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Antioxidant and Suppressive Effects of Ethanolic Extract Fractions from Safflower (*Carthamus tinctorius* L.) Flower on the Biosynthesis of Inflammatory Mediators from LPS-stimulated RAW 264.7 Cells

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Abstract The aim of this study was to elucidate the anti-inflammatory activity of safflower (*Carthamus tinctorius* L.) ethanolic extract fractions (CFEFs). Butanol fraction had the strongest antioxidant activity, and all CFEFs, except for chloroform fraction, partly inhibited lipopolysaccharide (LPS)-induced nitrite production in RAW 264.7 cells. In the cell-free system, hexane and butanol fractions chemically quenched nitric oxide (NO). In addition, the iNOS mRNA transcription was suppressed by ethanol extract and hexane fraction in LPS-stimulated RAW 264.7 cells. Taken together, the inhibitory effect of CFEFs on NO production from LPS-stimulated RAW 264.7 cells, might be due to both the chemical NO quenching activity and the suppression of iNOS mRNA transcription partially. The synthesis of prostaglandin E_2 (PGE_2) was potently inhibited by ethanol extract to below basal label, and the transcription of cyclooxygenase-2 (COX-2), an enzyme involving in PGE_2 synthesis, was partially suppressed by ethanol extract and hexane fraction. Based on these results, CFEFs may be useful as an alternative medicine for the relief and retardation of immunological inflammatory responses through the reduction of inflammatory mediators, including NO and PGE_2 production.

Keywords: *Carthamus tinctorius* L., nitric oxide, prostaglandin E_2, inducible nitric oxide synthase, cyclooxygenase-2

Introduction

Safflower (*Carthamus tinctorius* L.) which belongs to Compositae family, has been used for a long time period for edible oil production. Safflower seeds, rich in unsaturated fatty acid and α-linoleic acid, have been used for the promotion of bone formation and clinical treatments of osteoporosis and rheumatism in Korea (1). Aqueous methanolic extract and its ethyl acetate fraction of safflower seeds possessed the potent tyrosine inhibitory activity (2). Phenolic compounds in safflower seeds, such as conjugated serotonins, lignans, and flavones, stimulated bone formation, reduced bone loss, and increased plasma high density lipoprotein (HDL) cholesterol level in estrogen deficient rat (3-7). Most of researches for safflower have focused on seeds, and to date, there has been minimal research regarding the anti-inflammation of safflower. The flower of safflower has been used for the improvement of extravasated blood and the suppression of platelets-aggregation as traditional pharmaceuticals (8). Flower extracts of safflower contain more phenolic compounds and flavonoids than the other sections of safflower (8), and its antioxidant activity was superior to that of other part-extracts. However, its biological activity has not been scientifically proven.

Nitric oxide (NO) is involved in various physiological and pathophysiological responses. In inflammation, the production of NO from L-arginine results in the formation of L-citrulline and is catalyzed by the inducible NOS (iNOS or type II) in the host or isolated cells stimulated by bacteria or bacterial products such as lipopolysaccharide (LPS) and/or inflammatory cytokines (e.g., tumor necrosis factor (TNF)-α, interlukin (IL)-1β, interferon (IFN)-γ) (9). Induction of iNOS was observed in several cell types including macrophages, hepatocytes, vascular smooth muscle cells, glial cells, endothelial cells, and chondrocytes (9). NO regulates the inflammatory transcription factors in both prokaryotic and eukaryotic cells, and also is involved in nuclear factor (NF)-κB pathway, AP-1 pathway, and Jak-STAT pathway (10). Therefore, NO has been a target of intensive research and drug development. Additionally, prostaglandin E_2 (PGE_2), one of the inflammatory mediators, is mainly synthesized from arachidonic acid (AA) by cyclooxygenase-2 (COX-2) and contributes to vasodilation, pain, and fever (11). The inhibition of prostaglandin (PG) synthesis suppresses inflammation and confers analgesia (12). In inflammatory responses, COX-2, normally expressed at low levels, is strongly induced by pro-inflammatory agents, including LPS, tumor promoters, and growth factors (13). The COX-2/PGE_2 pathway has also been shown to be involved in the production of matrix metalloproteinase-1 (MMP-1) by LPS-stimulated human primary monocytes (14). Many studies have shown that PGE_2 is potent vasodilators that are present at high concentrations in inflammation sites (15). The PGE_2 exerts both pro- and anti-inflammatory effects, depending on receptor subtype, cell population, and context of activation (16).

