Machilus thunbergii extract inhibits inflammatory response in lipopolysaccharide-induced RAW264.7 murine macrophages via suppression of NF-κB and p38 MAPK activation

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Abstract: Machilus thunbergii Sieb. & Zucc. (Lauraceae) is a medicinal plant used to treat edema and pain in China and Taiwan. The anti-inflammatory effects of M. thunbergii were not clear. In this study, we evaluated the anti-inflammatory effects of M. thunbergii extract in LPS-stimulated RAW 264.7 cells. The cells were pretreated with various doses (12.5 µg/mL–100 µg/mL) of M. thunbergii extract and activated with LPS. We found that 25 µg/mL M. thunbergii extract inhibited nitric oxide (NO), prostaglandin E₂ (PGE₂), inducible nitric oxide synthetase (iNOS), and cyclooxygenase-2 (COX-2) expression, as well as levels of proinflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α). Furthermore, M. thunbergii extract also suppressed translocation of nuclear transcription factor kappa-B (NF-κB) subunit p65 proteins into the nucleus and induced a dose-dependent inhibition of the phosphorylation of p38 mitogen-activated protein kinase (MAPK). M. thunbergii extract also increased HO-1 and Nrf2 production in a concentration-dependent manner. Taken together, these results suggest that M. thunbergii extract has an anti-inflammatory effect because it reduces levels of proinflammatory cytokines and mediators via the suppression of NF-κB and p38 MAPK activation in RAW 264.7 cells.

Key words: Cyclooxygenase-2, inducible nitric oxide synthase, Machilus thunbergii, NF-κB, p38 MAPK

1. Introduction

As a part of innate immunity, macrophages recognize the lipopolysaccharide (LPS) of gram-negative bacteria and become activated, inducing signal transduction and expression of pro-inflammatory mediators (Peri et al., 2010). An appropriate inflammatory response enhances the host immune response to microbial invasion; however, an excessive inflammatory response can cause severe tissue injury (Wolff, 2011). Activated macrophages induce an inflammatory response through a series of complex processes (Kobayashi, 2010; Yoon et al., 2010). LPS-induced macrophages secrete proinflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and IL-1β (Guha and Mackman, 2001; Dinarello, 2002); express inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO); and synthesize cyclooxygenase-2 (COX-2) to induce prostaglandin E₂ (PGE₂) production (MacMicking et al., 1997; Wu, 2003). It is possible that inhibiting the macrophage-induced release of these inflammatory substrates could reduce tissue injury during the inflammatory process.

Nuclear transcription factor kappa-B (NF-κB) is a transcription factor that regulates the expressions of many genes (Oecckinghaus et al., 2011). In LPS-induced macrophages, NF-κB subunit p65 is activated and translocated into the nucleus where it binds the promoter of inflammatory-associated genes, leading to transcription of proinflammatory cytokines and mediators (Ruland,
2. Materials and methods

2.1. Machilus thunbergii collection and extraction

The aerial parts of Machilus thunbergii Sieb. & Zucc. (Lauraceae) were collected from Tao-Yuan, Taiwan, in July 2006 and identified by the taxonomist Mr Jun-Chih Ou, who previously worked with the National Research Institute of Chinese Medicine. The M. thunbergii samples (25 kg) were extracted with MeOH (140 L) at 50 °C for 72 h. The MeOH extract was evaporated under vacuum to 1178 g. The yield was 4.7%.

2.2. Cell line and culture medium

The murine macrophage cell line RAW 264.7 was purchased from the Bioresource Collection and Research Center (BCRC, Taiwan). RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen-Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (Biological Industries, Haemek, Israel) and 100 U/mL each of penicillin and streptomycin. The cells were incubated in an atmosphere of 5% CO₂ at 37 °C and were subcultured twice every week.

