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Digest

Synthesis and inhibitory effect of *cis*-guggulsterone on lipopolysaccharide-induced production of nitric oxide in macrophages

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ABSTRACT

Guggulsterone is a bioactive plant sterol naturally found in migratory plants. It exists in various forms, and its active compounds include the isomers *cis*-guggulsterone (E-GS) and *trans*-guggulsterone (Z-GS). In this study, the anti-inflammatory effects of these two isomeric pregnadienedione steroids were investigated against lipopolysaccharide-induced inflammatory reaction in RAW264.7 mouse macrophages. Our results showed that both guggulsterones inhibited the production of NO in macrophages treated with lipopolysaccharide, with IC50 values ranging from 3.0 to 6.7 μM. E-GS exerted higher efficacy than Z-GS, and its anti-inflammatory effects was mediated through inhibition of iNOS and COX-2 expression.

Inflammation is a series of immune responses that occur in immune cells for normal recovery of damaged cells and tissues. In addition, it is a defense mechanism against harmful stimuli, such as infection by microbial pathogens and tissue damage.[1,2] In normal inflammatory responses, the production of pro-inflammatory mediators is increased, decreasing anti-inflammatory mediators, and normal inflammatory response also has a self-limiting regulatory function.[3] Macrophages are known to be the main cells involved in the inflammatory response and are activated by stimulation or cytokines secreted by immune cells. Activated macrophages cause inflammation reactions such as pain, edema, and heat, and promote the migration of immune cells to the site of inflammation.[4]

Activation of immune cells during the inflammatory processes produces inflammatory mediators (such as nitric oxide (NO), prostaglandin (PGE_2)) and pro-inflammatory cytokines (such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α)).[5,6] Overexpression of inflammatory mediators and persistent inflammation lead to excessive immune reactions that further exacerbate inflammation, leading to various chronic diseases, such as arthritis, dementia, cardiovascular diseases, metabolic diseases, autoimmune diseases, and cancer.[7,8]

Therefore, inhibition of inflammatory mediators, such as NO, PGE_2, IL-1β, IL-6, and TNF-α, is important for the treatment of inflammatory diseases and various immune diseases.[9,10] Various steroids and non-steroidal anti-inflammatory drugs currently used as inhibitors of inflammatory responses have the potential to cause side effects when administered over a long period of time. Therefore, an anti-inflammatory agent derived from natural materials is considered as an alternative.[11]

Guggulsterone is a bioactive phytosterol found naturally in guggul plants (including *Commiphora kataf, C. erythraea, C. wightii, and C. mukul*).[12,13] Guggulsterone exists in various forms, among which the active forms are isomeric pregnadienedione steroids, namely *cis*-guggulsterone (E-GS) and *trans*-guggulsterone (Z-GS) (Fig. 1).[14] Guggulsterone is an antagonist of the bile acid pharmacokinetic (FXR) receptor, and inhibition of FXR expression by guggulsterone exerts anticancer activity in cancer cells.[15,16] In addition, guggulsterone has been shown to play an important role in nutritional metabolism by inhibiting cholesterol synthesis in the liver.[17] Guggulsterone has also shown anticancer properties, and isomer-specific antileukemic activities of the pregnadienedione structure have been identified in HL60 and U937 cells as well as in primary leukemic blasts *in vitro*.[14,18,19] However, to date, there have been no studies comparing the

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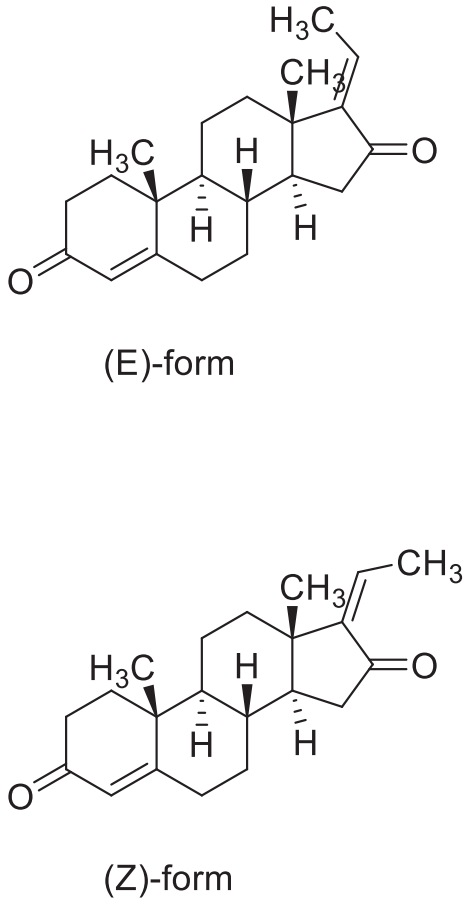

