

Diosmin suppresses the proinflammatory mediators in lipopolysaccharide-induced RAW264.7 macrophages via NF- κ B and MAPKs signal pathways

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Abstract. Diosmin is an unsaturated flavonoid glycoside, presents in citrus fruits. The aim of this study is to investigate the molecular mechanism of diosmin with respect to the NF- κ B and MAPKs signaling pathways. Firstly, 10, 20, 30, 40 and 50 μ M diosmin were treated to lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The anti-inflammatory effects of diosmin was displayed via measuring prostaglandin E₂ (PGE₂), nitric oxide (NO), interleukines (IL-6, IL-12), tumor necrosis factor α (TNF- α) production, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), IL-6, IL-12, TNF- α mRNA levels, and phosphorylation levels of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (I κ B- α) and mitogen-activated protein kinases (MAPKs); JNK, ERK, and p38 in LPS induced RAW264.7 macrophages. Our study showed that especially high concentrations of diosmin decreased NO, PGE₂, IL-6, IL-12, TNF- α production and mRNA levels of these mediators ($p < 0.05$). The expression of phosphorylated-JNK was significantly suppressed by diosmin at 40 and 50 μ M concentrations. Furthermore, diosmin significantly inhibited the expression of phosphorylated-ERK, p38, and p-I κ B- α in a dose-dependent manner. Our results suggest that diosmin is a potent anti-inflammatory agent and has potential for development into a therapeutic agent for inflammation-associated disorders.

Key words: Diosmin — Inflammation — Raw264.7 — Lipopolysaccharide — MAPKs — NF- κ B

Introduction

Inflammation is a kind of innate immunity that provides a defense against pathogens, damaged cells, and other dangerous molecules. Inflammatory cells, such as macrophages and neutrophils, let leukocytes and plasma components come to sites where infection or injury has occurred during inflammation to eliminate dangers (Nowarski et al. 2013; Lee et al. 2016). Although inflammation is important to the immune system, excessive activity of inflammatory cells can cause cancer, rheumatoid arthritis, multiple sclerosis, chronic asthma, psoriasis, and other diseases (Gautam and Jachak 2009; Nowarski et al. 2013; Ren et al. 2016). To treat those inflammation-driven diseases, inflammatory responses should be controlled.

Macrophages activated by lipopolysaccharide (LPS) can enhance the production of inflammatory mediators and cytokines, including nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-6, IL-12, and tumor necrosis factor- α (TNF- α) (Yun et al. 2014). The production of proinflammatory markers is closely linked to the activation of nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs) in macrophages (Jung et al. 2007). NF- κ B, a primary transcription factor, regulates the expression of various cellular gene-encoding factors for inflammatory responses. The MAPK signaling pathways, which include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, are known to induce COX-2 and iNOS expression in LPS-stimulated macrophage (Song et al. 2016). Moreover, Guha and Mackman (2001) have reported that MAPKs play a critical role in the activation of the NF- κ B signaling pathway. Therefore, compounds that regulate both the NF- κ B and MAPKs pathways have the potential to prevent inflammatory diseases (Song et al. 2016).

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Diosmin (3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside) is an unsaturated flavonoid glycoside, presents in citrus fruits (Melin et al. 1996). It is an oral phlebotropic drug used in the treatment of venous disease, that is, chronic venous insufficiency (CVI) and hemorrhoidal diseases (Cesarone et al. 2005). It might have potential in the treatment of neurodegenerative disease such as Alzheimer's diseases, and its anti-inflammatory and antiapoptotic activities have been demonstrated in neuronal cells (Dholakiya and Benzeroual 2011). Also it has antioxidant antihyperglycemic, and antimutagenic properties (Rajasekar et al. 2016). In rat models, Ahmed and colleagues (2016) showed a nephroprotective role for diosmin against alloxan-induced nephropathy, while Tahir et al. (2013) highlighted its beneficial effect against alcoholic liver injury. Moreover, diosmin could successfully improve cardiac function and exert antihyperlipidemic effects against isoproterenol-induced myocardial injury in rats (Queenthy and John 2013). However, there is no detailed research regarding the anti-inflammatory mechanism of diosmin. In this study, we aimed to explore the molecular mechanism of diosmin with respect to the NF- κ B and MAPKs signaling pathways.

Materials and Methods

Materials

RAW264.7 cells, a transformed macrophage cell line derived from the BALB/c mouse, were purchased from American Type Cell Culture (ATCC) (Rockville, MD, USA). Primary antibodies against β -actin, JNK, ERK, p38, phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (p-I κ B- α), phosphorylated-JNK, phosphorylated-ERK, and phosphorylated-p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A protein assay kit was obtained from Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). The enzyme-linked immunosorbent assay (ELISA) kits used to quantitate the levels of PGE₂ were purchased from Lifespan Biosciences (Seattle, WA, USA). Diosmin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-NAME, LPS, and Griess reagent were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Specific PCR primers for genes *IL-6*, *IL-12*, *TNF- α* , *iNOS*, *COX-2*, and *β -actin* were synthesized from PRZ BioTECH (Bilkent, Ankara, Turkey). All solvents used were of HPLC grade.

Cell culture

The RAW 264.7 murine macrophage cell line was purchased from ATCC (ATCC number TIB-71). The cells were grown

in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulphate, and 0.25 μ g/ml amphotericin B) to a subconfluent density. The cells were cultured at 37°C in a humidified incubator in an atmosphere of 5% CO₂ (Meng et al. 2013).

