

**Research Article**

# Leaf oil of *Cinnamomum Subavenium* Attenuates Inflammation Via NF- $\kappa$ B/I $\kappa$ B- $\alpha$ Signaling Pathway

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Inflammation is an initial host immune reaction mediated by a series of inflammatory mediators, such as nitric oxide (NO) and prostaglandin E2 (PGE2), which are produced by inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), respectively, and some related cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) [1]. However, excessive or persistent generation of these inflammatory mediators and cytokines may cause diseases including arthritis, diabetes, atherosclerosis and some types of cancer [2]. Thus, properly regulated inflammatory responses are necessary for a healthy immune function.

Nuclear transcription factor kappa-B (NF- $\kappa$ B), an important eukaryotic transcription factor, regulates the expressions of various genes involved in cellular proliferation, inflammatory and immune responses. NF- $\kappa$ B exists mainly as a heterodimer of the Rel family p50 and p65 subunits, which is normally localized in the cytosol as an inactive complex associated with the NF- $\kappa$ B inhibitory protein (I- $\kappa$ B) in unstimulated cells [3]. NF- $\kappa$ B binds to the I $\kappa$ B family in the form of homo- or heterodimers, and the phosphorylated I $\kappa$ Bs are subsequently ubiquitinated and degraded by the proteasome, leaving NF- $\kappa$ B free to translocate to the nucleus [4]. IKK- $\alpha$  and IKK- $\beta$ , the Kappa kinase (IKK), are responsible for phosphorylating I $\kappa$ Bs. Activation of the inhibitor of IKK complex, results in the breakdown of inhibitor of NF- $\kappa$ B (I $\kappa$ B) following inflammatory stimuli through direct phosphorylation of inhibitor of Kappa B $\alpha$  (I $\kappa$ B $\alpha$ ). NF- $\kappa$ B can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF or IL-1, viral infection or expression of certain viral gene products, UV irradiation, B or T cell activation, and by other physiological and nonphysiological stimuli [5].

*Cinnamomum subavenium*. Miq, which belongs to the family Lauraceae, is a medium sized evergreen tree, found in central to

southern mainland China, Burma, Cambodia, Taiwan, Malaysia and Indonesia. Its peel, fruit, and leaves have been used as folk medicine for the treatment of carcinomatous swelling, stomachache, chest pain, abdominal pain, hernia, diarrhea, rheumatism, nausea and vomiting [6]. Our previous study showed that CS-EO has potent anti-inflammatory effects, which was associated with the inhibition of iNOS, COX-2, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  expression an [7]. In this study, we investigated whether anti-inflammatory effects of the CS-EO by though NF- $\kappa$ B/I $\kappa$ B- $\alpha$  signaling pathway.

**Materials and Methods****Chemicals and Materials**

LPS (*Escherichia coli* serotype 0111: B4) was purchased from Sigma-Aldrich Co. (USA). The NF- $\kappa$ B inhibitor BAY 11-7082 was purchased from Beyotime Institute of Biotechnology (China). The p-I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\alpha$ , IKK- $\alpha$ , IKK- $\beta$  monoclonal antibodies were purchased from Cell Signaling Technology (USA).

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## Preparation of CS-EO

500 g of fresh leaves of *C.subavenium* were hydrodistilled for 4 h using a Clevenger-type apparatus. The collected oils were dried over anhydrous sodium sulfate and stored in amber vials at +4°C prior to analysis.

## Cell Culture

RAW 264.7 murine macrophages cell line was obtained from Cell Culture Center of Chinese Academy of Medical Sciences (Beijing, China). RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and were subcultured every 3 days.

## Preparation and Western Blot Analysis

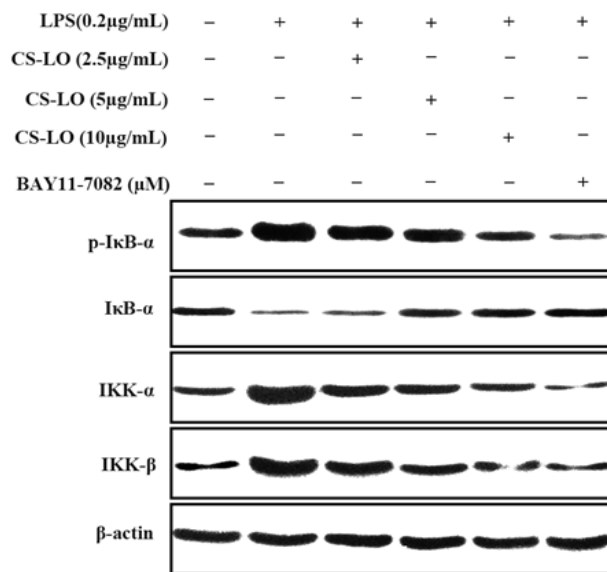
The stimulated RAW 264.7 cells were collected and washed with cold PBS, the cells were lysated in a cold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 10 mM NaF, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 20 mM Tris buffer (pH 7.9), 100 mM b-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)] and kept on ice for 30 min. Cell debris was removed by centrifugation, and supernatants were rapidly frozen. The protein was detected by BCA method (Pierce, USA). proteins from treated and untreated cell extracts were separated SDS-

polyacrylamide gel electrophoresis, and were electro blotted onto a PVDF membrane. The membranes were incubated overnight with locking solution (5% skim milk) at 48°C, followed by incubation for 4 h with a primary antibody. Blots were washed three times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software (Version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

