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Sesquiterpene Derivatives Isolated from *Cyperus rotundus* L. Inhibit Inflammatory Signaling Mediated by NF-κB

Salman Khan[1], Ran Joo Choi[1], Dong-Ung Lee[2], and Yeong Shik Kim[1,*]

[1]*Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Korea*
[2]*Department of Biotechnology, Dongguk University, Gyeongju, Korea*

Abstract – The immune system is finely balanced by the activities of pro-inflammatory and anti-inflammatory mediators or cytokines. Unregulated activities of these mediators can lead to the development of various inflammatory diseases. A variety of safe and effective anti-inflammatory agents are available with many more drugs under development. Of the natural compounds, the sesquiterpenes (nootkatone, α-cyperone, valencene and β-selinene) isolated from *C. rotundus* L. have received much attention because of their potential anti-inflammatory effects. However, limited studies have been reported regarding the influence of sesquiterpene structure on anti-inflammatory activity. In the present study, the anti-inflammatory potential of four structurally divergent sesquiterpenes was evaluated in lipopolysaccaride (LPS)-stimulated RAW 264.7 cells, murine macrophages. Among the four sesquiterpenes, α-cyperone and nootkatone, showed stronger anti-inflammatory and a potent NF-κB inhibitory effect on LPS-stimulated RAW 264.7 cells. Molecular analysis revealed that various inflammatory enzymes (iNOS and COX-2) were reduced significantly and this correlated with down-regulation of the NF-κB signaling pathway. Additionally, electrophoretic mobility shift assays (EMSA) elucidated that nootkatone and α-cyperone dramatically suppressed LPS-induced NF-κB-DNA binding activity using ^{32}P-labeled NF-κB probe. Hence, our data suggest that α-cyperone and nootkatone are potential therapeutic agents for inflammatory diseases.

Key words – *Cyperus rotundus*, α-Cyperone, Nootkatone, Valencene, β-Selinene, Sesquiterpenes

Introduction

C. rotundus L. belongs to the family Cyperaceae, also known as purple nutsedge. It is a widespread, perennial weed with slender, scaly creeping rhizomes, bulbous at the base and arising singly from the tubers (Lawal and Oyedeji, 2009). *C. rotundus* is a versatile plant, extensively used in traditional medicine around the world (Kilani *et al*., 2008a). A number of pharmacological and biological activities including anti-*Candida*, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antibacterial, antioxidant, cytotoxic as well as apoptotic, anti-pyretic and analgesic activities have been reported for this plant (Duarte *et al*., 2005).

Previous phytochemical investigation on *C. rotundus* revealed the presence of many novel sesquiterpenoids, alkaloids, flavonoids, tannins, starch, glycosides and furochromones (Raut and Gaikwad, 2006). The volatile oil constituents of *C. rotundus* were distinguished quantitatively with high amounts of sesquiterpenes (70%), with a low proportion of oxygenated monoterpenes (10%) and monoterpene compounds (5%) (Kilani *et al*., 2008b). The chemical composition of the volatile oils of *C. rotundus* has been extensively evaluated and four chemotypes (H-, K-, M- O-types) of the essential oils from various parts of Asia have been reported (Morimoto and Komai, 2005)

In all previous reports on the chemical composition of essential oils of *C. rotundus* from around the world, α-cyperone, cyperene, cyperotundone, β-selinene, nootkatone, α-pinene, β-pinene, limonene and valencene were found to be the major constituents identified (Kilani *et al*., 2008b). It is well-known that the composition of essential oils differs remarkably even within the same taxon, and the variations depend on genetic parameters, soil and growing conditions, environmental factors, process of distillation (Sefidkon *et al*., 1999), chemical methods, changing growth or harvest conditions of etheric plants and altering distillation technologies. Therefore, bioactivity of a given essential oil is dependent on its chemical composition (Kilani *et al*., 2008b). Owing to the new

*Author for correspondence
Tel: +82-2-880-2479; E-mail: kims@snu.ac.kr

attraction for natural products like essential oils (sesquiterpenes), despite their wide use as fragrances, it is important to develop a better understanding of their mode of action for new applications in human health, agriculture and the environment. Some of the essential oils constitute significant and effective alternatives to anti-inflammatory and anti-cancer activities (Carson *et al*., 2006). Currently, numerous essential oils have been qualified as natural antioxidants, and proposed as potential substitutes of synthetic antioxidants in specific sectors of food preservation (Vuotto *et al*., 2000).

