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Original Article

PECTINASE-MODIFIED RED GINSENG (GS-E3D) INHIBIT NF-KB TRANSLOCATION AND NITRIC OXIDE PRODUCTION IN LIPOPOLYSACCHARIDE-STIMULATED RAW 264.7 CELLS

S. E. CHUL HONG[1], MYEONG HWAN O. H.[1], HWAN LEE[1], YOUNG SIK PARK[1], N. A. YOUNG KIM[1], S. O. HEE PARK[1], JONG DAE PARK[1], JIN DONG JANG[2], SUN HWA KIM[2], EUN J. I. KIM[2], M. I. KYUNG PYO[1]*

[1]International Ginseng and Herb Research Institute, Geumsan, 312-804, Korea, [2]DOORI Cosmetics CO.,Ltd, Seoul, 137-071, Korea
Email: pmk67@ginherb.re.kr

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ABSTRACT

Objective: Red ginseng has been used as traditional medicines and functional foods in the world, because of its health benefits. The aim of this study was to elucidate the anti-inflammatory effect and mechanism of pectinase-modified red ginseng (GS-E3D) with a cellular model of lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

Methods: To study the anti-inflammatory effect of GS-E3D, the key inflammation mediators such as nitric oxide (NO),prostaglandin E_2 (PGE_2), inducible nitric oxide synthase (iNOS), Cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF-α), and interleukin (IL)-6 production as well as on nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) activation, were measured by using the enzyme linked immunosorbent assay (ELISA)and Western blotting.

Results: GS-E3D potently inhibited TNF-α and IL-6 and also diminished NO over-production, which was accompanied by the down-regulation of iNOS expression. GS-E3D effectively suppressed LPS-induced NF-κB activation through inhibiting the hyper-phosphorylation and degradation of IκB-α and phosphorylation of p38, ERK1/2 and JNK in MAPK signaling pathway.

Conclusion: GS-E3D has a potential to be as an anti-inflammatory agent for functional food or cosmetic materials targeting on the NF-κB p65 and MAPKs signaling pathways.

Keywords: GS-E3D, Lipopolysaccharide (LPS), Inflammation, Nuclear factor kappa B (NF-κB), Mitogen-activated protein kinases (MAPKs)

INTRODUCTION

Inflammatory response is mediated by oxidative stress occurring in the body which increase the gene expression of a particular cell to cause cell death, as well as responsible for the initiation of degenerative diseases or became attenuating the inflammatory response [1]. Macrophages are immune cells distributed to all tissues which are responsible for innate immune response, as the white blood cells have played a key role in the body's immune system. Macrophagesin inflammations are activated by bacterial lipopolysaccharide (LPS) of cell wall components of Gram-negative bacteria [2]. Activated macrophages produce inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), inducible nitric oxide synthase (iNOS), Cyclooxigenase-2 (COX-2), tumor necrosis factor (TNF-α),and interleukin (IL)-6 [3]. LPS stimulate a serial of signal transduction events which lead to the activation of pro-inflammatory cytokine such as nuclear factor kappaB (NF-κB) and mitogen-activated protein kinases (MAPKs) such as ERK, JNK and p38 [4].

Ginseng is used as a popular herbal medicine and functional food in the world andits pharmacological constituents, ginsenosides, exhibit various biological effects, including anti-inflammatory and antitumor activity [5-7]. Ginsenosides are about 40 types have been known up to now, but the main components of ginseng is six numbers, namely, ginsenoside Rg1, Re, Rb1, Rb2, Rc and Rd. Ginsenoside Rb1, Rb2 and R care transformed into ginsenoside Rd, F2 and compound K by microbial enzyme such as glycosidase [8]. Ginsenoside Rd exhibits potent anti-inflammatory, anti-obesity, and anti-ischemic effects [9-12]. In this study, we prepared commercial pectin lyase-modified red ginseng extracts (GS-E3D) enhanced with ginsenoside Rd for development of functional food or cosmetic materials and observed its anti-inflammatory effects and mechanism on LPS-stimulated RAW264.7 cells.