Fig. 1. Chemical structures of E-GS and Z-GS.

anti-inflammatory effects of isomeric guggulsterones in RAW264.7 macrophages.

In our recent study, we synthesized E-GS by using previously reported procedure.[20,21] The synthetic strategy to obtain E-GS has been used of compound 1 as starting material (Fig. 2). A compound 2 was generated by Wittig reaction using phosphoran ethyltriphenylbromide and potassium *t*-butoxide (*t*-BuOK) in THF. Without further purification, a compound 2 was transformed to a compound 3 by oxidation reaction using $Al(Oi\text{-}Pr)_3$. A compound 4 was obtained by oxidation of C-16 allylic position of a compound 3 using SeO_2 and *t*-BuOOH. E-GS was obtained by oxidation using manganese dioxide (MnO_2) relatively in high yield. We then investigated the inhibitory effect of synthesized E-GS on inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. The anti-inflammatory effect of E-GS and Z-GS at a nontoxic concentration to macrophages was confirmed because reduction in cell viability affects anti-inflammatory effects. Therefore, we examined the cytotoxicity of E-GS and Z-GS in RAW264 cells using the MTT assay. In RAW 264.7 cells, E-GS showed toxicity at 25 μM, whereas Z-GS at concentrations up to 25 μM exhibited no cytotoxic effect (Fig. 3A).

LPS as an external stimulus activates NF-κB through cell signaling, resulting in the expression of the inflammatory gene iNOS. NO is a highly reactive free radical produced mainly through conversion of L-arginine to L-citrulline by iNOS, a synthetic enzyme.[21,22] Moreover, it is an important substance in physiological and pathological reactions in the human body.[23,24] At an appropriate level, it plays a physiological role to control smooth muscle relaxation, platelet suppression, immunoregulation, neurotransmitter mediation, vasodilation, and blood pressure. However, excessive increase in NO by continuous inflammatory reaction causes pathological reactions, such as arthritis, bronchitis, multiple sclerosis, and immune diseases.[25,26] In this study, the inhibitory effect of E-GS and Z-GS on NO production was evaluated in RAW 264.7 cells with LPS-induced inflammation. Our results showed that E-GS and Z-GS inhibited NO production in LPS-induced RAW 264.7 cells in a dose-dependent manner (Fig. 3B). The inhibitory effects of E-GS and Z-GS on NO production were stronger than L-NANE (positive control) in LPS-activated RAW 264.7 macrophages. Moreover, the inhibitory effect of E-GS on NO production was stronger than that of Z-GS. Therefore, we further investigated the anti-inflammatory mechanism of E-GS.

In our recent study, we synthesized E-GS by using previously reported procedure with modifications.[20] In this study, we investigated the inhibitory effect of synthesized E-GS on inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. In the inflammatory response, iNOS and cyclooxygenase (COX)-2 enzymes play an important role in the production of NO.[27,28] Thus, protein expression in the cells