Various components of the essential oil from *C. rotundus* have been reported to display potent biological activities. Therefore, considering their chemical structures, we investigated the anti-inflammatory activity of four essential oils (sesquiterpenes) isolated from *C. rotundus*. In particular, four structurally divergent sesquiterpenes was evaluated in lipopolysaccaride (LPS)-stimulated RAW 264.7 cells. Among the four sesquiterpenes, α-cyperone and nootkatone exhibited strong anti-inflammatory effects in LPS-stimulated RAW 264.7 cells.

Experimental

Plant Material – Rhizome of *C. rotundus* were purchased at a local market in Seoul, Korea. The plant material was authenticated by Prof. J. H. Lee at Dongguk University. The plant was extracted and the individual compounds (Fig. 1) were isolated according as previously reported (Jin *et al*., 2011).

Cell culture – RAW 264.7 murine macrophages were obtained from American Type Culture Collection (USA). These cells were maintained at sub-confluence in a 95% air and 5% CO_2 humidified atmosphere at 37 °C. The medium used for routine subculture was Dulbecco's modied Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). The RAW 264.7 cells harboring pNF-κB-secretory alkaline phosphatase (SEAP)-NPT reporter construct (Moon *et al*., 2001a) were cultured under the same conditions except that the media was supplemented with 500 μg/mL geneticin.

MTT assay for cell viability – Cytotoxicity of nootkatone, α-cyperone, valencene and β-selinene on RAW 264.7 cells, was carried out by MTT assay. Briefly, RAW 264.7 cells were plated at a density of 1×10^4 per well in a 96-well plate and incubated at 37 °C for 24 h. The cells were treated with various concentrations of nootkatone, α-cyperone, valencene, β-selinene and/or vehicle alone 2 h before LPS (1 μg/mL) stimulation and then incubated at 37 °C for an additional 18 h. After incubation for 18 h, 10 μL of MTT (2 mg/mL in saline) solution was added to each well and incubated under the same conditions for another 2 h. Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in DMSO and the absorbance was measured at 595 nm by using an enzyme-linked immunosorbent assay (Molecular Devices, Emax, USA). Relative cell viability was calculated and compared with the absorbance of the untreated control group. All experiments were performed in triplicate.

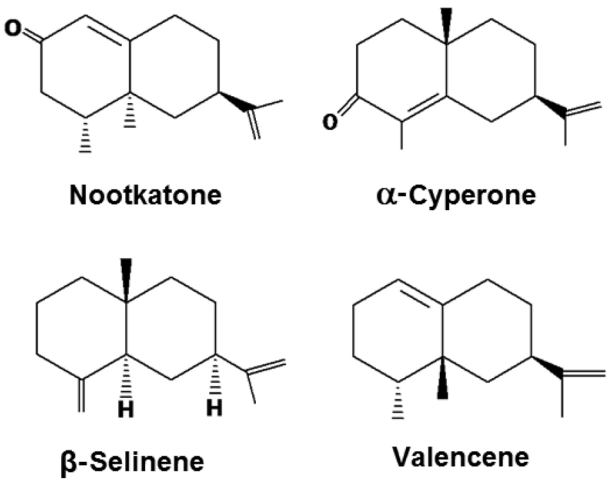

Fig. 1. Chemical structures of four sesquiterpene compounds isolated from *Cyperus rotundus* L.

Nitric oxide determination – The Inhibitory effect on NO production in RAW 264.7 cells was evaluated by measuring nitrite (Griess reaction) in the medium as an indicator of nitric oxide production, as described previously (Khan *et al*., 2011). In brief, 1×10^5 RAW 264.7 cells were plated in 24-well plates, incubated for 24 h and pretreated with different concentrations of nootkatone, α-cyperone, valencene, β-selinene or vehicle for another 2 h, then challenged with LPS (1 μg/mL) for an additional 18 h. Equal volumes of cultured medium and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in distilled water) were mixed, the absorbance at 540 nm was determined with a microplate reader (Molecular Devices, Emax, USA) and the absorption coefficient was calibrated using sodium nitrite solution as a standard. For this experiment, 2-amino-5, 6-dihydro-6-methyl-4H-1, 3-thiazine (AMT) was used as a positive control.

