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Anti-inflammatory Activity of an Ethanol Extract of *Caesalpinia sappan* L. in LPS-induced RAW 264.7 Cells

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Abstract

The anti-inflammatory activities of an ethanol extract of *Caesalpinia sappan* L. (CS) were investigated in LPS-induced RAW 264.7 cells. Result indicated that CS inhibited the LPS-induced NO production in a dose-dependent manner with an IC_{50} of 10.9 μg/mL. In addition, CS attenuated the iNOS mRNA and protein expression by inhibiting NF-κB activation. CS also suppressed the productions of IL-6 and MCP-1 in a dose-dependent manner, with IC_{50} values of 15.9 μg/mL and 5.47 μg/mL, respectively. In addition to the anti-inflammatory activities, CS decreased intracellular ROS formation in the same cells. In conclusion, CS inhibited the production of NO, IL-6 and MCP-1 via a suppression of the NF-κB activation and intracellular ROS generation.

Key words: *Caesalpinia sappan*, nitric oxide, iNOS (inducible nitric oxide synthase), NF-κB (nuclear factor kappa B), IL-6 (interleukin-6)

INTRODUCTION

Caesalpinia sappan L (CS) has been widely used in the treatment of tubuerculosis, diarrhea, dysentery, pain, and inflammation. Several recent studies reported a wide spectrum of physiological effects including: antioxidant activity (1), anticancer (2), anticonvulsant activity (3) and an anticomplementary activity (4). It was also demonstrated that brazilin isolated from CS has a suppressive effect of a iNOS expression in RAW 264.7 cells (5). However, there have been no studies on the effects of an ethanol extract of CS on nitric oxide (NO) formation through an inhibition of the NF-κB activation and intracellular ROS generation.

NO, which can be formed abnormally by an activation of bacterial lipopolysaccharide (LPS) in some cell types such as macrophages (6), is a potentially toxic gas and participates in many chemical reactions (7), and has been related with various disease such as atherosclerosis, inflammation, and carcinogenesis (8-10). NO is synthesized by an enzyme NO synthases (NOS), having 3 different isotypes (nNOS, iNOS, eNOS). Among them, iNOS is the main reason for the NO production at an inflammatory site.

In macrophages, LPS activates the transcription factor nuclear factor kappa B (NF-κB), which leads to the induction of the expression of many genes involved in immune responses, apoptosis, and the cell cycle. Above all, NF-κB is the most important factor in inflammatory diseases. When NF-κB is activated, it induces the expression of iNOS as well as synthesis of many pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β and IL-8 (11).

Therefore, we have investigated the effect of CS on NO, pro-inflammatory cytokines and chemokines production in LPS-stimulated RAW 264.7 cells to confirm the possibility that CS could be used as a material for the treatment of inflammatory diseases.

MATERIALS AND METHODS

Cell culture

RAW 264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) and held at 37°C with 5% carbon dioxide.

Reagents

CS was purchased from Duk Hyun Dang (Seoul, Korea). DMEM and FBS were purchased from Hyclone. LPS, Griess reagent, sodium nitrite and DMSO were purchased from Sigma-Aldrich (Korea). The mouse monoclonal anti-iNOS antibody, rabbit polyclonal anti-β-tubulin antibody and goat anti-mouse IgG-HRP conjugate were purchased from Santa Cruz Biotechnology.

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The goat anti-rabbit IgG-HRP conjugate was purchased from ZYMED. PreMix WST-1 was purchased from TAKARA BIO INC.

Sample preparation

CS (2 kg) was extracted with 70% ethanol for 6 hr, three times at room temperature. After filtration, the 70% ethanol extracts were evaporated to give ethanolic extracts (124 g).

Cytotoxicity assay

Tetrazolium salts are available to measure cell viability. Tetrazolium salts are cleaved to formazan dye by a succinate-tetrazolium reductase, which exists in a mitochondrial respiratory chain and is only active in viable cells. RAW 264.7 cells were cultured in a 96-well plate (5.0×10^4 cell/well) for 24 hr and subsequently treated with various concentrations of the CS ethanol extract for 48 hr. After the incubation period, 10 μL Premix WST-1 (TAKARA) was added into each well and incubated at 37°C and 5% CO_2 for 4 hr. The index of cell viability was determined by measuring the OD at 480 nm of formazan production using the ELISA reader. The reference wavelength was 650 nm.

