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*Full Length Research Paper*

# Inhibitory effect of *Sorbus commixta* extract on lipopolysaccharide-induced pro-inflammatory events in macrophages

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***Sorbus commixta*** **has been known as enthopharmacologically valuable plant in Korea, China and Japan. This plant has been reported to display numerous pharmacological activities such as anti-oxidative, anti-ice nucleation, anti-vascular inflammation, anti-lipid peroxidation, anti-atherogenic, and vasorelaxant effects. Although numerous pharmacological potentials have been demonstrated, immunomodulatory effect of this plant has not been fully elucidated yet. To evaluate its anti-inflammatory activity, macrophages activated by lipopolysaccharide (LPS) were employed and the production of inflammatory mediators was explored in terms of understanding its molecular inhibitory mechanism. 70% ethanol extract (Sc-EE) from** ***S. commixta*** **strongly suppressed the production of nitric oxide (NO) and prostaglandin (PG) $E_2$ but not tumor necrosis factor (TNF)-α. The extract also clearly diminished the mRNA levels of inducible NO synthase (iNOS) and cyclo-oxygenase (COX)-2, implying that the inhibition occurs at the transcriptional level. Indeed, Western blot analysis and luciferase activity assay revealed that Sc-EE remarkably suppressed AP-1 translocation and its activity, respectively. In agreement, this extract strongly suppressed the phosphorylation of JNK, a prime enzyme responsible for AP-1 translocation. Therefore, our results suggest that Sc-EE can be applied as an anti-inflammatory herbal medicine. To prove this possibility,** ***in vivo*** **efficacy test will be further continued in the following project.**

**Key words:** *Sorbus commixta*, macrophages, inflammatory mediators, AP-1 translocation, JNK activation.

## INTRODUCTION

Macrophages are the major immune cells performing the innate immunity. In particular, These cells are known to play a critical role in managing inflammation, a major innate immune response by producing pro-inflammatory cytokines [e.g. tumor necrosis factor (TNF)-α, and interleukin (IL)-1], and inflammatory molecules [e.g. nitric oxide (NO) and oxygen species intermediates (ROS)] (Allam and Anders, 2008). The activation of macrophages is mediated by interaction between pattern recognition receptors [e.g. Toll like receptor (TLR)-4] and their their ligands [e.g. lipopolysaccharide (LPS)] (Lin and Yeh, 2005). The molecular interaction of these molecules is linked to the transcriptional activation of NF-κB and AP-1, redox sensitive transcription factors activated by radicals generated (Pourazar et al., 2005). The up-regulation of these transcription factors requires the formation of total signaling complex including various protein kinases [Src, Syk, phosphoinositide 3-kinase (PI3K), and Akt (protein kinase B)] for NF-κB translocation and mitogen activated protein kinases (MAPKs) [such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38] for AP-1 translocation (Natarajan et al., 2002; Rahman et al., 2004). Since inflammation plays a critical role in causing various diseases such as cancer, auto-immune diseases, cardiovascular diseases, obesity, and diabetes, development of promising anti-inflammatory drugs without side effects could be valuable in terms of prevention or therapy of various inflammation-mediated

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diseases (Garcia-Lafuente et al., 2009).

*Sorbus commixta* (Malaceae) has been known as ethopharmacologically valuable plant in Korea, China and Japan, used as a tonic and for the control of cough, asthma, and other bronchial disorders (Sohn et al., 2005a). This plant has been reported to display numerous pharmacological activities such as anti-oxidative, anti-ice nucleation, anti-vascular inflammation, anti-lipid peroxidation, anti-atherogenic, and vasorelaxant effects (Bae et al., 2007; Kasuga et al., 2007; Sohn et al., 2005b; Yin et al., 2005). Although it has not been fully understood how the plant is able to modulate various biological activities, a potent radical scavenging activity, enhancement of NO-cyclic guanosine-3',5'-cyclic monophosphate-relevant pathway, or the inhibition of protein tyrosine phosphatase (PTP)1B have been considered as potential pharmacological targets of *S. commixta* (Kang et al., 2007; Na et al., 2009; Sohn et al., 2005a).