MATERIALS AND METHODS

Plant material and preparation of GS-E3D

The *Panax* (*P.*) *ginseng* used in this experiment was 4-year-old dried ginseng purchased from a local market (Wooshin Industrial Co., Ltd., Geumsan, Korea) and was deposited in the International Ginseng and Herb Research Institute (No. GS201104). GS-E3D was prepared according to early our report [13]. Briefly, red ginseng extract adjusted to 6 Brix were incubated with 10% pectin lyase (EC 4.2.2.10, Novozyme, #33095, Denmark) at 50 °C for 5 d in a shaking incubator (150 rpm). To terminate the reaction, processed extracts were heated at 95 °C for 10 min, and then freeze-dried for further experiment. The dried GS-E3D consisted of 120.2 mg/g crude saponin containing the following ginsenosides: 5.9 mg/g Rg1, 12.6 mg/g Re, 4.7 mg/g Rf, 30.2 mg/g Rb1, 14.0 mg/g Rc, 17.6 mg/gRb2, 2.5 mg/g Rb3, 27.7 mg/g Rd, 1.3 mg/g 20(S)-Rg3, 1.4 mg/g 20(R)-Rg3, 0.8 mg/g Rk1, and 1.5 mg/g Rg5.

Cell culture

RAW264.7 cells, a mouse peritoneal macrophage line, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco-BRL). The medium for RAW264.7 cells was routinely changed twice a day. RAW264.7 was passaged by trypsinization until they reached confluency level.

Cell viability assay

To determine the effect of GS-E3D on cell viability, RAW264.7 cells were seeded on 96-well plates at a density of 5×10^4 cells/well. GS-E3D was added at serially indicated concentrations. Control group was treated with an equal amount of DMSO, which resulted in a final concentration of 0.3% DMSO in the medium. After the treatment of GS-E3D at 24 h, 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) solution was added and then the cells were further incubated for another4 h at 37 °C. After removing the medium, 100 μl of DMSO was added to the cells. The absorbance was measured by using anenzyme linked immunosorbent assay (ELISA) microplate

reader at 450 nm. The control group consisted of untreated cells was considered as 100% of viable cells. Results are expressed as percentage of viable cells when compared with control groups.

Determination of NO and PGE_2 production

Inhibitory effect of GS-E3D on NO production by murine macro phage like RAW264.7 cells was evaluated using a method modified from previously reported [14]. Briefly, the RAW264.7 cells (2×10^5 cells/well) in 10% FBS-DMEM without phenol red were seeded in a 6-well plate for 24 h at 37°C. Cells were washed with 1×PBS, replaced with fresh media, and then treated with the varying concentrations of the GS-E3D for 30 min. After 30 min, LPS (1 µg/ml) was treated for 24 h at 37°C. After 24 h of LPS treatment, 100 µl of the medium was transferred in to a 96 well plate and then an equal amount of Griess reagent (1% sulfanilamide and 0.1% N-1-(naphthyl) ethylenediamine-dihydrochloride in 2.5% H_3PO_4) was added. The plate was incubated for additional 5 min at the room temperature and then the absorbance was measured at 540 nm using the ELISA microplate reader. The amount of NO was calculated using sodium nitrite standard curve. For the measurement of PGE_2 production, 50 µl of the supernatant of cultured medium was collected, and PGE_2 production was determined using PGE_2 ELISA monoclonal assay kit followed (Cayman Chemical, Ann Arbor, MI) with manufacturer's instructions.

Determination of TNF-α release

RAW264.7 cells (2×10^5cells/well) in 10% FBS-DMEM were seeded in a 6-well plate for 24 h at 37°C. Cells were washed with 1×PBS, replaced with fresh media, and then treated with the varying concentrations of GS-E3D for 30 min. After 30 min, LPS (1 µg/ml) was treated for 24 h at 37 °C. At 24 h after LPS treatment, 50 µl of the supernatant of cultured medium was collected, and TNF-α was determined using Human TNF-α ELISA kit (R&D systems, Minneapolis, MN) following with manufacturer's instructions.