To elucidate the mechanism for anti-inflammatory

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activities of safflower ethanolic extract fractions (CFEFs), we investigated the antioxidant and the inhibitory effects on NO and PGE_2 biosynthesis. The potential anti-inflammatory activity of flower of safflower might be proven scientifically through this report.

Materials and Methods

Reagents Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), N^G-monomethyl-L-arginine monoacetate (L-NMMA), and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture medium and reagents, such as Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-ethylenediamine tetraacetic acid (EDTA) were obtained from Gibco-Invitrogen Inc. (Grand Island, NY, USA). Ethanol and other reagents were used as first grade.

Plant material and the preparation of fractions Dried safflower was purchased from Kyungdong herbal market (Seoul, Korea) in May 2007, authenticated by Prof. Choon-Sik Jeong, College of Pharmacy, Duksung Women's University, Seoul, Korea, and deposited in Plant Resources Research Institute, Duksung Women's University. Seoul, Korea.

Dried flowers (30 g) were extracted with 100 mL of 70% ethanol (in water) for 24 hr at room temperature. Extracts were filtered through Whatman No. 1 filter paper, and were concentrated by evaporator under reduced pressure. The ethanol curd was dissolved in distilled water before being fractionated sequentially with hexane, chloroform, and *n*-butanol at room temperature. After removal of each solvent, 4 fractions were obtained, which are hereafter referred to as flower of safflower-ethanol extract (CFEE), -hexane fraction (CFHF), -chloroform fraction (CFCF), and -*n*-butanol fractions (CFBF). Extract fractions were redissolved in DMSO to 100 mg/mL, stored at −20°C, and used it as a stock.

Radical scavenging activity Scavenging effect of CFEFs on DPPH radicals was monitored according to the method described by Lee *et al.* (17). A 0.2 mL of methanolic solution containing CFEFs was mixed with 4 mL of methanol, and a methanolic solution of DPPH (1 mM, 0.5 mL) was added. The mixture was vortexed for 15 sec, left to stand at room temperature for 30 min, and the absorbance was read at 517 nm.

Reducing power The reducing power of CFEFs was determined by Fe^{3+} reduction (18). CFEFs (10-1,000 μg/mL) in distilled water were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 2,090×g for 10 min. A 2.5 mL of supernatant layer was added to 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the mixture was measured at 700 nm using UV-spectrophotometry (Agilent Technologies Inc., Santa Clara, CA, USA).

Cell culture RAW 264.7 murine macrophage cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in DMEM containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a 5% CO_2 humidified incubator at 37°C. For subculture, RAW 264.7 cells were rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of serum, which contains trypsin inhibitor, and was subdivided using 0.05% trypsin with 0.53 mM EDTA. They were used at passage numbers 10-20 for this study.

Cell viability assay Cell viability was examined using MTT assay. RAW 264.7 cells were plated at a density of 1×10^5 cells/well in 96-well tissue culture plate (Corning Inc., Corning, NY, USA), and incubated at 37°C for 3-4 hr. Plated cells were treated with indicated concentrations of CFEFs. After 24 hr incubation, MTT was added to all well at 0.5 mg/mL of concentration, and incubated for 4 hr at 37°C. After discarding all medium from the plates, 100 μL of DMSO was added to the all well. The plates were placed for 5 min at room temperature with a shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by UV-spectrophotometric plate reader (Emax, Molecular Devices Inc., Sunnyvale, CA, USA). Viability was defined as the ratio (expressed as %) of absorbance of treated cells to untreated cells.

Nitrite assay RAW 264.7 cells were plated at a density of 2×10^5 cells/well in a 96-well culture plate and incubated for 3-4 hr in a 5% CO_2 humidified incubator at 37°C. Plated cells were treated with LPS (1 μg/mL) for stimulation of nitrite-production and the indicated concentrations of CFEFs, followed by 24 hr incubation. LPS-stimulated nitrite-production from RAW 264.7 cells was measured by the Griess reaction (19). Briefly, 100 μL of each supernatant was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in distilled water), and the absorbance of the mixture was determined with a microplate reader (Emax, Molecular Devices Inc.) at 540 nm. In this experiment, 10 μM of L-NMMA, an iNOS inhibitor, was used as a positive control (20).

