Anti-inflammatory effects of *Calophyllum inophyllum* L. in RAW264.7 cells

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Abstract. *Calophyllum inophyllum* L. has been used as folk medicine in the treatment of ocular burn and it has demonstrated potential to be an anti-inflammatory agent. The aim of this study was to explore the anti-inflammatory activities of an acetone extract of *C. inophyllum* L. leaves (CIL). The CIL extract was tested on lipopolysaccharide (LPS)-induced RAW 264.7 cells to evaluate the effect of CIL extract on the expression of nitric oxide (NO) and inducible nitric oxide synthase (iNOS). Results showed that the CIL extract markedly suppressed the LPS-induced production of nitric oxide, as well as the expression of iNOS, cyclooxygenase (COX)-2 and nuclear factor-kappaB (NF-κB) in a dose-dependent manner. LPS-induced microRNA (miR)-146a expression was inhibited by CIL extract, while miR-155 and miR-424 expression was not affected as demonstrated using quantitative RT-PCR analysis. Taken together, these observations show that CIL extract has anti-inflammatory effect, which extends the potential application for prevention of inflammatory diseases, and its mechanism may be partially associated with blocking COX-2 and iNOS of RAW 264.7 cells.

Introduction

Inflammation is a vital defense mechanism for the organism in response to pathogen stimuli. Monocytes and macrophages are dominant at the locations of lipopolysaccharide (LPS) induced inflammation. Macrophages become active upon LPS stimulation, and several cellular mediators such as tumor necrosis factor-α (TNF-α), IL-1β, nitric oxide (NO) and cyclooxygenase (COX)-2 are released to regulate inflammation (1).

NO is also known as a short-lived free radical and one of key cellular mediator for inflammatory responses. There are three isoforms of NO synthases (NOS) in tissues to generate NO (2). The neural NOS and endothelial NOS isoforms are constitutively expressed in select tissues. Inducible NOS (iNOS), a third member of the NOS family, is known to have beneficial effects in response to inflammatory stimuli. The expression level of iNOS is elevated in response to LPS via a variety of transcription factors, particularly nuclear factor-kappaB (NF-κB) (3). Cyclooxygenase (COX)-2, another cellular inflammatory mediator, is undetectable in most normal tissues (4). Upon LPS stimulation, COX-2 expression is induced rapidly and transiently at inflammatory sites (5). Furthermore, NF-κB plays vital roles in the coordination of the expression of pro-inflammatory mediators and cytokines, including iNOS (6) and COX-2 (7). Once cells are stimulated by LPS, NF-κB becomes activated, dissociates with IκB and then translocates from cytosol to nucleus, which leads to induction of NF-κB downstream genes via binding to the NF-κB response element (8). MicroRNAs function as non-coding RNA molecules that regulate the expression of target genes involved in a wide range of biological processes. It has been shown that microRNAs are found in almost all eukaryotic cells. Mature microRNAs bind to the 3' untranslated regions of target genes and inhibit protein synthesis (9). Recently, microRNAs have been found to be involved in the growth and differentiation of immune cells (10). Previous studies showed that miR-146a and miR-155 were up-regulated in response to LPS stimulation. MiR-146 has been considered as a negative regulator in the innate immunity, while miR-155 regulated mature T cells and B cells (11). Moreover, miR-424 was found to be involved in the differentiation of macrophages (12).

Over-reactive and uncontrolled inflammatory responses can cause the tissues to remain in a chronic inflammatory status, which leads to a variety of diseases including rheumatoid arthritis, pulmonary fibrosis and even cancer (13). Previous studies have been shown that excessive NO production causes inflammation and carcinogenesis (14,15). Natural products have been used in drug discovery and development to regulate inflammation.

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Abbreviations: CIL, *Calophyllum inophyllum* L. leaves; NF-κB, nuclear factor kappaB; NO, nitric oxide; DMSO, dimetylsulfoxide; miRNA, microRNA; 1400W, *N*-(3-aminomethyl)-benzylacetamidine; COX-2, cyclooxygenase-2; LPS, lipopolysaccride

Key words: anti-inflammatory effect, microRNA, *Calophyllum inophyllum* L., traditional Chinese herbs