2.3. MTT assay for cell viability

RAW 264.7 cells (10⁵ cells/mL) were cultured in 96-well plates and treated with different doses of M. thunbergii extract for 24 h, as previously described (Gholami, 2014). The supernatant was removed, and cells were washed with PBS. Then MTT (50 mg/mL; Sigma, St Louis, MO, USA) was added to each well to determine cytotoxicity. Briefly, the plate was incubated for 4 h at 37 °C. The wells were washed with PBS, and isopropanol was added to dissolve the formazone crystal. Finally, the optical density of each well was measured at 570 nm using the microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

2.4. Determination of NO production

Nitrite levels were measured to determine NO production in culture supernatants of RAW 264.7 cells. Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with M. thunbergii for 1 h then incubated with LPS (1 µg/mL) for 24 h. Next 50 µL of supernatant were mixed with 50 µL of Griess reagent (Sigma) for 15 min at room temperature. The optical density of each well was measured at 570 nm using the microplate reader (Multiskan FC, Thermo).

2.5. Measurement of proinflammatory cytokine levels

RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different doses of M. thunbergii for 1 h and then LPS (1 µg/mL) was added for 8 h. Cytokines were measured by ELISA, as previously described (Liou et al., 2010; Nasir et al., 2014). The culture supernatants were measured using ELISA kits specific to IL-6 and TNF-α (R & D Systems, Minneapolis, MN, USA), and there were three independent experiments in each group (n = 9).

2.6. Determination of PGE₂ levels

PGE₂ was measured by ELISA (R & D Systems), as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with M. thunbergii for 1 h and then incubated with LPS (1 µg/mL) for 24 h. Samples were mixed with primary antibody for 1 h at room temperature. Next, PGE₂ conjugate solution was added, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added to assay the levels of PGE₂ by OD₄₅₀ measurements.

2.7. Preparation of total and nuclear protein

For total protein, macrophage cells (5 × 10⁶) were pretreated with different concentrations of M. thunbergii (12.5, 25, 50, or 100 µg/mL) for 1 h in 10-cm culture dishes (Corning, NY, USA). Then 1 µg/mL LPS was added for 24 h. Subsequently, the cells were lysed with protein lysis buffer (50 mM Tris–HCl, pH 8; 150 mM NaCl; 0.5% NP40; 0.1% SDS, 1 mM EDTA) containing a protein inhibitor cocktail (Sigma). To detect protein phosphorylation, cells (5 × 10⁶) were treated with M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.8. Preparation of nuclear protein

For nuclear protein, macrophages were treated with M. thunbergii (50 µg/mL) in the presence of actinomycin D (10 µg/mL) for 1 h, and then LPS (1 µg/mL) was added for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.9. Determination of AP-1 activity

AP-1 activity was determined using a chromogenic assay, as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different concentrations of M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.10. Determination of p38 MAPK activity

p38 MAPK activity was determined using a chromogenic assay, as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different concentrations of M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.11. Determination of NF-κB activity

NF-κB activity was determined using a chromogenic assay, as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different concentrations of M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.12. Determination of iNOS activity

iNOS activity was determined using a chromogenic assay, as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different concentrations of M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.13. Determination of COX-2 activity

COX-2 activity was determined using a chromogenic assay, as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different concentrations of M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.14. Determination of metalloproteinase-9 activity

Metalloproteinase-9 activity was determined using a chromogenic assay, as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with different concentrations of M. thunbergii (12.5 µg/mL–100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.15. Measurement of PGE₂ levels

PGE₂ was measured by ELISA (R & D Systems), as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with M. thunbergii for 1 h and then incubated with LPS (1 µg/mL) for 24 h. Samples were mixed with primary antibody for 1 h at room temperature. Next, PGE₂ conjugate solution was added, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added to assay the levels of PGE₂ by OD₄₅₀ measurements.

2.16. Preparation of total and nuclear protein

For total protein, macrophage cells (5 × 10⁶) were pretreated with different concentrations of M. thunbergii (12.5, 25, 50, or 100 µg/mL) for 1 h in 10-cm culture dishes (Corning, NY, USA). Then 1 µg/mL LPS was added for 24 h. Subsequently, the cells were lysed, and samples were incubated for 2 h at room temperature. Finally, the plate was washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).

