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 shown that excessive NO production causes inflammation and carcinogenesis (14,15). Natural products have been used in drug discovery and development to regulate inflammation.

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

**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), lipopolysaccharide (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 Perki-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 lipopolysaccharide (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, 1, 2.5, 5, and 10 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 miRNA and mRNA 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'-CTA Agg CCA ACC gTg AAA Ag-3', and β-actin anti-sense 5'-CAT CAA CCA gTA TTA Tgg CTC CT-3', and iNOS anti-sense 5'-TCC TGT TGT TTT TAG TTCT CTT T3' - 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. RNaseB was used as a reference gene control. Quantitative assays were performed in the
was prepared as described previously (28). Briefly, 5x10^5 RAW
Preparation of nuclear and cytosolic extract. The nuclear extract
normalized with the measurement of renilla luciferase activity.
(Molecular Devices, Sunnyvale, CA, USA). The values were
luciferase activity by using the SpectraMax L spectrometer
erase assay substrate in luciferase assay buffer II) to measure
instructions of the manufacturer. Briefly, cells were lysed with
dual-luciferase reporter assay system (Promega) according to the
luciferase activity was determined by a luminometer using a
with CIL extract for 1 h followed by stimulation with LPS and
normalize the reporter gene activity. Plasmids were transfected
virus promoter (Promega), was used as an internal control to
 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 cytomega-
avirus 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 transcription. 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 (lucif-
erase 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 incu-
 bation 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-
dioxogenase-conjugated 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
RAW267.4 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 IC50 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-

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)-benzylacetamide (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 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 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 ± SD from 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 1).

Macrophase 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 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 RAW264.7 model. First, the cytotoxicity of CIL extract in RAW264.7 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 pathophysiology 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 RAW264.7 cells.
The examination conditions and monitoring wavelength of hPLC analysis are described in Table I. The acetone extracts (right, 10 mg/ml) (UV-spectrum 215 nm), respectively. (B) hPLC chromatogram of oleanolic acid standard (left, 100 µg/ml) and CIL translocation in RAW 264.7 cells by western blot analysis. The plasm, binding to the inhibitory protein IκB in inflammatory and immune responses. It is present in the cytoplasm, where it 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 mediates the activation of NF-κB in RAW 264.7 macrophages. CIL extract exerts its potent anti-inflammatory activity by suppressing COX-2 promoter activity and expression. Our results demonstrate strong anti-inflammatory properties of CIL extract by inhibition of iNOS and COX-2 expression as well as miR-146a expression.

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References


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.