## Result

### Effects of CS-EO on NF-κB Activity in RAW 264.7 Cells

NF-κB transcription factor has been evidenced to play an important role in LPS-induced expression of inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α, IL-1β, and IL-6 [8]. Previous study showed CS-LO had potent anti-inflammatory properties, which was related to inactivation of NF-κB, so now we examined how CS-EO modulated translocation of NF-κB. The effect of CS-EO (2.5, 5, 10, 20, and 40 μg/mL) on the LPS-stimulated degradation of IκB-α in RAW 264.7 cells was analysed by Western blotting with anti-IκB-α antibody. As shows in **Figure 1**, LPS-induced IκB-α degradation was significantly blocked by CS-EO pretreatment. Furthermore, we



**Figure 1.** Effects of CS-EO on LPS-induced degradation of IκB-α, IKK-α, IKK-β and phosphorylation of IκB-α. (RAW 264.7 cells were pre-incubated with indicated concentrations of CS-EO for 2 h and stimulated with LPS (0.2 mg/mL) for 30 min. Total cellular proteins were then prepared and Western blotted for IκB-α, IKK-α, IKK-β and p-IκB-α using specific IκB-α, IKK-α, IKK-β and p-IκB-α antibodies. The β-actin protein was used as internal controls. NF-κB inhibitor of BAY 11-7082 (10 mM) was used as positive control.)

determined whether this I $\kappa$ B- $\alpha$  degradation was related to I $\kappa$ B- $\alpha$  phosphorylation by Western blotting. The result showed that CS-EO also significantly reduced LPS-induced I $\kappa$ B- $\alpha$  phosphorylation (Figure 1). IKK- $\alpha$  and - $\beta$  are upstream kinases of I $\kappa$ B in the NF- $\kappa$ B signal pathway [9], thus, the effects of CS-EO on LPS induced IKK- $\alpha$ , - $\beta$  activation were examined by immunoblotting using IKK- $\alpha$ , - $\beta$  antibodies. The result showed CS-EO inhibited the expression of IKK- $\alpha$  and IKK- $\beta$  (Figure 1). The  $\beta$ -actin protein was used as internal control.

LPS stimulation elicits a cascade leading to the activation of NF- $\kappa$ B. Activated NF- $\kappa$ B regulates the transcription of response gene encoding inflammation associated proinflammatory cytokines and enzymes such as iNOS, COX-2, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 [10].

### Funding Statement

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### Discussion

We used GC/MS to identify 39 compounds in CS-EO, which accounts for 36.82% monoterpenes, 45.52% sesquiterpenes, and 15.06% non-terpenoids of the total, which belong to a mixed type because of lack of a dominant compound [7]. The previous study demonstrated that CS-LO not only inhibited iNOS and COX-2 expression and the subsequent production of NO and PGE<sub>2</sub> but also reduced the expression of IL-1 $\beta$ , IL-6 and the inhibition of CS-LO was related to inactivation of NF- $\kappa$ B [7]. NF- $\kappa$ B dimmers is associated with cytoplasm I $\kappa$ B- $\alpha$  protein [11]. Once I $\kappa$ B- $\alpha$  is phosphorylated and degraded, NF- $\kappa$ B will be activated, and was followed by gene transcription [12]. In present study, the degradation and phosphorylation of I $\kappa$ B- $\alpha$  were inhibited in cells treated with CS-EO, and the I $\kappa$ B kinases (IKK- $\alpha$  and IKK- $\beta$ ) were also inhibited by CS-EO. IKK- $\alpha$  and IKK- $\beta$  (known as the I $\kappa$ B kinases) are responsible for phosphorylating I $\kappa$ Bs [13]. Our results showed IKK- $\alpha$  and IKK- $\beta$  were inhibited by CS-EO. On the basis of these findings, we suggest that the inhibition of IKK- $\alpha$  and IKK- $\beta$  by CS-EO underlies its inhibition of NF- $\kappa$ B activation.

### Conclusion

CS-LO attenuated LPS-induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation via suppressing the degradation of the inhibitor of  $\kappa$ B (I $\kappa$ B)  $\alpha$ , inhibitor of NF- $\kappa$ B kinase (IKK)  $\alpha$ , and IKK- $\beta$  and the phosphorylation of I $\kappa$ B- $\alpha$ . Hence, we considered that inhibition of CS-EO on the production of NO, PGE<sub>2</sub>, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 probably occurred via the NF- $\kappa$ B signaling pathway.

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