Calophyllum inophyllum Linn. (Guttiferae) is a medium to large tree distributed throughout Taiwan, India, and Australia (16-19). The active constituents of *C. inophyllum* is well known for containing xanthone, flavone, and terpene derivatives, some of which exhibit antitumor (20,21), and anti-HIV activities (22,23). The oil of *C. inophyllum* enhanced the healing of Ocular burn (24). The present study was undertaken to evaluate the anti-inflammatory effects of *C. inophyllum* in macrophage cells under LPS exposure. Given that NO and COX-2 are specific to inflamed tissue, the inhibition of NO over-production and COX-2 expression is important for evaluating the effects of anti-inflammatory drugs. However, the effect of *C. inophyllum* L. leaf (CIL) extract on LPS-induced RAW264.7 cells is still unclear. The purpose of this study was to investigate the anti-inflammatory actions of CIL extract on NO production, COX-2 expression and translocation of NF-κB. In addition, we also provide evidence suggesting that CIL extract also suppressed LPS-induced miR-146a expression.

Materials and methods

Chemicals and reagents. Celebrex, acetone, dimethylsulfoxide (DMSO), lipopolysaccride (LPS), and *N*-(3-aminomethyl)-benzylacetamidine (1400W) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Preparation of the Calophyllum inophyllum Linn extracts. The leaves of *C. inophyllum* L. (Guttiferae) were collected in Ping Tung Hsieng, Taiwan, in October, 2008, and a voucher specimen (2008) has been deposited in the Department of Biological Science and Technology, China Medical University. The dried leaves of *C. inophyllum* (1 kg) was ground, extracted with acetone at room temperature, and concentrated under reduced pressure to afford a brown residue (90 g). The series concentrations of CIL extract were further diluted with DMSO.

Quantitative analysis of active compounds of C. inophyllum by HPLC. Before analysis by HPLC, CIL extract was filtered through a 0.2 μm Millipore filter, and then total volume of 20 μl was loaded into the HPLC column. External standards were prepared as concentration of 100 μg/ml in HPLC grade-methanol and used to calculate the concentration of examined compounds. Reverse phase HPLC was performed on a Perkin-Elmer HPLC system (Perkin-Elmer, Waltham, MA, USA) equipped with Perki-Elmer Series 200 pump, Perki-Elmer 785A UV/VIS detector and Perk-Elmer Series 200 autosampler. Separations were accomplished on LiChroCART 250-4 C18 HPLC-cartridge (5 μm; Merck, Whitehouse Station, NJ, USA). The separation conditions of HPLC analysis of examined compounds are described in Table I.

Cell culture and treatment. The murine macrophage RAW 264.7 cells were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were incubated with CIL extract as indicated for 1 h and then stimulated with 1 μg/ml lipopolysaccride (LPS) (*Escherichia coli* 011:B4, Sigma Chemical Co., St. Louis, MO, USA) for 24 h.

Cell viability assay. Cell viability of RAW 264.7 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described elsewhere (25,26). Briefly, $1x10^4$ RAW 264.7 cells/well were seeded in 96-well plates and incubated with various concentrations of CIL extract (0-20 μg/ml) at 37˚C for 24 h and medium was completely removed. MTT was added to the cells, followed by incubation for 4 h at 37˚C. After incubation, the medium was discarded and the formazan crystals in viable cells were dissolved in 100 μl of fresh DMSO for 10 min. The absorbance was calculated at 590 nm using a microplate autoreader (Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was calculated by comparing the absorbance of the treated group to the LPS stimulated control group. All experiments were performed in triplicate.

Measurement of nitrite production. Nitric oxide (NO) production was determined by measurement of the accumulation of nitrite, the stable metabolite of NO in the culture medium. Nitrite was assayed colorimetrically after reaction with the Griess reagent as described previously (27). Briefly, $2x10^5$ cells per well were seeded onto 96-well plates and then treated with CIL extract (0, 1, 2.5, 5, and 10 μg/ml) at 37˚C for 1 h before stimulation with 1 μg/ml of LPS for 24 h in a final volume of 0.2 ml. The supernatant of LPS-induced RAW 264.7 cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 1 N hydrochloric acid) in a 96-well plate. Nitrite concentrations were calculated by comparison with OD550 of standard solutions of sodium nitrite in culture medium. All determinations were performed in triplicate.