SDS-PAGE and western blot

The cells were washed with 1 x phosphate-buffered saline (PBS), and lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA), and centrifuged at 15,000 x g at 4°C for 20 min. The supernatant containing protein concentration was determined by Bradford protein assay. Equal amount of proteins were subjected to SDS-PAGE and then transferred onto PVDF membrane. The membranes were blocked for non-specific binding with 5% non-fat dry milk in tris-buffered saline containing 0.05 % Tween 20 (TBS-T) for 1hat room temperature and then probed with the primary antibodies overnight at 4 °C, followed by incubation with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1h at room temperature. Chemi-luminescence was detected with ECL Western blotting substrate (GE Health care, Pittsburgh, PA, USA) and visualized in polaroid film.

RESULTS AND DISCUSSION

***In vitro* cytotoxicity of GS-E3D against RAW264.7 cells**

The cell viability was determined by MTT assay. RAW264.7 cells were treated with various concentrations of GS-E3D for 24 h. As shown in fig. 1A, extracts did not exhibit cytotoxicity at the range of 25-200 µg/ml against RAW264.7 cells. This dose-range was used for the treatment of GS-E3D for further experiments.

GS-E3D inhibits the production of NO and PGE_2 by suppressing iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells

NO is generated from L-arginine by nitric oxide synthase. iNOS is strongly induced by the bacterial toxins and body's inflammatory cytokines [15]. A significant increase of NO by iNOS under pathological conditions, along with other inflammatory mediators causing tissue damage, is a major mediator of inflammatory damage [16,17].

Therefore, compounds which are capable of inhibiting the production of NO by inhibiting iNOS expression or activity may be used as an anti-inflammatory substance. Pro-inflammatory macrophage cytokine activated the cyclooxygenase-2 (COX-2) which plays an important role in the inflammatory phase by increasing the PGE_2 subsequently produced inflammatory reaction causes an inflammatory response.

To determine whether GS-E3D inhibit the production of NO induced by LPS which plays a central role in the inflammatory response, RAW264.7 cells were pretreated with the GS-E3D for 30 min and then stimulated with LPS (1 µg/ml). After stimulation for 24 h, the cell medium was harvested, and the production of NO was measured using the Griess assay. RAW264.7 cells unstimulated by LPS and treated with GS-E3D alone without LPS secreted basal level of NO, while the stimulation of LPS without GS-E3D resulted in an increased level of NO production. GS-E3D significantly inhibited the production of LPS-induced NO in a concentration-dependent manner.