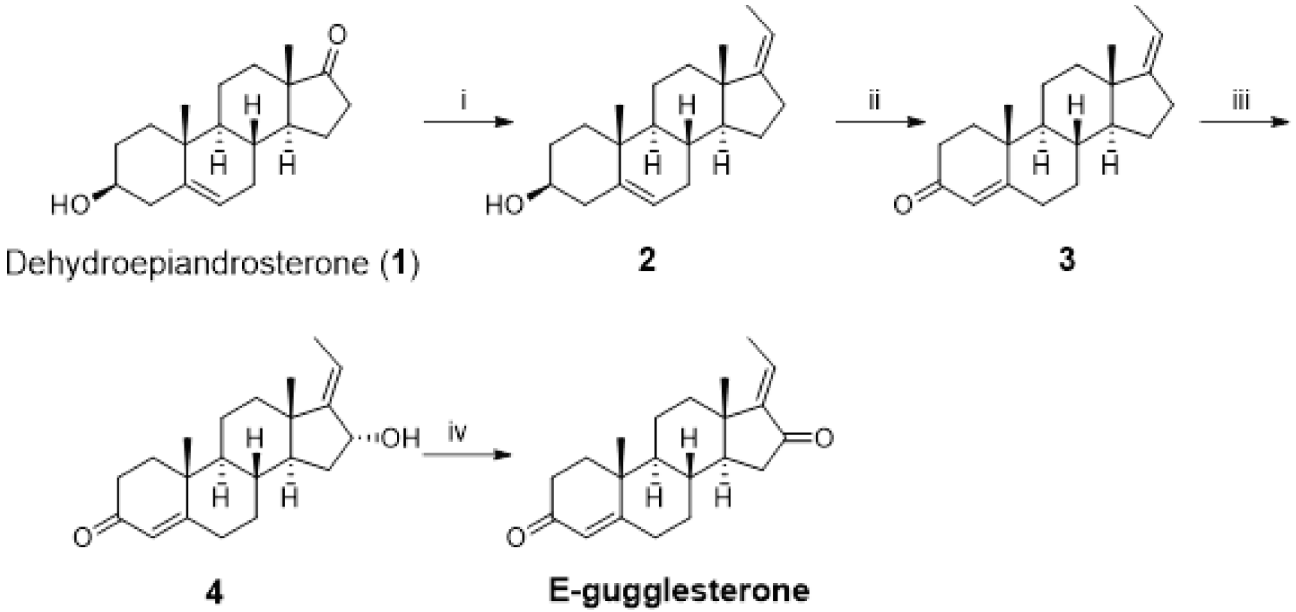

Fig. 2. Synthetic route of guggulsterone. Reagent and conditions: (i) $EtPPh_3Br$, *t*-BuOK, THF, 0 °C–80 °C, 3 h; (ii) $Al(Oi\text{-}Pr)_3$, cyclohexanone, benzene, reflux, 4 h, 92% (two steps); (iii) SeO2, *t*-BuOOH, CH_2Cl_2, room temperature, 1 h, 95.5%; (iv) MnO_2, CH_2Cl_2, 3 h, 97.6%.