Quantification of mRNA and miRNA expression level by quantitative real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA (1 μg) was heated at 70˚C for 10 min and reversely transcribed using reverse transcriptase 200 U (Promega, Madison, WI, USA). The mixture was then incubated at 37˚C for 60 min, heated at 95˚C for 10 min and stored at -20˚C until use. Real-time PCR was performed 40 cycles with primers for iNOS and β-actin as an internal control using the ABI PRISM 7300 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Cycles consisted of 30 sec of denaturation at 95˚C, 30 sec of annealing at 60˚C, 1 min of extension at 72˚C, followed by 10 min of elongation at 72˚C. Data were collected by the Sequence Detection Software (SDS; Version 1.3.1, Applied Biosystems) and analyzed using the threshold cycle relative quantification method. Primers were designed with computer assistance according to the gene bank. The sequence of the primers are as follows; iNOS sense primer 5'-CAT CAA CCA GTA TTA TGG CTC CT-3', and iNOS antisense 5'-TCC TGT TGT TTC TAT TTC CTT TGT T -3'; β-actin sense 5'-CTA AGG CCA ACC GTG AAA AG-3', and β-actin antisense 5'-ACC AGA GGC ATA CAG GGA CA-3'. The cycle threshold (Ct) values were determined in at least three independent experiments for each sample. Results were normalized to the endogenous gene β-actin.

MicroRNA quantification. TaqMan miRNA assays (Applied Biosystems) were used to quantify mature miRNA of miR-146a, miR-155 and miR-424. RNU6B was used as a reference gene control. Quantitative assays were performed in the

Table I. HPLC separation conditions for identifying marked components within CIL extract.[a]

Compounds	Mobile phase	Wavelength (nm)	RT (min)	Contents (mg/g of CIL)
Amentoflavone	MeOH:H_3PO_4 (0.11%, pH 2.2) = 25:75	330	10.39	84.72±1.46
Oleanolic acid	ACN:H_3PO_4 (0.11%, pH 2.2) = 28:72	215	10.15	1.05±0.01

[a]ACN, acetonitrile; RT, retention time. All samples were loaded at a total volume of 10 μl into the HPLC cartridge; a flow rate of 1.0 ml/min was used to perform HPLC analysis.

7900HT system according to the manufacturer's instructions (Applied Biosystems). Briefly, 10 ng of RNA samples extracted either normal parts or tumor of same oral patient were mixed and reacted with reverse transcription master mix reagents (Applied Biosystems) at 16˚C for 30 min, 42˚C for 30 min, and 85˚C for 5 min. Each of PCR cycle contained the denaturation step at 95˚C for 15 sec and the extension step at 60˚C for 1 min for a total of 40 cycles. miRNA expression values were normalized using RNU6B following the $2^{-\Delta\Delta Ct}$ method and analyzed by using ABI 7900HT SDS 2.2 software. All reactions were run in triplicate.

Transient transfection. Transfection followed the manufacturer's protocol (Thermo Scientific Open Biosystems, Huntsville, AL, USA). Briefly, $3x10^5$ RAW 264.7 cells/well were incubated in 6-well plates overnight. Medium was changed into the DMEM without 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin before transfection. Solution A (2 μg of DNA and 50 μl of DMEM) was mixed with solution B (10 μl of Arrest-In transfection reagent and 50 μl of DMEM) at room temperature for 20 min. Transfection reagents were equally added into cells. Cells were harvested after 48-h transfection.

Luciferase reporter gene assays. pCOX-2-LUC plasmid was used to quantify COX-2 promoter activity. pRL-CMV, a renilla luciferase reporter plasmid under the control of the cytomegavirus promoter (Promega), was used as an internal control to normalize the reporter gene activity. Plasmids were transfected into RAW264.7 cells. After 24-h transfection, cells were treated with CIL extract for 1 h followed by stimulation with LPS and analyzed the luciferase activity after 48-h transfection. The luciferase activity was determined by a luminometer using a dual-luciferase reporter assay system (Promega) according to the instructions of the manufacturer. Briefly, cells were lysed with 1X PLB (passive lysis buffer) for 15 min. PLB lysate (20 μl) was added into a 96-well plate and mixed with 80 μl of LAR II (luciferase assay substrate in luciferase assay buffer II) to measure luciferase activity by using the SpectraMax L spectrometer (Molecular Devices, Sunnyvale, CA, USA). The values were normalized with the measurement of renilla luciferase activity.

Preparation of nuclear and cytosolic extract. The nuclear extract was prepared as described previously (28). Briefly, $5x10^5$ RAW 264.7 cells were incubated with or without concentrations of CIL extract for 1 h, and then treated with LPS (100 ng/ml) for 30 min. After LPS treatment for 24 h, cells were harvested, washed with ice-cold PBS, and then centrifuged at 2500 g for 5 min at 4˚C. Cell pellets were added to 100 μl lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P 40, 1 mM dithiothreitol, 0.5 mM PMSF) and vortexed mildly. Samples were incubated for 10 min on ice and centrifuged at 2500 g for 5 min at 4˚C. The supernatant was collected as a cytosolic fraction. Pellets containing crude nuclei were resuspended in 100 μl extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF) and incubated for 30 min on ice, and centrifuged at 15,000 g for 10 min. The supernatant containing nuclear extracts was collected and stored at -80˚C until required.