NO measurement

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL), and held at 37°C with 5% CO_2 in a 100 mm petri dish for 24 hr (2×10^5 cell/mL). Cells were then treated with various concentrations of the CS ethanol extract for 2 hr. Thereafter cells were treated with LPS (1 μg/mL) for 18 hr. The cell supernatants were collected at the end of the culture to measure nitrite, which was used to estimate NO production (12). Equal volumes of Griess reagent (100 μL; Sigma-Aldrich) were added to each cell supernatant (100 μL), and their absorbance was measured at 570 nm. The concentration of nitrite (μM) was calculated from a standard curve drawn with a known concentration of sodium nitrite dissolved in DMEM. The results are presented as the mean±SD of 4 replicates of one representative experiment and this experiment was repeated three times with similar results.

Quantitative real-time PCR

The analyses of iNOS mRNA in the RAW 264.7 cells were performed on total RNA. After stimulation with various concentrations of the CS ethanol extract for 2 hr, LPS (1 μg/mL) was added for 18 hr. Total RNA from the RAW 264.7 cells was isolated using the RNeasy Kit according to the manufacturer's instructions (Qiagen). The Advantage RT-for-PCR kit was used for reverse transcription according to the manufacturer's protocol (Clontech). Chromo4 real-time PCR detection system (Bio-Rad) and iTaq^{TM} SYBR^{R} Green supermix (Bio-Rad) were used for the RT-PCR amplification of iNOS and β-actin ('housekeeping gene' encoding beta-actin) with the following conditions: 50 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 30 s. For a normalization of the cycling threshold values obtained with the experimental samples. All the reactions were repeated at least three times independently to ensure the reproducibility of the results. Primers for iNOS, IL-6 and β-actin were purchased from Bioneer corp (Korea) and PCR was performed on the cDNA using the following sense and anti-sense primers: iNOS, forward primer 5'-TCCTAC ACCACACCAAACTGTGTGC-3', reverse primer 5'-CTCCAATCTCTGCCTATCCGTCTC-3'; β-actin, forward primer 5'-TGAGAGGGAAATCGTGCGTGAC-3', reverse primer 5'-GCTCGTTGCCAATAGTGATGACC-3'.

Western blotting

The analyses of iNOS and β-tubulin in the RAW 264.7 cells were performed on whole cell lysates. After stimulation with various concentrations of the CS ethanol extract for 2 hr, LPS (1 μg/mL) was added for 18 hr. Cells were collected by scraping and centrifuged at 2,000 rpm for 3 min. Then the cells were washed with PBS. Cells were lysed for 20 min at -20°C with a lysis buffer (Protein extraction solution, iNtRON Biotechnology) containing a complete protease inhibitor cocktail. The supernatants were collected, and the protein concentrations in cell lysates were determined by the Bio-Rad Protein Assay (Bio-Rad). Equal amounts of protein (50 μg) were mixed with a gel loading buffer at a ratio of 3:1, boiled for 5 min, and centrifuged at 12,000 rpm for 1 min. Each sample was loaded and electrophoresed on a 8% SDS-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Hybond ECL Nitrocellulose, Amersham). The membranes were washed once with a washing buffer (PBS, 0.05% Tween 20) and blocked with a blocking buffer (PBS, 5% skim milk, 0.05% Tween 20) for 1 hr. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4°C. Mouse monoclonal antibody anti iNOS and rabbit polyclonal anti β-tubulin were diluted at 1:800 in the blocking buffer. After the incubation, the membranes were washed three times with the washing buffer and they were incubated for 1 hr at room temperature with HRP-conjugated anti-mouse secondary antibody for iNOS and HRP-conjugated anti-rabbit secondary antibody for β-tubulin diluted at 1:2000 in the blocking buffer. The membranes were washed three times with the washing buffer, and the protein bands were detected by an chemiluminescence system

(Amersham Pharmacia).

