

Original Research Article

Aralia elata (Miquel) Seemann Suppresses Inflammatory Responses in Macrophage Cell by Regulation of NF-kappa B Signalling

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Abstract

Purpose: To investigate the antioxidant and anti-inflammatory effects of *Aralia elata* extract (AEE) in lipopolysaccharide (LPS)-stimulated Raw264.7 cells.

Methods: Antioxidant activity was measured using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. Cell viability was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells were stimulated with LPS to study protein expression and production of inflammatory mediators, determined by Western blot analysis.

Results: AEE significantly inhibited DPPH-generated free radicals showing maximum inhibition at 40 µg/mL ($p < 0.001$). AEE alone did not exhibit any signs of cytotoxicity to RAW264.7 cells up to 200 µg/mL concentration. The LPS-induced increase in the production of nitric oxide was concentration-dependently suppressed with half-maximal concentration (IC_{50}) of 91.5 µg/mL of AEE ($p < 0.05$ at 10 µg/mL, $p < 0.01$ at 20 µg/mL and $p < 0.001$ at 40 µg/mL, respectively). AEE also inhibited dose-dependently the LPS-induced increase in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions with IC_{50} of 72.5 µg/mL. Furthermore, the production of pro-inflammatory cytokines, viz, tumor necrosis factor- α by LPS-stimulation in RAW264.7 cells was inhibited dose-dependently with IC_{50} of 34.9 µg/mL by AEE pretreatment. Mechanistic studies revealed that AEE acts by regulation of nuclear factor kappa-B signaling pathway in LPS-stimulated RAW264.7 cells.

Conclusion: This study shows, for the first time, that AEE possesses antioxidant and anti-inflammatory effects and can be developed as a potential therapeutic agent for ameliorating macrophage-mediated inflammation.

Keywords: *Aralia elata*, Anti-inflammatory activity, Macrophage cells, Inducible nitric oxide synthase, nuclear factor kappa-B signaling

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INTRODUCTION

Aralia elata is widely distributed in the northeast of Korea and China. The young shoots are eaten as vegetables for various dishes in Korea. A constituent of *Aralia elata* has been established widely to have effects on diabetes and rheumatism [1]. However, studies on its

beneficial effects on microglia-mediated inflammatory diseases have not been reported. The aim of this study was to investigate whether *Aralia elata* ethanol extract (AEE) exhibits protective effects on LPS-activated inflammatory processes in RAW264.7 cells.

Nitric oxide (NO) is involved in a variety of pathophysiological responses. Therefore NO has been a target of intensive research and drug development. COX-2, normally expressed at low levels, is strongly induced by proinflammatory agents, including LPS, tumor promoters, and growth factors. The COX-2 pathway has also been shown to be involved in the production of matrix metalloproteinase-1 (MMP-1) by LPS-stimulated human primary monocytes [2]. NF- κ B pathway may play an important role in the development of chronic pain following peripheral tissue inflammation. Functionally active NF- κ B exists mainly as a heterodimer consisting of subunits of the Rel family p50 and p65, which are normally sequestered in the cytosol as an inactive complex due to binding with inhibitors of κ B (I κ B s) in unstimulated cells [3]. NF- κ B is translocated to the nucleus, where it binds to κ B binding sites in the promoter regions of target genes and then induces the transcription of pro-inflammatory mediators [4].

The present study is aimed to investigate whether AEE possesses antioxidant and anti-inflammatory activities by regulating NF- κ B signaling in LPS-stimulated murine RAW264.7 macrophage cells.

EXPERIMENTAL

Preparation of *Aralia elata* extracts (AEE)

The dried whole plant of *A. elata* (5 kg) collected in June 2014 and purchased from a local market in South Korea. It was authenticated by Prof Jong-Bo Kim, a taxonomist at Konkuk University, South Korea. A voucher specimen (AE-KU2014) has been kept in our laboratory herbarium. The powdered material was extracted with 10 volumes (v/w) of 70 % ethanol at room temperature for 72 h and filtered. The filtered extract was concentrated in a rotary evaporator (EYELA NVC-2000, Tokyo, Japan) under reduced pressure and lyophilized.