Western blot analysis. Proteins were separated by SDS-PAGE and transferred onto PVDF (Millipore, Billerica, MA, USA) as described previously (29-31). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing 5% non-fat dry milk and 0.1% Tween-20 in PBS. The membrane was incubated with specific primary antibodies to COX-2 or NF-κB (Abcam, Cambridge, UK) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:7000 dilution, Abcam). For internal controls, the same membranes were incubated with mouse anti-β-actin and anti-PCNA for 1 h followed by incubation with horseradish-peroxidase-linked goat anti-mouse IgG for 1 h. Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). The intensity of the bands was scanned and quantified with Adobe Photoshop software.

Statistical analysis. Data are expressed as mean ± standard deviations (SD) from three different experiments. Statistical analysis was carried out using the Student's t-test. It was considered statistically significant at $^{*}p<0.05$, and $^{**}p<0.01$.

Results and Discussion

Effect of CIL extract on cell proliferation of LPS-induced RAW264.7 cells. After 24-h treatment with CIL extract at the indicated concentrations (0-25 μg/ml) in RAW264.7 cells, CIL extract was found to inhibit RAW264.7 cell proliferation in a dose-dependent manner, and the IC_{50} value of CIL extract for 24 h was 14 μg/ml (Fig. 1). Therefore, sample treatments between 1 and 10 μg/ml were used in the subsequent experiments.

CIL extract inhibits LPS-induced NO production and iNOS gene expression in RAW 264.7 macrophage cells. We investi-

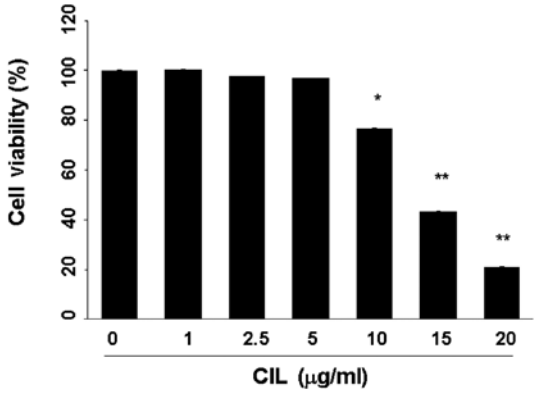

Figure 1. Effects of *C. inophyllum* L. leaf (CIL) extract on the proliferation of the murine macrophage cell line RAW 264.7 cells. Cells were treated with various concentrations of CIL for 24 h, and cell viability was measured by MTT assay. Values are expressed as the mean ± SD. $^{*}p<0.05$; $^{**}p<0.01$ as compared with the LPS-induced cells.

gated the inhibitory effect of CIL extract on LPS-induced NO production in RAW264.7 cells. Cells were preincubated with CIL extract at the indicated concentrations for 1 h and then stimulated with LPS for 23 h. Supernatants were collected for determination of nitrite production. CIL extract inhibited NO production in RAW264.7 cells in a dose-dependent manner as compared to controls, and the anti-inflammatory agent *N*-(3-aminomethyl)-benzylacetamidine (1400W) as the positive control (Fig. 2A). Quantitative RT-PCR showed that the iNOS mRNA expression was almost undetectable in unstimulated cells but were markedly augmented by LPS. Upon LPS stimulation, iNOS expression was induced ~25-fold, and CIL extract blocked iNOS expression which was induced by LPS 7.6-fold in RAW264.7 cells (Fig. 2B). The LPS-induced iNOS expression was inhibited 11.5-fold by the iNOS inhibitor 1400W at 10 μM (Fig. 2B).