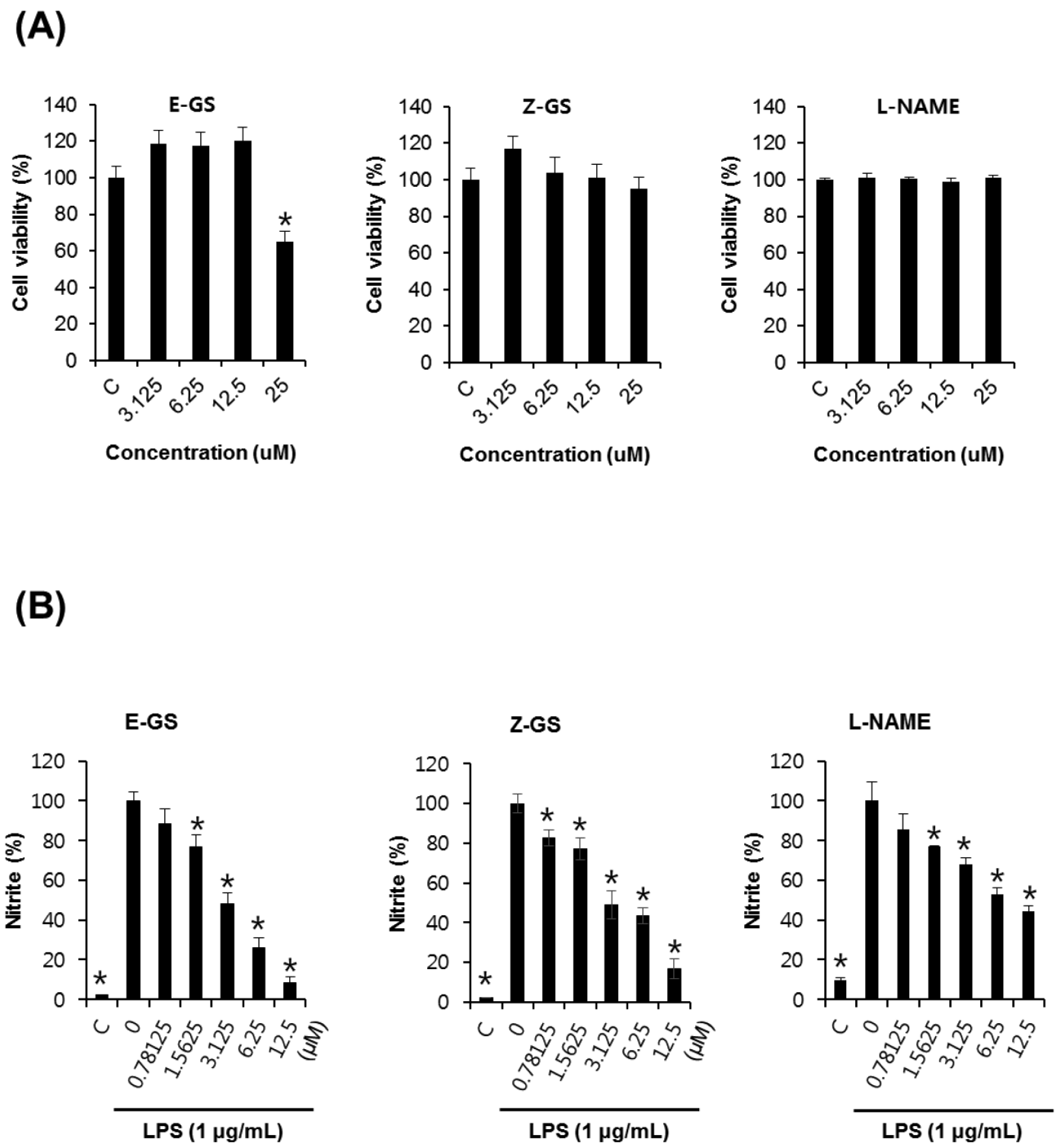

Fig. 3. Cytotoxic and anti-inflammatory effects of E-GS and Z-GS in RAW 264.7 cells. (A) Cells were treated with L-NAME (positive control), E-GS and Z-GS at various concentrations 0–25 μM) 24 h, and cell viability was evaluated using the MTT assay. (B) Inhibitory effects of L-NAME (positive control), guggulsterone E and Z on nitric oxide (NO) production in LPS-activated RAW264.7 mouse macrophages. The cells were pretreated with the samples at the indicated concentrations for 1 h and then exposed to 1 μg/mL LPS for 24 h (mean ± SD). L-NAME, N(G)-nitro-L-arginine-methyl ester. C, medium containing 0.5% DMSO (control vehicle). *$P < 0.05$ compared to the LPS-treated group.

was confirmed using Western blotting to confirm whether the inhibition of NO production by E-GS was related to iNOS and COX-2 protein expression. As shown in Fig. 4, Western blotting data showed that the expression of iNOS and COX-2 decreased in the cells treated with E-GS, compared to that in the control. In addition, E-GS inhibited TNF-α and IL-6 production in LPS-induced RAW 264.7 cells in a concentration-dependent manner (Fig. 5). These results showed that E-GS, which exhibited the most potent inhibitory effect on NO production, exerted its anti-inflammatory effects by downregulating iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages.

