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Citrus unshiu flower inhibits LPS-induced iNOS and COX-2 via MAPKs in RAW 264.7 macrophages

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ABSTRACT

In the present study, we investigated the effects of *Citrus unshiu* flower on regulatory mechanisms of cytokines and nitric oxide (NO) involved in immunological activity of RAW 264.7 macrophages. Our results indicated that ethyl acetate fraction of *Citrus unshiu* flower (CUF-EA) downregulated LPS-induced nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, thereby reducing the production of NO and prostaglandin E_2 (PGE_2) in LPS-activated RAW 264.7 macrophages. Furthermore, CUF-EA suppressed LPS-induced production of pro-inflammatory cytokines such as interleukin IL-6, and tumor necrosis factor (TNF)-á. To elucidate its anti-inflammatory mechanisms, CUF-EA was investigated as an inhibitor of phosphorylation of mitogen-activated protein (MAP) kinase in LPS-stimulated RAW 264.7 macrophages. As expected, the phosphorylation of MAP kinases (p38, ERK1/2 and JNK) in LPS-stimulated RAW 264.7 macrophages was suppressed by CUF-EA in a dose-dependent manner. These results suggest that the anti-inflammatory properties of CUF-EA might results from inhibition of NO, PGE_2, iNOS, COX-2, IL-6 and TNF-á expressions through the down-regulation of phosphorylation of MAPKs in RAW 264.7 macrophages.

Key words: *C. unshiu*, lipopolysaccharide, mitogen-activated protein kinases, nitric oxide, prostaglandin E_2

INTRODUCTION

Citrus unshiu Markovich (Rutaceae) is a seedless and easy-to-peel fruit cultivated primarily on Jeju Island, Korea after introduction from Japan in the 1910s. *C. unshiu* constitutes more than 30% of the total fruits produced in Korea. The peels, called Jin-pi, have also been used in Traditional Korean Medicine to improve gastroenteric disorders, asthma, and loss of appetite[1-2]. There are

reports that *C. unshiu* peels and their constituents have diverse pharmacological activities, including anti-anxiety[3], recovery of liver function[4-5], anti-inflammatory[6], anti-allergic[7-8], anticancer[9], and anti-diabetic[10]. However, anti-inflammatory effects of the extracts of *C. unshiu* flowers have not yet been described. Therefore, the anti-inflammatory activities of the extracts of *C. unshiu* flower were investigated in this study.

Inflammation is the result of the host's immune response to pathogenic challenges or tissue injuries and functions to restore tissue structure and function to normal states. Inflammation is tightly controlled and self-limiting, with down-regulation of pro-inflammatory proteins and up-regulation of anti-inflammatory proteins[11-14]. However, if uncontrolled, the inflammatory mediators become involved in the pathogenesis of many inflammatory disorders such as rheumatoid arthritis, diabetes, and cancer [15-16]. There have been many attempts to derive new anti-inflammatory agents from natural compounds [17-19].

Inflammatory stimulants such as lipopolysaccharide (LPS) activate production of a variety of inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines including TNF-á, IL-1â, and IL-6. The production of NO and PGE_2 by macrophages is a current research interest for the development of new anti-inflammatory agents.

This study was designed to explore the anti-inflammatory effects of *C. unshiu* flower by measuring its effects on the production of pro-inflammatory factors (TNF-α, IL-6, NO, and PGE_2). Furthermore, we sought to elucidate the mechanisms of these anti-inflammatory effects by investigating the role of mitogen-activated protein kinase (MAPK) pathways in RAW 264.7 murine macrophages. To the best of our knowledge, this is the first report of the anti-inflammatory activity of the *C. unshiu* flower.

MATERIALS AND METHODS

Plant material

C. unshiu flowers were collected from the Namwon area (Jeju Island, Korea) in May 2011. The specimen was identified by Dr Young Hun Choi, Citrus Research Station, National Institute of Horticulture and Herbal Science, Jeju, Korea. The specimen was deposited at the Herbarium of the Citrus Research Station.