CIL extract suppresses mRNA levels of COX-2. QPCR was performed to determine whether the inhibitory effects of the CIL extract on the pro-inflammatory mediators (NO) were related to the modulation of the expression of iNOS and COX-2. After the transient transfection of RAW 264.7 cells with pCOX-2-LUC and pRL-CMV, the expressions of firefly luciferase and Renilla luciferase, respectively, were used to quantify the COX-2 promoter activity. RAW264.7 cells were treated with the various concentrations of CIL extract (0, 1, 2.5, 5 and 10 μg/ml). As shown in Fig. 1, CIL extract did not exhibit cytotoxicity against RAW264.7 cells <2.5 μg/ml. The basal level of COX-2 promoter activity was set in the absence of LPS stimulation. LPS significantly induced up to 203% in the production of COX-2. CIL extract significantly reduced to 128% at 1 μg/ml, and 82% at 2.5 μg/ml in the production of LPS-induced COX-2 promoter activity (Fig. 3).

CIL extract inhibits LPS-induced NF-κB activation. Since p65 and p50 are the major components of NF-κB activated by LPS in the macrophage (32,33), the levels of NF-κB/p65 in the nuclear extract were determined by western blot analysis (Fig. 4A). RAW 264.7 cells were incubated with LPS in the presence or absence of the CIL extract with various concentrations for 24

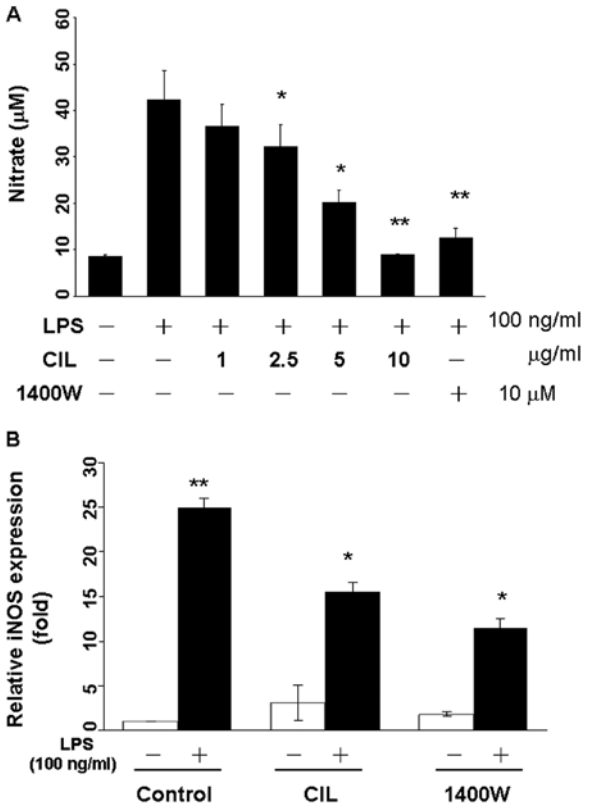

Figure 2. The inhibitory effect of CIL extract on production of nitric oxide (NO) and the expression of iNOS in LPS-induced inflammation. (A) The NO production of CIL extract in RAW 264.7 cells. LPS^{+} indicated cells were induced by LPS (100 ng/ml) for a 24-h treatment. LPS^{-} indicated cells were treated in the absence of LPS. iNOS inhibitor (1400W) at 10 μM was the positive control. $^{*}p<0.05$; $^{**}p<0.01$ as compared with the LPS-induced cells. (B) Real-time RT-PCR analysis of iNOS mRNA expression the LPS-induced cells were determined and data were normalized to β-actin control. iNOS inhibitor (1400W) at 10 μM was the positive control.

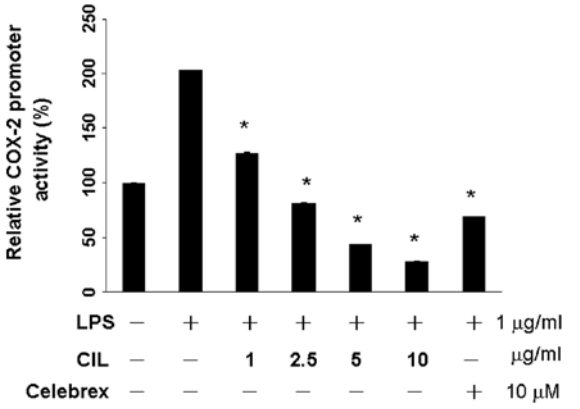

Figure 3. Effect of CIL extract on LPS-induced COX-2 activation. The p COX-2-LUC and the pRL-CMV-LUC were co-transfected into RAW 264.7 cells for 24 h and then cells were treated with DMSO or the indicated concentrations (μg/ml) of CIL extract for 1 h before stimulation with LPS (1 μg/ml) for another 24 h. The firefly and Renilla luciferase activities in the cell lysates were determined. The former activity was normalized to the respective latter activity. Data represent as the mean ± SD from at least three independent experiments. $^{*}p<0.05$ as compared with the LPS-induced cells.