This is not the first study of the isomer-specific activities of the pregnadienedione structures. Comparison of antileukemic activities

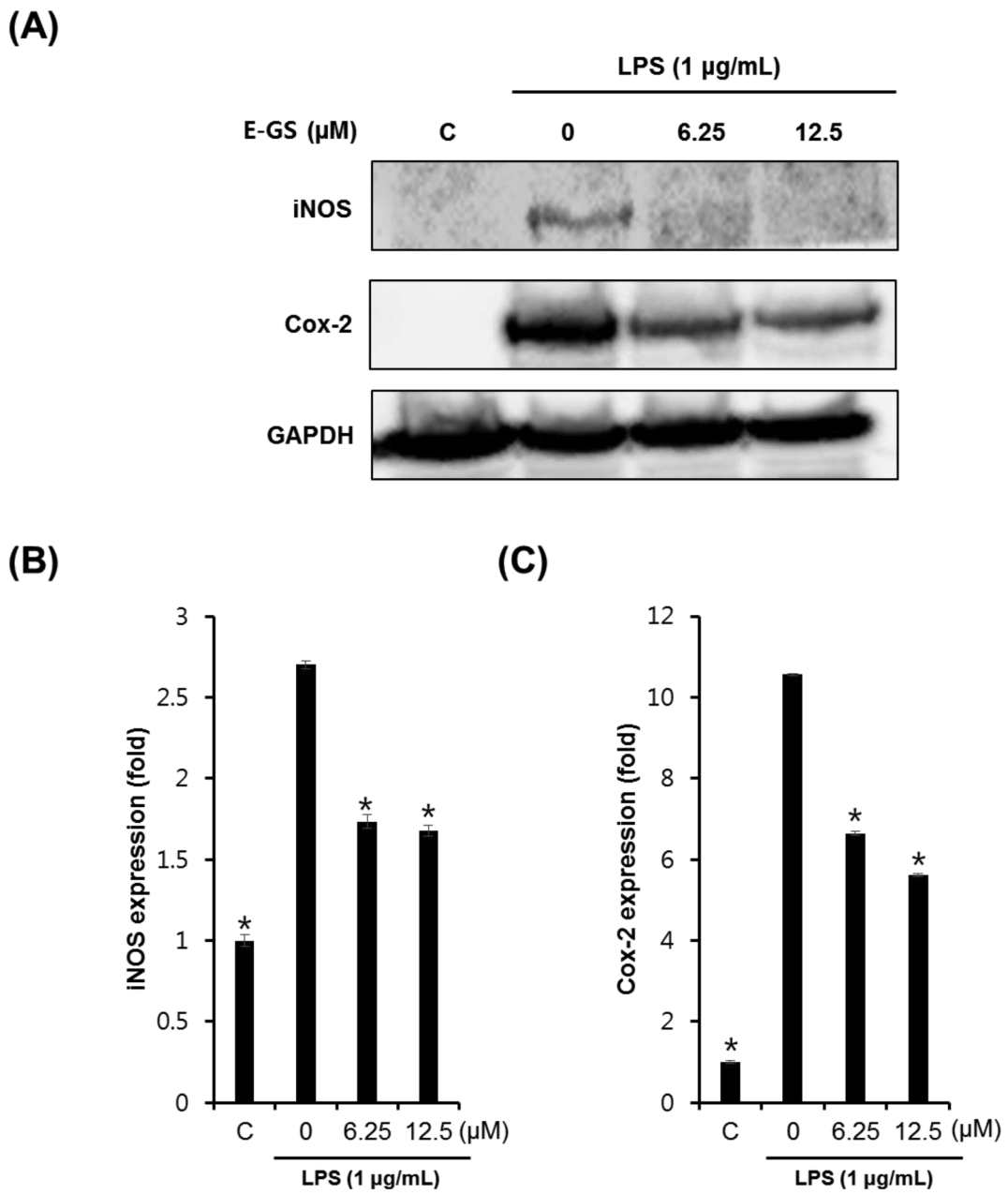

Fig. 4. Effects of E-GS on LPS-induced iNOS and COX-2 protein expression in RAW264.7 mouse macrophages. (A) Representative western blots for iNOS, COX-2, and GAPDH protein expression, (B) quantitative graph for iNOS, (C) quantitative graph for COX-2 (mean ± SD). Effect of E-GS on LPS-induced iNOS and COX-2 protein expression was compared with the LPS-treated control (0 μg/mL). iNOS, inducible nitric oxide synthase. COX-2, cyclooxygenase-2. C, medium containing 0.5% DMSO (control vehicle). *$P < 0.05$ compared to the LPS-treated group.

between E-GS, Z-ES, and 16-dehydroprogesterone showed that only E-GS induced a rapid depletion of glutathione levels and oxidation of the mitochondrial phospholipid cardiolipin; in addition, 16-dehydroprogesterone and Z-GS induced differentiation of HL60 and NB4 cells.[19] Therefore, there were differences in isomer-specific potencies and mechanisms of action between E-GS and Z-ES, and these differences are of particular interest in the studies of guggulsterones.