Nonetheless, immunomodulatory effect of this plant and its mechanism are still largely unelucidated, although it has been traditionally used for long time. In this study, therefore, we investigated the effect of 70% EtOH extract of *S. commixta* (Sc-EE) on the modulation of LPS-induced inflammatory responses mediated by macrophages.

## MATERIALS AND METHODS

### Materials

Lipopolysaccharide (LPS) and phorbal-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). SP600125 was from Calbiochem (La Jolla, CA). 70% EtOH extract was prepared by conventional extraction methods (Lee et al., 2007). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All other chemicals were of reagent grade. Anti-phospho or total antibodies to extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), p65 (NF-κB), c-Jun, c-fos, β-actin and γ-tublin were from Cell Signaling (Beverly, MA).

### Cell culture

RAW 264.7 and HEK293 cells obtained from American Type Culture Collection (Rockville, MD) were cultured with RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), glutamine and antibiotics (penicillin and streptomycin) at 37°C with 5% $CO_2$.

### NO, $PGE_2$ and TNF-α production

The inhibitory effect of Sc-EE on NO, $PGE_2$ and TNF- α production was determined as previously described (Lee et al., 2009). Sc-EE solubilized with vehicle (100% DMSO) was diluted with RPMI1640. RAW264.7 cells ($2\times10^6$ cells/ml) were incubated with LPS (1 μg/ml) in the presence or absence of Sc-EE for 6 (TNF-α) or 24 ($PGE_2$ and NO) h. Supernatants were assayed for NO, $PGE_2$ and TNF- α contents using Griess reagent, $PGE_2$ EIA, and TNF- α ELISA kits (Amersham, Little Chalfont, Buckinghamshire, UK).

### MTT assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Cho et al., 2000).

### Extraction of total RNA and semi-quantitative RT-PCR amplification

The total RNA from the LPS treated-RAW264.7 cells was prepared by adding TRIzol Reagent (Gibco BRL), according to manufacturer's protocol. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase as reported previously (Hong et al., 2003). The primers (Bioneer, Daejeon, Korea) were used as previously reported (Lee et al., 2006a).

### Radical scavenging activity

The radical scavenging activity of Sc-EE was performed using DPPH assay and Griess assay (Bai et al., 2005). Briefly, various concentrations of Sc-EE were incubated with DPPH (0.2 mM) or SNP (10 mM) for 30 min or 3 h and then scavenging effects were determined by spectrophotometric analysis.

### Preparation of cell lysate and immunoblotting

For total protein extraction: RAW 264.7 cells were harvested, washed with cold PBS and lysed in lysis buffer (20 mM TRIS-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin, 1 mM benzimidine and 2 mM phenylmethane sulphonyl-fluoride) for 30 min rotating at 4°C. Lysates were clarified by centrifugation at 16,000 × g for 10 min at 4°C. For nuclear protein extraction: Nuclear proteins were obtained through three steps. After the treatment, cells were harvested and lysed in 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40.25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin and 100 μM 1,4-dithiothreitol) on ice for 4 min. Cells lysates were centrifuged ate 14,000 rpm for 1 min at 4°C. In the second step, the pellet was washed with the wash buffer, which was the same as the lysis buffer excluding Nonidet P-40. In the final step, the nuclei were incubated with an extraction buffer (500 mM KCl, 10% glycerol, 10 mM HEPES, 300 mM NaCl, 0.1 mM 1,4-dithiothreitol, 0.1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and centrifuged at 14,000 rpm for 5 min. Supernatant was collected as nuclear protein extract. Soluble cell lysates were immunoblotted and phospho-ERK levels were visualized as previously reported (Lee et al., 2008).