h. The expression of NF-κB/p65 in the nucleus was markedly increased upon exposure to LPS alone 2.3-fold, but the extract or

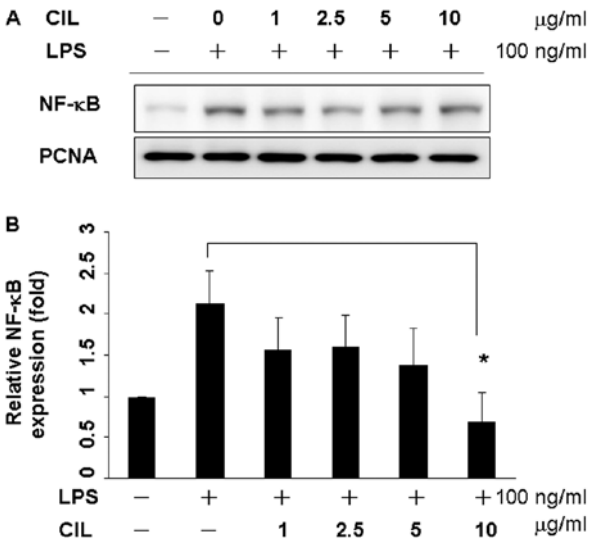

Figure 4. Inhibitory effect of CIL extract on LPS-induced NF-κB activation. (A) RAW 264.7 cells were cultured with the indicated concentrations of CIL extract and 100 ng/ml of LPS. Nuclear extract fraction of cell lysates were analyzed by western blot analysis. PCNA was the internal control. (B) The pNF-κB-LUC and the pRL-CMV-LUC co-transfected cells were treated with DMSO or the indicated concentrations (μg/ml) of CIL extract for 1 h before stimulation with LPS for another 24 h. The firefly and Renilla luciferase activities in the cell lysates were determined. The former activity was normalized to the respective latter activity. Values are expressed as the mean % of three independent experiments. *p<0.05 as compared with the LPS-induced cells.

CIL extract inhibited LPS-mediated nuclear translocation of NF-κB/p65 in a dose-dependent manner (Fig. 4B) (p<0.05), indicating that CIL extract could inhibit the nuclear translocation of NF-κB/p65.

CIL extract attenuates LPS-induced microRNA-146a, 155, and 424 expressions. When RAW264.7 cells were exposed to LPS, expressions of microRNA-146a and miR-155 were up-regulated 6.97- and 77.23-fold, respectively (Fig. 5A and B). After treatment with 10 μg/ml CIL extract for 24 h, the expression of microRNA-146a was reduced to 4.26-fold (Fig. 5A). However, there was no significant change for LPS or CIL extract in the miR-424 expression level (Fig. 5C).

Quality of extraction procedure of CIL extract by HPLC analysis. Two of the marked components of CIL extract, including amentoflavone and oleanolic acid, were identified by HPLC analysis to be indicator compounds for quality check of extraction procedure of each batch (Fig. 6). Using HPLC, the contents of amentoflavone and oleanolic acid were calculated to be 84.72 and 1.05 mg/g of CIL extract, respectively (Table I).

Macrophage activation is important to the progression of multiple diseases through the release of inflammatory mediators. Lipopolysaccharide (LPS)-induced RAW264.7 macrophages are widely used *in vitro*, because LPS is a pathogen that triggers toll-like receptor 4 (TLR4) and activates various inflammatory signals (34). Natural products have played a significant role in drug discovery and development, especially agents against several diseases that have existed from antiquity to the present. Since inflammation is closely linked to the promotion of certain tumors, substances with potent anti-inflammatory activities are