The specimen materials for extraction were freeze-dried and then ground into a fine powder by using a blender. The dried powder (1.5 kg) was extracted with 80 % ethanol (EtOH; 10 L) at room temperature for 3 days and then evaporated under a vacuum. The evaporated EtOH extract (20 g) was suspended in water (1 L) and fractionated with ethyl acetate (EA; 1 L). The yield and recovery of EA fractions were 2.4 g and 6.2 %, respectively.

Cell culture

RAW 264.7 murine macrophages were purchased from the Korean Cell Line Bank (Seoul, Korea). These cells were maintained at sub-confluence in a 95% air and 5% CO_2 humidified atmosphere at 37°C. The medium used for routine subculture was Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL).

LDH assay for measuring cytotoxicity

RAW 264.7 macrophages (1.8×10^5 cells/mL) were plated in 24-well plates and incubated for 18 h prior to treatment with the indicated concentrations of extract samples for 2 h. Macrophages were then challenged with LPS (1 µg/mL) for an additional 18 h. The release of lactate dehydrogenase (LDH) from the macrophages was used to assess cytotoxicity by using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). This method determines LDH activity from the production of NADH during the conversion of lactate to pyruvate. The optical density of the solution was measured at a wavelength of 490 nm by using an ELISA plate reader (Bio-TEK Instruments Inc., Vermont, WI, USA). The cytotoxicity percentage was determined relative to the control group. All experiments were performed in triplicate.

Nitric oxide determination

Nitrite concentration in the culture medium was measured as an indicator of nitric oxide production according to the Griess reaction method,

as previously described[19]. In brief, RAW 264.7 macrophages (1.8×10^5 cells/mL) were plated in 24-well plates and incubated for 24 h. Macrophages were pre-treated for 2 h with 10 μμ 2-amino-4-methylpyridine (positive control), 20 μμ dexamethasone (negative control), or CFU extracts in water, EtOH, or EA or a water fraction at concentrations of 25, 50 and 100 μg/mL), Macrophages were then challenged with LPS (1 μg/mL) for an additional 18 h. Equal volumes of culture medium and Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphtylethylenediamine dihydrochloride in distilled water) were mixed. The absorbance at 540 nm was determined with an ELISA plate reader (Bio-TEK Instruments Inc., Vermont, WI, USA). The absorption coefficient was calibrated using a sodium nitrite solution standard.

Measurement of PGE_2 Production

RAW 264.7 macrophages were plated in a 24-well cell culture plate at a density of 1.8×10^5 cells/well and incubated for 18 h. Macrophages were then stimulated with and LPS (1 μg/mL) in the presence of CFU-EA (25, 50 and 100 μg/mL) or in the absence of CFU-EA (control) for 24 h. The culture medium was collected and assayed with ELISA kit (R&D Systems, Minneapolis, MN, USA). Culture medium was incubated in a goat anti-mouse IgG coated plate with acetylcholinesterase linked to PGE_2 and PGE_2 monoclonal antibody for 18 h at 4 °C. The plate was emptied and rinsed five times with wash buffer contained in the kit. Two hundred milliliters of substrate reagent was added to each well and incubated for 1 h at 37 °C. The plate was read at 405 nm on an ELISA plate reader (Bio-TEK Instruments Inc., Vermont, WI, USA). The PGE_2 concentration of each sample was determined according to the standard curve.

Measurement of pro-inflammatory mediators

The secretion of TNF-α and IL-6 in macrophage cultures was measured using a commercial mouse TNF-α or IL-6 ELISA kit (R&D Systems Inc., Minneapolis, MI, USA). Macrophages (1.8×10^5 cells/mL) were cultured in 24-well plates in the presence or absence of CFU-EA (25, 50 and 100 μg/mL).and LPS (1 μg/mL), and then incubated for 24 h. Supernatant samples were obtained 16 h later and frozen until subjected to ELISA analysis following the manufacturer's protocol. The TNF-á and IL-6 production in colon homogenates was quantified by reading the absorbance at 540 nm on an ELISA plate reader (Bio-TEK Instruments Inc., Vermont, WI, USA).