### Statistic analysis

The Student's *t*-test and one–way ANOVA were used to determine the statistical significance between values of the various experimental and control groups. P values of 0.05 or less were considered to be statistically significant.

## RESULTS AND DISCUSSION

Inflammation causes numerous diseases such as cancer, diabetes, atherosclerosis and obesity (Ferencik et al., 2007; Guzik et al., 2006). Due to this, numerous trials to develop anti-inflammatory drugs without toxicity and side

effects have been performed. Natural products traditionally used for long time are regarded as attractive anti-inflammatory candidates in terms of safety. Our laboratory therefore has been focused on developing anti-inflammatory drugs using naturally-occurring compounds or plants for a decade.

Inhibitory effect of Sc-EE has been evaluated using *in vitro* inflammatory models. Thus, we tested its modulatory effect on the production of inflammatory mediators such as $PGE_2$, NO, and TNF-α. Under non-toxic concentrations (0 to 400 µg/ml) (Figure 1), Sc-EE strongly blocked the release of $PGE_2$ and NO, but not TNF-α (Figures 2A, B and C). The inhibition of NO and $PGE_2$ production was occurred at the transcriptional levels, according to Figure 3. Thus, mRNA levels of inducible NO synthase (iNOS) and cyclo-oxygenase (COX)-2 were decreased significantly (Figure 3).

Because transcriptional up-regulation of inflammatory mediators is mostly regulated by transcription factors (Hume et al., 2007), we next examined whether Sc-EE was able to modulate the activation of transcription factors. To do this, the activation of NF-κB and AP-1, representative redox-sensitive transcription factors activated in response to LPS (Adcock, 1997), were tested under Sc-EE pre-treatment conditions. First, whether Sc-EE was capable of blocking the nuclear translocation of p65, a subunit of NF-κB (Baldwin, 1996), was examined. As Figure 4A shows, this extract did not block the translocation of p65 up to 300 µg/ml. In contrast, the translocation of c-Jun but not c-fos, components of AP-1 (Dokter et al., 1993), was remarkably blocked by Sc-EE (Figure 4B), suggesting that AP-1 may be a target transcription factor. To confirm this effect, luciferase assay was employed using constructs with binding sites for AP-1 or NF-κB. Sc-EE suppressed the activity of luciferase with AP-1 (Figure 4C) binding element but not NF-κB (Figure 4B).

The inhibitory mechanism by which this extract suppressed only AP-1 activation was finally explored in terms of both redox-system regulatory potential and signaling events involved in AP-1 translocation. According to Figures 5A and 5B, anti-oxidative potential of Sc-EE seemed not to be critical in this anti-inflammatory response. Thus, the facts that Sc-EE did not exhibit scavenging activity against SNP-induced radical generation (Figure 5B) unlike DPPH assay (Figure 5A), unlike previous papers (Bae et al., 2007; Lee et al., 2006b), and that translocation inhibition of redox sensitive transcription factor was appeared in the case of AP-1 but not NF-κB support this possibility. However, Figure 5C strongly depicts that early signaling events participated in AP-1 translocation can be targeted in Sc-EE-mediated anti-inflammatory responses. Of MAPKs tested, interestingly, strong inhibition was observed in the phosphorylation of JNK and weak suppression was also seen in ERK phosphorylation. Relevance of JNK pathway in inflammatory responses was also confirmed with SP600125 treatment. This compound also blocked AP-1 translocation and