Determination of DPPH radical scavenging activity

The anti-oxidant activity of the AEE was determined using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the AEE and a DPPH methanolic solution as described previously [5]. Briefly, a sample solution of 60 μ l of each AEE, was added to 60 μ l of DPPH (60 μ M) in methanol. After mixing vigorously for 10 s, the mixture was then transferred into a 100 μ l Teflon

capillary tube and the scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer (Jeol Ltd., Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly after 2 min. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 , and temperature, 298 °K.

Cell cultures and viabilities

RAW264.7 microglia cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % FBS (Hyclone, Logan, UT, USA) containing 100 U/mL of penicillin (Invitrogen), and 100 μ g/mL streptomycin (Invitrogen) in 37 °C in humidified atmosphere of 5 % CO₂. In all experiments, cells were pre-treated with the indicated concentrations of AEE for 1 h before the addition of LPS (1 μ g/mL, Sigma-Aldrich, St. Louis, MO, USA) in serum free RPMI1640. An equal volume of sterile water was added to all control treatments. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay as described previously. Cells were incubated with various concentrations of AEE for 24 h followed by MTT for 4 h, and then 100 μ l of isopropanol (in 0.04 N-hydrochloric acids) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using the Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was calculated as relative absorbance compared to control.

Nitric oxide assay

The amount of NO production in the medium was detected with the Griess reaction [6]. Each supernatant was mixed with the same volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthyl ethylenediamine dihydrochloride in water). The absorbance of the mixture at 540 nm was determined with an ELISA reader (Bio-Tek Instrument, Winooski, VT, USA), nitrite concentration was determined using sodium nitrite as a standard.

Nuclear protein extraction and Western blot analysis

Cells were washed in cold PBS three times and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 % (v/v) Tergitol- type NP-40 (NP-40), 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 2 mM Sodium orthovanadate (Na₃VO₄) and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany)

at 4 °C. The lysate was clarified by centrifugation at 10,000 x g for 20 min at 4 °C to remove insoluble components. Cell lysates were normalized for protein content using bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded onto 10 % poly acryl amide gel electrophoresis (PAGE) and separated by standard sodium dodecyl sulphate (SDS)-PAGE procedure. Proteins were transferred to an NC membrane (S&S, Dassel, Germany) and blocked with 5 % non-fat dry milk in TBS. To detect protein expression, the blots were probed with specific antibodies for iNOS (1:1000), COX-1 (1:1000), COX-2 (1:1000), NF- κ B, I κ B- α (1:1000), anti-p-I κ B- α and β -actin (1:2000) followed by a 1 h incubation with horseradish peroxidase-conjugated secondary antibodies (1:1000 – 2000)(Bio-Rad, Hercules, CA, USA) with β -actin as internal control. The immuno reactive proteins on the membrane were detected by chemiluminescence using the West-Save substrate (Lab-Frontier, Seoul, Korea) on x-ray film. The antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase (COX) - 1, COX-2, nuclear factor kappa-B (NF- κ B), I kappa B-alpha (I κ B- α) and β -actin were purchased from Cell Signaling Technology Inc (Beverly, MA, USA).

TNF- α assay

RAW264.7 microglia cells (1×10^5 cells/well) were cultured on 96 well plates and treated with the AEE at indicated concentrations for 1 h and stimulated with LPS (1 μ g/mL). At 24 h post LPS treatment, the production of TNF- α was determined in cell supernatant using assay kits (BD Biosciences, San Jose, CA, USA) as per the

manufacturer's instructions. The TNF- α assay was performed at room temperature and optical absorbance was measured at 450 nm using ELISA reader.