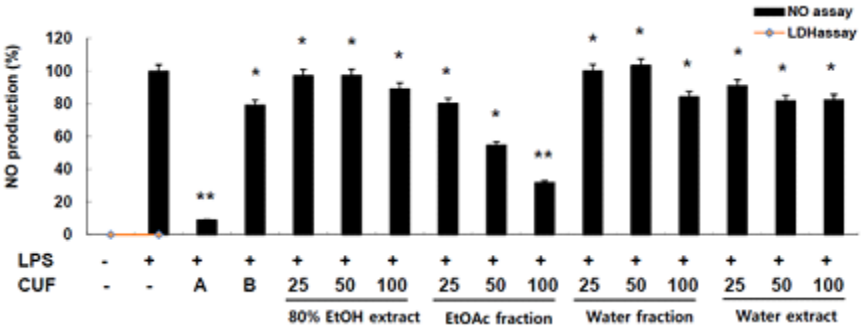
Western blot analysis

RAW 264.7 macrophages were pre-incubated for 18 h in a 6-well cell culture plate at a density of 1.0×10^6 cells/well before being stimulated with LPS (1 μg/mL) in the absence or presence of CFU-EA (25, 50 and 100 μg/mL) extracts for 24 h. After incubation, the macrophages were collected and washed twice with cold phosphate-buffered saline (PBS). Macrophages were lysed in a lysis buffer (1x RIPA [Upstate USA Inc., NY, USA], 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin) and kept on ice for 1 h. The cell lysates were centrifuged at 15,000 rpm and 4 °C for 15 min, and the supernatants were stored at -70 °C until use. Protein concentrations were then determined using a Bradford Assay (Bio-Rad, Richmond, CA, USA). Aliquots of the lysates (30~50 μg of protein) were separated on an 8~12 % SDS polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to polyvinylidene fluoride (PVDF) membranes (BIO-RAD, HC, USA), blocked with 5 % non-fat milk in TBS-T buffer, and blotted with each primary antibody (1:1,000, except for iNOS (1:5,000) and β-actin (1:10,000)) and the corresponding secondary antibody (1:5,000 or 1:10,000) according to the manufacturer's instructions. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences, NJ, USA).

RESULTS

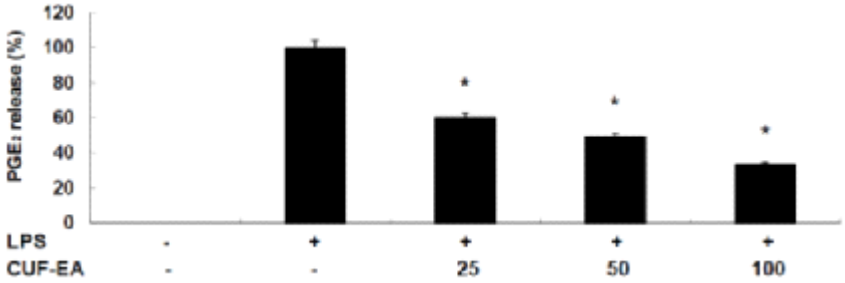
Effect of CUF-EA on cell viability and inhibition of NO production

We initially examined the inhibitory effects of the extracts of *C. unshiu* flower (CUF) on the production of the inflammatory mediator, NO. The amount of produced NO was indicated by amount of nitrite (a stable metabolite of NO) that accumulated in LPS-exposed cells in the presence of each type of CUF. The ethyl acetate fraction of *C. unshiu* flower extract (CUF-EA) strongly inhibited