NF-κB secretory alkaline phosphatase (SEAP) reporter gene assay – The NF-κB SEAP inhibitory activities of nootkatone, α-cyperone, valencene and β-

selinene were determined in LPS stimulated RAW 264.7 macrophages. NF-κB-dependent reporter gene transcription was analyzed by a secretory alkaline phosphatase (SEAP) assay, as previously described with some modifications (Noh *et al*., 2006). In brief, 1×10^5 RAW 264.7 macrophages transfected with pNF-κB-SEAP-NPT encoding four copies of the -κB sequence and SEAP gene as a reporter were pre-incubated with different concentrations of nootkatone, α-cyperone, valencene, β-selinene for 2 h and challenged with LPS (1 μg/mL) for an additional 18 h. Aliquots of the cell-free culture medium were heated at 65 °C for 5 min and then added to assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 500 μM 4-methylumbelliferyl phosphate; MUP) in the dark at 37 °C for 1 h. The fluorescence from the products of the SEAP/MUP was measured using a 96-well microplate fluorometer (Molecular Devices, Gemini XS, USA) with excitation/emission at 360/449 nm, respectively. *N-p*-tosyl-L-phenylalanyl chloromethyl ketone (10 μM; TPCK) was used as a positive control.

Western immunoblot analysis – RAW 264.7 macrophages were pretreated with the indicated concentrations of nootkatone, and α-cyperone, or vehicle for 2 h and stimulated with LPS (1 μg/mL) for 18 h (COX-2, and iNOS). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (USA). Ten micrograms of total protein for iNOS, and 5 μg for COX-2 were separated on an 8% SDS-PAGE gel. After electrophoresis, the proteins were electro-transferred to nitrocellulose membranes (Whatman GmbH, Germany), blocked with 5% non-fat milk in TBS-T buffer and, blotted with each primary antibody (1:1000) and its corresponding secondary antibody (1:5000) according to the manufacturer's instructions and detected with WEST-SAVE Up™ luminol-based ECL reagent (LabFrontier, Korea). The target bands were quantified using UN-SCAN-IT™ software (Silk Scientific Corp, USA).

Electrophoretic mobility shift assay (EMSA) – EMSA was performed to investigate the inhibitory effect on NF-κB DNA binding, as previously described (Khan *et al*., 2011). Briefly, nuclear extracts prepared from LPS-treated cells were incubated with ^{32}P-end-labeled 22-mer double-stranded NF-κB consensus oligonucleotide (Promega, USA; sequence: 5'-AGT TGA GGG GAC TTT CCC AGG C-3') for 30 min at room temperature and the DNA protein complexes were separated from the free oligonucleotides on 6% native polyacrylamide gels. The signals obtained from the dried gel were quantitated with an FLA-3000 apparatus (Fuji) using the BAS reader version 3.14 and Aida version 3.22 softwares (Amersham Biosciences, USA). The binding conditions were optimized by Shin *et al*. (Shin *et al*., 2008).

Data analysis – The results, unless otherwise stated, were expressed as means ± standard deviation (S.D.) from three different experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was applied to assess statistical significance of the differences among the study groups (SPSS version 10.0, Chicago, USA). A value of $P < 0.05$ was chosen as the criterion for statistical significance.

Results and Discussion

C. rotundus is used to treat poor appetite, diarrhea, dysentery, fever, parasites, gastritis, indigestion and sluggish liver (Duarte *et al*., 2005). It also helps in the treatment of convulsions, moodiness and depression, premenstrual syndrome (PMS) including associated pain and cramps, menopause and heart palpitations. This herb contains volatile oils, alkaloids, flavonoids and triterpenes among others (Raut and Gaikwad, 2006). To explore the principle mechanism underlying this anti-inflammatory effect, the action of nootkatone and α-cyperone on inflammation related to macrophage function was evaluated. The cytotoxic effect of these four sesquiterpene analogues (nootkatone, α-cyperone, valencene, and β-selinene) in LPS-stimulated RAW 264.7 macrophages was initially measured using a MTT assay (Fig. 2). No cytotoxic effect was observed up to 75 μM and therefore, non-toxic concentrations were used in the following experiments.

