments were described as mean ± standard deviation (Significance of results, * : p<0.05, ** : p<0.01, *** : p<0.001 compared to normal).

Discussion

Existing anti-inflammatory agents used for the treatment of inflammatory diseases can cause side effects such as gastrointestinal disorders, renal function deterioration, and cardiovascular disorders when taken for a long period of time. Therefore, research is underway to develop anti-inflammatory agents extracted from natural resources that can minimize these side effects²¹. Similarly, chemically synthesized single-component antioxidants previously listed may have side effects due to administration of high-concentration components to the human body²². Natural substances extracted from plants showed antioxidant and anti-inflammatory effects, and it was reported that low-concentration components are absorbed while being friendly to the human body, resulting in fewer side effects²³.

The *Lonicerae* Flos is a dried flower bud or freshly blooming flower of *Lonicera japonica* Thunb of the Caprifoliaceae family. It is reported to be used in the treatment of inflammatory diseases such as upper respiratory tract infections such as otitis media, tonsillitis, laryngitis, cutaneous suppuration, viral conjunctivitis, influenza, pneumonia, purulent infections^{18,24}. The *Viola* Herba is a dried perennial herb belonging to the Violaceae family, Violet, and the close root of its close relatives, and contains salicylic acid, glycosides, alkaloids, flavonoids, and various types of saponins¹⁶. It has been reported to be effective in inflammatory diseases such as rheumatoid arthritis and atopic dermatitis^{16,17}. The *Citri* Pericarpium is the dried peel of the mature tangerine fruit of the tangerine tree, an evergreen small tree belonging to the Unhyeongdae family,

and is used as a rich resource for inducing natural flavonoids²⁵. It contains various components such as vitamin C, folic acid, pectin and terpenes, dietary fiber, carotenoids, and flavonoids. Among them, the main compounds of citrus flavonoids include flavanones, naringin and hesperidin, and their non-glycoside forms, naringenin and hesperetin. It has Antibacterial, antioxidant, anticancer, anti-inflammatory, and anti-obesity effects¹⁹. Through the above effects, it has been reported that it is used in various diseases such as asthma, viral hepatitis, alcoholic liver disease, obesity, high blood pressure, and arteriosclerosis²⁶. Therefore, in this study, antioxidant and anti-inflammatory effects were confirmed by measuring the antioxidant content, antioxidant function evaluation, biomarker production amount, gene expression level, and protein expression level of LCVC.

Various studies related to inflammation, such as inhibition of inflammatory mediators, inflammatory cytokines, and inflammatory signaling pathways, are being conducted. LPS-treated RAW 264.7 macrophages model was mostly used to demonstrate these kinds of experiments. Adequate oxidative stress is a fundamentally necessary function for maintaining intracellular homeostasis, but abnormally excessive oxidative stress is closely related to the pathological condition of various chronic inflammatory diseases²⁷. Since antioxidants play an important role in controlling inflammation that occurs in chronic diseases²⁸, various experiments were conducted to specifically demonstrate antioxidant efficacy. As a result, it was confirmed that LCVC contains polyphenols and flavonoids, which are known to act as

antioxidants in vivo (Table 3), and DPPH radical scavenging activity, ABTS radical scavenging activity, FRAP activity corresponding to the measurement methods of antioxidant efficacy showed good results. Overall, it was confirmed that LCVC had an antioxidant effect (Table 4).

Cell viability was measured to establish an appropriate concentration range. LCVC was not toxic when the concentration was 50, 100, or 200 $\mu\text{g/ml}$ compared to the control group. Experiment was conducted based on the above concentration (Table 5).

ROS overexpresses inflammation-related genes to amplify the inflammatory signal of macrophages, and moves these macrophages and additional neutrophils to the site of inflammation to additionally secrete inflammatory mediators and cytokines^{29,30}. In other words, the regulation of ROS is also an important indicator to control inflammation. In order to check the level of intracellular ROS, which is a strong potential factor that induces inflammation, the change in ROS production after LCVC treatment was measured. The result showed that the level of ROS were significantly decreased which means LCVC has antioxidant effect (Table 6).