Cell viability assay

MTT assay was performed to determine the cytotoxicity and cell viability of diosmin on RAW 264.7 macrophages. The 100 μ l of RAW 264.7 macrophages was seeded in triplicate into 96-well plates (2×10^4 cells/well) and incubated for 24 h (Lee et al. 2013). The macrophages were treated with various gradient concentration of diosmin with serial dilutions at 2.5, 5, 7.5, 10, 20, 30, 40, 50, 100, 150, 200, 250, and 500 μ M and then incubated for 24 h. Briefly, thereafter, 10 μ l of MTT solution (10 mg/ml) in phosphate-buffered solution (pH 7.4) was added to each well and then followed by incubation for another 3 h. The medium was removed and the purple formazan crystals formed were dissolved by adding 100 μ l dimethyl sulfoxide (DMSO). The plate was swirled gently to mix well and kept in dark condition at room temperature for 30 min. The absorbance was determined by using Microplate Reader 680 XR (Bio-Rad Laboratories, California, USA) at 570 nm wavelength.

The percentage of surviving cells was calculated using the following formula: Cell viability (%) = (mean absorbance in test wells/mean absorbance in control wells) \times 100 (Baek et al. 2015).

NO production assay

RAW264.7 macrophages were plated in a 96-well plate (2×10^4 cells/well) overnight, followed by the addition of 1 μ g/ml LPS for 24 h in the presence or absence of different concentration of diosmin with increasing concentration at 10, 20, 30, 40, and 50 μ M. To analyze NO production, 100 μ l of supernatant was incubated with equal volume of Griess solution (0.1% N-1-naphthylethylendiamide-dihydrochloride (NED), 1% sulphanilamide, and 2.5% phosphoric acid) in room temperature for 10 min in dark condition and absorbance was then read at 540 nm. Since NO content was reflected by the amount of nitrite, a calibration curve was generated using sodium nitrite. The amount of nitrite in the supernatants was calculated based on the calibration curve. The percentage inhibition of NO production is evaluated using the formula $1 - \{(\text{nitrite amount of fraction-treated}) / (\text{nitrite amount of vehicle})\} \times 100$ (Baek et al. 2016).

Determination of PGE₂ levels

To assess whether diosmin could inhibit the production of PGE₂ in LPS-induced RAW 264.7 macrophages, the cells

were pretreated with diosmin for 1 h before incubation with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h. After incubation for 24 h, the cell culture medium was harvested, and the production of PGE_2 was measured using an ELISA. The levels of PGE_2 were determined using PGE_2 ELISA kit (Lifespan Biosciences, Seattle, WA, USA) according to the manufacturer's instruction (Yi et al. 2016). Briefly, cells were grown at a density of 2×10^4 cells/well and treated with different concentrations of diosmin for 1 h before incubation with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h. Following incubation, 50 μl of medium was added to wells of antibody coated 96-well plates. Then, 50 μl of mouse anti- PGE_2 and 50 μl of diluted conjugate were added and incubated for 1 h at room temperature on well plate shaker. The reaction mixture was removed from each well and washed four times with washing buffer. Subsequently, 150 μl of 3,3',5,5'-tetramethylbenzidine (TMB) enzyme substrate were added into each well and incubated for 30 min at room temperature (RT) on microplate shaker. After adding 100 μl of stop solution, the OD was measured at 450 nm using Microplate Reader 680 XR (Bio-Rad Laboratories, California, USA). The data were expressed as the means of at least three independent experiments. Each value was expressed as the mean \pm SD of triplicate experiments.

Cytokine determination

The levels of IL-6, IL-12, and TNF- α were determined using Lifespan Biosciences ELISA kits (Seattle, WA, USA) according to the manufacturer's instructions (Rajapakse et al. 2008). Briefly, the cells were grown at a density of 2×10^4 cells/well and treated with different concentrations of diosmin (10–50 μM) for 1 h before incubation with LPS (1 $\mu\text{g}/\text{ml}$) for the indicated times. Following incubation, 50 μl of medium was added to wells of antibody-coated 96-well plates. Then, 50 μl of biotinylated antibody reagent was added and incubated for 3 h at RT. The reaction mixture was removed from each well and washed four times with washing buffer. Subsequently, 100 μl of streptavidin conjugated to horseradish peroxidase was added and incubated for 30 min at RT. After the same washing step as above, 100 μl of TMB substrate solution was added and incubated for 30 min at room temperature. After adding 50 μl of stop solution, the optical density was measured at 450 nm using Microplate Reader 680 XR (Bio-Rad Laboratories, California, USA).