Stimulation of macrophages by endotoxin (bacterial LPS) results in the expression of iNOS (Xie and Nathan, 1994). Nitric oxide production plays a key role in the process of macrophages stimulation and is co-related to inflammatory responses. Hence, the suppression of NO production by inhibition of iNOS expression and/or enzyme activity may be a very important therapeutic target in the development of anti-inflammatory potentials. In the present study, we found that nootkatone and α-cyperone inhibit LPS-induced NO production in RAW 264.7 cells. The effect was measured after LPS (1 μg/mL) stimulation by measuring the released nitrite in the culture medium. Cells were treated with various concentrations of nootkatone, α-cyperone, valencene and β-selinene 2 h before LPS stimulation. After 18 h of incubation, RAW 264.7 macrophages in the resting state produced 4.1 ± 19 μM nitrite. When LPS (1 μg/mL) was added, NO production in the culture media was considerably increased to 39.76 ± 0.9 μM. Pre-treatment with nootkatone and α-cyperone dramatically inhibited LPS-induced

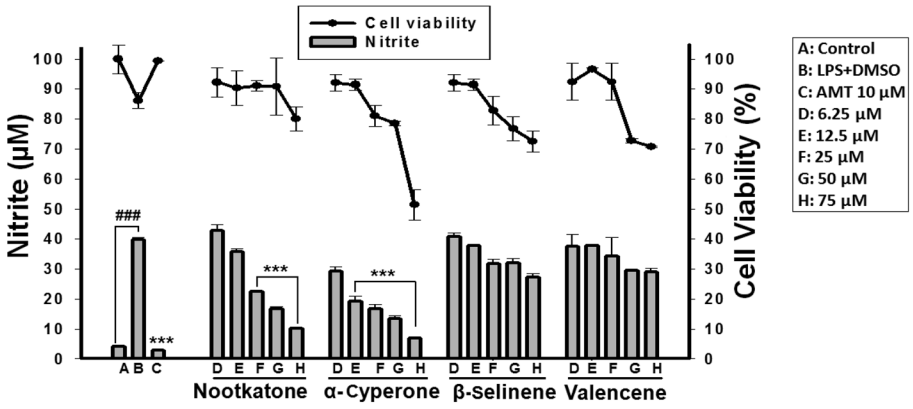

Fig. 2. Effects of nootkatone, α-cyperone, valencene β-selinene and valencene β-selinene on cell viability and NO production in RAW 264.7 macrophages using MTT and Griess reagent assays as described in "Materials and Methods". Cont, vehicle control; LPS, (LPS + DMSO) cells treated with LPS; AMT (10 μM), 2-amino-5, 6-dihydro-6-methyl-4H-1, 3-thiazine, was used as a positive control.

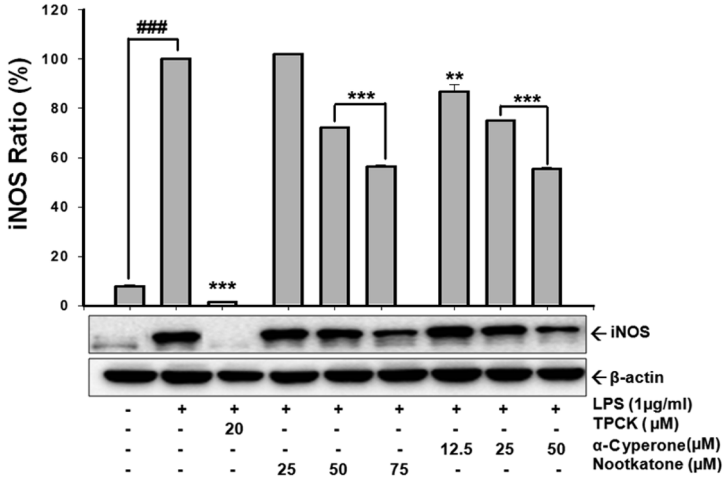

Fig. 3. Inhibition of iNOS protein expression by nootkatone and α-cyperone in LPS-stimulated RAW 264.7 macrophages. Total lysates were subjected to Western immunoblot analysis, as described in "Materials and Methods". Relative ratios (%) of proteins are expressed as the means ± S.D. of three individual experiments. The iNOS signal was normalized to β-actin. *N*-*p*-tosyl-$_L$-phenylalanyl chloromethyl ketone (TPCK, 20 μM) used as a positive control.

Table 1. IC_{50} values of the nootkatone, α-cyperone, β-selinene, and valencene

Compounds	IC_{50} (μM)		
	Cell Viability	Nitric Oxide	NF-κB SEAP
Nootkatone	> 75	30.0	38.8
α-cyperone	> 75	18.2	17.6
β-selinene	> 75	> 75	> 75
Valencene	> 75	> 75	> 75

elevation of nitrite concentration in the medium with IC_{50} values of 30 μM and 18.21 μM, respectively (Table 1, Fig. 2). In comparison, valencene and β-selinene sesquiterpenes did not significantly inhibit NO production. Since the cytotoxicity was not observed in the tested concentrations after treatment of nootkatone and α-cyperone. These data imply that reduction in NO production by nootkatone and α-cyperone was not due to toxic reaction on the cells. AMT (10 μM) was used as a positive control (Fig. 2).

