Bornyl Cinnamate Inhibits Inflammation-Associated Gene Expression in Macrophage Cells through Suppression of Nuclear Factor-κB Signaling Pathway

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Key words
Liquidamber formosana
Altingiaceae
bornyl cinnamate
anti-inflammatory activity
NF-κB

Abstract
Formosan sweetgum (Liquidamber formosana) is an endemic tree species. Various parts of this tree are used as a traditional Chinese medicine for treating pain, inflammation, and rheumatic disorders. In this study, we investigated the anti-inflammatory potential of bornyl cinnamate, a cinnamic acid derivative from the essential oil of L. formosana. Pretreatment with bornyl cinnamate significantly inhibited lipopolysaccharide-induced proinflammatory molecules, including nitric oxide, prostaglandin-E2, tumor necrosis factor α, and interleukin-1β production, in murine macrophage RAW 264.7 cells. RT-PCR and immunoblotting analysis revealed that the inhibition of the proinflammatory molecules occurred through the downregulation of their corresponding mediator genes. Immunofluorescence and luciferase reporter assays revealed that the inhibition of proinflammatory genes by bornyl cinnamate was caused by the suppression of nuclear translocation and transcriptional activation of the redox-sensitive transcription factor nuclear factor κB. In addition, bornyl cinnamate increased the protein stability of the inhibitor of nuclear factor κB, an endogenous repressor of nuclear factor κB, through inhibition of its phosphorylation and proteasomal degradation. Furthermore, bornyl cinnamate significantly blocked the lipopolysaccharide-induced activation of I-κB kinase α, an upstream kinase of the inhibitor of nuclear factor κB α. Taken together, these results suggest that bornyl cinnamate could inhibit proinflammatory molecules through the suppression of the redox-sensitive nuclear factor κB signaling pathway.

Abbreviations
BC: bornyl cinnamate
BSA: bovine serum albumin
COX-2: cyclooxygenase-2
CC: curcumin
DAPI: 4',6-diamidino-2-phenylindole dihydrochloride
FITC: fluorescein isothiocyanate
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
IKK: I-κB kinase
iNOS: inducible nitric oxide synthase
IkB: inhibitor of nuclear factor κ-B
IL-1β: interleukin-1β
LPS: lipopolysaccharide
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB: nuclear factor κB
NO: nitric oxide
PGE2: prostaglandin-E2
TNF-α: tumor necrosis factor α

Introduction
Inflammation is a necessary part of the body’s response to many pathological conditions including tissue injury and microbial invasion. The inflammatory response is tightly regulated, with disruption leading to morbidity and a reduced life span [1, 2]. Macrophages play a functional role in coordinating the immune response to invading pathogens through phagocytosis and cytokine secretion [3]. Activation of macrophages by endotoxins, such as lipopolysaccharides, the major constituents of the outer cell wall of gram-negative bacteria, has been widely used to investigate the mechanism of inflammation in both cell culture and animal models [4]. The activated macrophages subsequently trigger the activation of the redox-sensitive transcription factor NF-κB...
through the inhibition of its endogenous repressor, I-κB. The cascade involves the phosphorylation of two serine residues (Ser32 and Ser36) on I-κBa, which results in its polyubiquitination and subsequent degradation by the 26S proteasome, permitting the unmasking of the nuclear localization signal and the translocation of the activated NF-κB into the nucleus. The translocated NF-κB bind to the DNA binding domain and transcribe a number of proinflammatory genes such as iNOS, COX-2, TNF-α, and IL-1β, which promote the production of proinflammatory molecules NO, PGE2, TNF-α, and IL-1β [5-7]. Overproduction of these molecules elicits many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and hepatitis [8]. Therefore, inhibition of these proinflammatory molecules represents an ideal target for minimizing the burden of inflammatory diseases.