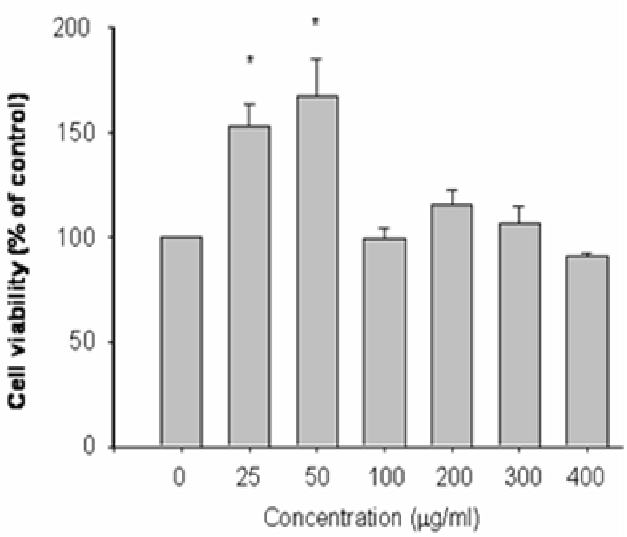


**Figure 1.** Effect of Sc-EE on the viability of RAW264.7 cells. RAW264.7 cells ($1 \times 10^6$) were treated with Sc-EE for 24 h. The viability of RAW264.7 cells was determined by MTT assay. * $p<0.05$ represents significant difference compared to normal.

$PGE_2$production (Figure 6), suggesting that JNK can be a target of Sc-EE.

So far, which kinds of components in this plant can act as inhibitors of JNK/AP-1 activation pathway is not fully understood. Two compounds, lupenone and lupeol were identified as inhibitor of PTP1B with $IC_{50}$ values of 13.7 and 5.6 µM, respectively (Na et al., 2009). These compounds have been reported to block NF-κB activation (Saleem et al., 2004). However, Sc-EE did not block p65/NF-kB translocation (Figure 4A), suggesting that these compounds may not be the major principles. None of the reports on the inhibitory effects of lupenone and lupeol on AP-1 translocation has been published. Therefore, whether these compounds are major principles with anti-inflammatory activity and suppressive effect on AP-1 and JNK activation should be tested. Otherwise, other potential components should be identified based on AP-1 translocation conditions.

## Conclusion

In this study, we found that 70% ethanol extract from *S. commixta* was able to suppress the production of NO and $PGE_2$ but not TNF-α at the transcriptional levels. The anti-inflammatory effect of Sc-EE seemed to be due to AP-1 translocation inhibition, according to immunoblotting analysis with nuclear fraction and luciferase assay. Indeed, Sc-EE strongly suppressed the phosphorylation of JNK, a prime signal to stimulate AP-1 translocation. Therefore, our results suggest that Sc-EE can be applied as an anti-inflammatory herbal medicine. Further *in vivo* efficacy test will be continued in the next project.