The inhibition of NO by nootkatone and α-cyperone was consistent with inhibition of both LPS-induced iNOS expression as well as iNOS transcriptional activity (Fig. 2). To evaluate whether the inhibitory effect of nootkatone and α-cyperone on NO production occurred by inhibition of corresponding gene, iNOS protein was determined by Western blot analysis. After LPS treatment for 20 h, iNOS expression was significantly increased in RAW 264.7 cells (Fig. 3). However, agreeing with the inhibitory effect on NO generation, LPS-induced iNOS expression was drastically suppressed by nootkatone and α-cyperone, based on densitometric analysis (Fig. 3).

Since NO can induce COX-2 expression, and COX-2 is also an enzyme that regulates inflammation, we therefore examined whether the expression of COX-2 can be also inhibited by nootkatone and α-cyperone. Nootkatone and α-cyperone dramatically inhibited the LPS-induced COX-2 expression at the transcriptional level (Fig. 4).

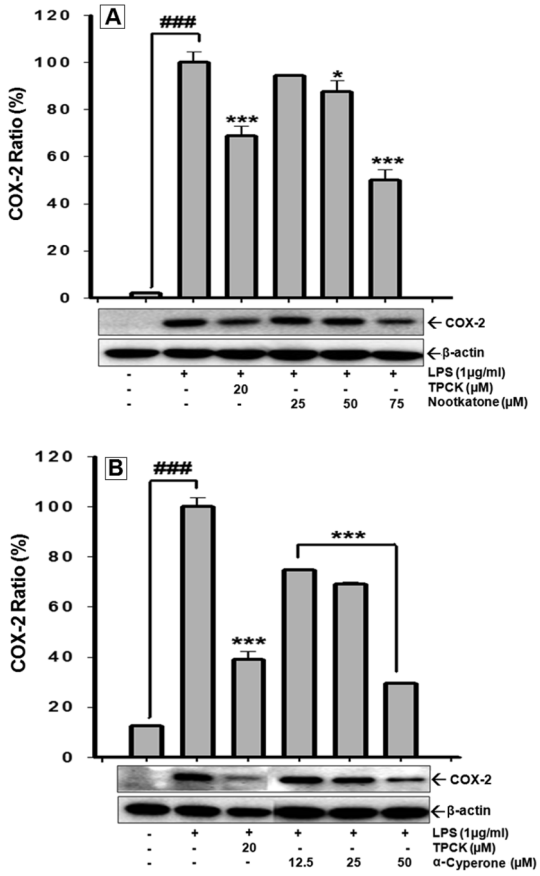

Fig. 4. Inhibition of COX-2 protein expression by nootkatone and α-cyperone in LPS-stimulated RAW 264.7 macrophages. Total lysates were subjected to Western blot analysis, as described in "Materials and Methods". Relative ratios (%) of proteins are expressed as the means ± S.D. of three individual experiments. The COX-2 signal was normalized to β-actin. *N-p*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK, 20 μM) used as a positive control.

NF-κB plays a significant role in LPS-induced expression of proinflammatory proteins including iNOS and COX-2 (Xie and Nathan, 1994). In order to investigate the molecular mechanism of sesquiterpene-mediated inhibition of iNOS and COX-2 transcription, NF-κB transcriptional activity was measured using the reporter gene assay. RAW 264.7 cells were stably transfected with a NF-κB-SEAP-NPT plasmid containing four copies of κB sequence fused to SEAP as the reporter (Moon *et al*., 2001b). LPS treatment of the transfected cells for 18 h increased SEAP expression about 5.4-fold over the basal levels (Fig. 5). Pretreatment with nootkatone and α-cyperone significantly inhibited LPS-induced SEAP expression in a concentration-dependent manner, with maximum inhibition at 75 μM (70%) at 50 μM (94.3 ± 1.3%), respectively. In contrast, valencene and β-selinene exhibited no inhibitory effect (Fig. 5). As a positive control, TPCK had a significant inhibitory effect on NF-κB activation at the transcription level (Fig. 5).