Clinically used anti-inflammatory drugs exhibit several side effects on humans and a high cost of treatment, as in the case of biologics. Plant extracts and derived compounds offer promising options for the development of drugs for treating inflammatory diseases [9]. *Liquidambar formosana* Hance (Altingiaceae) is an endemic deciduous tree native to Taiwan. *L. formosana* also produces a balsamic exudate named resina liquidambaris, which has numerous medical applications in Asian folk medicine, such as a promoter of blood circulation, alleviator of blood stasis, analgesic, anti-inflammatory, and wound-healing agent [10,11]. A recent report shows that essential oil from the leaves of *L. formosana* inhibits LPS-induced inflammation in murine macrophage cells [12]. However, the active ingredients of the exudates involved in the protective effects of *L. formosana* were poorly understood. BC (Fig. 1) is one of the major compounds in the balsamic exudate of *L. formosana* [10]. Biological effects of BC have been rarely studied, and the compound may be related to other organic molecules found in plants and microbes that also exhibit anti-inflammatory and antioxidant properties. For example, BC was identified in the rhizomes of the kava kava plant (*Piper methysticum* Forst.), which has been adopted in traditional treatment for its anti-inflammatory and anxiolytic properties [13]. A previous study by Lobitz et al. [14] reported that BC derivatives from *Verbesina turcensis* showed potent anti-inflammatory and antioxidant effects in human polymorphonuclear granulocytes. In the present study, we investigated whether BC could attenuate an inflammatory response in LPS-challenged RAW 264.7 macrophage cells, and if so, how it exerted this effect. Results of this study showed that pretreatment with BC significantly inhibited the LPS-induced production of proinflammatory molecules through the downregulation of their corresponding mediators via suppressing the transcriptional activation of NF-κB in murine macrophage cells.

**Results and Discussion**

Complementary and alternative therapies have been attractive, as accumulating evidence elucidated the efficacy, safety, and functional mechanisms of herbal medicine. *L. formosana* is used in traditional Chinese medicine for treating rheumatoid arthritis, dermatosis, inflammation, and wounds. A previous study shows that essential oil isolated from *L. formosana* inhibits LPS-induced proinflammatory genes in murine macrophage cells [12]. BC isolated from the essential oil of *L. formosana* also showed potent antifungal activity against *Lenzites betulina* [10]. In this study, we found that BC treatment inhibits LPS-induced proinflammatory molecules through the suppression of the NF-κB signaling pathway. Therefore, we believe that BC could be responsible for the anti-inflammatory effect of the essential oil of *L. formosana*. Natural products and phytocompounds have a fever-related cytotoxic effect on immune cells. Thus, prior to in vitro anti-inflammatory assessment, cytotoxicity of BC to the macrophages was examined. RAW 264.7 cells were incubated with increasing concentrations (2.5–100 µg/mL) of BC for 4 h. The cell viability was determined by the MTT colorimetric assay as described in Materials and Methods. Data are reported as mean ± SD of three independent experiments; * p < 0.05, ** p < 0.01, and *** p < 0.001 show significant differences between the control and sample-treated groups. (Color figure available online only.)

![Fig. 1 Structure of bornyl cinnamate.](https://example.com/fig1.png)