Extracellular NO scavenging assay Extracellular NO radical scavenging activity was measured by the modified protocol (21). Briefly, sodium nitroprusside, a NO donor, generates NO when interacts with oxygen in aqueous solution at physiological pH, and NO is measured by Griess reaction. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM in PBS) and CFEFs were incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture and 0.5 mL of Griess reagent were mixed. The absorbance of the chromophore formed was evaluated at 540 nm. Hemoglobin (Hb, 5 μM) was used as a NO chemical scavenger (22).

Measurement of PGE_2 RAW 264.7 macrophage cells were cultured in 24-well culture plates. After reaching confluence, LPS-induced cells were treated with CFEFs,

and then incubated in a humidified incubator at 37°C for 48 hr. After 48 hr incubation, the supernatant were collected, and frozen at −74°C. The production of PGE_2 was measured using commercially available assay (PGE_2: R&D Systems, Wiesbaden, Germany). As a blank we measured cell culture medium from wells without cells that had been treated the same way as the samples according to the procedure described by the manufacturer. Briefly, the supernatant (100 μL) of cell culture that treated with CFEFs were added to the plate, and mixed with 50 μL of a primary mouse monoclonal PGE_2-antibody and PGE_2-conjugated with horseradish peroxidase (HRP), and were incubated for 2 hr on the shaker at room temperature. Then, the supernatant was discarded and washed 4 times. The mixture with hydrogen peroxide and chromogen (tetramethylbenzidine), a substrate, was added to plate and incubated for 30 min at room temperature under light protection. Sulfuric acid (200 μL, 2 N) was added to all well for termination of reaction, and the absorbance was read at 450 nm within 30 min.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis For the transcription assay of iNOS and COX-2 gene, the LPS-stimulated RAW 264.7 cells were incubated with CFEFs for 24 hr in a 5% CO_2 humidified incubator at 37°C. NMMA (10 μM) and celecoxib (20 μM) were used as a positive control for a selective inhibitor iNOS and COX-2, respectively. Total RNA was isolated from RAW 264.7 cells using RNeasy kit (Qiagen Inc., Valencia, CA, USA) after treated with CFEFs. RT-PCR was performed using One-Step RT-PCR kit (Qiagen Inc.) and primers at a final concentration of 1 μM. For PCR of iNOS and COX-2 in RAW 264.7 cells, the primers were used as followed (23): iNOS forward primer: 5'-CCCTTCCGAAGTTTCTGGCAGC-3', iNOS reverse primer: 5'-GGCTGTCAGAGCCTCGTGGCTT-3', COX-2 forward primer: 5'-GGAGAGACTATCAAGATA GTGATC-3', COX-2 reverse primer: 5'-ATGGTCAGTAG ACTTTTACAGCTC-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, the house-keeping gene, for RAW 264.7 cells were used to determine PCR efficiency and the quantitation; GAPDH forward primer: 5'-TGAAGGTCGGTGTGACGGATTTGGC-3', GAPDH reverse primer: 5'-CATGTAGGCATGAGGTCCACCAC-3'. Thermal cycling was performed according to modified method (23). For cDNA synthesis and predenaturation, 1 cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. For PCR reactions of iNOS and COX-2 gene were cycled 25 times between 94°C (for denaturation) for 45 sec; 60°C (for annealing) for iNOS, 55°C (for annealing) for COX-2 and GAPDH for 1 min; and 72°C (for extension) for 2 min. The last 1 cycle was performed for the final extension at 72°C for a 10 min using the thermal cycler (MJ Mini; Bio-Rad Inc., Hercules, CA, USA). RT-PCR product was stored at 4°C until agarose gel separation. Transcriptional change were calculated using an electrophoresis image quantify program (Bio-Rad Inc.).

Statistical analysis The Student's *t*-test was used to determine the statistical significance of differences between values for a variety of experimental and control groups.