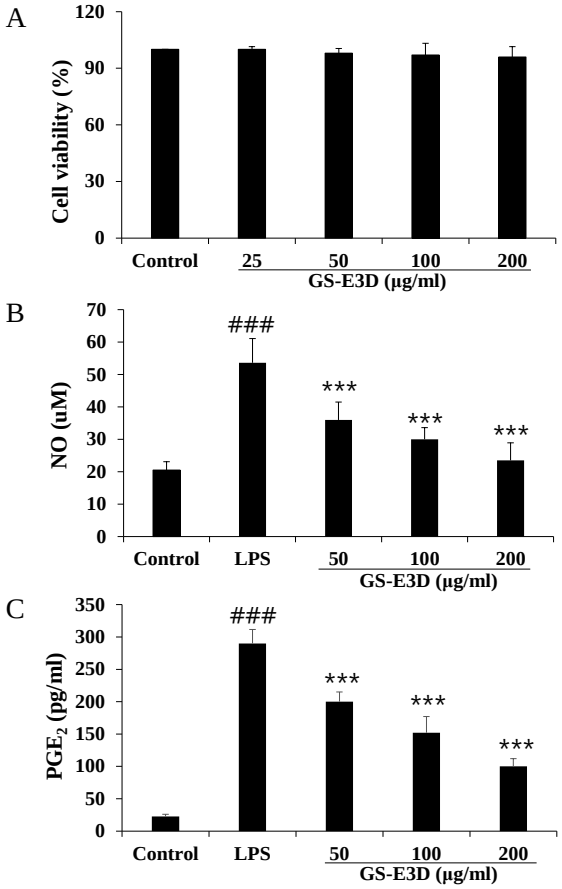

Fig. 1: Cytotoxicity (A) and inhibitory effect of GS-E3D on NO (B) and PGE2 (C) production in LPS-stimulated RAW 264.7cells. The cells were pretreated with the varying concentrations of GS-E3D and then treated with LPS (1 µg/ml). Data are expressed as the mean±SEM. (n=5). ### p<0.001 compared to control group. *** p<0.001 compared to LPS group (ANOVA followed by Tukey's test)

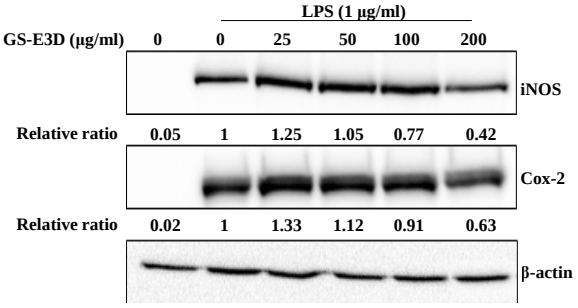

Fig. 2: Inhibitory effect of GS-E3D on iNOS and COX-2 protein expression in LPS-stimulated RAW264.7 cells. The cell was co-treated with GS-E3D (25-200 µg/ml) and with or without LPS (1µg/ml) for 24 h. In western blot, each well was loaded with 25 µg protein. % relative density of expressed iNOS and COX-2 bands in western blot was calculated by the density using the Chemi Doc XRS+with image lab software (Bio-Rad)

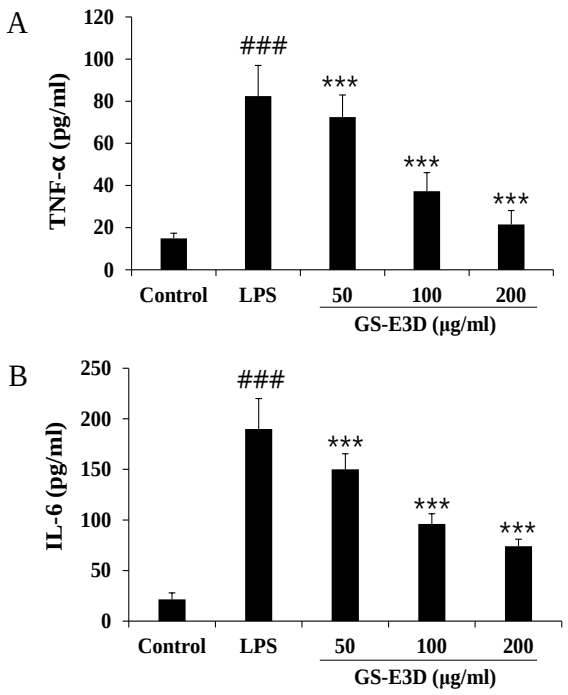

Fig. 3: Inhibitory effect of GS-E3D on TNF-α (A) and IL-6 (B) release in LPS-stimulated RAW264.7 cells. The cells were co-treated with GS-E3D (50-200 μg/ml) and with or without LPS (1 μg/ml) for 24 h. TNF-α and IL-6 release were decreased in LPS-induced RAW 264.7 cells after treatment with GS-E3D in a dose-dependent manner. Data are expressed as the mean±standard error of the mean (SEM) (n=5). ### p<0.001 compared to control group. *** p<0.001 compared to LPS group (ANOVA followed by Tukey's test)