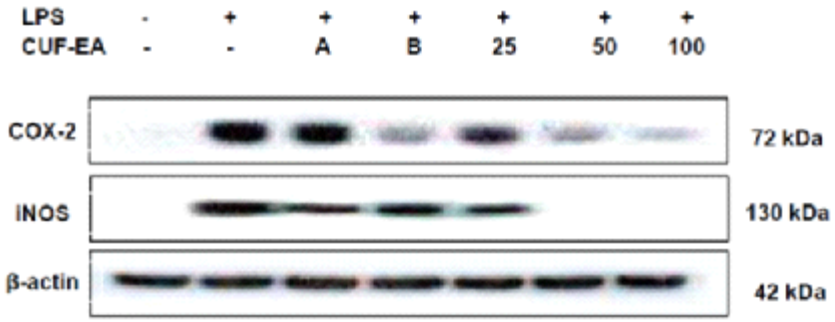
NO production was assayed in the culture medium of macrophages (1.8 x 105 cell/mL) stimulated with LPS (1 ìg/ml) for 24 h in the presence of CFU extracts in water, EtOH, or EA or a CFU water fraction at concentrations of 25, 50 and 100 µg/mL. The positive control (A) was 10 µM 2-amino-4-methylpyridine and the negative control (B) was 20 µM dexamethasone. Data were expressed as means ± S.D. of three determinations. *, P<0.05; **, P<0.01 compared to positive control.

Fig. 1: Effects of *Citrus unshiu* flower (CUF) on cytotoxicity and nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages



Macrophages (1.8 x 10^5 cell/mL) were pre-incubated for 18 h and then stimulated with LPS (1 µg/ml) for 24 h in the presence or absence of CFU-EA (25, 50 and 100 µg/mL). PGE_2 production was analyzed with an ELISA assay. The data were expressed as means ± S.D. of three determinations. *, P<0.05; **, P<0.01 compared to the positive control (LPS alone).

Figu. 2: Effects of the ethyl acetate fraction of *Citrus unshiu* flower (CUF-EA) on LPS-stimulated PGE2 production



Macrophages (1.8 x 10^5 cell/mL) were pre-incubated for 18 h, and then cells were stimulated with LPS (1 ìg/mL) for 24 h in the presence of CFU-EA (25, 50 and 100 ìg/mL). The positive control (A) was 10 ìÌ 2-amino-4-methylpyridine and the negative control (B) was 20 ìÌ dexamethasone. iNOS and COX-2 protein levels were determined by immunoblotting with actin as a positive control.

Fig. 3: Effects of CUF-EA on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 macrophages

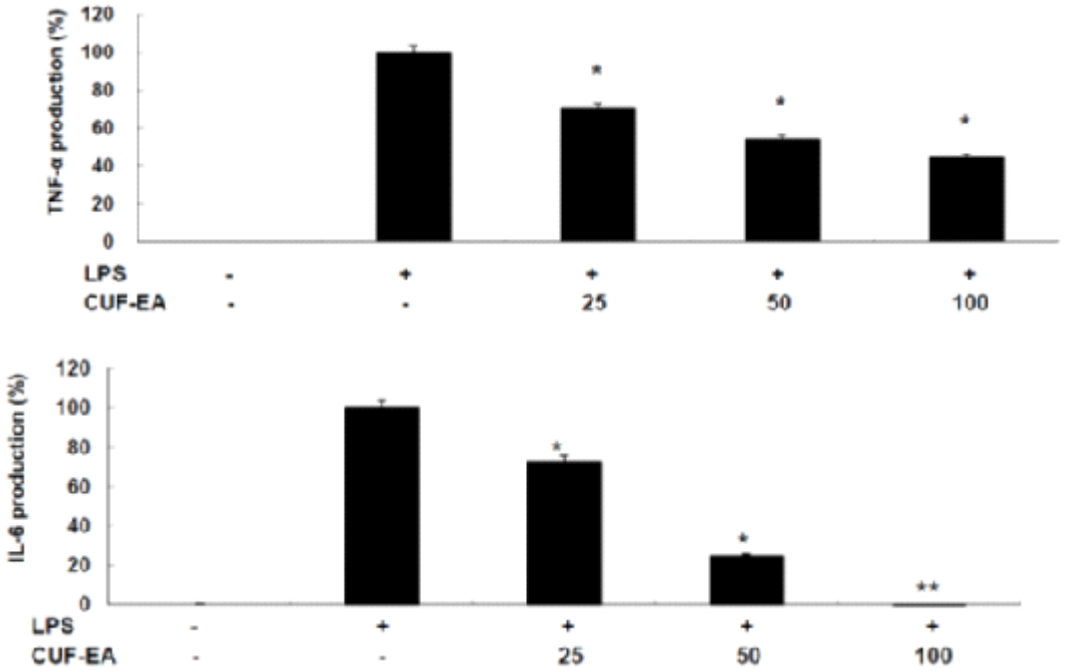
LPS-induced NO production (Fig. 1). In contrast, the 80% EtOH and water extracts of *C. unshiu* did not inhibit LPS-induced NO production. In addition, the cytotoxicity of extracts of *C. unshiu* were evaluated by a lactate dehydrogenase (LDH) assay after the cells were incubated for 24 h in the presence of extracts of *C. unshiu* and LPS. As shown in Figure 1, there was no cytotoxicity. Thus, the inhibitory effect of all CUF-EA concentrations on NO production could not be attributed to cytotoxic effects. These results suggest that the CUF-EA exerted anti-inflammatory effects.