Measurement of mRNA expression levels

RAW264.7 cells (5×10^4 cells/well) were seeded in 24-well plates. After reaching approximately 70% confluence, each well was treated with various concentrations of diosmin together with LPS (1 $\mu\text{g}/\text{ml}$). After 4 h incubation, total RNA was isolated using TRIzol reagent (Invitrogen,

Carlsbad, CA, USA). Real-time RT-PCR was performed with a Roche LightCycler 480 II device using SYBR Green PCR master mix (Invitrogen, Carlsbad, CA, USA). PCR amplification was carried out using the following primers: *IL-6*, forward (5'-CCT CTG GTC TTC TGG AGT ACC-3') and reverse (5'-ACT CCT TCT GTG ACT CCA GC-3') (Zhu et al. 2015); *IL-12*, forward (5'-TGC TCA TGG CTG GTG CAA AG-3') and reverse (5'-TGG ACT TCG GTA GAT GTC TTC-3') (Saito et al. 2006); *TNF- α* , forward (5'-ATG AGC ACA GAA AGC ATG A-3') and reverse (5'-AGT AGA CAG AAG AGC GTG GT-3') (Zhu et al. 2015); *iNOS*, forward (5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3') and reverse (5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'); *COX-2*, forward (5'-GTC TGA TGA TGT ATG CCA CAA TCT G-3') and reverse (5'-GAT GCC AGT GAT AGA GGG TGT TAA A-3') and *β -actin* (as a mouse housekeeping gene), forward (5'-AGG TCA TCA CTA TTG GCA AC-3') and reverse (5'-ACT CAT CGT ACT CCT GCT TG-3') (Berkoz and Allahverdiyev 2017). All mRNA expression was normalized using *β -actin*. Relative expression levels were calculated using the comparative method.

Western blotting

RAW264.7 macrophages were cultured in DMEM containing 10% (v/v) FBS at 70–80% confluency. The cells were incubated with 1 $\mu\text{g}/\text{ml}$ LPS for 24 h. The cells were then lysed with $1 \times$ cell lysis buffer (Cell Signaling Technology), and protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal quantities of total protein were loaded onto a 10% SDS polyacrylamide gel (Bio-Rad Laboratories) for separation. The separated proteins were transferred to Immobilon P membranes (Millipore, Billerica, MA, USA). Nonspecific proteins were blocked with 5% fat-free milk for 1 h, before the primary antibody was treated at 4°C overnight. Protein bands were detected with a chemiluminescence detection kit (GE Healthcare, NJ, USA) after hybridization with an HRP-conjugated secondary antibody (Cell Signaling Technology, Massachusetts, USA). The p-I κ B- α protein level was expressed as a relative value to that of β -actin. p-JNK, p-ERK, and p-p38 levels were expressed as a relative value to that of JNK, ERK, and p38, respectively. Relative protein levels were quantified by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as means \pm standard deviation (SD) of three replicate determinations. Statistical analysis was performed using the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey *post hoc*

test for multiple comparison. The p value of less than 0.05 was considered as a significant difference. Statistical analysis was conducted using SPSS statistics software (version 15.0, SPSS Inc, Chicago, IL, USA).

Results

We first investigated the cytotoxicity of diosmin to determine the applicable treatment concentration range. MTT reduction assay was used to access the cytotoxicity effect of diosmin at concentration ranging from the lowest to highest (2.5–500 μM) on RAW 264.7 macrophages. The cytotoxicity potential of diosmin on macrophages was presented in Figure 1. A decrease in cell viability by more than 15% when compared to control cells was considered to be cytotoxic as determined by MTT assay. The results showed that increasing concentrations of diosmin have caused reduction of cell viability. However, diosmin did not exhibit any toxicity to macrophages at concentrations ranging from 10 to 50 μM . According to the cytotoxicity investigations, the concentrations at 10, 20, 30, 40, and 50 μM were chosen for further anti-inflammatory experiments.

We measured NO and PGE₂ production in LPS-induced RAW264.7 macrophages for determining the possible anti-inflammatory effects of diosmin (Fig. 2). The level of NO was markedly increased after treatment with LPS for 24 h; however, diosmin significantly suppressed the release of NO compared with the control exposed to LPS only ($p < 0.001$). Treatment with diosmin at concentration of 40 μM decreased NO production by approximately 36%, indicating that this concentration of diosmin possesses highest inhibition of NO production in RAW264.7 ($p < 0.05$). However, 10 and 20 μM diosmin showed no statistically significant effect on NO generation compared to control group treated with LPS only ($p > 0.05$) (Fig. 2A).

The production of PGE₂ was significantly increased after treatment with LPS for 24 h; however, diosmin significantly suppressed the production of PGE₂ compared with the control exposed to LPS only ($p < 0.001$). Of particular note, treatment with diosmin at concentration of 50 μM decreased PGE₂ production by approximately 33%, indicating that this concentration of diosmin possesses highest inhibition of PGE₂ production in RAW264.7 ($p < 0.05$). However, 10 and 20 μM diosmin showed no statistically significant effect on PGE₂ generation compared to control group treated with LPS only ($p > 0.05$) (Fig. 2B).

The effect of diosmin on the expression of cytokines, including IL-6, IL-12, and TNF- α , was investigated using LPS-induced RAW264.7 cells (Fig. 3). As shown in Figure 3, very low levels of cytokines were observed in LPS-untreated RAW264.7 cells, whereas macrophages treated with LPS alone for 24 h significantly ($p < 0.001$) showed an enhanced release of IL-6, IL-12, and TNF- α . Diosmin treatment significantly suppressed IL-6, IL-12, and TNF- α production at all concentrations ($p < 0.05$). Diosmin at 40 μM concentration exhibited the highest inhibitory effect of IL-6 ($p < 0.05$) (Fig. 3A). Moreover, diosmin at 50 μM concentration showed highest inhibitory effect of IL-12 ($p < 0.001$) (Fig. 3B). The release of TNF- α was significantly decreased by treatment with diosmin in a dose-dependent manner ($p < 0.05$). Diosmin at 50 μM concentration exhibited highest inhibitory effect of TNF- α ($p < 0.001$) (Fig. 3C).