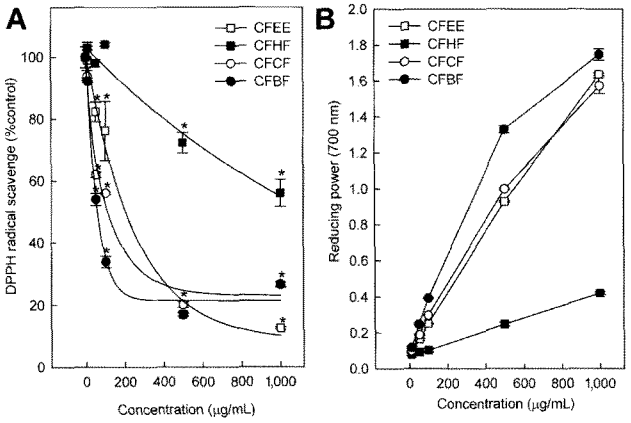

Fig. 1. Antioxidant activities of safflower ethanolic extract fractions (CFEFs). A. Scavenging effect on DPPH radicals; B. Reducing power of different amounts of CFEFs. *Significantly different as compared to control ($p<0.05$).

Data was expressed as mean±standard errors (SEM) of at least triplicate.

Results and Discussion

Free radical scavenging activity and reducing power of CFEFs Antioxidant activities of CFEFs were summarized in Fig. 1. To investigate the IC_{50} of each CFEFs for the quenching DPPH free radical scavenge, the dose-response relationship was studied (Fig. 1A). CFEFs had the hydrogen donating activity to DPPH radical in concentration-dependent manner. IC_{50} value of the reference antioxidant ascorbic acid and butylated hydroxytoluene (BHT) were found experimentally to be <0.156 and 1.758 μg/mL, respectively. CFEE showed the free radical scavenging activity reaching an IC_{50} of 202.7 μg/mL. All the fractions showed a significant scavenging activity, except for CFHF. The best activity was found in CFBF with an IC_{50} of 58.12 μg/mL, followed by CFCF with an IC_{50} of 101.8 μg/mL.

Reducing power of CFEFs determined by Fe^{3+} reduction assay. The reducing power of CFEFs for Fe^{3+} was increased in concentration-dependent manner (Fig. 1B). These results were totally consistent with DPPH radical scavenging results. CFBF had a superior reducing power capacity to others. Pyrogallol (100 μg/mL) had high reducing power, 1.851±0.056, as a control. Taken together, the antioxidant activities, tested by DPPH radical scavenging and reducing power, were decreased in the order of CFBF>CFCF> CFHF in CFEFs. Based on these results, the polar compounds in CFBF may be due to the antioxidant activity in CFEE. We may speculate that at proper concentrations these CFEFs may act as electron donors and may react with free radicals to convert them to more stable products and terminate radical chain reactions.

The cell and tissue injury associated with acute and chronic inflammation is due to the toxicity of ROS generated and released by activated phagocytes (24). NADPH oxidase in phagocytes is responsible for the production of superoxide anion and represents a major host defense mechanism of phagocytes against invading microorganisms. Superoxide initiates the formation of other ROS, such as hydroxyl radical and oxygen singlet, which are known as strong

Table 1. Cell viabilities of CFEFs[1)] for RAW 264.7 murine macrophage cells

Fractions[2)] (μg/mL)	RAW 264.7 macrophage cells (% control)						
	Control	6.25	12.5	25	50	250	500
CFEE	99.98±3.56	110.60±6.61	98.19±6.35	80.99±19.35	108.73±9.38	113.84±2.85	99.13±3.37
CFHF		92.74±14.80	90.57±17.01	92.51±7.16	92.59±5.78	94.88±20.19	64.67±7.36*
CFCF		102.20±9.23	92.80±9.37	97.19±2.05	100.85±9.85	45.75±3.53*	12.32±1.51*
CFBF		131.30±1.61	102.13±9.22	110.14±3.52	107.28±4.95	127.20±6.64	118.21±9.95

[1)]CFEFs, safflower ethanolic extract fractions.
[2)]CFEE, safflower-ethanol extract; CFHF, -hexane fraction; CFCF, -chloroform fraction; CFBF, *n*-butanol fractions.
*Significant different as compared to control, $p<0.05$.