2.17. Determination of PGE₂ levels

PGE₂ was measured by ELISA (R & D Systems), as previously described (Liou and Huang, 2011). Briefly, RAW 264.7 cells (2 × 10⁶ cells/mL) were pretreated with M. thunbergii for 1 h and then incubated with LPS (1 µg/mL) for 24 h. Samples were washed, and substrate solution was added. The optical density was measured at 450 nm using the microplate reader (Multiskan FC, Thermo).
µg/mL) and cultured for 24 h at 37 °C in 10-cm culture dishes. Then cells were cultured with 1 µg/mL LPS for 30 min to detect phosphorylated-p38, phosphorylated-ERK 1/2, and phosphorylated-JNK. In addition, nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagent kits (Pierce, Rockford, IL, USA) to detect NF-κB subunit p65. Protein concentrations were determined with the BCA protein assay kit (Pierce).

2.8. Western blot analysis
Protein samples (10 µg) were separated on 10% SDS polyacrylamide gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA), as previously described (Wang et al., 2014). The membranes were incubated with primary antibodies, including COX-2, HO-1, iNOS, p65, PCNA, (Santa Cruz, CA, USA), phospho-ERK 1/2, ERK, phospho-JNK, JNK, phospho-p38, p38, Nrf2 (Millipore), and β-actin (Sigma), overnight at 4 °C. The membranes were washed and incubated with secondary antibodies for 1 h. Finally, the membranes were incubated with solution from the ECL detection kit (Millipore) for 3 min and exposed with the BioSpectrum 600 (UVP, Upland, CA, USA).

2.9. Statistical analysis
Statistical analyses were performed using one-way ANOVA followed by the post-hoc Dunnett’s test. All values were expressed as mean ± SD of at least three independent experiments, and a P value less than 0.05 was considered statistically significant.

3. Results
3.1. Cytotoxicity of M. thunbergii in RAW264.7 macrophages
Prior to other experiments, the cytotoxicity of M. thunbergii was determined by the MTT assay (Figure 1a). Cell viability was not significantly altered by doses of M. thunbergii up to 100 µg/mL. Therefore, cells in all experiments were treated with concentrations of M. thunbergii from 12.5 to 100 µg/mL.

3.2. M. thunbergii inhibited the levels of proinflammatory cytokines in LPS-stimulated RAW264.7 macrophages
To assess the effects of M. thunbergii on proinflammatory cytokine production by LPS-stimulated RAW264.7 macrophages, culture supernatants were harvested and measured by ELISA (Figure 2). Treatment with M. thunbergii dose-dependently inhibited the levels of IL-6 and TNF-α in RAW264.7 cells. M. thunbergii concentrations ≥50 µg/mL inhibited IL-6, while concentrations ≥25 µg/mL suppressed TNF-α secretion. Obviously, the extract of M. thunbergii suppressed levels of proinflammatory cytokines in LPS-activated macrophages.

3.3. Effect of M. thunbergii on LPS-induced NO and PGE2 production
To investigate the effects of M. thunbergii on NO and PGE2 production, RAW264.7 cells were pretreated with various doses of M. thunbergii (12.5, 25, 50, or 100 µg/mL) and exposed to LPS (1 µg/mL) for 24 h; then the levels of nitrite, the stable metabolite of NO, and PGE2 were measured.
LPS-treated cells exhibited increased NO production (approximately 13-fold) compared with unstimulated cells. However, pretreating cells with *M. thunbergii* significantly reduced NO levels in a dose-dependent manner (Figure 3a). *M. thunbergii* treatment also significantly and dose-dependently decreased PGE₂ secretion in RAW264.7 macrophages (Figure 3b).

### 3.4. Effects of *M. thunbergii* on LPS-induced iNOS and COX-2 protein levels

Next, we determined whether the inhibitory effects of *M. thunbergii* on the levels of NO and PGE₂ were related to suppression of iNOS and COX-2 expression (Figures 3c and 3d). The protein levels of iNOS and COX-2 were significantly increased by LPS, and pretreatment with *M. thunbergii* significantly suppressed iNOS and COX-2 protein expression in a dose-dependent manner. Thus, inhibition of NO and PGE₂ production by *M. thunbergii* likely resulted from inhibition of iNOS and COX-2, respectively.