RT-PCR was performed to determine whether the inhibitory effects of diosmin on mRNA expression of *iNOS*, *COX-2*, *IL-6*, *IL-12*, and *TNF- α* (Fig. 4). As shown in Figure 4A, diosmin at concentrations of 40 and 50 μM significantly ($p < 0.05$) reduced the expression of *iNOS* compared to control group treated with LPS only. Especially, 40 μM diosmin inhibited the expression of *iNOS* by more than 19.5%. As shown in Figure 4B, diosmin at concentrations of 30, 40 and 50 μM significantly ($p < 0.05$) reduced the expression

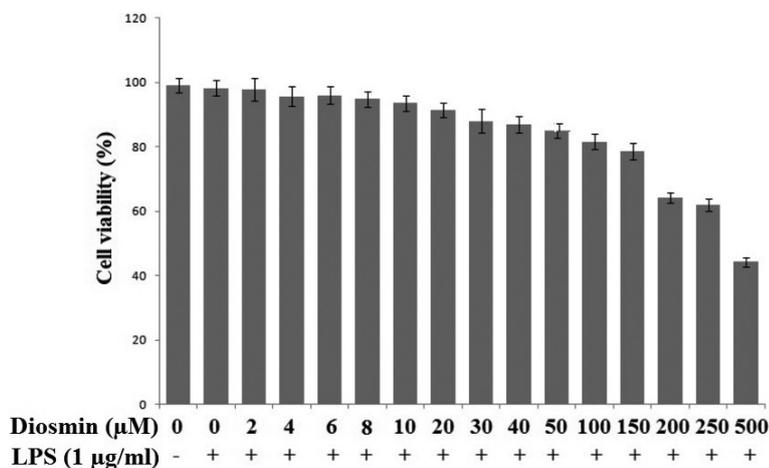


Figure 1. Effect of diosmin on the cell viability of RAW264.7 cells. Cells were plated in 96 well plate at density of 2×10^4 cells/well. The cells were pretreated with various concentrations of samples for 24 h and cytotoxicity was determined by MTT assay.

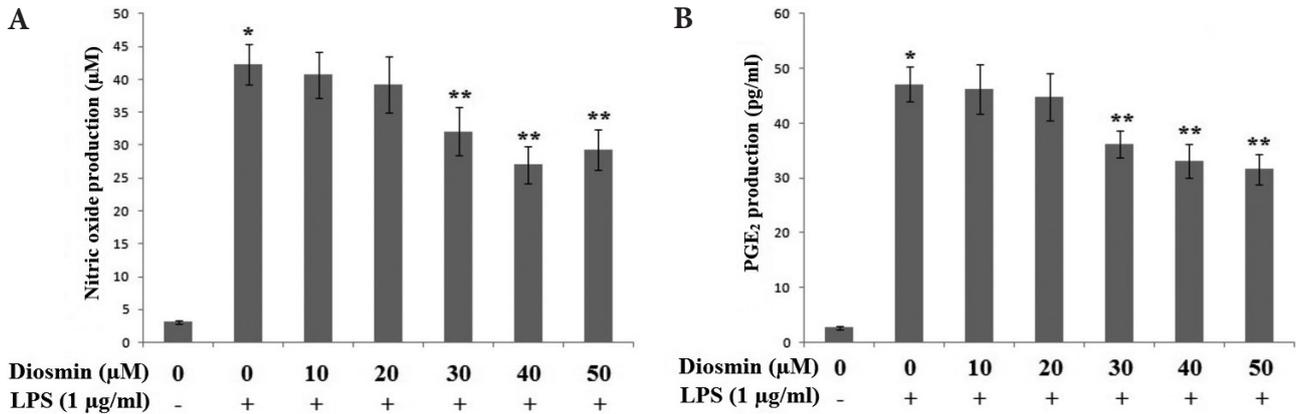


Figure 2. Inhibitory effects of diosmin on NO (A) and PGE₂ (B) production in LPS-induced RAW264.7 macrophages. RAW264.7 macrophages were incubated for 24 h, and exposed to diosmin together with LPS (1 μg/ml) for 24 h. Data represent the means ± SD of three independent experiments. * $p < 0.001$ in comparison to untreated controls; significant difference was determined using unpaired Student's t -test; ** $p < 0.05$ in comparison to cells treated with LPS alone. (One-way ANOVA followed by Tukey's test for multiple comparison).

of COX-2 compared to control group treated with LPS only. Especially, 50 μM diosmin inhibited the expression of COX-2 by more than 22.5%. The effect of diosmin on the expression of cytokines, including IL-6, IL-12, and TNF-α, was investigated using LPS-induced RAW264.7 cells. As shown in Figures 4C, D, and E, almost undetectable expres-

sions of cytokines (IL-6, IL-12, and TNF-α, respectively) were observed in LPS-untreated RAW264.7 cells, whereas macrophages treated with LPS alone for 24 h significantly showed an enhanced expression of IL-6, IL-12, and TNF-α ($p < 0.05$). Diosmin inhibited the expression of IL-6 in a dose-dependent manner, but diosmin at concentration of