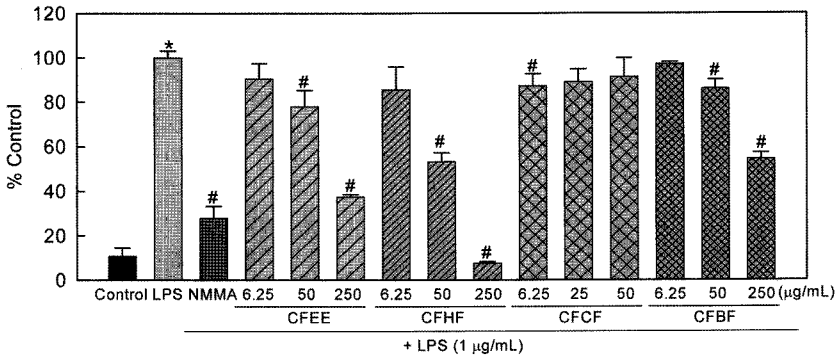

Fig. 2. Effects of CFEFs on NO production from LPS-stimulated RAW 264.7 murine macrophage cells. *,#Significantly different as compared to control and only LPS-treated group, respectively ($p<0.05$).

oxidant products, and hydrogen peroxide and hypochlorous acid (25). Free radical scavenging activity and reducing power play the critical role for quenching superoxide anions and maintaining the cellular redox homeostasis against harmful oxidants and free radicals in cells. Also, the reducing power is generally associated with the presence of reductones, which have been shown to exert an antioxidant effect by donating a hydrogen atom and breaking the free radical chain (26). Reductones are reported to react with certain peroxide precursors, which preventing peroxide formation (27). In healthy blood, several enzymes, such as glutaredoxin (GRX) and glutathione reductase (GRD), and chemical compounds, such as ascorbic acid, vitamin E, and glutathione, play as antioxidant and reducing enzyme system for scavenging harmful superoxides and free radical compounds (28). In inflammation and rheumatoid arthritis (RA), the cellular reducing power may support to quench superoxide anions secreted from cells inside synovial membrane and prevent the inflammation responses in joint-cells. From this result, the free radical scavenging activity and reducing power of CFEFs will help the relief on inflammatory symptoms, caused by harmful radical compounds.

Cytotoxicity of CFEFs for RAW 264.7 murine macrophage cells Cytotoxic effect of CFEFs for RAW 264.7 murine macrophage cells were shown in Table 1. CFEE, CFHF, and CFBF did not affect the cell viability within tested concentration (0-250 μg/mL, IC_{50}>500 μg/mL), whereas CFCF showed a significant cytotoxicity at more than 50 μg/mL of concentration (IC_{50}=227.1 μg/mL). Based on this result, CFEFs (6.25-250 μg/mL) was used for further studies, including the inhibition of NO and PGE_2 production from LPS-stimulated RAW 264.7 cells. However, in case of CFCF, the concentration range for the further studies was adjusted to 6.25-50 μg/mL, due to its cytotoxicity.

Effect of CFEFs on the NO production from LPS-stimulated RAW 264.7 cells To investigate the anti-inflammatory effect of CFEFs, we examined whether CFEFs could modulate NO synthesis in LPS-stimulated cultures of the murine macrophages of the RAW 264.7 cells. CFEFs potently inhibited LPS-induced nitrite production in a dose-dependent manner, as shown in Fig. 2. We observed LPS-stimulated nitrite production from RAW 264.7 cells, as compared to the control. Also, L-NMMA (10 μM) inhibited nitrite production significantly (approximately 81.49%) as a positive control. CFEE (250 μg/mL) inhibited approximately 70% of NO production (IC_{50}=163.9 μg/mL) from LPS-induced RAW 264.7 cells. However CFHF (250 μg/mL), more fractionated from CFEE, had remarkable inhibitory effects, which are below basal level (IC_{50}=31.54 μg/mL) at 250 μg/mL. This was superior to inhibitory effect of NMMA. In addition, CFBF showed approximately 52% of NO inhibition at 250 μg/mL from LPS-induced RAW 264.7 cells, while CFCF had no suppressive effect on NO production.

NO, one of the important inflammatory mediators, is also related to the pathophsiology of inflammation joint disease and plays a key role in cartilage catabolism mediated by inflammation. Also, the inflammatory transcription factors, NF-κB pathway, Ap-1, Jak-STAT pathway, and nitric oxide synthase (NOS) and cellular adhesion molecule (CAM) protein transcription were regulated by NO (10). NO may represent key regulatory molecules in the inflammatory process in rheumatoid arthritis. Also, NO act to either up-

regulate or down-regulate COX activity (29), while PGs can affect iNOS activity (30). This finding shows that CFHF and CFBF declined significantly NO production from LPS-induced RAW 264.7 cells, and, in turn, might affect the NO-mediated inflammatory responses.