Effect of CUF-EA on LPS-induced PGE_2 production

To evaluate the effects of CUF-EA on LPS-induced PGE_2 production in RAW 264.7 macrophages, the production of PGE_2 was measured using ELISA. Stimulation of cells with LPS (1 μg/mL) resulted in a significant increase in PGE_2 production compared with un-stimulated control cells. Furthermore, pretreatment with CUF-EA (10, 50, and 100 μg/mL) markedly inhibited LPS-induced PGE_2 production (Figure 2).

Inhibitory Activities of CUF-EA on LPS-Activated iNOS and COX-2 Expression

To understand whether CUF-EA can inhibit LPS-induced activation of iNOS and COX-2 protein, western blotting was performed. As shown in this experiment, the iNOS protein was strongly induced by LPS and CUF-EA showed a decrease in iNOS protein expression in a concentration-dependent manner. In order to clarify whether the inhibitory effect of CUF-EA on PGE_2 release could result from a decreased COX-2 protein level, we further examined the effect of CUF-EA on LPS-induced COX-2 protein levels by Western blot analysis. As shown in this experiment, the COX-2 protein was strongly induced by LPS. CUF-EA slightly suppressed the induction of COX-2 (Figure 3).



Macrophages (1.8 x 10^5 cell/mL) were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) for 24 h in the presence or absence of CFU-EA (25, 50 and 100 μg/mL). TNF-α and IL-6 production were analyzed by ELISA. The data are expressed as means ± S.D. of three determinations. *, $P<0.05$; **, $P<0.01$ compared to positive control (LPS alone).

Fig. 4: Effects of CUF-EA on production of pro-inflammatory cytokines in LPS-induced RAW 264.7 macrophages