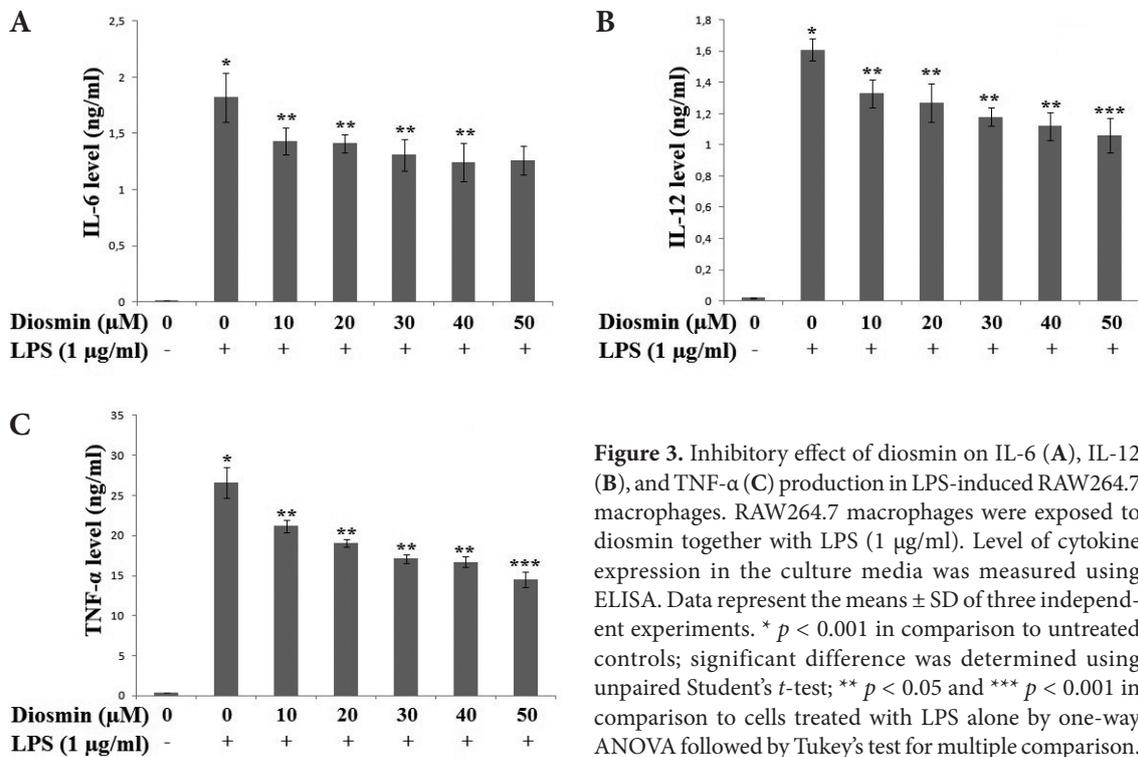


Figure 3. Inhibitory effect of diosmin on IL-6 (A), IL-12 (B), and TNF-α (C) production in LPS-induced RAW264.7 macrophages. RAW264.7 macrophages were exposed to diosmin together with LPS (1 μg/ml). Level of cytokine expression in the culture media was measured using ELISA. Data represent the means ± SD of three independent experiments. * $p < 0.001$ in comparison to untreated controls; significant difference was determined using unpaired Student's t -test; ** $p < 0.05$ and *** $p < 0.001$ in comparison to cells treated with LPS alone by one-way ANOVA followed by Tukey's test for multiple comparison.

40 μM showed the highest inhibitory effect on *IL-6* expression (Fig. 4C) ($p < 0.05$). Diosmin inhibited the expression of *IL-12* and *TNF- α* in a dose-dependent manner ($p < 0.05$), and diosmin at concentration of 50 μM showed the highest inhibitory effect on *IL-12* and *TNF- α* expression (Fig. 4D and E) ($p < 0.05$).

The effect of diosmin on the activation of NF- κB was investigated by analyzing the inhibition of I $\kappa\text{B-}\alpha$ phosphorylation in LPS-induced RAW264.7 cells. As shown in Figure 5, the activation of I $\kappa\text{B-}\alpha$ *via* phosphorylation significantly increased in LPS-stimulated RAW264.7 cells, whereas di-

osmin inhibited the activation of I $\kappa\text{B-}\alpha$ ($p < 0.05$). It was found that treatment with diosmin at 50 μM significantly triggered the highest inhibitory effect on phosphorylation of I $\kappa\text{B-}\alpha$ ($p < 0.001$).

The inhibitory effects of diosmin on LPS-induced MAPKs (JNK, ERK, and p38) phosphorylation were investigated (Fig. 6). The phosphorylation levels of JNK, ERK, and p38 were increased in LPS-induced RAW264.7 macrophages, as shown in Figures 6A, B, and C, respectively. Diosmin at 40 and 50 μM concentrations significantly suppressed the expression of phosphorylated-JNK compared with control