Effects of CFEFs on the chemical NO scavenge and the suppression of iNOS mRNA transcription in LPS-induced RAW 264.7 murine macrophage cells The inhibition of NO production by CFEFs (Fig. 2), can be explained by two possibilities. One explanation is the chemical NO quenching by CFEFs in the cell-free system (Fig. 3A). Sodium nitroprusside (SN), a NO donor, produces nitrite ions in aqueous solution at physiological pH. The chemical quenching of NO by CFEFs was measured by Griess reaction. CFHF and CFBF showed partially chemical NO quenching (maximum 36%), whereas Hb, used as a NO scavenger, showed the most NO scavenge (approximately 96%, Fig. 3A). NMMA, an iNOS inhibitor, did not have the chemical quenching in the cell-free system at all (data not shown). It could be expected that the chemical NO scavenging effect of CFBE was based on the potent antioxidant property (Fig. 1). From this result, the inhibition of NO production by CFHF and CFBF in LPS-induced RAW 264.7 cells was might be due to partially chemical scavenge of NO produced by iNOS reaction.

The other explanation for the inhibition of NO production from LPS-stimulated RAW 264.7 cells, is the suppression of iNOS mRNA transcription by CFEFs. We observed the up-regulation of iNOS mRNA transcription in LPS-stimulated RAW 264.7 cells significantly (Fig. 3B). NMMA, an iNOS inhibitor, suppressed approximately 88% of up-regulated iNOS gene transcription. CFEE and CFHF markedly inhibited iNOS mRNA transcription from LPS-induced RAW 264.7 cells. Especially CFHF suppressed iNOS mRNA transcription under the basal level at 250 μg/mL concentration. Otherwise, CFCF and CFBF did not appear the inhibition of iNOS mRNA transcription in LPS-stimulated RAW 264.7 cells. Taken together, the inhibitory effect of CFHF showing highest inhibition on NO production (Fig. 2), is both due to partially chemical quenching of NO and the suppression of iNOS mRNA transcription in LPS-stimulated RAW 264.7 cells. The inhibition of NO production by CFEE and CFBF is mainly due to only iNOS mRNA transcription in LPS-stimulate RAW 264.7 cells and only chemical quenching of NO as an antioxidant property, respectively.

NO production and iNOS expression are considered to be associated with the pathogenesis of several diseases such as inflammation and carcinogenesis. Therefore, the regulation of iNOS in tissues might be important for the treatment of inflammation. Expression of iNOS is closely related with the up-regulation of NF-κB (31). NF-κB site was identified in the promoter region of iNOS gene. NF-κB, an inducible transcription factor, is activated in response to various extracellular cytokines (32), LPS (33), and oxidative stresses (34). Presumably, the inhibitory activity of CFEE and CFHF on iNOS mRNA transcription might be due to the regulation of NF-κB. Its reaction product NO is involved in various diseases, such as psoriasis and atopic inflammation.

