

***Ageratum conyzoides* leaf extract inhibit inflammatory response via suppression of NF- κ B and MAPKs pathway in LPS-induced macrophages**

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ABSTRACT

Ageratum conyzoides (Asteraceae) has been widely used in traditional medicine in several countries for the treatment of skin diseases, ulcer wound, diarrhea, fever, pain and inflammation. Leaf extracts from *Ageratum conyzoides* have been shown anti-inflammatory activity in several *in vivo* models. However, the mechanism of its action has not been described yet. In this study, we determined the anti-inflammatory activity and the molecular mechanism of the ethanol extract of *Ageratum conyzoides* leaves (ACE) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage model. ACE exhibited an inhibitory effect on inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO) and cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E₂ (PGE₂) production with IC₅₀ values of 23.4 and 18.5 μ g/ml, respectively. ACE showed no significant cytotoxic effect determined by MTT assay. ACE attenuated the expression of iNOS and COX-2 at mRNA as well as protein levels in a concentration-dependent manner. Additionally, ACE suppressed the level of nuclear factor-B (NF-B) translocation and phosphorylation of p38 kinase, extracellular receptor kinase (ERK) and c-jun NH2 terminal kinase (JNK) of mitogen-activated protein kinases (MAPKs). These results indicate that ACE inhibits inflammatory response, at least in part, by inhibition of NO and PGE₂ production through suppression of iNOS and COX-2 expression via a signaling pathway that involves NF-B nuclear translocation and MAPKs phosphorylation. These findings provide the scientific evidence to justify the anti-inflammatory therapeutic use of *Ageratum conyzoides* leaves in traditional medicine.

Keywords: *Ageratum conyzoides*, Nitric oxide, Prostaglandin E₂, iNOS, COX-2, Macrophage

1. INTRODUCTION

Inflammation is a critically important aspect of host responses to infection and injury. In response to inflammatory stimuli such as LPS, macrophages secrete various pro-inflammatory mediators including inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO) and cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E₂ (PGE₂) which are involved in eradication of infection and injury. However, excessive or prolonged secretion of these mediators is implicated to the pathogenesis of a variety of diseases, including asthma, atherosclerosis, cancer, diabetes, inflammatory bowel diseases, and rheumatoid arthritis [1, 2]. Therefore, inhibition of NO and PGE₂ production is a promising target in the development of anti-inflammatory agents. Expression of iNOS and COX-2 in LPS-stimulated macrophages is mainly regulated by nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPKs) pathways [3-5].

Ageratum conyzoides L. (Asteraceae) is annual herb used in traditional medicine in many countries in the world. The leaves of *A. conyzoides* have been used in the treatment of chronic pain and various inflammatory diseases [6]. *A. conyzoides* leaf extracts have been shown to exert anti-inflammatory activity in several *in vivo* models [7-9]. However, the molecular mechanisms of its action have not been described. The purpose of this study was to investigate underlying anti-inflammatory mechanism of *A. conyzoides* leaf extract in LPS-induced RAW 264.7 macrophages.

2. MATERIALS AND METHODS

Preparation of extract

Leaves of *A. conyzoides* were collected from Ban Ang-Ed official community forest, Chantaburi Province. The plants were cleaned with tap water, dried and finally powdered. The powdered were soaked in 95% ethanol in a ratio 1:10 for 5 days with occasional shaking, after that ethanol extract was filtered through filter paper. Plant residues were re-extracted with 95% ethanol 2 times. The filtrate were pooled and evaporated by rotary evaporator until dryness before storage at -20°C.

Cell viability assay

Cell viability of RAW 264.7 macrophage were assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as determined by Buapool et al, 2013 [5].

Measurement of nitric oxide production

Nitrite, an oxidation product of NO, in the cultured media is an index of NO production determined by Griess reaction according to the method reported by Buapool et al, 2013 [5].

Determination of PGE₂

PGE₂ produced from LPS-activated RAW 264.7 cells was quantified using PGE₂ competitive enzyme immunoassay kit (R&D Systems, USA.).

Western blotting analysis

Levels of iNOS, COX-2 and β -actin proteins in macrophages were determined by Western blotting analysis as described by Buapool et al, 2013 [5].