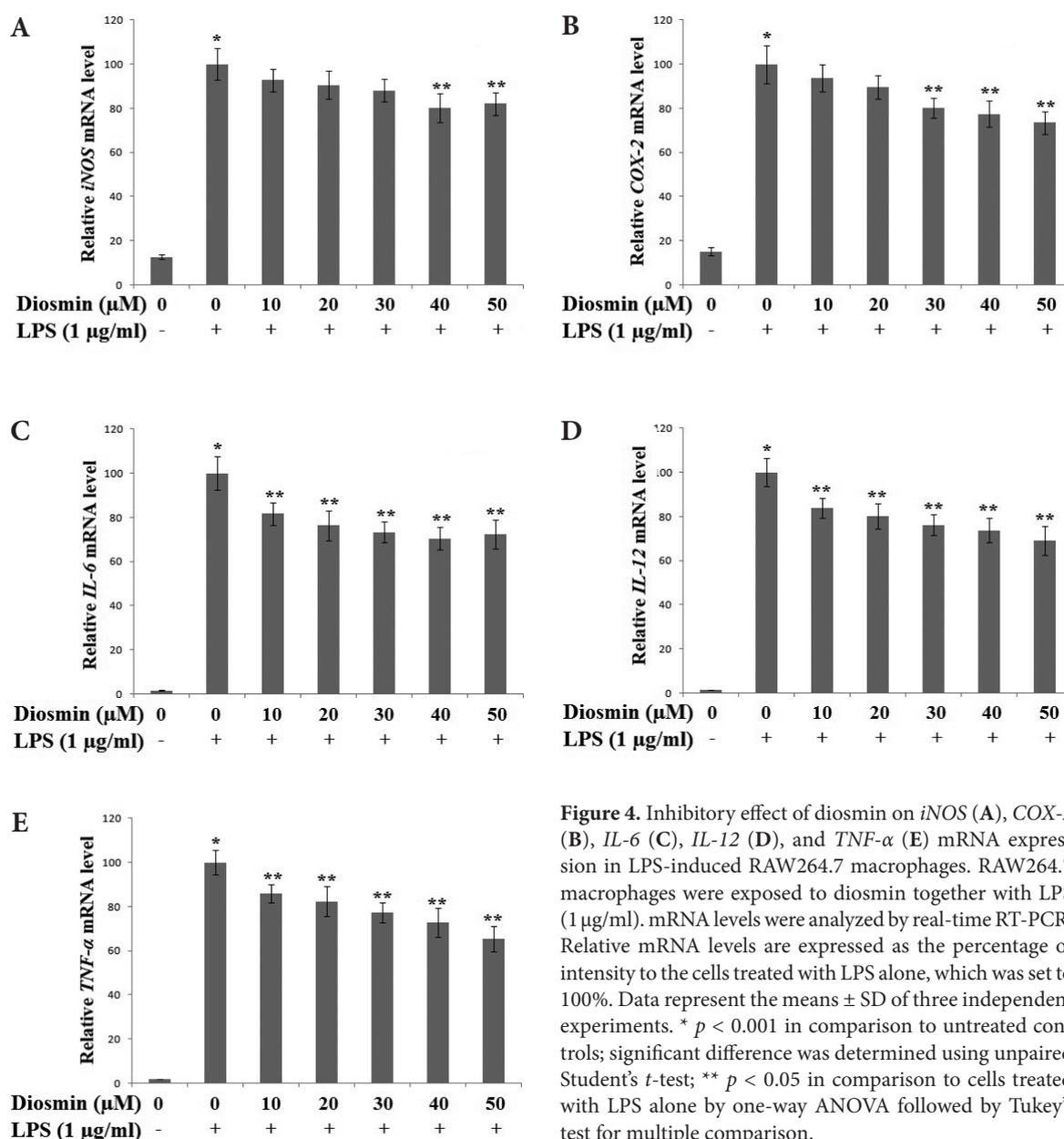


Figure 4. Inhibitory effect of diosmin on *iNOS* (A), *COX-2* (B), *IL-6* (C), *IL-12* (D), and *TNF- α* (E) mRNA expression in LPS-induced RAW264.7 macrophages. RAW264.7 macrophages were exposed to diosmin together with LPS (1 $\mu\text{g/ml}$). mRNA levels were analyzed by real-time RT-PCR. Relative mRNA levels are expressed as the percentage of intensity to the cells treated with LPS alone, which was set to 100%. Data represent the means \pm SD of three independent experiments. * $p < 0.001$ in comparison to untreated controls; significant difference was determined using unpaired Student's *t*-test; ** $p < 0.05$ in comparison to cells treated with LPS alone by one-way ANOVA followed by Tukey's test for multiple comparison.

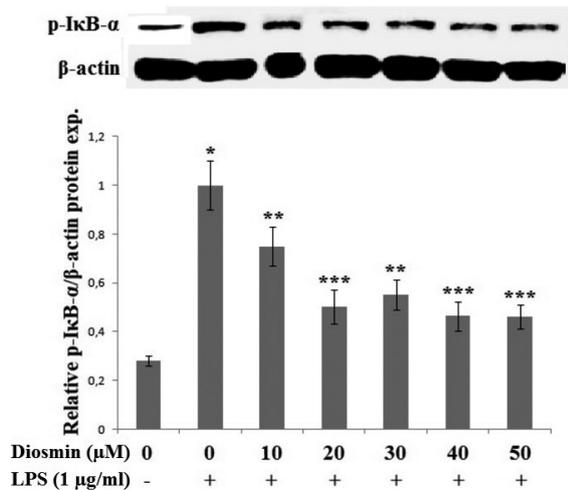


Figure 5. Effect of diosmin on phosphorylation of IκB-α in LPS-stimulated RAW264.7 macrophages. RAW264.7 macrophages were incubated for 24 h and exposed to LPS (1 μg/ml) with diosmin for 24 h. Levels of IκB-α and phosphorylated IκB-α were determined using Western blot assay. Relative protein levels are expressed as the fold of intensity to the cells treated with LPS alone, which was set to 1.00. Data represent the means ± SD of three independent experiments. * $p < 0.001$ in comparison to untreated controls; significant difference was determined using unpaired Student's *t*-test; ** $p < 0.05$ and *** $p < 0.001$ in comparison to cells treated with LPS alone by one-way ANOVA followed by Tukey's test for multiple comparison.

group treated with LPS only ($p < 0.001$), but this suppression didn't statistically significant on the other concentrations ($p > 0.05$) (Fig. 6A). Moreover, diosmin significantly inhibited the expression of phosphorylated-ERK and p38 in a dose-dependent manner, in an other saying, diosmin at concentration of 50 μM showed the highest suppression effect on the phosphorylation of ERK and p38 ($p < 0.001$) (Fig. 6B,C).