Immunochemistry staining

RAW 264.7 cells growing on a 22×22 mm^2 cover slip were fixed by 4% paraformadehyde in PBS at RT for 20 min. The cells incubated in 1% Triton X-100 (Sigma, USA) were added to increase the cell membrane permeability. The cells were blocked by 10% goat serum (Vector, USA) in PBS for 1 hr. The primary antibody was diluted to 1:100 using 1% goat serum. The cells were incubated in the primary antibody at 4°C overnight. Following three washes with the washing buffer (PBS, 0.05% tween 20), the cells were stained with fluorescence labeled secondary antibody and then the nuclei were stained with 300 ng/mL propridium iodide (Invitrogen, USA) following washing excess antibody. The slips were mounted and observed under a ZEISS LSM 510 META confocal microscope. In this study, mouse NF-kappaB p65 (Abcam, Cambrige, UK) was employed as a primary antibody. The secondary antibody was goat anti-mouse IgG Alexa 488 (Molecular probe, USA).

Measurement of MCP-1 and IL-6 by ELISA

The RAW 264.7 cells (2×10^5 cell/mL) were incubated for 24 hr and then the CS ethanol extract was added for 2 hr. After that, LPS (1 μg/mL) was added for 4 hr. To determine the resultant cytokines, the supernatants were harvested, and then the levels of MCP-1 and IL-6 were measured by ELISA (R&D systems).

Intracelluar ROS measurement

RAW 264.7 cells (2×10^5 cell/mL) were incubated for 24 hr and then the CS ethanol extract was added for 2h. After that, LPS (1 μg/mL) was added for 18 hr. Cells were incubated with 10 μmole/L 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, carboxy-H_2DCFDA (molecular probe, Invitrogen) for 30 min and then the cells were washed with PBS buffer, after which they were trypsinized and harvested. They were washed with PBS once more and resuspended in 1 mL PBS. Intracellular ROS was detected with a flow cytometer (Cytomics FC 500, BECKMAN).

Statistical methods

Significance between pairs of mean values was determined by the Student's t-test. A $p<0.05$ was considered significant for all the analyses.

RESULTS AND DISCUSSIONS

Cytotoxicity

To explore the potential use of the CS ethanol extract in LPS-induced RAW 264.7 cells as NO, ROS and iNOS regulators, we first tested the cytotoxic effects of the ethanol extract of CS on the RAW 264.7 cells by a Premix WST-1 (TAKARA). As shown in Fig. 1, The ethanol extract of CS showed a cytotoxicity at concentrations higher than 60 μg/mL.

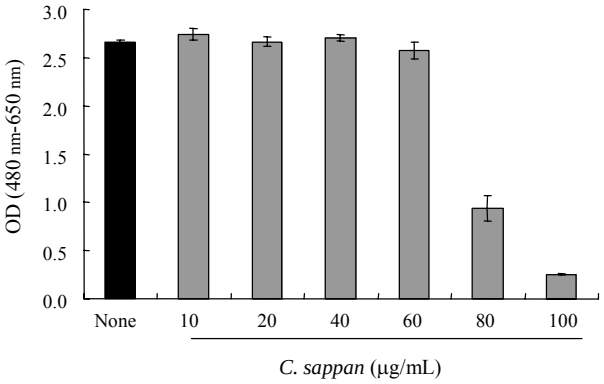

Fig. 1. Cytotoxicity of the CS ethanol extract on RAW 264.7 cells.

Effect of CS on an LPS-induced NO formation

In the present study, we wanted to establish whether CS could be used as a material to alleviate the inflammatory diseases such as rheumatoid arthritis. Therefore, we first checked to see whether CS could regulate LPS-induced NO formation. Overproduction of NO is highly associated with inflammation (13). LPS (1 μg/mL) markedly increased the production of NO from the basal level of 0.81 ± 0.04 μM to 17.01 ± 0.06 μM for an 18 hr incubation period. When the ethanol extract of CS was added to the RAW 264.7 cells stimulated with LPS (1 μg/mL), a concentration-dependent decrease in nitrite production in the cell medium was observed and the value of IC_{50} was 10.9 μg/mL ($p<0.001$, n=4) (Fig. 2).