Real time-reverse transcription-polymerase chain reaction (Real time RT-PCR) analysis

Total RNA was reverse transcribed into cDNA using 5x iScriptTM Reverse Transcription Supermix. The cDNA was used for real-time quantitative PCR which conducted in a CFX96TM Real-Time System (Bio-Rad) using 2x iTaqTM Universal SYBR[®] Green Supermix. The fold increase or decrease of iNOS and COX-2 were determined relative to a control after normalized to a housekeeping gene (EF-2) using formula $2^{-\Delta\Delta CT}$.

Nuclear protein extraction

For analyses of NF- κ B p65 subunit level, nuclear protein extracts were isolated according to the method reported by Srisook et al., 2011 [10].

3. RESULTS AND DISCUSSION

This study was performed to investigate the mechanism of anti-inflammatory effect of *A. conyzoides* leaves in LPS-induced RAW 264.7 macrophages. The ethanol leaf extract of *A. conyzoides* (ACE) inhibited the production of NO and PGE₂ in a dose-dependent manner with IC₅₀ values of 22.69 ± 0.14 and 25.92 ± 5.72 µg/mL, respectively (Figure 1). ACE at concentrations 3.125 to 50 µg/mL did not significantly affect cell viability when compared to unstimulated control cell, indicating that the reduction of NO and PGE₂ production was not attributed to cell cytotoxicity. As shown in Figure 2, iNOS and COX-2 proteins were increased dramatically in cells treated with LPS and ACE attenuated the expression of protein iNOS and COX-2 in a dose-dependent manner. In accord, we found that ACE attenuated the level mRNA of iNOS and COX-2 in a dose-dependent manner (Figure 3). The results show that inhibition of NO and PGE₂ by ACE as a result of the suppression of iNOS and COX-2 mRNA and protein syntheses.

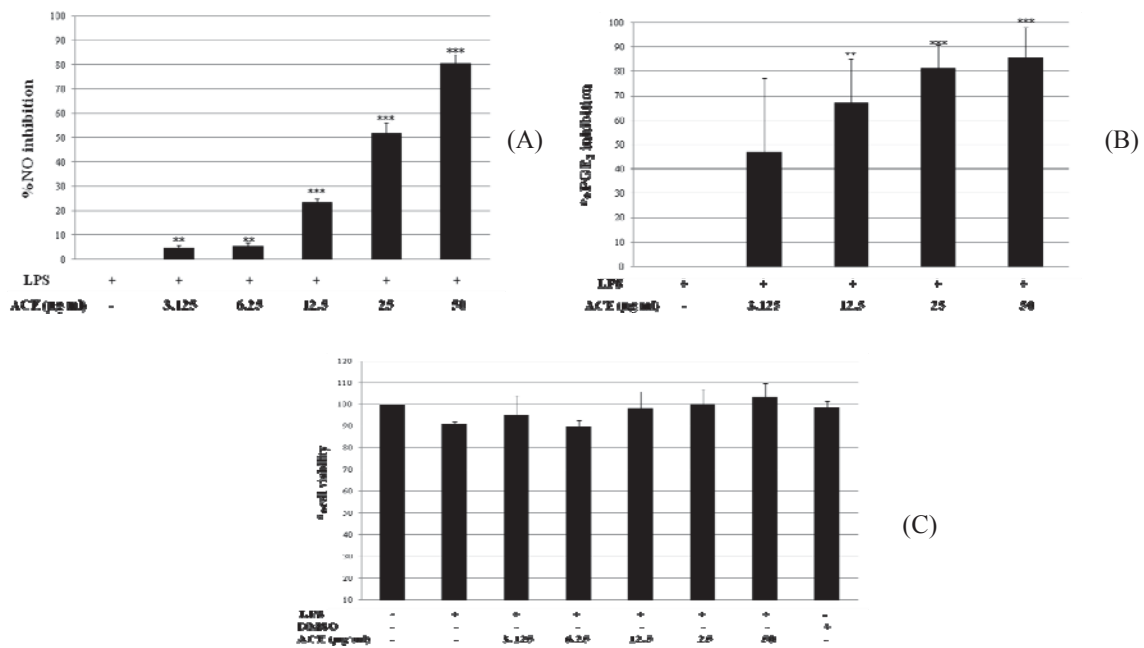


Figure 1. Inhibitory effects of ACE on NO (A) and PGE₂ (B) production in LPS-induced RAW 264.7 macrophages. Cells were incubated with ACE and LPS for 24 h. Accumulated nitrite and PGE₂ concentrations present in the medium were determined. (C) Viability of cells was determined using the MTT assay. Each column represents the mean ± SD of at least three independent experiments with triplicate samples. ***p* < 0.01, *** *p* < 0.001 vs. LPS alone.