**Fig. 2** Effect of bornyl cinnamate on macrophage cell viability. A RAW 264.7 cells were incubated with increasing concentrations (2.5–100 µg/mL) of BC for 24 h. B Cells were pretreated with BC (5–20 µg/mL) or curcumin (CC, 10 µg/mL) for 2 h, and then stimulated with LPS (1 µg/mL) for 24 h. The cell viability was determined by the MTT colorimetric assay as described in Materials and Methods. Data are reported as mean ± SD of three independent experiments; * p < 0.05, ** p < 0.01, and *** p < 0.001 show significant differences between the control and sample-treated groups. (Color figure available online only.)
Macrophages have long been considered to be important immune effector cells and the most sensitive targets of bacterial endotoxin LPS [15]. Upon stimulation of macrophages with LPS, bioactive products such as proinflammatory cytokines, chemokines, and adhesion molecules are released [16], NO, a prominent proinflammatory molecule, acts as an intracellular messenger, which regulates cellular functions such as vasodilation and elimination of pathogens and tumor cells [17]. However, the overproduction of NO is associated with several pathological processes including inflammation [17]. Induction of RAW 264.7 cells with LPS caused synthesis and secretion of NO into the culture media, which can be measured by a stable nonvolatile breakdown product, nitrite [18]. In this study, we found that untreated control cells released trace amount of NO (9.6 µM). Upon stimulation with LPS, NO production was markedly increased to 35.8 µM, whereas pretreatment with BC significantly decreased LPS-induced NO production to 34.2, 23.5, and 15.8 µM by 5, 10, and 20 µg/mL BC, respectively. A similar inhibitory effect was also observed in curcumin, a known anti-inflammatory agent in treated cells. Since PGE2 is one of the stable prostanoids secreted by activated macrophages [19], we examined the PGE2 level in culture media. Cell incubation with LPS for 24 h markedly increased PGE2 production from 53.8 pg/mL to 582 pg/mL. On the other hand, PGE2 production by LPS was significantly diminished by BC. Twenty µg/mL of BC inhibited nearly 50% of PGE2 (249.5 pg/mL) production in RAW 264.7 cells.

It has been reported that activated macrophages secrete vast amounts of TNF-α and interleukins including IL-1β, IL-6, IL-12, and IL-18 [20]. Indeed, BC treatment significantly as well as dose-dependently inhibited LPS-induced TNF-α and IL-1β production in RAW 264.7 cells. As shown in Fig. 3C, cells incubated with LPS showed a remarkable increase of TNF-α from 66.8 pg/mL to 1788 pg/mL, whereas BC pretreatment significantly as well as dose-dependently decreased the elevated TNF-α. Furthermore, BC treatment dose-dependently inhibited the LPS-induced IL-1β production in RAW 264.7 cells. A similar inhibitory effect of curcumin was also noted in LPS-treated cells.

Fig. 3 Effect of bornyl cinnamate on lipopolysaccharide-induced nitric oxide, prostaglandin-E2, tumor necrosis factor α, and interleukin-1β secretion in macrophage cells. RAW 264.7 cells were preincubated with BC (5–20 µg/mL) or curcumin (CC, 10 µg/mL) for 2 h and then stimulated with LPS for 24 h. A The nitrite concentration in the culture media was determined by the Griess reagent assay. B–D PGE2, TNF-α, and IL-1β levels in the culture media were measured by commercially available assay kits as described in Materials and Methods. Data are reported as mean ± SD of three independent experiments. *P < 0.05, **p < 0.01, and ***p < 0.001 show significant differences between the control and LPS-only treated groups. (Color figure available online only.)
Since BC was found to inhibit the LPS-induced elevation of NO, PGE₂, TNF-α, and IL-1β, we hypothesized that the inhibition may be associated with the downregulation of their corresponding mediator genes iNOS, COX-2, TNF-α, and IL-1β, respectively. As we expected, pretreatment with BC significantly inhibited the LPS-induced protein expression levels of iNOS, COX-2, TNF-α, and IL-1β in a dose-dependent manner (Fig. 4A). To further confirm this effect at the transcriptional level, RT-PCR analysis was performed. As shown in Fig. 4B–E, LPS-induced increases in mRNA levels of iNOS, COX-2, TNF-α, and IL-1β were significantly as well as dose-dependently inhibited by BC. A similar effect was also observed in curcumin-treated cells.

NF-κB, a redox-sensitive transcription factor, can be activated in macrophage cells stimulated with endotoxin LPS or other inflammatory agents that trigger the transcriptional activation of the responsive genes iNOS, COX-2, TNF-α, and IL-1β [21]. Thus, we examined whether BC treatment affects LPS-induced NF-κB transcriptional activity in RAW 264.7 cells by luciferase reporter gene analysis. LPS treatment caused a 6.2-fold increase in the NF-κB reporter activity, whereas pretreatment of cells with 5, 10, and 20 µg/mL of BC dose-dependently reduced the LPS-induced increase in NF-κB reporter activity 5-fold, 3.6-fold, and 2.7-fold, respectively (Fig. 5A).