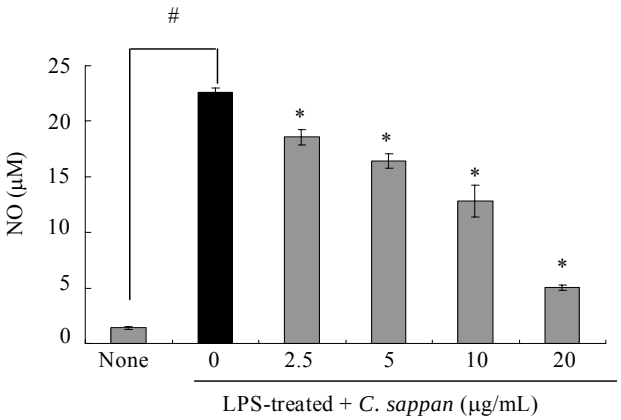

Fig. 2. Inhibition of NO production by CS in RAW 264.7 cells. RAW 264.7 cells were treated with various concentrations of CS for 2 hr prior to the addition of LPS (1 μg/mL), and the cells were further incubated for 18 hr. Data shown are the mean±SD (n=4). $^{\#}p<0.001$ versus a media alone-treated group. $^{*}p<0.001$ versus LPS alone-treated group.

Effect of CS on the LPS-induced iNOS expression level

Since the accumulation of NO in LPS activated macrophages is highly correlated with an enhanced iNOS expression, we investigated that as well. As shown in Fig. 3, the ethanol extract of CS attenuated the LPS-induced iNOS mRNA and protein expression levels. Therefore, an inhibition of the LPS-induced NO formation by the ethanol extract of CS was due to the suppression of the iNOS mRNA and protein expression levels in the RAW264.7 cells.

Effect of CS on an LPS-induced NF-κB activation

To clarify mechanism of the inhibition of LPS-induced iNOS expression by the CS ethanol extract, we investigated the NF-κB activation by an immunochemistry staining. The transcription factor NF-κB usually consists of two subunits: p50 and p65. When NF-κB is activated, it moves from the cytoplasm to the nuclei. So we checked the location of p65 with antibody. With LPS treatment there a translocation of p65 to the nuclei was observed (Fig. 4B). And we found that the CS ethanol extract inhibited the translocation of p65 to the nuclei (Fig. 4C). Therefore, the ethanol extract of CS suppressed the LPS-induced NO formation by inhibiting NF-κB activation in the RAW 264.7 cells.

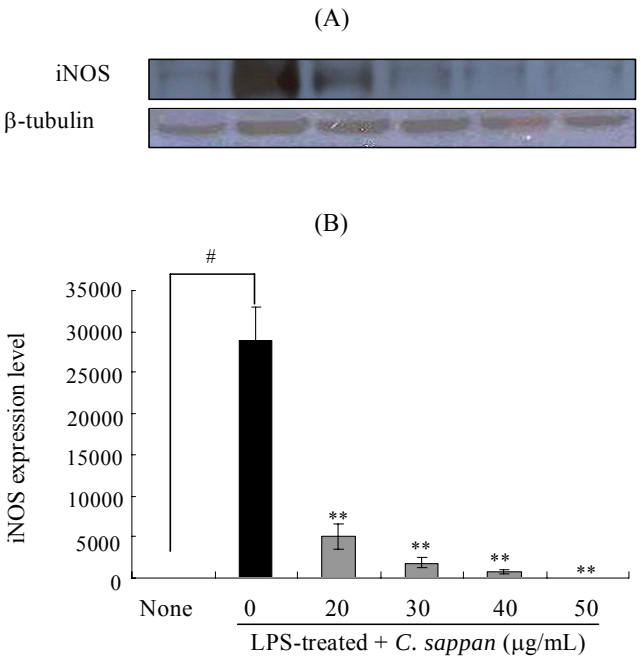

Fig. 3. Effects of CS on the LPS-induced iNOS expressions levels, (A) western blotting, (B) quantitative real time PCR. RAW 264.7 cells were treated with various concentrations of CS for 2 hr prior to the addition of LPS (1 μg/mL), and the cells were further incubated for 18 hr. Data shown are the mean ± SD (n=2). #$p < 0.001$ versus media alone-treated group. **$p < 0.001$ versus LPS alone-treated group.