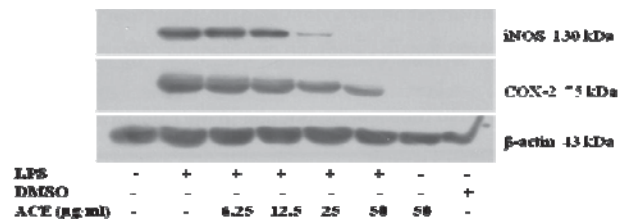


Figure 2. Effect of ACE on iNOS and COX-2 expression in LPS-induced RAW 264.7 macrophages. Cells were stimulated with ACE and LPS for 24 h. iNOS and COX-2 proteins were detected by Western blot analysis.

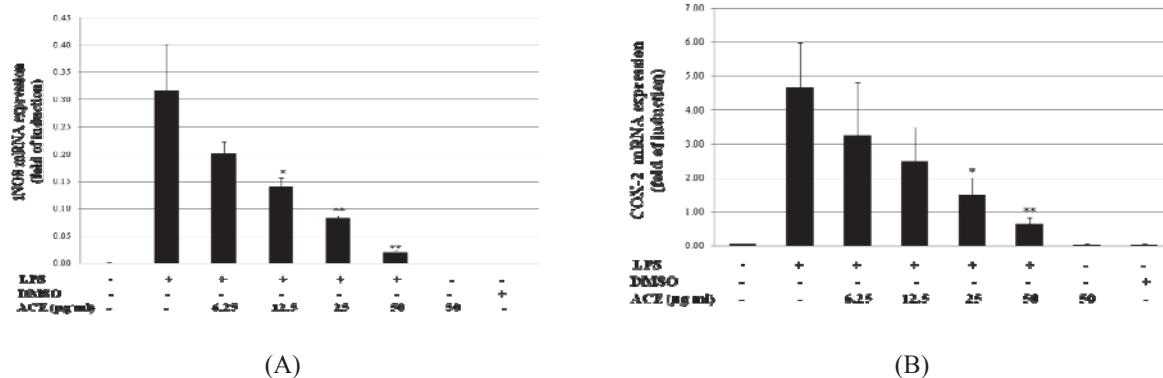


Figure 3. Effect of ACE on iNOS (A) and COX-2 (B) mRNA expression in LPS-induced RAW 264.7 cells. Cells were treated with LPS and ACE for 9 h. Total RNA were isolated, iNOS and COX-2 mRNA levels were determined by real-time RT-PCR. Each column represents the mean \pm SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. LPS alone.

In unstimulated cells, transcription factor NF- κ B is bound to inhibitor of κ B (I κ B) in cytoplasm. Upon activation, NF- κ B is rapidly translocated to the nucleus, subsequently facilitates the transcription of target genes, involved in inflammation, such as pro-inflammatory cytokines, chemokines, and inducible enzymes such as iNOS and COX-2 [3, 11]. We next determined the effect of ACE on the level of NF- κ B p65 subunit since p65 protein is a subunit of the most common heterodimer NF- κ B. LPS induced NF- κ B p65 subunit translocation in nucleus, but ACE suppressed the level of NF- κ B p65 subunit (Figure 4A). The other major signal pathway of iNOS and COX-2 expression stimulated by LPS is the mitogen-activated protein kinase (MAPKs) pathway composed of p38 kinase, extracellular receptor kinase (ERK), and c-jun NH₂ terminal kinase (JNK) [4, 5]. As shown in Western blot analysis data in Figure 4B, LPS induced the phosphorylation of ERK and p38 MAPK while ACE suppressed the phosphorylated ERK and p38 MAPK in a dose-dependent manner. These results indicate that ACE inhibited NO and PGE₂ production through suppression of iNOS and COX-2 expression, at least in part, via a reduction of NF- κ B p65 subunit nuclear translocation and ERK and p38 MAPK phosphorylation.

The extracts from *A. conyzoides* have been shown anti-inflammatory effects in inflammation models of animal which associated with the secretion of inflammatory mediators including PGE₂ and NO [7-9]. The obtained results from LPS-induced PGE₂ and NO release in RAW 264.7 macrophages indicate that the mechanism of anti-inflammatory effect of *A. conyzoides* on several *in vivo* models [7-9] might be attributed to the inhibition of PGE₂ and NO. Literature review reports indicate the presence of kaempferol and quercetin in *A. conyzoides* [6] which have been shown to possess anti-inflammatory activities [12]. Thus these compounds might be attributed to the potent anti-inflammatory activity of ACE in LPS-induced macrophages. However, the bioactive compounds which responsible for anti-inflammatory property of ACE should be identified.