The process of transcriptional activation of NF-κB is mediated through the nuclear translocation of the p65/p50 complex, which is regulated by phosphorylation and degradation of its repressor IκB [21]. Therefore, the nuclear translocation of p65, an active subunit, was examined by immunofluorescence analysis. In control cells, NF-κB proteins were localized in the cytoplasm, while upon LPS stimulation, NF-κB proteins were largely accumulated in the nucleus. However, BC pretreatment significantly blocked LPS-induced nuclear translocation of NF-κB in a dose-dependent manner (Fig. 5B). Interestingly, BC treatment also significantly reduced the cytosolic NF-κB level in macrophage cells. In addition, Western blot analysis using specific antibodies that can detect IκBα phosphorylation at Ser32/36 residues showed that a dramatic increase in phosphorylation of IκBα was found after LPS treatment, whereas pretreatment with BC significantly blocked LPS-induced IκBα phosphorylation, which is directly proportional to the significant increase of cytosolic IκBα by BC (Fig. 5C). Furthermore, BC treatment significantly inhibits LPS-induced phosphorylation of IKKα in a dose-dependent manner (Fig. 5C). However, the total IKKα level was not affected by either LPS or BC.
In conclusion, this study demonstrates that bornyl cinnamate inhibited the production of proinflammatory molecules NO, PGE2, TNF-α, and IL-1β via the downregulation of their corresponding genes in LPS-induced RAW 264.7 macrophage cells. This inhibitory action resulted from the suppression of NF-κB transcriptional activation, since NF-κB is one of the critical transcription factors regulating the transcription of many inflammation-associated genes. Thus, inhibition of NF-κB by bornyl cinnamate would be a possible therapeutic approach to the treatment of inflammation-associated diseases.

Materials and Methods

Chemicals and reagents

Bornyl cinnamate was prepared according to the protocol described previously [10]. The purity was above 99%, as confirmed by HPLC and 1H-NMR analysis. DMEM, FBS, glutamine, and penicillin/streptomycin were obtained from Gibco® Life Technologies, Inc. LPS, MTT, BSA, curcumin (purity: 96%), and Griess reagent were purchased from Sigma-Aldrich. DAPI was obtained from Calbiochem. Antibodies against iNOS and COX-2 were purchased.
from Cayman Chemical. Antibodies against IKKα, phos-IKKα, IκBα, phos-IκBα, and NF-κB were obtained from Cell Signaling Technology, Inc. All other chemicals and solvents used in this study were of reagent grade or HPLC grade and supplied by either Merck or Sigma-Aldrich.

Cell culture and cell viability assay
RAW 264.7 murine macrophage cells were obtained from ATCC and cultured at 37°C in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 4 mM glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL) in an incubator with a humidified atmosphere of 5% CO2 as recommended by ATCC. Cell viability was determined by the MTT colorimetric assay as described previously [22]. RAW 264.7 cells (2 × 10^5 cells/well) were seeded in a 96-well culture plate. Cells were incubated with various concentrations of BC (2.5–100 µg/mL) for 24 h. For other samples, cells were pretreated with BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then incubated with LPS (1 µg/mL) for 24 h. Cells were pretreated with various concentrations of BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then stimulated by LPS (1 µg/mL) for 1 to 24 h. After incubation, the culture medium was removed and stored at −80°C, which can be used for determining intercellular NO. The medium-deprived cells were incubated with MTT (10 µg/mL) in 100 µL of fresh DMEM for 1 h at 37°C. The MTT-generated violet formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm (A(570)) using an ELISA microplate reader (µQuant, Bio-Tek Instruments, Inc.). Cell viability (%) was calculated as: (A(570) of treated cells/A(570) of untreated cells) × 100.