### 3.5. *M. thunbergii* affected NF-κB translocation into the nucleus in LPS-stimulated RAW264.7 macrophages

We investigated whether *M. thunbergii* could suppress NF-κB (active subunit p65) translocation from cytosol to nucleus in LPS-stimulated RAW264.7 cells (Figure 4). In unstimulated cells, NF-κB was constant in the cytosol and hardly translocated into the nucleus. However, the nuclear translocation of NF-κB subunit p65 increased in LPS-stimulated RAW264.7 cells. Interestingly, pretreatment with various doses of *M. thunbergii* (25, 50, or 100 µg/mL) significantly suppressed NF-κB p65 nuclear translocation, compared with the LPS group. Hence, *M. thunbergii* displayed a substantial influence on NF-κB p65 translocation into the nucleus.

### 3.6. *M. thunbergii* affected activation of MAPK pathways in LPS-stimulated RAW264.7 macrophages

To determine whether MAPK signaling pathways were suppressed by *M. thunbergii*, RAW264.7 cells were treated with various doses of *M. thunbergii* for 24 h and then were stimulated with LPS for 30 min. Evaluation of the phosphorylation of MAPK-signaling molecules, including ERK1/2, JNK, and p38, by western blot revealed that *M. thunbergii* did not suppress phosphorylation of ERK1/2 and JNK compared with the LPS-stimulated macrophages (Figure 5). However, LPS-stimulated macrophages that were pretreated with *M. thunbergii* exhibited significant and dose-dependent decreases in the phosphorylation levels of p38. These results indicated that only phosphorylation of p38 MAPK is effectively blocked by *M. thunbergii* in LPS-activated macrophages.

### 3.7. Effect of *M. thunbergii* on LPS-induced HO-1 and Nrf2 expression in RAW264.7 cells

Heme oxygenase (HO)-1 has an antioxidant role and anti-inflammatory function in the inflammatory process (An et al., 2012). We found that *M. thunbergii* could modulate HO-1 and Nrf2 expression compared with normal macrophages (Figure 6).

### 4. Discussion

In response to LPS released by gram-negative bacteria, activated macrophages release proinflammatory cytokines and mediators that cause acute or chronic inflammation (Schett, 2008). LPS combines with LPS binding protein to bind CD14, a macrophage receptor, inducing activation of Toll-like receptor-4 (TLR4) (Kawai and Akira, 2007; Peri et al., 2010). This complex induces an intracellular signaling cascade, including activation of myeloid differentiation factor 88 (MyD88) and TNF receptor-activated factor 6 (TRAF6) (Zanoni and Granucci, 2010), which activates the IκB kinase (IKK) complex and MAPK signaling pathways.
The activated IKK complex gives rise to phosphorylation of IκB and NF-κB (subunits p50 and p65) (Schmid and Birbach, 2008). Finally, activated p65 can translocate from cytosol to nucleus, where it binds the promoter to activate transcription of proinflammatory-associated genes. The MAPK signaling pathway also modulates activation of NF-κB (Huang et al., 2010). Therefore, suppression of the translocation of NF-κB p65 or of MAPK activation may be useful for modifying the production of proinflammatory cytokines and mediators in LPS-activated macrophages.

We found that *M. thunbergii* extract suppressed the levels of IL-6, TNF-α, NO, and PGE₂ in LPS-activated RAW 264.7 murine macrophages. In addition, production of iNOS and COX-2 proteins was inhibited, decreasing production of proinflammatory mediators via suppression of activated NF-κB and the p38 phosphorylation pathway. Therefore, *M. thunbergii* extract has good ability to inhibit inflammatory response.

Activated macrophages induce iNOS proteins to produce NO (MacMicking et al., 1997). The extra NO helps activated macrophages to kill microorganisms; therefore, iNOS expression is closely related to protection against pathogen invasion. However, overproduction of NO can cause severe tissue or cell injury in acute and chronic diseases (Kobayashi, 2010). Additionally, COX-2 expression is linked to production of PGE₂, a
Figure 4. Inhibitory effect of *M. thunbergii* on nuclear translocation of NF-κB in RAW 264.7 cells. Cells were pretreated with different doses of *M. thunbergii* (12.5–100 µg/mL) for 1 h, and then LPS (1 µg/mL) was added for 1 h. PCNA in the nucleus and β-actin in cytosol were used as internal controls. The densitometry values of three independent experiments were analyzed and expressed as mean ± SD.