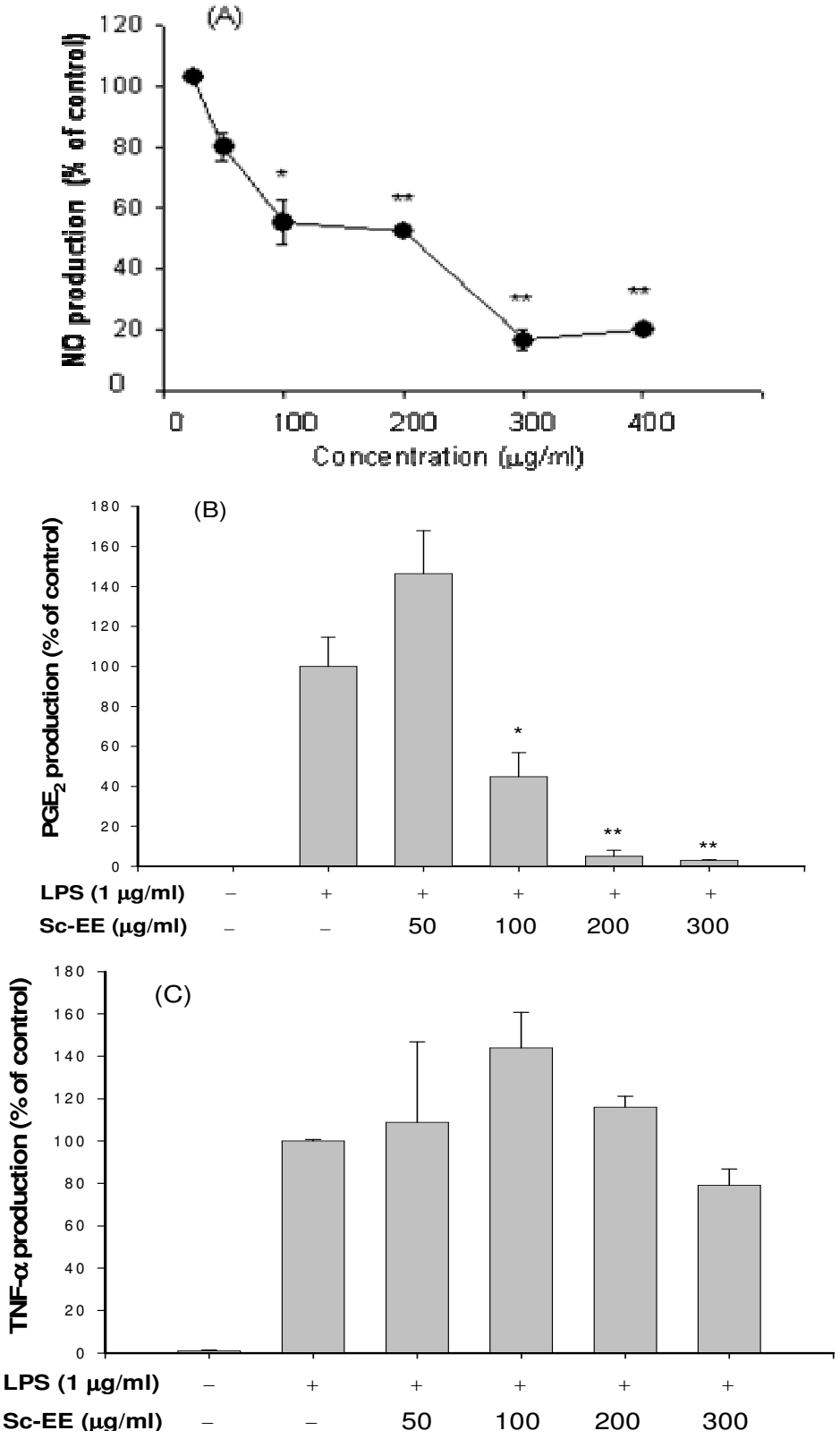


**Figure 2.** Effect of Sc-EE on the production of NO, $PGE_2$ and TNF-α in LPS-activated RAW 264.7 cells. (A, B, and C) RAW264.7 cells ($2\times10^6$ cells/ml) were incubated with indicated concentrations of Sc-EE in the presence of LPS (1 μg/ml) for 24 (NO), 24 ($PGE_2$), and 6 h (TNF-α). NO, $PGE_2$, and TNF- α levels in culture supernatant were determined by Griess reagent, EIA and ELISA.
$^{*}p<0.05$ and $^{**}p<0.01$ represent significant difference compared to LPS alone.

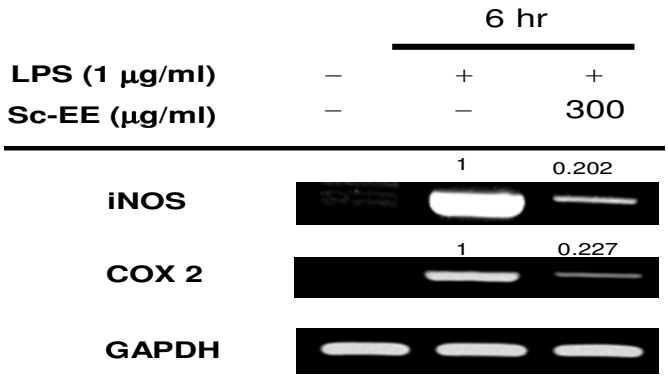


**Figure 3.** Effect of Sc-EE on the mRNA levels of inflammatory genes in RAW264.7 cells under LPS stimulation. RAW264.7 cells ($5\times10^6$ cells/ml) were incubated with Sc-EE in the presence of LPS (1 μg/ml) for 6 h. The mRNA levels of COX-2 and iNOS were determined by RT-PCR.