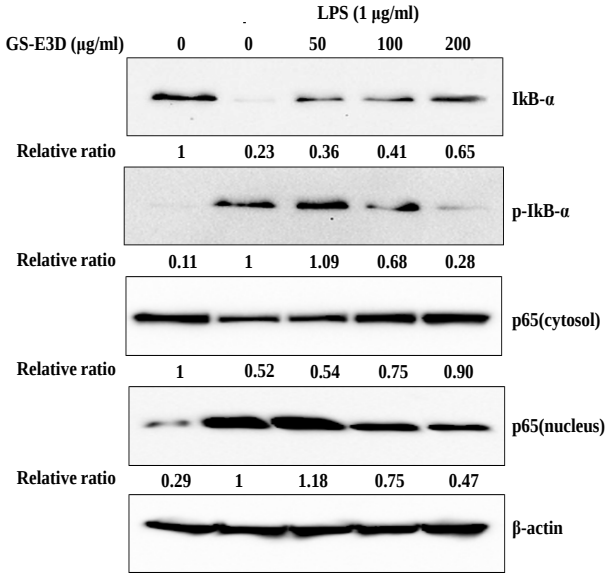

Fig. 4: Inhibitory effect of GS-E3D against NF-κB activation in LPS-stimulated RAW264.7 cells. Western blot analysis shows the inhibitory effect of GS-E3D on phosphorylation of IκBα in cytosol and activation of NF-κB p65 in the nucleus. The cells were co-treated with GS-E3D (50-200 μg/ml) and with or without LPS (1 μg/ml) for 30 min. In western blot, each well was loaded with 25 μg protein. % relative density of expressed bands in western blot was calculated by the density using the ChemiDoc XRS+with image lab software (Bio-Rad)

At the dose of 100 μg/ml GS-E3D, NO production induced by LPS was strongly reduced to approximately basal levels compared with RAW264.7 cells unstimulated by LPS and treated with GS-E3D alone without LPS (fig. 1B). To further understand the inhibitory effects of GS-E3D on NO production, western blot analysis was performed to determine the expression of iNOS protein at 24 h after LPS stimulation. Under unstimulation of LPS, iNOS protein was not detectable. However, GS-E3D attenuated the iNOS expressions in LPS-stimulated RAW264.7 cells in a concentration-dependent manner (fig. 2).

This implies that GS-E3D inhibited NO production by suppressing iNOS expression in LPS-stimulated RAW264.7 cells. PGE_2 is an inflammatory mediator which is produced from the conversion of arachidonic acid by cyclooxygenase. In a variety of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount PGE_2 at inflammatory sites [18]. Therefore, we evaluated the inhibitory effects of GS-E3D against PGE_2 production in LPS-stimulated RAW264.7 macrophages. When macrophages were stimulated with LPS (1 μg/ml) without the GS-E3D for 24 h, RAW264.7 macrophages produced PGE_2 of 294 pg/ml in the culture medium. However, GS-E3D significantly inhibited the production of LPS-induced PGE_2 by 31% at 50 μg/ml, 45% at 100 μg/ml, and 68% at 200 μg/ml. RAW264.7 cells unstimulated by LPS and treated with GS-E3D alone without LPS secreted basal level of PGE2 (fig. 1C).

As the previously mention, GS-E3D is pectin lyasetreated ginseng extract for enhancing ofginsenoside Rd. The contents of ginsenoside Rd in GS-E3D was higher 10 fold than that of ginseng extract (not treated with pectin lyase). Inhibitory effect of GS-E3D against NO and PGE2 production induced by LPS were significantly higher than those of ginseng extract (data was not shown). The difference of inhibitory effect against NO and PGE2 production between GS-E3D and ginseng extract maythink due to difference of ginsenoside Rd contents.