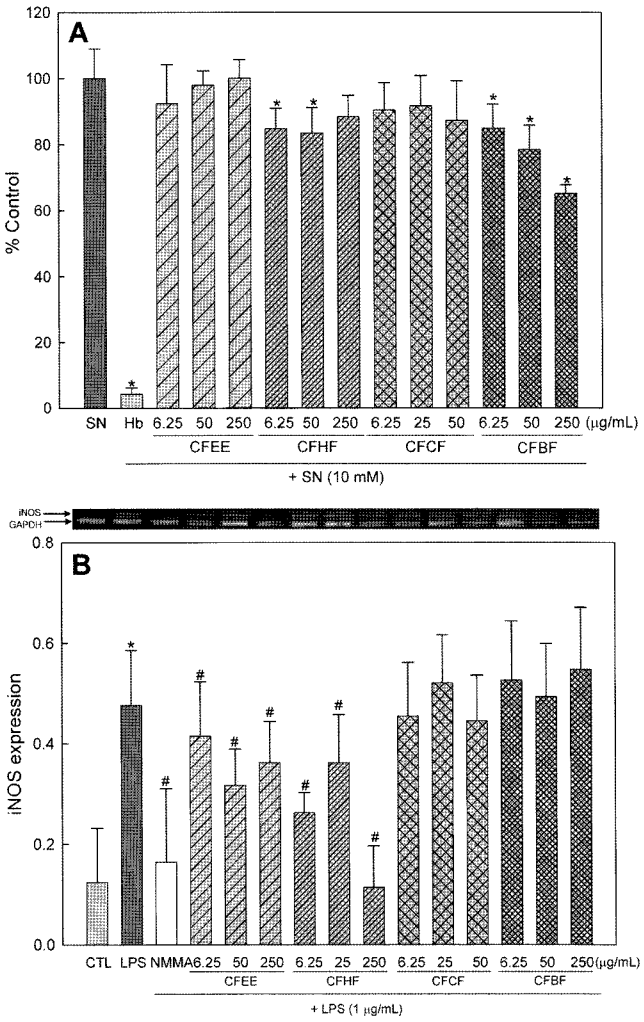

Fig. 3. Effects of CFEFs on the chemical NO quenching and the suppression of iNOS mRNA transcription. A. Chemical NO scavenging of CFEFs in the cell-free system. *Significantly different as compared to SN ($p<0.05$). B. Suppression of iNOS mRNA transcription by CFEFs in LPS-induced RAW 264.7 cells. *,#Significantly different as compared to control and to the LPS-treated group, respectively ($p<0.05$). CTL, control; SN, Sodium nitroprusside; Hb, hemoglobin.

Effects of CFEFs on the production of PGE_2 and the suppression of COX-2 mRNA transcription in LPS-induced RAW 264.7 murine macrophage cells The biosynthesis of PGE_2, a typical inflammatory and rheumatoid arthritis mediator, was monitored from LPS-induced RAW 264.7 cells for the indication of inflammatory response (Fig. 4A). LPS stimulated significantly PGE_2 production from RAW 264.7 cells, and celecoxib (10 μM), which is a selective COX-2 inhibitor, inhibited approximately 89.6% of PGE_2 synthesis. CFEE inhibited PGE_2 synthesis in a concentration-dependent manners (IC_{50}=4.95), and showed almost complete inhibitions, which was superior effect to response of celecoxib. Additionally, CFHF suppressed approximately 60% of PGE_2 synthesis from LPS-induced RAW 264.7 cells, while CFCF and CFBF did not show the inhibitory effect on PGE_2 production with a significant difference.

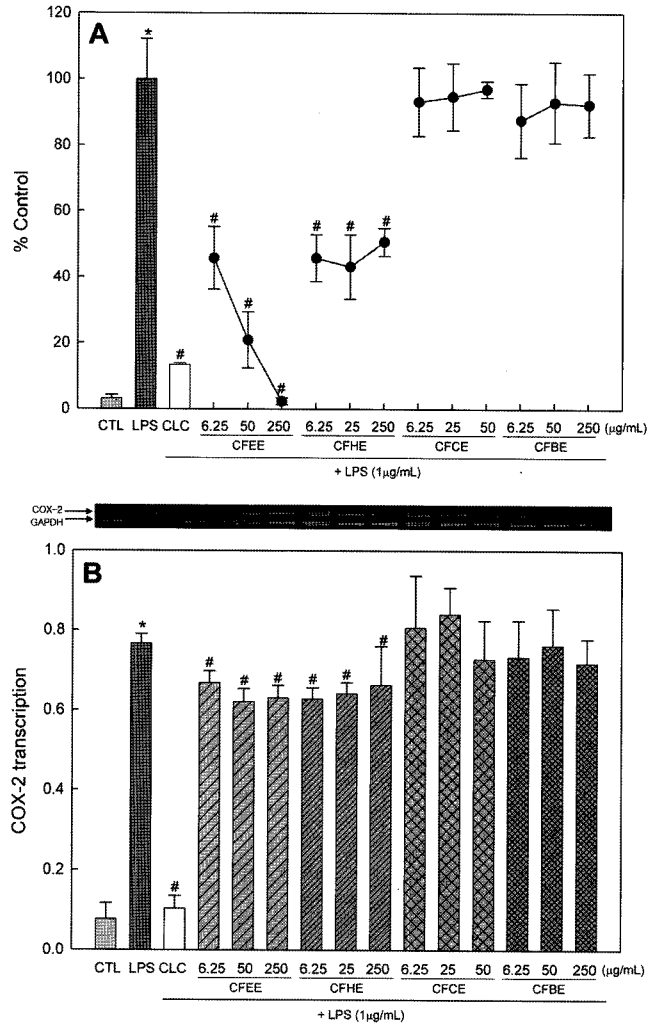

Fig. 4. Inhibition of CFEFs on the production of PGE_2 and COX-2 transcription in LPS-induced RAW 264.7 macrophage cells. A. Effect of CFEFs on the production of PGE_2 from LPS-stimulated RAW 264.7 macrophage cells, B. Suppressive effect of CFEFs on COX-2 mRNA transcription in LPS-stimulated RAW 264.7 macrophage cells. *,#Significantly different as compared to control and to the LPS-treated group, respectively ($p<0.05$). CTL, control; CLC, celecoxib.