Statistical analysis

All data are presented as mean \pm SEM (at least n = 3). Statistical analysis was performed using SAS statistical software (SAS Institute, Cray, NC, USA) based one-way analysis of variance, followed by Dunnett's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of AEE on DPPH radical scavenging activity

DPPH is a stable free radical donor, which has been widely used to test free radical scavenging effect of natural anti-oxidants. In this study, the capacity of AEE to scavenge DPPH was measured by ESR spectrometry. As shown in Fig 1A, AEE exhibited significant DPPH radical scavenging activity in a dose-dependent manner showing a maximum effect at 1mg/mL of concentration. The ESR spectroscopy data at 0.01, 0.1 and 1 mg/mL was represented in Fig 1B.

Effect of AEE on LPS-induced NO production

The effects of AEE on NO production were examined in LPS-stimulated RAW264.7 cells.

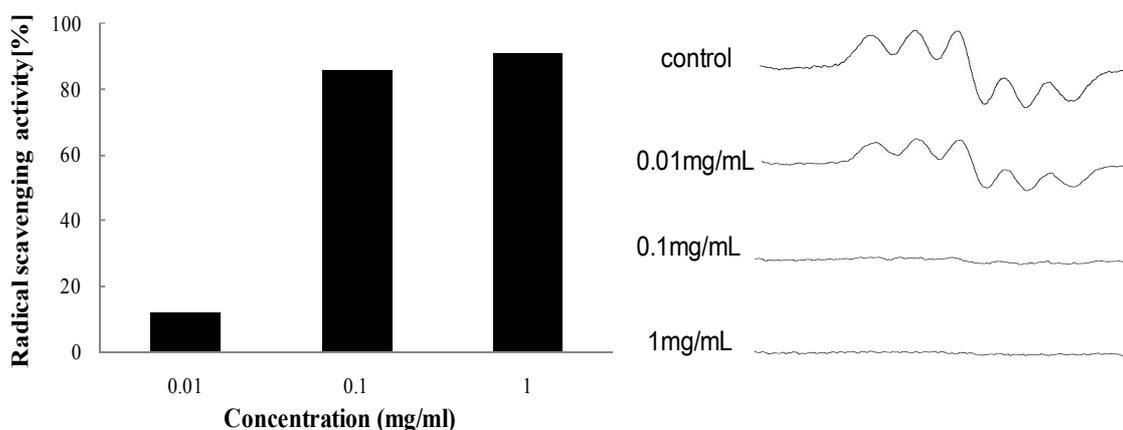


Fig 1: Effect of AEE on DPPH radical scavenging activity. The capacity to scavenge DPPH free radical by different concentrations AEE (A) and ESR spectra (B) was measured. The scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. AEE = *Aralia elata* ethanol extract

Further, AEE treatment did not exhibit any significant cytotoxicity in RAW264.7 cells treated for 24 h at concentrations up to 200 $\mu\text{g}/\text{mL}$, and in all cases the viability was found above 96 % by MTT assay (Fig. 2). As shown in Fig 3, treatment of LPS resulted in the excessive production of NO. Pretreatment of AEE significantly suppressed the LPS-induced NO production in a concentration-dependent manner (Fig 2). The maximum effect was shown at 100 $\mu\text{g}/\text{mL}$ ($p < 0.001$).

Effect of AEE on LPS-induced expressional levels of iNOS and COX-2

To know the effect of AEE on iNOS and COX levels, RAW264.7 cells were stimulated with LPS (1 $\mu\text{g}/\text{mL}$) in the presence or absence of AEE (20, 40 and 80 $\mu\text{g}/\text{mL}$). LPS strongly induced RAW264.7 cells showing an increased expression of iNOS. However, treatment with AEE exhibited a broad spectrum of inhibitory effect on the expression of iNOS induced by LPS in RAW264.7 cells (Fig 4).