* P < 0.05, ** P < 0.01; compared to the LPS-treated group.

Figure 5. Effect of *M. thunbergii* on LPS-induced phosphorylation of MAPK. The cells were stimulated with or without LPS (1 µg/mL) for 30 min. Protein samples were analyzed by western blot with phospho-specific antibodies. The total MAPK levels were used as internal controls. The densitometry values of three independent experiments were analyzed and expressed as mean ± SD.

* P < 0.05, ** P < 0.01; compared to the LPS-treated group.
highly inflammatory substance that can accumulate and cause swelling, increased pain (Jiao et al., 2009), and tissue or cell damage. In the present study, M. thunbergii significantly decreased overproduction of PGE_2 and NO in LPS-activated murine macrophages. We suggest that M. thunbergii extract suppressed production of these proinflammatory molecules via suppression of iNOS and COX-2 expression.

Proinflammatory cytokines IL-6 and TNF-α are secreted by activated macrophages (Zanoni and Granucci, 2010). IL-6 induces the synthesis of C-response proteins to enhance the inflammatory response (Valledor et al., 2010). Low levels of TNF-α can injure tissue and enhance fibroblast growth, and high levels in conjunction with sepsis can injure both inflammatory and noninflammatory tissue (Yan and Hansson, 2007). M. thunbergii suppressed the levels of IL-6 and TNF-α, which would decrease the inflammatory response to injured tissues or cells.

Production of proinflammatory cytokines and mediators is closely related to the transcription factor NF-κB (Ruland, 2011), which plays a critical role in inflammation, immune disease, apoptosis, and tumorigenesis (Brasier, 2006). Research has shown that suppression of NF-κB activation decreases the ability of NF-κB to bind promoters of inflammatory-associated genes and thus decreases levels of proinflammatory cytokines and mediators (Bennyeriah and Karin, 2011; Ruland, 2011). We found that M. thunbergii significantly suppressed p65 translocation and decreased expression of IL-6, TNF-α, iNOS, and COX-2, thereby inhibiting p65 translocation in LPS-activated macrophage. HO-1, an antioxidant enzyme, could protect against cell injury in inflammatory response and oxidative stress (An et al., 2012). HO-1 could convert heme to iron, biliverdin, and CO.

Hence, HO-1 is recognized as a key factor in the cellular defense system. Nrf2 is a transcription factor translocated into the nucleus for HO-1 expression. Recent studies found that HO-1 could decrease the production of inflammatory mediators in activated macrophages. In the present study, M. thunbergii promoted the nuclear translocation of Nrf2 and increased HO-1 expression. We thought that M. thunbergii enhanced HO-1 for antioxidant effect and decreased iNOS and COX-2 expressions for anti-inflammatory response.

A previous study found that major MAPKs, including ERK 1/2, JNK, and p38, were important for regulating the expression of proinflammatory cytokines or mediators in the signaling pathways (Cargnello and Roux, 2011). However, M. thunbergii only significantly inhibited phosphorylation of p38 and did not decrease the phosphorylation of ERK 1/2 or JNK. Phosphorylation of p38 can enhance and stabilize the required NF-κB transcription factor-related gene (Cargnello and Roux, 2011; Cuadrado and Nebreda, 2010), as well as TNF-α mRNA, enhancing production of TNF-α (Cargnello and Roux, 2011). We suggest that M. thunbergii not only suppressed p65 translocation into the nucleus, but also decreased phosphorylation of p38 in order to destroy the stability of TNF-α mRNA and decrease the level of TNF-α.

In conclusion, our data suggest that M. thunbergii has anti-inflammatory effects that include inhibiting the production of proinflammatory cytokines and mediators,
as well as suppressing the iNOS/NO and COX-2/PGE₂ pathways by blocking the p38 MAPK and NF-κB pathways. In the future, we will try to isolate specific compounds from *M. thunbergii* to clarify the details of the anti-inflammatory mechanism and the effects of *M. thunbergii*.

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