Determination of nitric oxide, prostaglandin-E2, tumor necrosis factor α, and interleukin-1β levels in culture media
Accumulation of intercellular NO was determined by the Greiss reaction assay as described previously [22]. Cells supernatant derived from the cell viability assay was mixed with an equal volume of Griess reagent and incubated for 30 min at room temperature. The intercellular level of NO was determined by the Griess reaction assay as described previously [22]. RAW 264.7 cells were cultured at a density of 1 × 10^4 cells/well in a 12-well plate. The culture supernatant was determined by the Greiss reaction assay as described previously [22]. Culture supernatant was mixed with an equal volume of Griess reagent (R&D Systems) and incubated with serum-free DMEM that did not contain antibiotics or curcumin (10 µg/mL) for 2 h, after which they were incubated with or without LPS for 2 h. The relative fluorescence intensity was quantified using a luminance ELISA reader at 405 nm (A(405)). The fold increase of luciferase activity was calculated as: (A(405) of treated cells/A(405) of untreated cells) × 100.

Luciferase reporter assay
The NF-κB transcrptional activity was measured using a dual-luciferase reporter assay system (Promega). RAW 264.7 cells were cultured in 24-well plates that had reached 70–80% confluence and incubated with serum-free DMEM that did not contain antibiotics for 5 h. The cells were then transfected with either a pcDNA vector or an NF-κB with β-galactosidase using Lipofectamine 2000 (Invitrogen). Cells were treated with BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h, after which they were incubated with or without LPS for 2 h. The relative fluorescence intensity was quantified using a luminance ELISA reader at 405 nm (A(405)). The fold increase of luciferase activity was calculated as: (A(405) of treated cells/A(405) of untreated cells) × 100. The luciferase activity was normalized to β-galactosidase activity in the cell lysates.

Immunofluorescence staining
RAW 264.7 cells at a density of 1 × 10^4 cells/well were cultured in an 8-well glass Tek chamber and pretreated with BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then incubated with the presence or absence of LPS for 2 h. The cells were then fixed in 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, washed, blocked with 10% FBS in PBS, and then incubated with anti-NF-κB primary antibody in 1.5% FBS for 2 h, followed by incubation with FITC (488 nm)-conjugated secondary antibody for 1 h in 6% BSA. Cells were stained with 1 µg/mL of DAPI for 5 min. The stained cells were washed with PBS and visualized using a confocal microscope at 630× magnification.
Gene Primer Sequence Reference

INOS Forward 5’-TCCTACACACACACACAAAC-3’ [24]
Reverse 5’-TCCTACACACACACACAAAC-3’

COX-2 Forward 5’-CCTCTGCATGCTCTTTCC-3’ [24]
Reverse 5’-TCACTATTTATGGTCAAAATCC-3’

TNF-α Forward 5’-GAGGCTGAGGCTGGCAGAGGAG-3’ [25]
Reverse 5’-GCGGCAAGCAGGAAAGA-3’

IL-1β Forward 5’-CTGCTGCTCAGCAGCCATAT-3’ [26]
Reverse 5’-TGTGGTGCTTGGCTTCTCTTGT-3’

GAPDH Forward 5’-TCAACGCGACAGTCAAGG-3’ [24]
Reverse 5’-ACTCCAGCACATACCTCAGG-3’

Statistical analysis
Statistical analysis was performed using one-way ANOVA followed by Duncan’s test for multiple comparisons and a Student’s t-test for single comparison. The data are reported as mean ± SD. The numbers of independent experiments assessed are given in the figure legends.

Acknowledgements
This study was supported by the Ministry of Science and Technology, Taiwan (NSC-103-2911-I-005-301, NSC-102-2911-I-005-301) and the Ministry of Education, Taiwan under the ATU plan.

Conflict of Interest
The authors declare that there are no conflicts of interest.

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