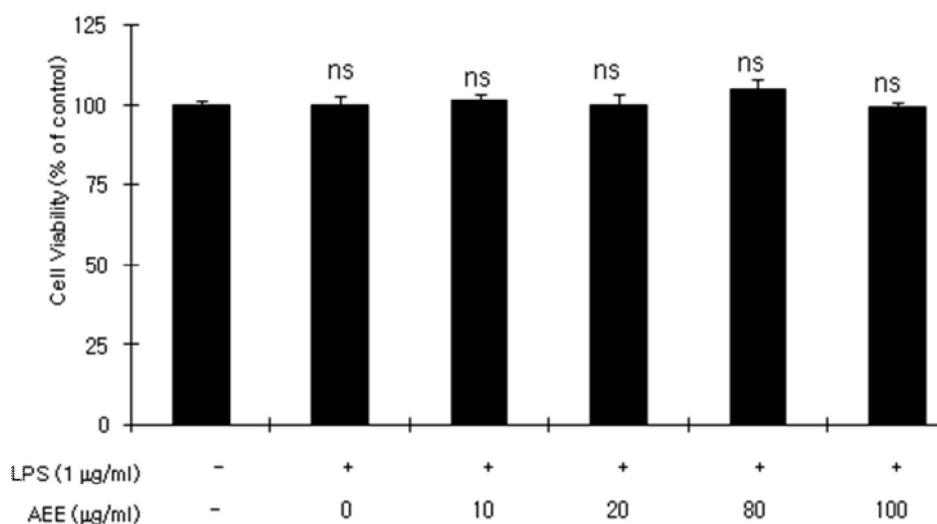


Fig 2: Effects of AEE on the viability of RAW264.7 cells. Viability in AEE treated cells was determined using MTT assay. The results are displayed as percentage of control samples. Data are presented as mean \pm SEM ($n = 3$) for three independent experiments. ^{NS} Not significant; AEE = *Aralia elata* ethanol extract

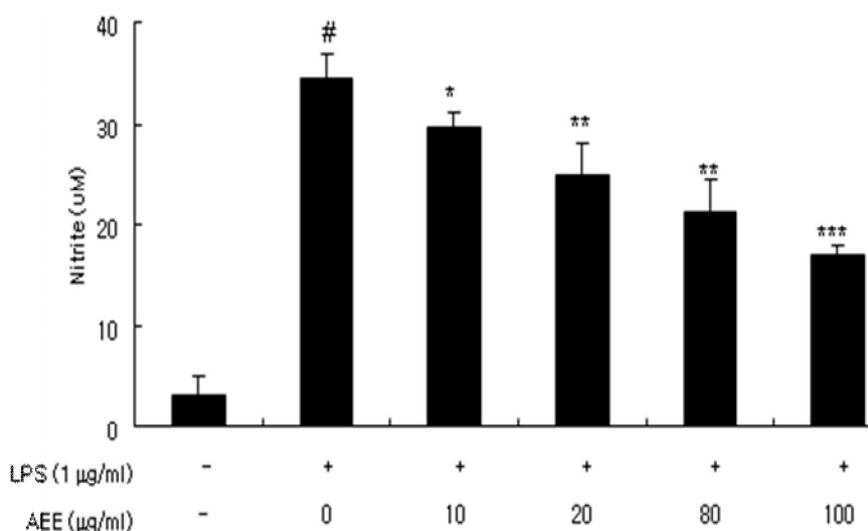


Fig 3: Effect of AEE on NO Production in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with AEE at various concentrations (10, 20, 40, 80, and 100 $\mu\text{g}/\text{mL}$) with or without LPS (1 $\mu\text{g}/\text{mL}$) for 24 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as the mean \pm SEM ($n = 3$) for three independent experiments. # $p < 0.001$, when compared with control group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, when compared with LPS alone treated group by one-way analysis of variance, followed by Dunnett's multiple range tests. AEE = *Aralia elata* ethanol extract