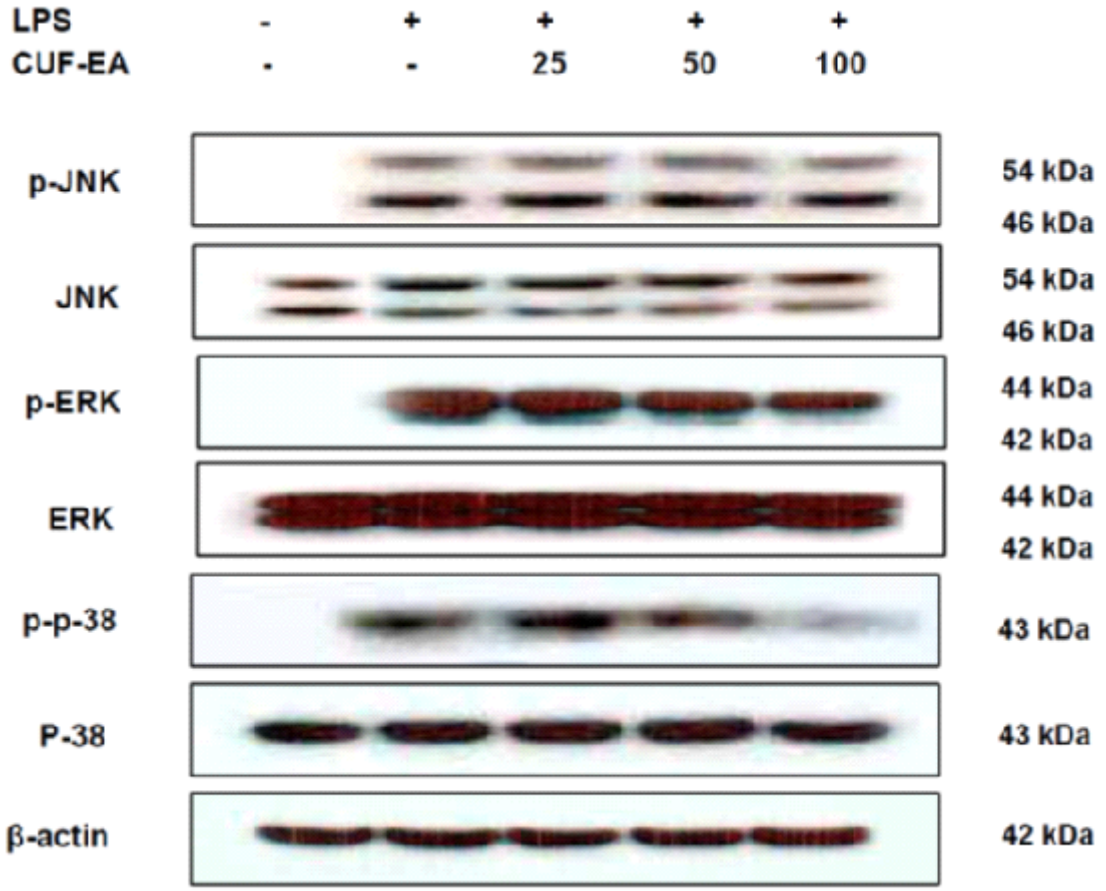
Effect of CUF-EA on LPS-induced TNF-á and IL-6 production

In this study, we used ELISA kits to measure the level of TNF-α (Figure 4A) and IL-6 (Figure 4B) in the culture supernatants. Compared with the control group, treatment of RAW 264.7 cells with LPS alone led to a significant increase in cytokine production. However, compared with the LPS group, the levels of TNF-α and IL-6 in CUF-EA-pretreated, LPS-stimulated cells were reduced significantly in a dose dependent manner.

Suppression of MAPK phosphorylation by CUF-EA in LPS-stimulated RAW 264.7 macrophages

Because CUF-EA most markedly inhibited the secretion of inflammatory mediators, we focused on the mechanism by which CUF-EA inhibits LPS-induced inflammation in macrophages. MAPKs mediate various biological processes and the cellular response to external stress signals. Furthermore, they are involved in regulating the synthesis of inflammatory mediators, making them potential targets for anti-inflammatory therapeutics[20].

We examined the effect of CUF-EA on MAPK phosphorylation in RAW 264.7 cells activated by LPS. As shown in Figure 5, LPS-induced phosphorylation of JNK, ERK, and p38 were all inhibited by CUF-EA treatment in a dose-dependent manner.



Macrophages (1.0 x 10^6 cell/mL) were pre-incubated for 18 h and then stimulated with LPS (1 μg/mL) for 24 h in the presence or absence of CFU-EA (25, 50 and 100 μg/mL). p-JNK, JNK, p-ERK, ERK, pp-38, and p-38 were determined by immunoblotting with β-actin as a positive control.