The synthesis of PGE_2 is regulated by COX-2 in acute inflammatory responses. Therefore the effect of CFEFs on COX-2 mRNA transcriptional level was investigated in LPS-induced RAW 264.7 cells. The transcription of COX-2, one of the important enzymes in inflammatory pathogenesis, was not observed almost in basal RAW 264.7 cells. However LPS increased COX-2 gene transcriptional levels significantly in RAW 264.7 cells, and LPS-stimulated COX-2 expression was suppressed by celecoxib (20 μM, 96%), a selective inhibitor of COX-2, depicted in Fig. 4B. CFEE and CFHF had a similar exacerbation (approximately 15-22% inhibition) in COX-2 mRNA transcription. However CFCF and CFBF did not show the suppression of COX-2 mRNA transcription with a significant difference, as compared to LPS-treated group. Kim *et al.* (8) reported that lots of luteolin, luteroilin-7-glucoside, and acacetin were detected in flower extract. And acacetin showed the suppressive effects on LPS-induced up-expression of iNOS and COX-2 in murine macrophage cells (35). The present results are entirely consistent to the suppression of PGE_2 synthesis by CFEFs and might be due to phenolic compounds such as acacetin in CFEFs.

COX, the rate-limiting enzyme in the conversion of arachidonic acd (AA) to PGs, exists as 2 isoforms. COX-1 is constitutively produced and detectible in most human tissues. In contrast, COX-2, normally expressed at low levels, is strongly induced by inflammatory stimuli, including LPS, tumor promoters, and growth factors. At inflammatory sites, such as human atherosclerotic lesions, high levels of COX-2 have been found compared with low levels in unaffected arteries (36). The expression of COX-2 is a key element in the pathophysiology of several inflammatory disorders, and its regulation differs between cell types. COX-2 is expressed during inflammatory disease in many cells, including fibroblasts and macrophages, and mediates the release of large quantities of pro-inflammatory PGs at the site of inflammation. Additionally the peroxidase and prostaglandin E synthase (PGES) are involved in the biosynthesis of PGE_2. However their roles in rheumatoid arthritis are currently unclear.

COX-2 and NO represent key regulatory molecules in the inflammatory process in rheumatoid arthritis. Many studies have demonstrated that some inducible enzyme (COX and iNOS)/cytokines and their reaction products are involved in chronic inflammatory disease. The suppression of COX-2 transcription by CFEE and CFBF could be caused by inhibition of Akt activation, or by inhibition of AA release causing suppression of PGs synthesis (37). PGs are produced by COX, and play a role in the nociceptive mechanism (38). The reduction of writhing responses in mice is probably related to the reduced synthesis of the inflammatory mediators by inhibition of cyclooxygenases and/or lipoxygenases (39). Additionally, in rheumatoid arthritis, it is known that the third phase of the edema-induced by carrageenan, in which the edema reaches its highest volume, is characterized by the presence of PGs (40). Besides, in the carrageenan-induced rat paw edema model, prostanoids are produced through the serum expression of COX-2 by a positive feedback mechanism (41).

This report elucidated that the potential analgesic and anti-rheumatoid arthritis activities of CFEFs might be due to the inhibition of PGE_2 synthesis. Additionally, our results raise the possibility that CFEFs may retard inflammatory process in rheumatoid synovium by suppression of PGE_2 synthesis.

It is concluded that CFEE and CFHF have suppressed NO production and the transcription of iNOS and COX-2 mRNA by LPS-stimulated RAW 264.7 macrophage cells. The potential analgesic and anti-inflammatory activities were confirmed by investigating the anti-inflammatory activities. Taken together, CFEFs, especially CFEE and CFHF may be useful as an alternative medicine for the relief and retardation of immunological inflammatory responses and its action may occur through the reduction of inflammatory mediators, including NO and PGE_2.

Acknowledgments

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