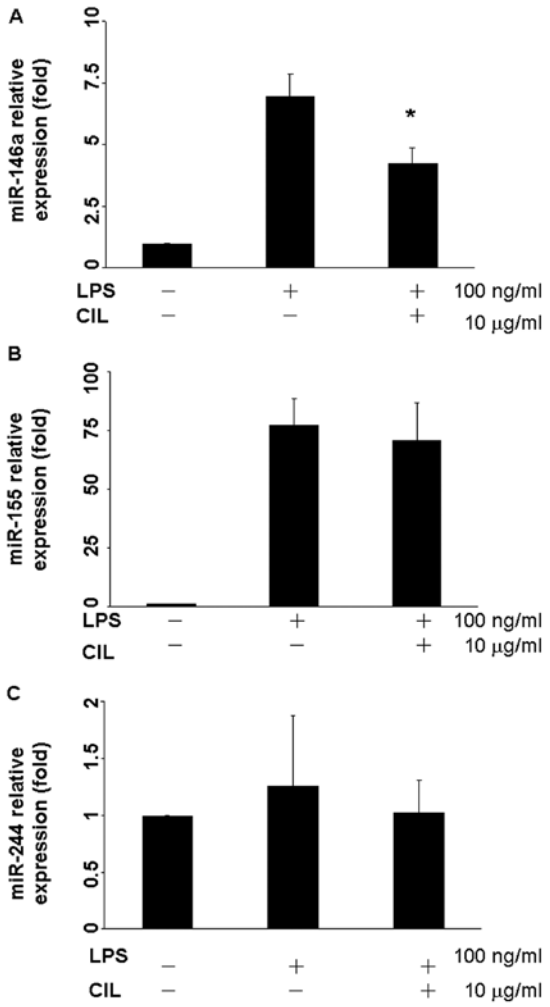

Figure 5. miR-146a, miR-424, and miR-155 expression levels in response to CIL extract in LPS-induced RAW264.7 cells. RAW264.7 cells were stimulated with LPS (100 ng/ml) in the presence (+) or absence (-) of CIL extract for 24 h. Expression of miR-146a (A), microR-155 (B), and miR-424 (C) was assayed by real-time PCR, and data were normalized to U6 control. The fold increase in the expression of these miRNAs versus non-stimulated cells is shown. Data represent the mean ± SD from at least three independent experiments. *p<0.05 as compared with the LPS-induced cells.

anticipated to exert chemopreventive effects on carcinogenesis (35).

In the present study, we prepared acetone extract from CIL extract and examined its effects on the LPS-induced inflammation in a murine macrophage cell line RAW 264.7 model. First, the cytotoxicity of CIL extract in RAW 264.7 cells were evaluated by MTT assay, and it was observed that CIL extract did not affect cell viability <2.5 μg/ml. Many lines of evidence have indicated that NO is a potent proinflammatory mediator and may have a multi-faceted role in mutagenesis and carcinogenesis (35). The massive amounts of NO produced in response to bacterial LPS or cytokines play an important role in the inflammatory condition. Improper activation or upregulation of iNOS or COX-2 has been shown to be associated with the pathophysiologies of certain types of human cancer as well as inflammatory disorders. Therefore, aberrant or excessive expression of iNOS is often implicated in the oncogenesis and pathogenesis of cancer. Indeed, we found that CIL extract demonstrated the inhibition of the NO production in LPS-stimulated RAW 264.7