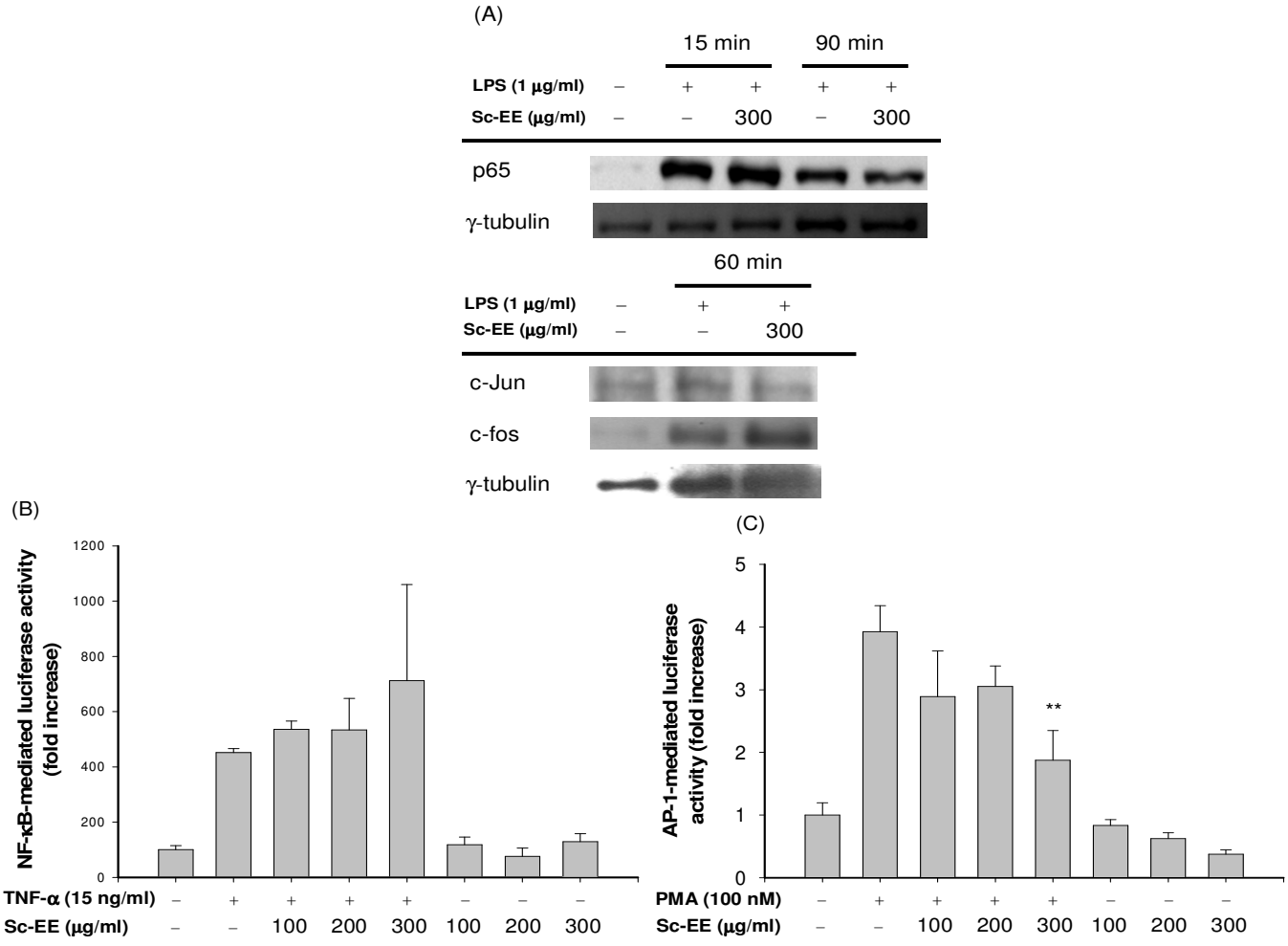


**Figure 4.** Effect of Sc-EE on the transcriptional activation of AP-1 and NF- κB. (A) RAW264.7 cells ($5\times10^6$ cells/ml) pre-treated with Sc-EE for 1 h were stimulated in the absence or presence of LPS (1 μg/ml) for 30 min. After preparation of nuclear fraction, the protein levels of c-Jun, c-fos and γ tublin were determined by immunoblotting analysis with their total protein antibodies. (B and C) HEK293 cells co-transfected with the plasmid constructs, NF-κB-Luc or AP-1-Luc (each 1 μg/ml), and β-gal (as a transfection control) were treated with Sc-EE in the presence or absence of PMA (100 nM) or TNF-α (10 ng/ml) for 18 h. Luciferase activity was determined by luminometry.
**: $p < 0.01$ compared to control.