NO, one of the major inflammatory mediators, is a by-product of the metabolic process in which L-arginine is converted to L-citrulline through Nitric Oxide Synthase (NOS)³¹. NOS is divided into three types: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). PGE2 is involved in a variety of bodily functions to maintain homeostasis³², and under normal circumstances, it plays an essential role in repairing damaged tissues and improving the

human's ability to recover¹). However, excessive production of inflammatory mediators accelerates the inflammatory response, leading to the onset and progression of various inflammatory diseases such as rheumatoid arthritis and multiple sclerosis²⁹. As a result of the experiment, the NO level was significantly decreased at all concentrations of LCVC, and the PGE2 level was significantly decreased at all concentrations except 50 $\mu\text{g/ml}$ (Table 7,8). iNOS is involved in the production of NO and COX-2 in the production of PGE2. The result of measuring the gene expression levels of iNOS and COX-2 was significantly reduced at all concentrations except 50 $\mu\text{g/ml}$ amount of LCVC (Table 8). It means that LCVC reduced inflammatory mediators such as NO and PGE2 by controlling the expression of genes such as iNOS and COX-2 at the transcriptional stage. The inhibition of the production of Inflammatory mediators such as NO and PGE2, and the inhibition of iNOS and COX-2 gene expression play an important role in the treatment of inflammatory diseases. The results shows that LCVC has potential to be the key to treat with those diseases.

The inflammatory cytokines such as IL-1 β and IL-6 TNF- α increase the expression of iNOS and COX-2. These markers significantly increase the production of NO and PGE2³³. It means that Inhibiting the production of inflammatory cytokines is an important function in regulating inflammatory mediators. As a result of measuring the production amount following LCVC treatment, IL-1 β was significantly decreased at all concentrations except 50 $\mu\text{g/ml}$, IL-6, TNF- α level was significantly decreased at all

concentrations (Table 8). The expression level of the corresponding gene was also significantly decreased concentration dependently by treatment amount of LCVC (Table 9). It means that the production of inflammatory cytokines is suppressed by decreased genetic expression.

Nrf2 is a primary transcription factor in response to oxidative stress and plays an important role in maintaining intracellular homeostasis through physiological defense mechanisms against various inflammatory and oxidative stimuli³⁴. Under normal oxidation-reduction conditions, Nrf2 exists in the cytoplasm in a state of binding to the inhibitory protein Keap1. When activated by an external stimulus or oxidative stress, it is separated from the Keap1 protein and migrates to the nucleus. In the nucleus, Nrf2 binds to the antioxidant response element (ARE) in a dimer state with the small maf protein and promotes the expression of antioxidant genes. Also, it controls the expression of antioxidant proteins such as HO-1 and decrease macrophage M1 phenotype and ROS to protect the body from oxidative stress from damage and infection³⁵. HO-1 decomposes heme into iron, carbon monoxide and biliverdin. Among those, biliverdin is converted to bilirubin by biliverdin reductase, which becomes a powerful antioxidant³⁶. HO-1 and NQO1 are a representative target gene of Nrf2 which suppresses excessive secretion of TNF and IL by controlling Nrf2 pathway. It has anti-inflammatory effects against oxidative stress³⁷. To acknowledge whether the anti-inflammatory response of LCVC is related to the activation of the Nrf2/HO-1 signaling pathway, we observed whether the expression of Nrf2, Keap1, HO-1,

and NQO1 was changed according to the amount of LCVC treatment. As a result, Nrf2 and HO-1 were significantly increased at all concentrations except 50 $\mu\text{g/ml}$, Keap1 expression significantly decreased at all concentrations, and NQO1 increased at all concentrations (Table 9). Similarly, as a result of measuring the protein expression levels of the above indicators, Nrf2 and HO-1 showed a significant increase at all concentrations except 50 $\mu\text{g/ml}$, and Keap1 showed a decrease, and NQO1 showed a significant increase at all concentrations, showing the same trend (Table 10). It means that the effect of blocking the inflammatory response of LPS through its antioxidant effect is related to the activation of the Nrf2/Ho-1 signaling system.