Discussion

Inflammation involves many complex interactions between cellular and inflammatory mediators. During the inflammatory process, significant amounts of NO and prostaglandin E2 are produced *via* the expression of *iNOS* and *COX-2* (Lu et al. 2017). The excessive production of proinflammatory mediators by macrophages can induce variety of inflammation-related disorders; thus, agents that suppress the production of these factors may have the potential to protect against inflammatory diseases (Jung et al. 2007; Yun et al. 2014; Song et al. 2016). We investigated the inhibitory effects of diosmin on LPS-induced proinflammatory media-

tors in RAW264.7 cells. The results revealed that the inhibition of LPS-induced NO and PGE₂ production by diosmin was accompanied by a reduction in *iNOS* and *COX-2* gene expressions, respectively. Macrophage activation induced by LPS resulted in the secretion of typical proinflammatory cytokines including IL-6, IL-12, and TNF-α (An et al. 2006). TNF-α is a key mediator in the inflammatory process and stimulates other cytokines such as IL-6 and IL-12. Additionally, the cytokines IL-6 and IL-12 are known to upregulate the production of *iNOS* in macrophages (Jana et al. 2003), while IL-12 also promotes NF-κB activation in macrophages (Pahan et al. 2001). These cytokines induce the expression of *iNOS* and NO, and overexpression of proinflammatory mediators can result in tissue injury and multiple organ failure (Amirshahrokhi and Khalili 2016). The upregulated IL-6, IL-12, and TNF-α levels in LPS-induced RAW264.7 cells were significantly decreased with treatment of diosmin compared with control group treated with LPS only. 40 and 50 μM diosmin were more potent inhibitor of the cytokines in this study. Diosmin at all concentrations significantly reduced the mRNA expression of *IL-6*, *IL-12*, and *TNF-α* compared with control group treated with LPS only. Furthermore, diosmin at concentrations of 40 and 50 μM showed higher inhibitory effect on *IL-6*, *IL-12*, and *TNF-α* mRNA expression. We have estimated that, diosmin may inhibit posttranscriptional pathway of *IL-6* and *IL-12* proteins.

LPS activates typical inflammatory signaling pathways, including the NF-κB signaling pathway. NF-κB is a mammalian transcription factor that regulates proinflammatory mediators and cytokines in LPS-induced macrophages and exists as a heterodimer comprising p50 and p65 subunits known to mediate the expression of genes associated with immune modulation (Gao et al. 2013). In unstimulated macrophages, NF-κB remains inactive in the cytoplasm bound to IκB-α. The activation of NF-κB by LPS stimulation occurs through phosphorylation and subsequent degradation of IκB-α, followed by nuclear translocation of free NF-κB (Liu et al. 2002). Following activation, NF-κB regulates gene transcription of *iNOS*, *COX-2*, *IL-6*, *IL-12*, and *TNF-α* (Kim et al. 2010). Our results show that diosmin significantly reduced LPS-induced phosphorylation of IκB-α. These results demonstrate that NF-κB activation by phosphorylation of IκB-α is suppressed by diosmin. 50 μM diosmin showed the highest inhibitory effect on phosphorylation of IκB-α.

The activation of MAPK pathways in macrophages is strongly associated with the inflammatory response and activates downstream proinflammatory cytokines and mediators. The MAPKs include ERK, p38, and JNK, and their signaling pathways play a crucial role in biological processes in addition to typical inflammatory signaling (Sui et al. 2014). MAPK signaling can be activated by LPS, which upregulates the production of NO in RAW264.7 cells (Diao et al. 2014).

The activation of ERK in response to LPS in turn leads to the upregulation of *iNOS* and proinflammatory cytokines (Han et al. 2013). Furthermore, LPS-induced *COX-2*, *iNOS*, and *TNF- α* expression in macrophages is regulated by p38 and JNK (Kim et al. 2010). In this study, the phosphorylation levels of p38 and ERK were significantly attenuated by treatment of diosmin compared with RAW264.7 cells treated with LPS only. Increased phosphorylation level of JNK was significantly reduced by 40 and 50 μ M diosmin treatment. These results demonstrate that diosmin is a potent inhibitor of MAPK signaling, resulting in a greater reduction of proinflammatory mediators and cytokines. Blockade of NF- κ B and MAPK signaling pathways by diosmin could cause potential inhibition of LPS-induced *COX-2*, *iNOS*, NO, and cytokine production.