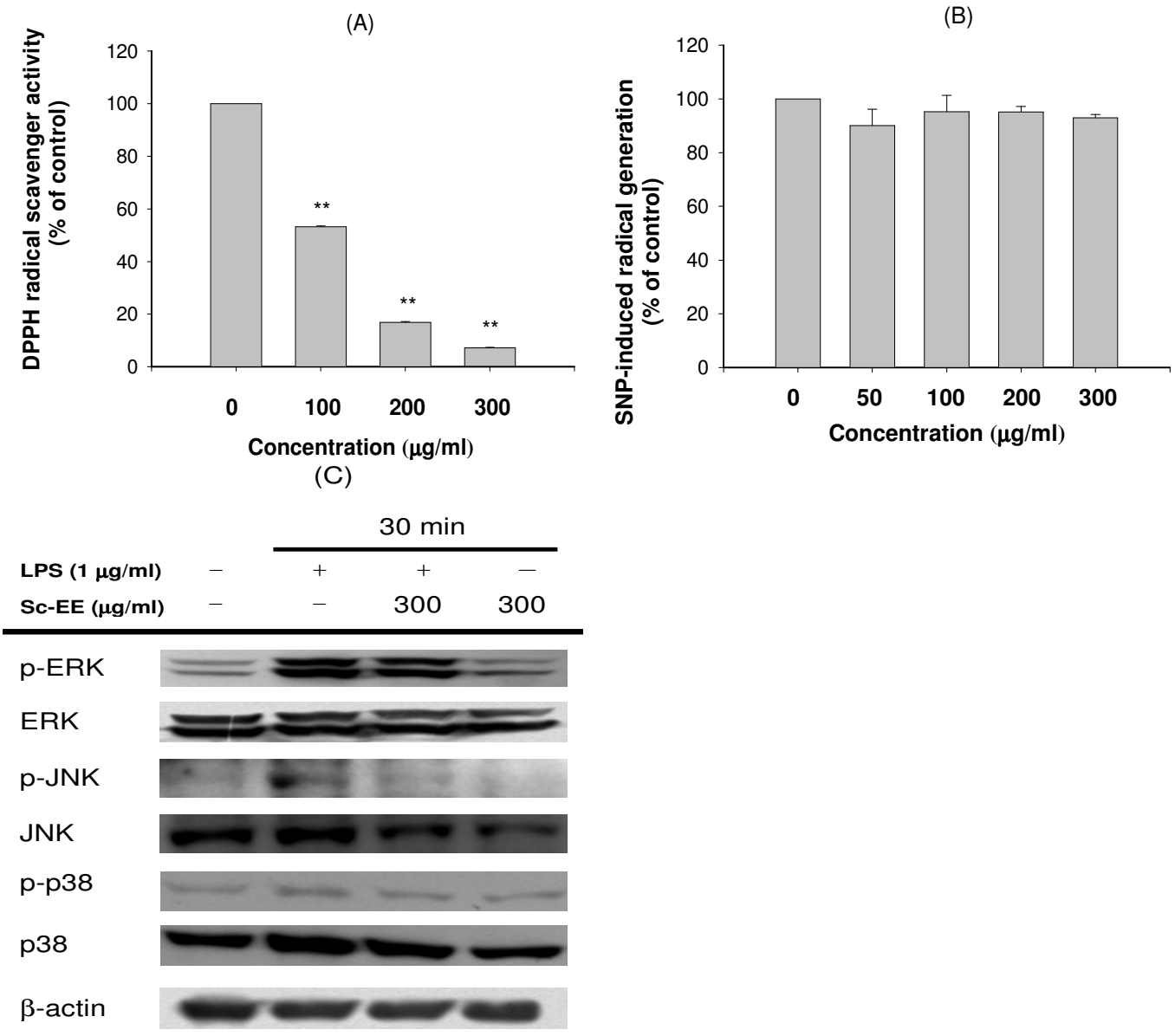


**Figure 5.** Inhibitory mechanism of Sc-EE-mediated AP-1 translocation inhibition. (A and B) Radical scavenging activity of Sc-EE was determined by DPPH assay and Griess assay as described in Materials and methods. (C) RAW264.7 cells ($5\times10^6$ cells/ml) pretreated with Sc-EE (300 μg/ml) were stimulated with LPS (1 μg/ml) for 30 min. After immunoblotting, the levels of phospho- or total ERK, p38, and JNK were identified by their antibodies. The results show one experiment out of three. Data represent mean ± SEM of three independent observations performed in triplicate.