The MAPKs signaling pathway induces the production of inflammatory mediators and cytokines in macrophages, and plays a key role in the activation of the ROS production stimulation pathway. MAPKs-family proteins such as ERK, JNK, and p38 stimulate TLR4 of macrophages or monocytes which are one of the cells involved in the inflammatory response in the body, to produce inflammatory mediators such as IL-1 β , TNF- α , IL-6, and NO. When it functions normally, various physiological functions such as body defense, signal transduction, and vasodilation occur but if it occurs acutely or excessively, tissue damage and genetic mutation septic shock may occur⁶. That is, targeting the MAPKs signaling pathway can be effective therapeutic agents for inflammatory diseases by blocking various inflammatory stimulus signals. In order to confirm the effect of LCVC treatment on the activity of MAPKs family proteins induced by

LPS, the expression level of ERK JNK p38 protein was checked. As a result, the expression levels of ERK and p38 had no difference with the control group, and the expression level of JNK showed a significant decreased concentration-dependently by LCVC (Table 10). It means that the anti-inflammatory effect of LCVC is related with the regulation of the MAPKs signaling system.

In this study, by checking the level of biomarker production, gene expression, and protein expression, it was confirmed that *Lonicerae* Flos, *Citri* Pericarpium, and *Violae* Herba Complex (LCVC) have anti-inflammatory and antioxidant effects through the mechanisms of suppressing the production of inflammatory mediators and cytokines. In addition, in this process, it was confirmed that the Nrf2/HO-1 signaling pathway, which is a major pathway of the anti-inflammatory mechanism, was activated, and the activation of the MAPKs signaling pathway was decreased. Considering the above experimental results, LCVC has antioxidant and anti-inflammatory effects through the regulation of multiple signal transduction pathways so it could be considered of being used in the treatment of inflammatory diseases in the future. However, this study have limitations as it was the result of cell experiment in mice, and the analysis has not been conducted by comparing the efficacy of individual herbs because of insufficient studies and differences in experimental methods and conditions. In the future, we are expecting for this study to be used effectively for efficacy verification by comparing the efficacy in individual herbs or combination with various

herbs. Also, various anti-oxidative and anti-inflammatory studies to be conducted on herbs with *Lonicerae* Flos, *Citri* Pericarpium, and *Violae* Herba Complex.

Conclusion

LCVC (*Lonicerae* Flos, *Citri* Pericarpium and *Violae* Herba Complex) was administered to in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages and the conclusions are as follows.

1. LCVC contains polyphenol and flavonoid, which are antioxidants. DPPH radical scavenging activity, ABTS radical scavenging activity, and FRAP activity were increased concentration-dependently.
2. Cell viability showed proliferation of cells concentration-dependently at a concentration of 50 $\mu\text{g/ml}$ or more, and less than 90% at a concentration of 400 $\mu\text{g/ml}$ or more.
3. ROS and NO production decreased concentration-dependently.
4. As for the biomarkers, IL-6 and TNF- α were significantly reduced concentration-dependently at all concentrations, and PGE2 and IL-1 β at concentrations of 100 $\mu\text{g/ml}$ or higher.
5. The gene expression levels of IL-1 β , IL-6, TNF- α , and Keap1 were significantly decreased concentration-dependently at all concentrations, and NQO1 was increased. iNOS and COX-2 decreased concentration-dependently at 100 $\mu\text{g/ml}$ or higher, while Nrf2 and HO-1 increased.
6. Protein expression level was significantly decreased concentration-dependently at all concentrations of JNK, and NQO1 was increased. Nrf2 and HO-1 were significantly increased

concentration-dependently at 100 $\mu\text{g/ml}$ or higher, and Keap1 was decreased. ERK and p38 showed no significance

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ORCID

Hong Kyoung Kim <https://orcid.org/0000-0002-8893-5112>