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

Authors
Kanthasamy Jayabal Senthil Kumar1, Justine Li2, Muthuraj Gokila Vani1, Yu-Hsin Hsieh3, Yueh-Hsiung Kuo4, 5, Sheng-Yang Wang1,6, 7

Affiliations
The affiliations are listed at the end of the article

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
glyceraldehyde-3-phosphate dehydrogenase
IKK: I-κB kinase
iNOS: inducible nitric oxide synthase
Ik-B: 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
PGΕ2: 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-κBα, 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]. Liquidamber 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, antifungal activity against Lenzites betulina [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 turcubensis 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 24 h. The cell viability significantly decreased with increasing concentrations of BC (2.5–100 µg/mL) for 24 h. Consequently, more than 20 µg/mL of BC had a substantial cytotoxic effect, therefore all subsequent experiments were executed with concentrations ranging from 5 to 20 µg/mL of BC. Moreover, incubation of the cells with BC (5–20 µg/mL) and LPS (1 µg/mL) showed a similar pattern of cell survival.

![Fig. 1](structure_of_bornyl_cinnamate.png)

**Fig. 1** Structure of bornyl cinnamate.

![Fig. 2A](cell_viability_a.png)

**Fig. 2A** 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.

![Fig. 2B](cell_viability_b.png)

**Fig. 2B** Effect of bornyl cinnamate on macrophage cell viability.
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 again 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–E 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 LPS-only and BC/curcumin treatment groups. No “*” available. Does this caption maybe belong to Fig. 4?
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 with BC significantly blocked LPS-induced nuclear translocation of NF-κB in a dose-dependent manner (Fig. 5A). 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 nuclear translocation of NF-κB in a dose-dependent manner (Fig. 5B). 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 primary antibody overnight, followed by FITC secondary antibody for 1 h. The cellular DNA was stained with DAPI (1 µg/ml) and images were captured by a confocal microscope (magnification ×200). Arrows indicate NF-κB protein levels in the cytoplasm. C. Cells were preincubated with BC (5–20 µg/ml) or curcumin (10 µg/ml) for 2 h and then treated with or without LPS for 1 h. The phosphorylated and total protein expression levels of IκBα and IKKa were determined by Western blot analysis. The housekeeping protein β-actin served as an internal loading control. Data are reported as mean ± SD of three independent experiments. * p < 0.01 indicates a significant difference between the control and LPS-only treated groups; ** p < 0.001, *** p < 0.001 show significant differences between the LPS-only and BC/curcumin treatment groups. (Color figure available online only.)

Fig. 5 Effect of bornyl cinnamate on lipopolysaccharide-induced transcriptional activation of nuclear factor κB in macrophage cells. A. RAW 264.7 cells were cotransfected with NF-κB harboring luciferase reporter construct. After transfection, cells were preincubated with BC (5–20 µg/ml) or curcumin (CC, 10 µg/ml) for 2 h and then stimulated by LPS for 2 h. Luciferase activity was determined and normalized with β-gal activity. The histogram shows the relative luciferase activity (fold increase). B. The nuclear localization of NF-κB in macrophage cells was determined by immunofluorescence staining. RAW 264.7 cells were seeded in an 8-well Tek chamber and allowed to adhere for 24 h. Then the cells were preincubated with BC (5–20 µg/ml) or curcumin (10 µg/ml) for 2 h and stimulated with LPS (1 µg/ml) for 2 h. After treatment, cells were fixed with paraformaldehyde and then incubated with NF-κB primary antibody overnight, followed by FITC secondary antibody for 1 h. The cellular DNA was stained with DAPI (1 µg/ml) and images were captured by a confocal microscope (magnification ×200). Arrows indicate NF-κB protein levels in the cytoplasm. C. Cells were preincubated with BC (5–20 µg/ml) or curcumin (10 µg/ml) for 2 h and then treated with or without LPS for 1 h. The phosphorylated and total protein expression levels of IκBα and IKKa were determined by Western blot analysis. The housekeeping protein β-actin served as an internal loading control. Data are reported as mean ± SD of three independent experiments. * p < 0.01 indicates a significant difference between the control and LPS-only treated groups; ** p < 0.001, *** p < 0.001 show significant differences between the LPS-only and BC/curcumin treatment groups. (Color figure available online only.)
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 × 10⁵ 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 the medium-deprived cells 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 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 (A₅₇₀) using an ELISA microplate reader (μQuant, Bio-Tek Instruments, Inc.). Cell viability (%) was calculated as: (A₅₇₀ of treated cells/A₅₇₀ 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 Griess reaction assay as described previously [22]. Culture 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 nitrate, a major stable product of NO and measured at 540 nm using an ELISA microplate reader. A standard curve was constructed using known concentrations of sodium nitrate. On the other hand, RAW 264.7 cells at a density of 4 × 10⁵ cells/well in a 12-well plate were pretreated with or without BC (5–20 μg/mL) or curcumin (10 μg/mL) for 2 h and then incubated with or without LPS for 24 h. The PGE₂, TNF-α, and IL-1β concentrations in the culture media were determined using an ELISA kit (R&D Systems) according to the manufacturer’s protocols.