NF-κB, as a transcriptional factor that controls the expression of enzymes including iNOS and COX-2 which contribute to the pathogenesis of the inflammatory process (Nath and Powledge, 1997). To investigate whether nootkatone and α-cyperone can inhibit LPS-induced NF-κB DNA binding activity, the EMSA analysis was performed (Fig. 6). LPS (LPS + DMSO) alone induced strong NF-κB DNA binding activity. Nootkatone and α-cyperone suppressed the NF-κB DNA binding activity in a dose-dependent manner as shown in (Fig. 6). Parthenolide (20 μM), a sesquiterpene lactone, was used as the positive control and exhibited potent

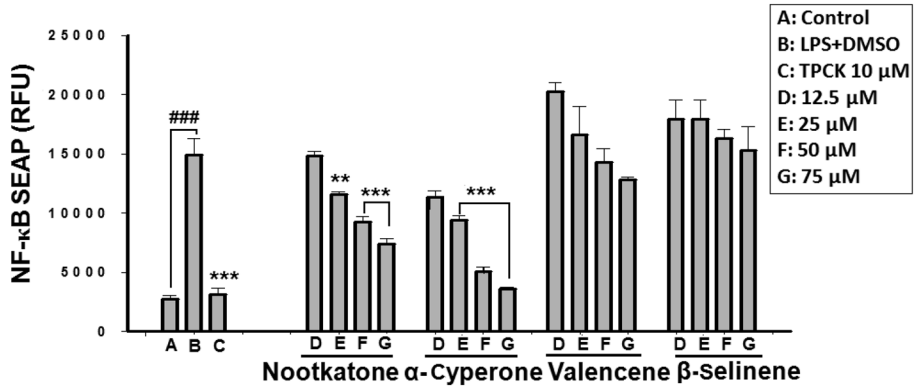

Fig. 5. Effect of nootkatone, α-cyperone, valencene and β-selinene on LPS-induced NF-κB dependent alkaline phosphatase (SEAP) in transfected RAW 264.7 macrophages. Data were derived from three independent experiments and expressed as means ± SD. Cont, vehicle control; LPS (LPS + DMSO), LPS stimulation; TPCK (10 μM), *N-p*-tosyl-L-phenylalanyl chloromethyl ketone, used as a positive control.

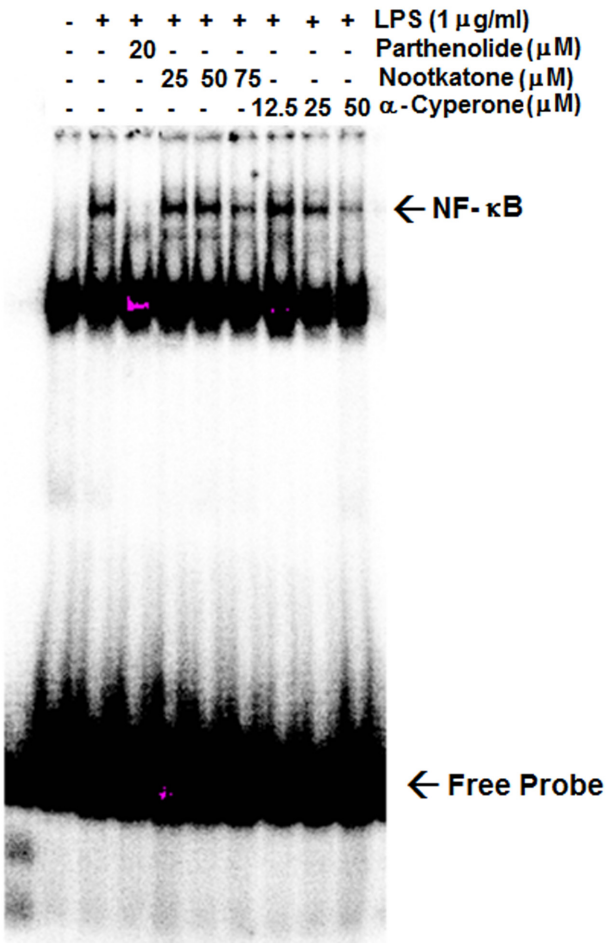

Fig. 6. Electrophoretic mobility shift assay (EMSA) for NF-κB-DNA binding in LPS-stimulated RAW 264.7 cells after nootkatone and α-cyperone treatment. EMSA was carried out as described in "Material and Methods". Parthenolide (20 μM) was used as a positive control.

inhibition of NF-κB- DNA in the nucleus (Fig. 6). These results suggest that the inhibitory effect of nootkatone and α-cyperone on the activation of NF-κB may be important for the inhibition of iNOS and COX-2 expression.

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