To determine whether inhibition of PGE2 production by GS-E3D was mediated by regulation of COX-2 expression, western blottinganalysis was performed. As shown in fig. 2, the expression of COX-2 protein was significantly increased in macrophages treated with LPS (1 μg/ml) alone compared to the unstimulated cells. However, GS-E3D significantly inhibited the production of LPS-induced COX-2 protein by 10% at 100 μg/ml, and 37% at 200 μg/ml. This implies that GS-E3D potentially inhibited PGE2 production by suppressing COX-2 expression in LPS-stimulated RAW264.7 cells.

GS-E3D inhibits the release of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells

TNF-αand IL-6 are known to be pro-inflammatory cytokines, however inflammatory reaction is accelerated by these large amount of cytokines. Therefore, inflammation on RAW 264.7 cells by LPS stimulation with or without GS-E3D was studied. In order to investigate the effect of GS-E3D capable of inhibiting the medium was subject to investigate the production of IL-6 and TNF-α(fig 3). As a result, in the RAW 264.7 cells, LPS activated the production of TNF-α (82.5 pg/ml) and IL-6 (182.6 pg/ml) are significantly increased as compared to the control group (14.8 pg/ml TNF-α, 21.5 pg/ml IL-6). However, production of IL-6 and TNF-αactivated by LPS was significantly inhibited by GS-E3D in a concentration dependent manner. GS-E3D at the dose of 100 ug/mlinhibited the production of TNF-α(37.3 pg/ml) and IL-6 (96.0 pg/ml). However, at the dose of 200 ug/ml, the inhibitory effect of GS-E3D on TNF-α(21.9 pg/ml) and IL-6 (74.0 pg/ml) further supported its potential inhibitory activity.

Inflammation promoting cytokines may play a role which regulates a variety of immune and inflammatory responses in the body. The macrophages stimulated by LPS of the bacterium produce a large amount of TNF-α, thereby sustained inflammatory responses by inducing the production of IL-1β and IL-6 [19]. TNF-α induced by LPS to promote the onset of inflammation reaction and its continued generation leads to chronic inflammation [20, 21]. IL-1β is a major inflammatory cytokine produced from macrophages and an important cytokine in the initiation and enhancement of the inflammatory response to bacterial infection [22]. IL-6 is considered as important inflammatory cytokine macrophage responsible for promoting gender while generated in the acute immune response [23]. Thus GS-E3D

observed in the present study is to show that GS-E3Dclaim suppress the generation of IL-1β, IL-6, TNF-α induced by LPS stimulation, which is the initial step in the inflammatory response caused by LPS-stimulated and that the inhibition was successfully attained.

Inhibitory effect of GS-E3D on activation of NF-κB and MAPK signaling pathway

To evaluate whether the inhibitory effect of GS-E3D on iNOS expression is related to blocking NF-κB activation, we performed western blot for kB -α degradation and kB -α phosphorylation, since the phosphorylation and subsequent degradation κBf I -α is an essential step in NF-κBactivation by various stimuli [24]. As shown in Fig. 4, LPS induced kB -α degradation at 30 min after LPS stimulation. However GS-E3D dose-dependently suppressed κB -α degradation induced by LPS. Moreover, to determine whether the effect of GS-E3D against κB -α degradation was mediated by blocking I -α phosphorylation, we evaluated the inhibitory effect of GS-E3D against the phosphorylation of kB -α induced by LPS, and found that GS-E3D significantly reduced kB -α phosphorylation. From these results, it is thought that GS-E3D inhibits LPS-induced NF-κB activation. To further investigate whether the inhibition of GS-E3D on the expression of iNOS via blocking NF-kB activation is related to the modulation of MAPK pathway, we evaluated the effects of the GS-E3D on phosphorylation of p38, ERK1/2 and JNK in LPS-stimulated RAW264.7 cells by western blot (fig.5). Hyper-phosphorylation of p38, ERK1/2 and JNK was observed in RAW264. 7 cells treated with LPS alone compared to the untreated cells. However, GS-E3D suppressed LPS-induced phosphorylation of p38, ERK1/2 and JNK. These results suggest that GS-E3D blocks the phosphorylation of p38, ERK1/2 and JNK to suppress the inflammatory response in LPS-stimulated RAW264.7 cells. Our data strongly indicate that GS-E3D possesses the anti-inflammatory effect through suppressing NF-κB and MAPK signaling pathway. NF-κB pathway is regulated by protein signal transduction, such as phosphoric acid group MAPKs and Akt are involved in a variety of intracellular signal transduction [25-27]. In many studies, p38, JNK, MAPKs, such as the ERK reveals to play an important role in the activation of NF-κB from the LPS in macrophages [15, 28].