Fig. 5: Effects of CUF-EA on the protein levels of p-38, JNK, and ERK in LPS-induced RAW 264.7 cells

DISCUSSION

C. unshiu is a kind of citrus fruit in the Rutaceae family. It is composed of rind and sarcocarp and includes various bioactive substances such as essential oils, carotenoids, cellulose, pectin, limonoid. In recent studies, it has been reported that flavonoids are also contained in *C. unshiu* peel extracts that have anti-oxidant, anti-cancer, and anti-inflammatory activity. In addition, the flavonoid content changes during maturation of *C. unshiu* and is high in premature *C. unshiu*. However, anti-inflammatory effects of the extracts of *C. unshiu* flowers have not yet been described. The present study was designed to elucidate the pharmacological and biological effects of the flower of *C. unshiu* on the production of pro-inflammatory cytokines and inflammatory mediators in macrophages.

Inflammation is an immune response to external stimuli such as bacterial infections. During the inflammatory response, macrophages produce pro-inflammatory cytokines (TNF-α, IL-1β) in response to external agents, leading to excessive generation of NO and PGE_2 through increased synthesis of iNOS and COX-2[21].

Our results indicated that each type of *C. unshiu* extract we used in this study reduced the production of inflammatory mediators stimulated by LPS. Especially, the ethyl acetate fraction of *C. unshiu* flower (CUF-EA) showed the most significant suppression of NO production. These results suggest that CUF-EA may have a most potent therapeutic effect on the LPS-induced inflammation.

We examined the anti-inflammatory mechanism of CUF-EA in LPS-stimulated RAW 264.7 cells. Similar to NO, excessive production of PGE_2 is correlated with many inflammatory disorders[22]. Macrophages activated by the inflammatory response produce excessive COX-2, which catalyzes PGE_2 production; the produced PGE_2 in turn promotes the inflammatory response[23]. Therefore, newly developed selective COX-2 inhibitors have a common therapeutic action on acute and chronic inflammation—suppression of PGE_2 production—and have been used as useful anti-inflammatory drugs[24]. Our results demonstrate that CUF-EA decreases the production of PGE_2 as well as COX-2 in LPS-stimulated RAW264.7 cells.

We confirmed that iNOS expression plays an important role in the generation of NO by western blotting. Further, CUF-EA dose-dependently down-regulated iNOS expression. Among the pro-inflammatory cytokines, TNF-α and IL-6 regulate the inflammatory response both in vivo and in vitro and are known to interact with each other[25]. In the present study, CUF-EA strongly inhibited the LPS-induced production of TNF-α and IL-6.

LPS-mediated activation of mammalian cells occurs through its binding to TLR-4 and activation of downstream signaling pathways, including MAPK pathways[26]. MAPK also plays an important role in the regulation of cellular responses that control growth and differentiation, cytokine production, and response to stress[27]. LPS stimulation induces the phosphorylation and activation of three types of MAPKs: JNK, ERK, and p38 MAPK. p38 and JNK constitute a part of the stress response pathway and are activated by cellular stress induced by the presence of inflammatory cytokines[28]. ERK upregulates iNOS expression and the production of proinflammatory cytokines, including TNF-α and IL-6 in LPS-stimulated macrophages[29].

In this study, CUF-EA inhibited the phosphorylation of ERK1/2, JNK1/2, and p38 in LPS-stimulated RAW 264.7 cells. In conclusion, the results of the present study provide the first evidence that CUF-EA inhibits LPS-induced NO and PGE_2 production in macrophages. CUF-EA exerted these effects via inhibition of MAPK phosphorylation, which led to the production of pro-inflammatory cytokines (TNF-α and IL-6). Furthermore, CUF-EA is considered to have superior efficacy in ameliorating inflammatory-mediated diseases, and it is expected to be utilized as a preventive therapeutic. However, further studies are required to determine the mechanisms underlying its anti-inflammatory effect and to identify the active components.

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