Preparation of protein fraction and Western blot analysis
RAW 264.7 cells (1 × 10⁶ cells/dish) were seeded in a 6-cm cell culture dish and preincubated with or without BC (5–20 μg/mL) or curcumin (10 μg/mL) for 2 h and then stimulated with LPS (1 μg/mL) for 1 to 24 h. The total cell lysates were obtained by mammalian protein extraction reagent (Cayman Chemical). The protein content in each fraction was determined by Bio-Rad protein assay reagent, with BSA as the standard. Equal amounts (50 μg) of denatured protein samples were electrophoresed by 8–15% SDS-PAGE, followed by a transfer onto PVDF membranes overnight. The membranes were blocked with 5% nonfat dry milk for 30 min at room temperature and reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h and developed using the enhanced chemiluminescence substrate (Millipore). Images were captured with a VL Chemi-Smart 3000 gel documentation system (Vilber Lourmat).

RNA extraction and Q-PCR analysis
RAW 264.7 cells (1 × 10⁶ cells/dish) were seeded in a 6-cm cell culture dish and preincubated with or without BC (5–20 μg/mL) or curcumin (10 μg/mL) for 2 h and then stimulated with LPS (1 μg/mL) for 12 h. RNA extraction and quantitative polymerase chain reaction (Q-PCR) were performed as described previously [23]. In brief, total RNA was extracted from cultured macrophage cells using Trizol reagent according to the supplier’s instructions (Invitrogen Corporation). For cDNA preparation, total RNA (5 μg) was incubated at 37°C for 90 min using a first-strand cDNA synthesis kit (Invitrogen Corporation), Q-PCR analysis for iNOS, COX-2, and GAPDH mRNA were performed using the Applied Biosystems detection instrument and software. This system incorporates a gradient thermocycler and a 48-channel optical unit. For the quantitative analysis of mRNA expression, the same system was employed using a DNA binding dye, SYBR Green, for the detection of PCR products. The melting point, optimal conditions, and specificity of the reaction were first determined using a standard procedure. The working stock solution of SYBR Green was 1:100 (Invitrogen Corporation). Quantitative PCR was carried out in a 48-well plate with 10 pmol forward and reverse primers and the working solution SYBR green, using a PCR master mix, under the following conditions: 95°C for 5 min, followed by 40 cycles at 95°C for 1 min, 55°C for 45 s, 72°C for 30 s. GAPDH, a housekeeping gene, was chosen as an internal control. The sequences of the primers are summarized in Table 1. The copy number of each transcript was calculated as the relative number normalized by the GAPDH copy number.

Luciferase reporter assay
The NF-κB transcriptional 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 (A₄₀₅). The fold increase of luciferase activity was calculated as (A₄₀₅ of treated cells/A₄₀₅ 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 × 10⁴ 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% PBS 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.
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.

Affiliations
1 Department of Forestry, National Chung Hsing University, Taichung, Taiwan
2 Department of Neurobiology and Behavior, Department of Psychology, College of Arts and Sciences, Honors College, Stony Brook University, New York, USA
3 Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California, Davis, CA, USA
4 Graduate Institute of Chinese Pharmaceutical Science, China Medical University, Taichung, Taiwan
5 Department of Biotechnology, Asia University, Taichung, Taiwan
6 Agricultural Biotechnology Center, National Chung-Hsing University, Taichung, Taiwan
7 Agricultural Biotechnology Research Institute, Academia Sinica, Taipei, Taiwan

References
3 Gwyer Findlay E, Hussell T. Macrophage-mediated inflammation and disease: a focus on the lung. Mediat Inflamm 2012; 2012: 140937
17 Ricciardolo FL. Multiple roles of nitric oxide in the airways. Thorax 2003; 58: 175–182

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>Forward</td>
<td>[24]</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward</td>
<td>[24]</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>[25]</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>[26]</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA3PDH</td>
<td>Forward</td>
<td>[24]</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Primers used for Q-PCR analysis.