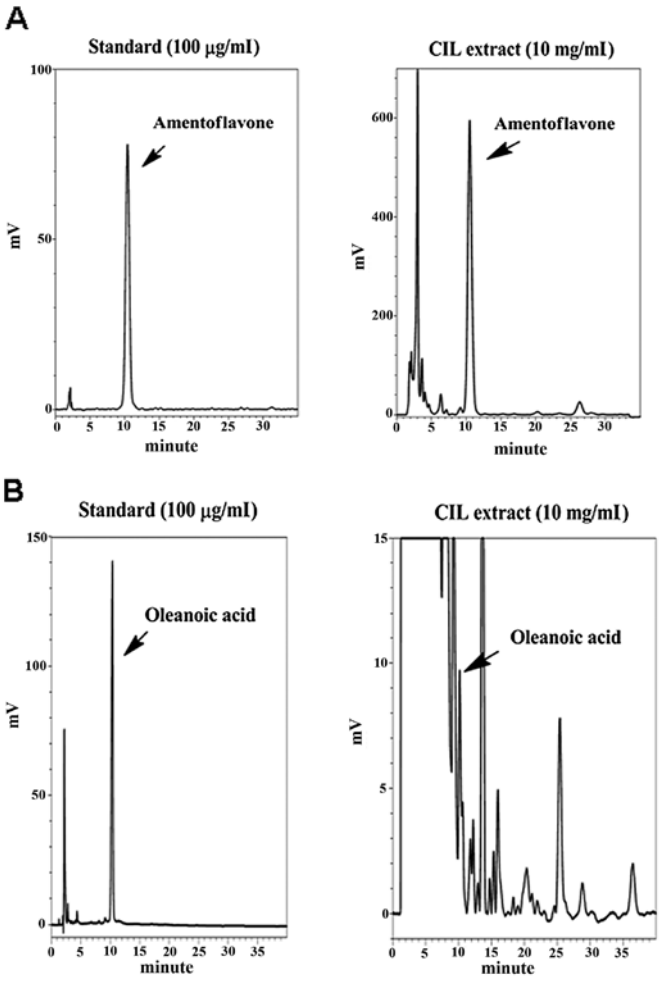

Figure 6. Representative HPLC chromatogram of the marked compounds in CIL extract. (A) HPLC chromatogram of amentoflavone standard (left, 100 μg/ml and CIL extract (right, 10 mg/ml) (UV-spectrum 330 nm), respectively. (B) HPLC chromatogram of oleanolic acid standard (left, 100 μg/ml) and CIL extract acetone extracts (right, 10 mg/ml) (UV-spectrum 215 nm), respectively. The examination conditions and monitoring wavelength of HPLC analysis are described in Table I.

macrophages. The mRNA expression levels of iNOS in LPS-stimulated cells were examined by western blot analyses and quantitative RT-PCR compared with the specific iNOS inhibitor 1400W. Our results suggest that CIL extract blocks the transcription level of iNOS. Moreover, CIL extract at 1 μg/ml also suppressed the LPS-induced COX2 promoter activity in RAW 264.7 cells. Taken together, CIL extract suppressed the production of NO and downregulated the expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells.

NF-κB is a transcription factor that plays a critical role in inflammatory and immune responses. It is present in the cytoplasm, binding to the inhibitory protein IκB in unstimulated cells (36,37). When the cells are exposed to the stimulants such as LPS, IκB is phosphorylated and liberates NF-κB, resulting in NF-κB translocation into the nucleus. Nuclear NF-κB then binds to the promoters of pro-inflammatory mediators, resulting in the induction of their gene expression (38). Here, we have elucidated that CIL extract diminished the LPS-induced NF-κB nuclear translocation in RAW 264.7 cells by western blot analysis. The nuclear translocation and DNA binding of NF-κB is essential for the LPS-mediated NO production and COX2 expression (39). These findings suggest that CIL extract may prevent inflammation by suppressing the NF-κB-mediated inflammatory gene.

Up to date, miRNAs have been demonstrated to be dysregulated in cancer (40) and aberrantly expressed in such inflammatory diseases as rheumatoid arthritis (41). Thus, miRNAs may form a key link between inflammation and cancer; however, the induction of specific miRNAs, including miR-146a and miR-155, as a key step in tumor progression is still unclear. While miR-146a has previously been reported in response to various microbial components and cytokines, much is still not known about its biological significance. In this study, miR-146a expression was induced by LPS stimulation in RAW 264.7 cells. Consistent with previous findings, exposure of THP-1 monocytes to various bacterial inflammatory insults, such as LPS and endotoxin, resulted in rapid and continuous expression of mature miR-146a (42). Our results show that LPS-induced miR-146a expression was inhibited by the CIL extract. Notably, during LPS stimulation, several miRNAs, including miR-146a, miR-155 and miR-424 are upregulated. The biological significance of miR-155 and miR-424 to LPS-induced inflammation has been widely investigated. The new data are focused on the expression of miR-146a, miR-155 and miR-424 in LPS-induced inflammatory responses. CIL extract reduced miR-146a expression, instead of miR-155 and miR-424 expression. This finding highlights the importance of further studies on whether miR-146a and miR-155 can be used as a therapeutic intervention for controlling immune response and defining the role of miR-146a, miR-155 and miR-424 in LPS-induced inflammatory responses.

Furthermore, amentoflavone and oleanolic acid are two major components isolated from CIL extract (20,40,43). Flavonoids are naturally occurring polyphenolic compounds that have many biological properties, including antioxidative, anti-inflammatory and neuroprotective effects (44). Oleanolic acid is a triterpenoid compound that is widely found in vegetables, medicinal herbs, and other plants. It has been shown that oleanolic acid has potent antioxidant and anti-inflammatory effects (45,46).

Collectively, we have demonstrated that CIL extract inhibits LPS-induced NO production and iNOS expression which mediated through the inhibition of NF-κB activation in RAW 264.7 macrophages. CIL extract exerts its potent anti-inflammatory activity by suppressing COX-2 promoter activity and expression. Our results demonstrate the strong anti-inflammatory properties of CIL extract by inhibition of iNOS and COX-2 expression as well as miR-146a expression.

Acknowledgements

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