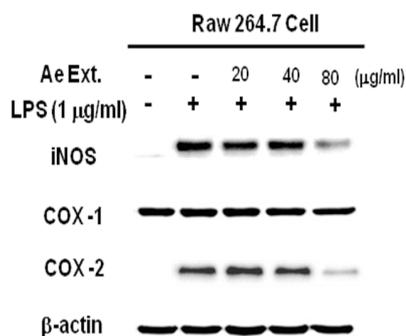


Fig 4: Effect of AEE on iNOS, COX-1 and COX-2 protein expression levels in LPS-stimulated RAW264.7 cells. The expression levels of iNOS, COX-1 and COX-2 production in the LPS-stimulated Raw264.7 cells by various concentrations (20, 40 and 80 µg/mL) of AEE was monitored by immunoblot analyses with the specific antibodies against iNOS, COX-1 and COX-2. The internal control used was β-actin. AEE = *Aralia elata* ethanol extract

Effect of AEE on NF-κB levels

AEE inhibited the LPS-induced phosphorylation and degradation of IκB-α, and nuclear translocation of p65 NF-κB in a concentration dependent manner (Fig 5).

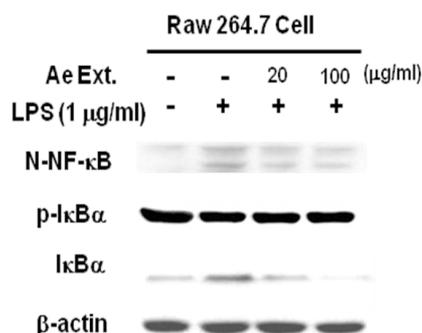


Fig 5: Effect of AEE on NF-κB activity in LPS stimulated Raw 264.7 cell. The expression levels of IκB-α, and nuclear translocation of p65 NF-κB in the LPS-stimulated Raw 264.7 cells by indicated concentrations (20 and 100 µg/ml) of the AEE was monitored by immunoblot analyses with the specific antibodies. The internal control used was β-actin. Data are presented as the mean ± SEM (n = 3) for three independent experiments. AEE = *Aralia elata* extract

Effect of AEE on TNF-α production in LPS-stimulated RAW264.7 cells

To determine the effect of AEE on the expression of pro-inflammatory cytokines such as TNF-α, extracellular release of cytokines was examined using ELISA assay. As shown in Fig. 6, TNF-α

levels increased significantly after LPS treatment (1 µg/mL) when compared to those in untreated cells ($p < 0.001$). However, AEE significantly inhibited TNF-α production in a concentration-dependent manner in LPS-stimulated RAW264.7 cells ($p < 0.05$ at 20 µg/mL and $p < 0.01$ at 40 and 80 µg/mL, respectively).

DISCUSSION

Inflammation is partially ascribed, to release of toxic free radicals and ROS. DPPH radical scavenging assay is one of the widely used methods for screening the free radical scavenging activities of several agents in a relatively short period of time. In the present study, AEE significantly scavenged the DPPH free radicals.

In this study a pro-inflammatory stimulus by LPS on RAW 264.7 cells resulted in excessive production of NO. Earlier studies showed that prolonged activation of RAW 264.7 cells led to increased release of NO by iNOS. NO, an important regulatory mediator involved in cell survival and death exerts a number of pro-inflammatory effects during several physiological and pathological processes leading to increased inflammatory reaction. It was well known that COX-1 is constitutively expressed in many cell types and COX-2 is normally not present in most cells, but its expression is induced in response to inflammatory cytokines linked to pathological events [7]. COX-2 is upregulated in response to various inflammatory stimuli including LPS in RAW 264.7 cells. Therefore, agents that inhibit the release of NO and attenuate iNOS and COX-2 expression could be beneficial for preventing and delaying the progression of inflammatory disease. Data from our study clearly showed that AEE attenuated LPS-induced iNOS and COX-2 expression and downstream NO production. However, AEE has no influence on the constitutive COX-1 expression.