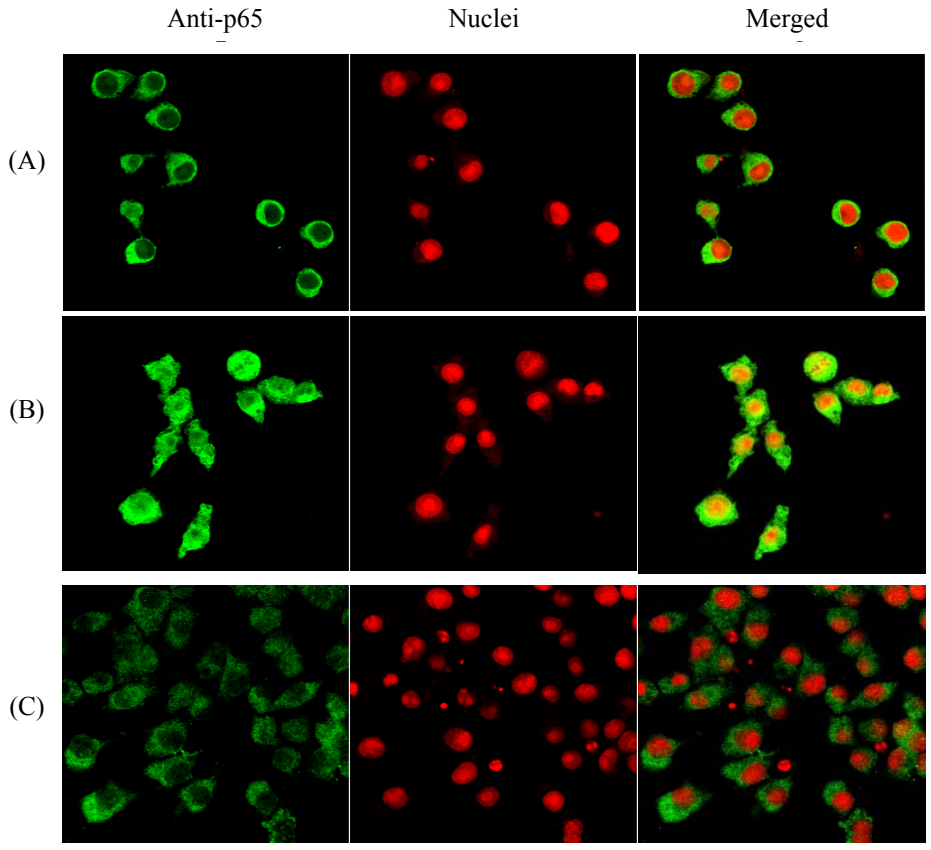

Fig. 4. Intracellular localization of p65 in RAW 264.7 cells. Cells were seeded onto 22×22 mm^2 cover slips and fixed by 4% paraformadehyde in PBS at RT for 20 min. Cells were incubated in 1% Triton X-100 (Sigma, USA) and blocked by 10% goat serum in PBS for 1 hr. Cells were incubated in the primary antibody at 4°C overnight. Following three washes, cells were stained with fluorescence labeled secondary antibody and then the nuclei were stained with propridium iodide. A: none, B: LPS only, C: LPS+CS 50 μg/mL.