Inhibitory effects of GS-E3D for activation of NF-κB induced by LPS stimulation in the results of the present study indicate that the process is related to the phosphorylation of MAPKs phosphate group signal transduction. In addition GS-E3D could inactivate the Nrf2 path to mediate the anti-inflammatory activity. These results, in LPS-stimulated RAW264.7 cells, was demonstrated that inflammatory mediators such as NO, as well as the production of inflammatory cytokines and promoting gender expression is suppressed by the GS-E3D. These inhibitory effects of GS-E3D has been shown to inactivate NF-κB by inhibiting the degradation pathway of IκB. GS-E3Dwas also found to affect also the upper MAPKs and Akt signaling pathway of NF-κB activation. The anti-inflammatory effect and its mechanism studies of ginsenoside Rd, main compound of GS-E3D, remain to be further.

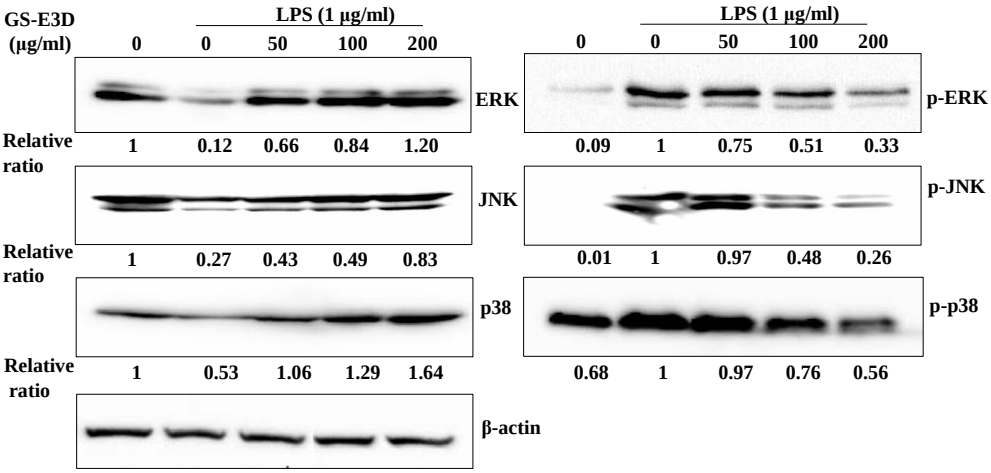

Fig. 5: Inhibitory effect of GS-E3D against MAPKs activation in LPS-stimulated RAW264.7 cells. Western blot analysis shows the inhibitory effect of GS-E3D on the phosphorylation of MAPKs such as p38MAPK, ERK1/2 and JNK. Cells were pre-treated with GS-E3D for 24 h and then 1 μg/ml of LPS was treated for 5 min. In western blot, each well was loaded with 25 μg protein. Relative % density of expressed bands in western blot was calculated by the density using the Chemi Doc XRS+with image lab software (Bio-Rad).

CONCLUSION

The present findings suggest that GS-E3D revealed anti-inflammatory effects by suppressing the NF-κB p65 and MAPKs signaling pathways and could be used as a functional food or a cosmetic materials.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest

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