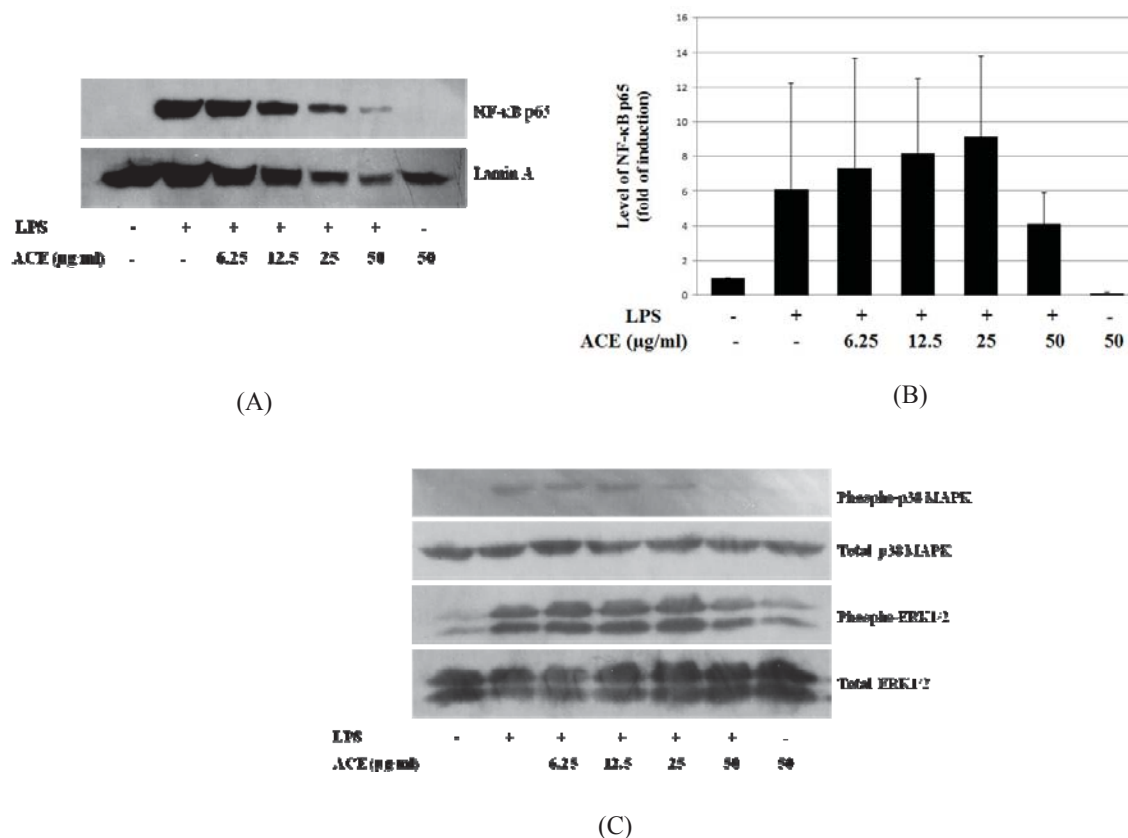


Figure 4. Effect of ACE on the nuclear translocation of subunit p65 of NF-κB and phosphorylation of MAPKs in RAW 264.7 macrophages. Cells were pre-treated with the indicated concentrations of ACE for 30 min, and stimulated with LPS for 1 h for NF-κB and 30 min for MAPKs. The nuclear extracts were analyzed the levels of NF-κB p65 subunit (A). Graph shows the mean ± SD of results of densitometric analyses of NF-κB p65 subunit which were normalized to Lamin A densitometric values (n = 3) (B). Whole cell extracts were determined the phosphorylated ERK1/2 and p38 MAPK level by Western blot analysis (C).

4. CONCLUSIONS

These obtained results demonstrate that the mechanism of anti-inflammatory activity of ACE is, at least in part, the inhibition of NO and PGE₂ production by inhibition of iNOS and COX-2 expression, via NF-κB and MAPKs signaling pathway. These findings support the uses of *Ageratum conyzoides* leaves in traditional medicine.

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