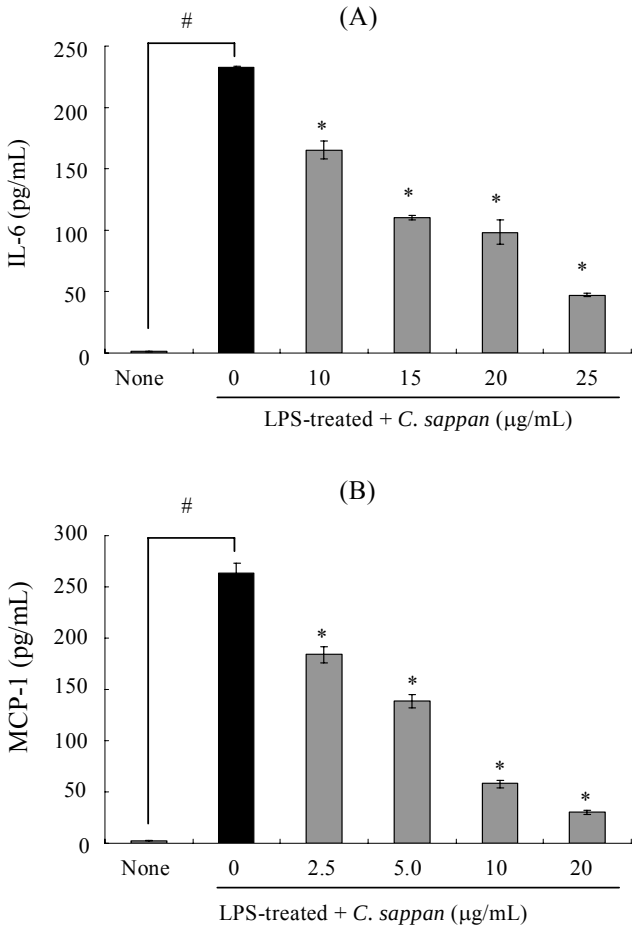

Fig. 5. Effects of CS on LPS-induced production of pro-inflammatory cytokines and chemokines in the RAW 264.7 cells by ELISA analysis, (A) IL-6, (B) MCP-1. Cells were treated with various concentrations of CS for 2 hr prior to the addition of LPS (1 μg/mL), and the cells were further incubated for 4 hr. Data shown are the mean ± SD (n=3). $^{\#}p < 0.001$ versus media alone-treated group. $^{*}p < 0.001$ versus LPS alone-treated group.

Effect of CS on an LPS-induced pro-inflammatory cytokine and chemokine

In the early state in an inflammation, chemokines provoke the influx of immune cells to an inflammatory site and the cells cause a disruption of the balance between the pro-inflammatory cytokines (14-16). IL-6 is a typical pro-inflammatory cytokine and MCP-1 is a kind of chemokine related involved in inflammation (17,18). Therefore, we checked to see whether CS also could inhibit the LPS-induced production of the IL-6 and MCP-1. As shown in Fig. 5, the CS ethanol extract reduced the LPS-induced production of IL-6 and MCP-1 in a dose-dependent manners with IC_{50} values of 15.9 μg/mL and 5.47 μg/mL respectively. Therefore CS inhibited not only the LPS-induced NO formation but also the LPS-induced production of IL-6 and MCP-1.

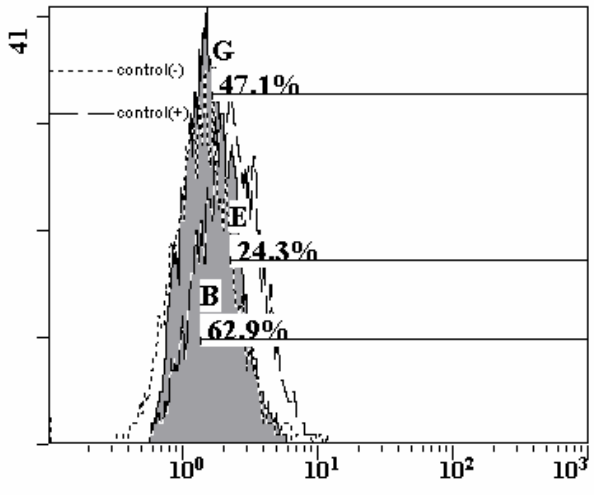

Fig. 6. Detection of the intracellular ROS generation in the RAW 264.7 cells by a flow cytometer. Carboxy-H_2DCFDA fluorescent signals after incubation with 50 μg/mL CS for 18 hr with or without LPS (1 μg/mL). B: none, E: LPS, G: LPS+ CS.

Effect of CS on LPS-induced intracellular reactive oxygen species

Since intracellular ROS could also cause NF-κB activation (19,20), we checked successively to see whether CS could remove those directly. To examine the LPS-induced ROS production in the RAW 264.7 cells, we used a carboxy-H_2DCFDA probe (Invitrogen). By monitoring intracellular oxidation of the dye carboxy-H_2DCFDA, we found that LPS increased intracellular ROS formation in the RAW 264.7 cells (Fig. 6). The increased intracellular ROS production by LPS was attenuated by pre-incubating the RAW 264.7 cells with the CS ethanol extract (50 μg/mL). The CS ethanol extract decreased the intracellular ROS level by 60% over the positive control (Fig. 6). Therefore, CS inhibited the LPS-induced NO formation via two mechanisms, one is a suppression of the NF-κB activation and the other is the removal of the intracellular ROS.

In the methanolic extract of CS, many kinds of compounds such as brazilin, brazilein, sappanchalcone, and protosappanins A, B and C were isolated and tested for antioxidant and anti-inflammatory activities (21), but another phenolic compounds from CS such as 3-deoxysappanchalcone, sappanone B, 3-deoxysappanone B and 3'-deoxy-4-O-methylepisappanol were not tested for anti-inflammatory activities (22).

In conclusion, we suggest that the ethanol extract of CS has anti-inflammatory activities and has the potential for development as a functional food and/or drug for inflammatory diseases such as rheumatoid arthritis.

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