Some flavonoids from natural products are known to inhibit and/or reduce the progression of inflammation (Kim et al. 2004). Flavonoids appear to exhibit anti-inflammatory properties *via* the modulation of reactive oxygen species that induce the activation of NF- κ B and subsequent release of cytokines (Martínez-Flórez et al. 2005). Hajimahmoodi et al. (2014) have reported that diosmin has a powerful

DPPH radical scavenging, reducing power, superoxide anion scavenging activity, and directly scavenging intracellular radicals. We therefore speculate that diosmin, which exhibits a potent antioxidant property, may show an anti-inflammatory effect.

In conclusion, in this study, anti-inflammatory effects of diosmin was investigated in LPS-stimulated RAW264.7 macrophages. Our findings indicate that diosmin at high concentrations (40 and 50 μ M) exhibits more potent inhibition of the production of proinflammatory mediators including cytokines, NO, and PGE₂ in LPS-stimulated RAW264.7 macrophages. These outcomes are mediated by the suppression of NF- κ B activation by preventing I κ B- α degradation and MAPK phosphorylation in LPS-stimulated RAW264.7 macrophages. Taken together, our results suggest that diosmin is a potent anti-inflammatory agent and has potential for development into a therapeutic agent for inflammation-associated disorders.

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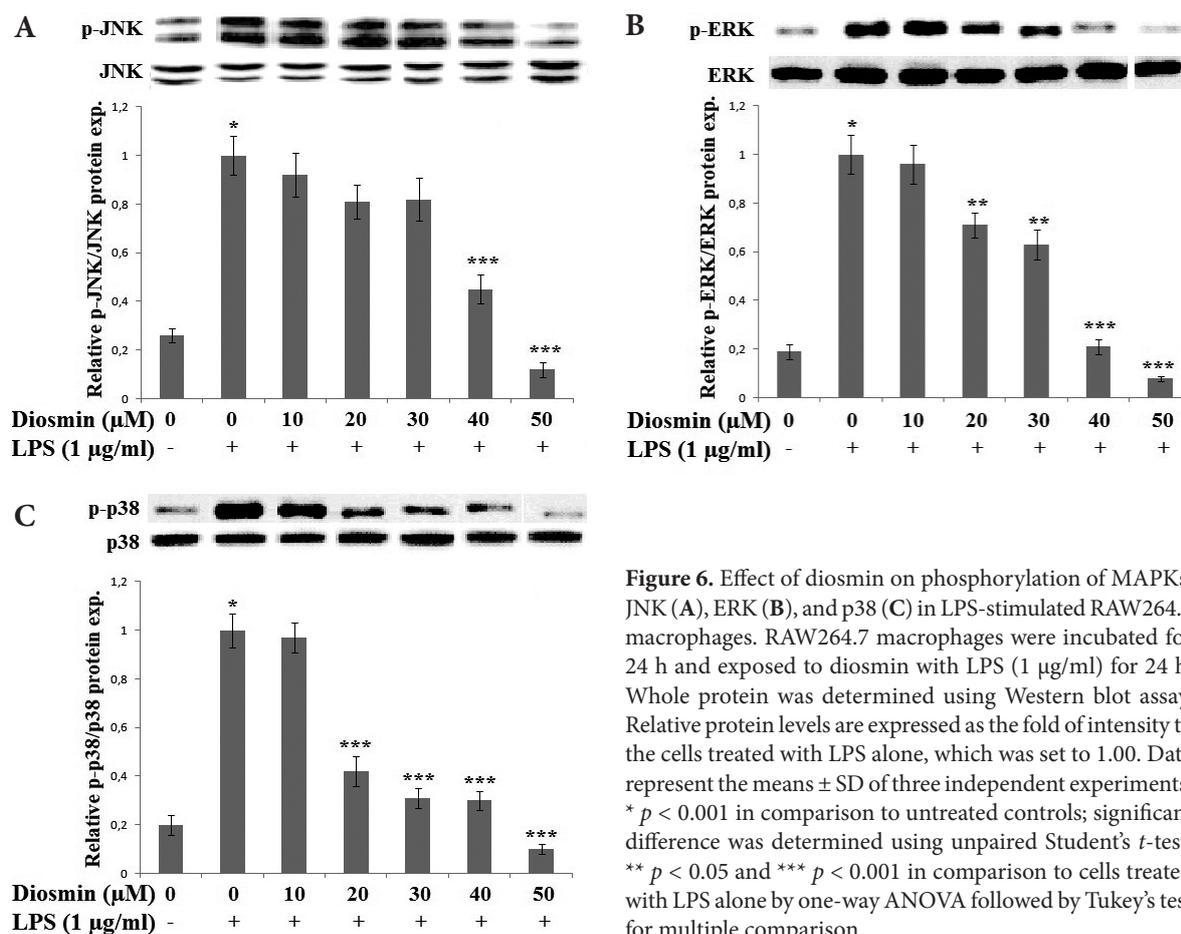


Figure 6. Effect of diosmin on phosphorylation of MAPKs; JNK (A), ERK (B), and p38 (C) in LPS-stimulated RAW264.7 macrophages. RAW264.7 macrophages were incubated for 24 h and exposed to diosmin with LPS (1 μ g/ml) for 24 h. Whole protein was determined using Western blot assay. Relative protein levels are expressed as the fold of intensity to the cells treated with LPS alone, which was set to 1.00. Data represent the means \pm SD of three independent experiments. * $p < 0.001$ in comparison to untreated controls; significant difference was determined using unpaired Student's *t*-test; ** $p < 0.05$ and *** $p < 0.001$ in comparison to cells treated with LPS alone by one-way ANOVA followed by Tukey's test for multiple comparison.

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