**: $p<0.01$ represents significant difference compared to control.

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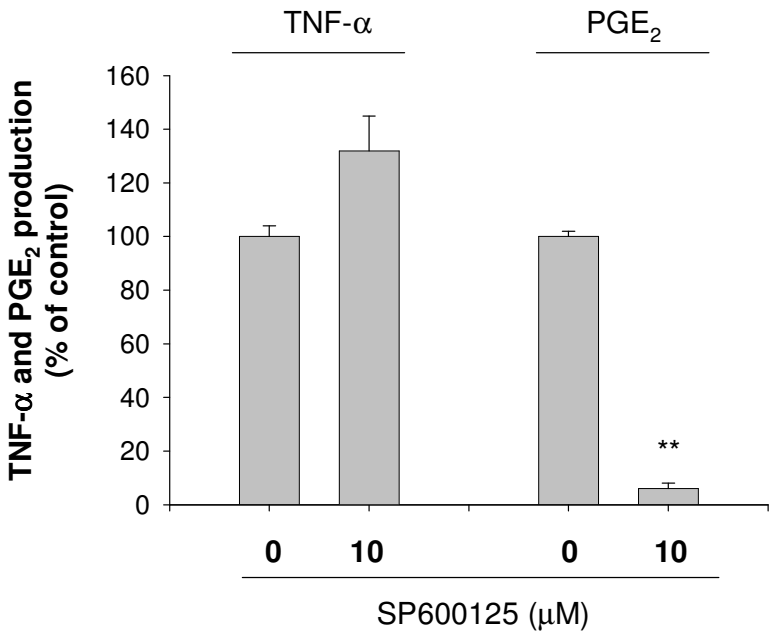


**Figure 6.** Effect of SP600125 on TNF-α and $PGE_2$ production. RAW264.7 cells ($2\times10^6$ cells/ml) were incubated with indicated concentrations of Sc-EE in the presence of LPS (1 μg/ml) for 24 ($PGE_2$), and 6 h (TNF-α). $PGE_2$ and TNF-α levels in culture supernatant were determined by EIA, and ELISA. $^{**}p<0.01$ represents significant difference compared to LPS alone.

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