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 play central roles in inflammation. In particular, increased levels of brain TNF-α and IL-6 has been associated with severe cognitive impairments, neuronal damage and neuro-inflammation [8]. Therefore, the effects of AEE on pro-inflammatory cytokine TNF-α production in LPS-stimulated RAW 264.7 cells were evaluated. LPS-stimulation increased the levels of TNF-α in RAW 264.7 cells. However, pretreatment with AEE suppressed the increased TNF-α production indicating that AEE may convincingly be an effective anti-inflammatory agent.

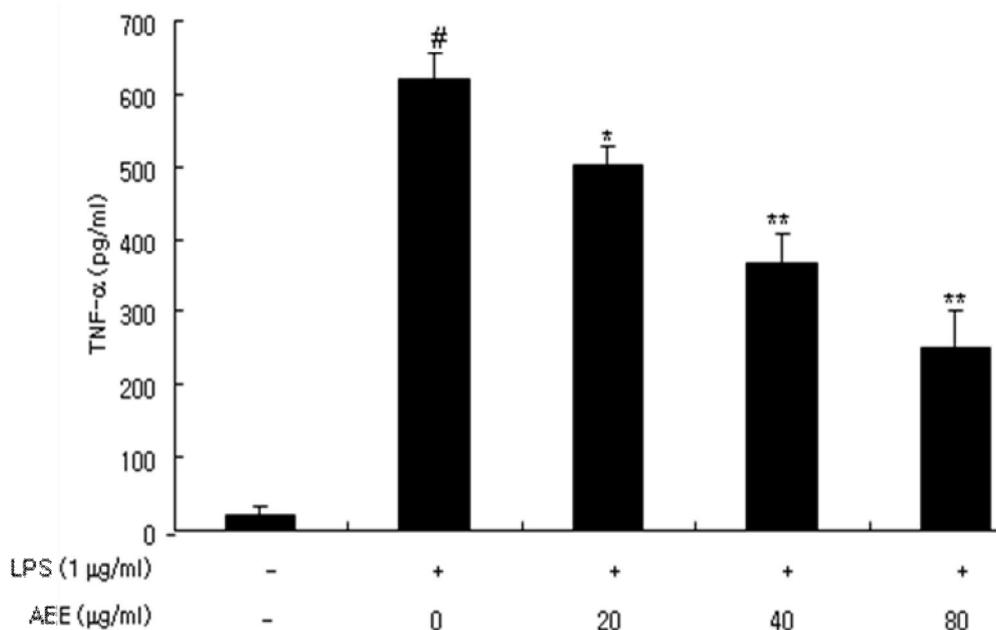


Fig 6: Effect of AEE on TNF- α production in LPS-stimulated RAW264.7 cells. Suppression of pro-inflammatory cytokine TNF- α expression by AEE was measured with ELISA test. RAW264.7 cells were treated with AEE at 20, 40 and 80 $\mu\text{g}/\text{mL}$ with or without LPS (1 $\mu\text{g}/\text{mL}$) for 24 h. The TNF- α in the culture supernatant was evaluated using a murine TNF- α ELISA kit. Data are presented as mean \pm SEM (n = 3) for three independent experiments. [#] $p < 0.001$, when compared with control group. ^{*} $p < 0.05$, and ^{**} $p < 0.01$, when compared with LPS alone group by Student t-test. **Note:** AEE = *Aralia elata* ethanol extract; TNF- α = Tumor necrosis factor-alpha; LPS = Lipopolysaccharide)

NF- κB , a mammalian transcription factor, activated by LPS, is known to control the expression of cell survival genes as well as pro-inflammatory enzymes and cytokines [9]. Our result showed that AEE inhibited the LPS induced phosphorylation/degradation of I κB - α and translocation of NF- κB /p65 sub unit in a concentration-dependent manner. Considering the above data, we can conclude that NF- κB is a major target of AEE. However, the exact molecular target of AEE on NF- κB activation remains to be elucidated.

CONCLUSION

The findings of this study indicate that AEE inhibits anti-inflammatory responses via NF- κB signaling in LPS-stimulated RAW264.7 cells. Further, the antioxidant activity of AEE may be partly involved in the observed effects.

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