In conclusion, we investigated the anti-inflammatory effects of E-GS and Z-GS, which are active forms of guggulsterone. NO production in LPS-treated macrophages was inhibited by both E-GS and Z-GS. The related mechanisms were also investigated using E-GS, which exerted the most potent anti-inflammatory effect. The anti-inflammatory effect of E-GS was mediated by downregulation of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. Further studies on the analysis of additional mechanisms as well as in vivo efficacy tests related to anti-inflammatory effects of E-GS are needed to clarify therapeutic strategies for the development as an anti-inflammatory agent.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

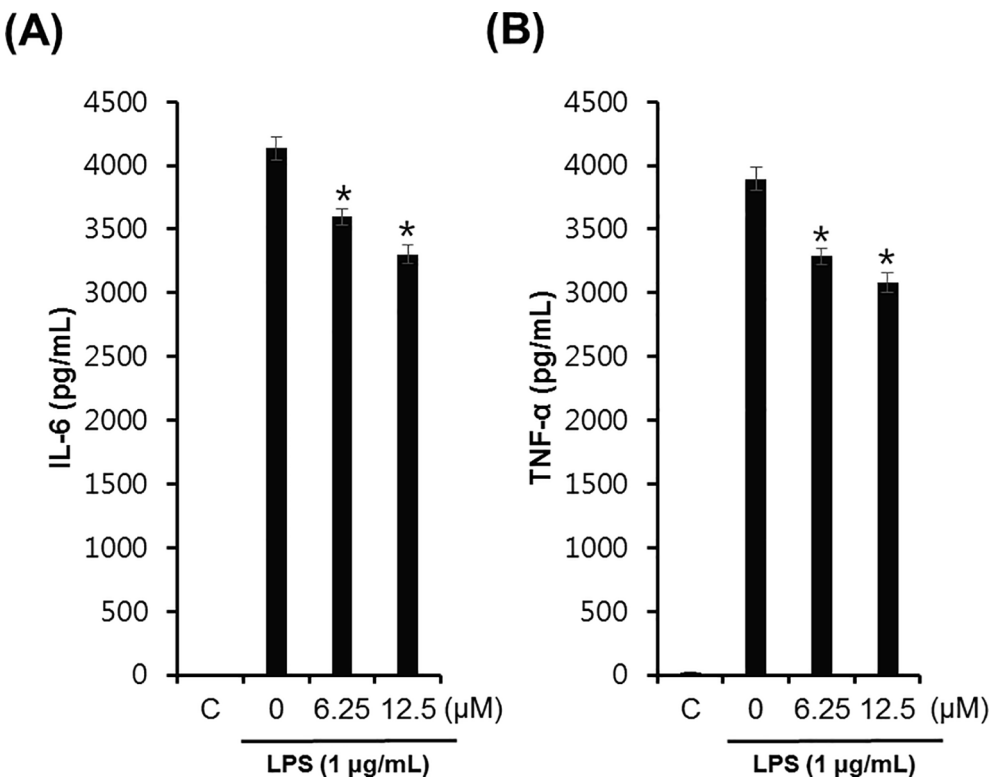

Fig. 5. Effects of E-GS on LPS-induced interleukin (IL)-6 and tumor necrosis factor (TNF)-α production in RAW264.7 mouse macrophages. RAW264.7 cells were treated with various concentrations of E-GS (0, 6.25 and 12.5 μM) for 2 h prior to the addition of LPS (1 μg/mL), and the cells were further incubated for 18 h. The culture supernatant was measured by using enzyme-linked immunosorbent assay (ELISA). Effect of E-GS on LPS-induced IL-6 and TNF-α production was compared with the LPS-treated control (0 μg/mL). C, medium containing 0.5% DMSO (control vehicle). *$p < 0.05$ vs. the LPS-treated group.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.126962.

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