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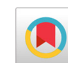

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Article: Bioactive Materials

Anti-inflammatory activity of 6-*O*-phospho-7-hydroxycoumarin in LPS-induced RAW 264.7 cells

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Abstract Esculetin (also known as 6, 7-dihydroxycoumarin) a type of coumarin, has been exhibited anti-inflammatory and anti-aging effects. Biorenovation is the microbe-mediated enhancement of biological efficacies and structurally diversified compounds relative to their substrate compounds. The production of different kinds of esculetin derivatives using *Bacillus* sp. JD3-7 and their effects on lipopolysaccharide (LPS)-triggered inflammatory response in RAW 26.7 cells were assessed. One of the biorenovation products, identified as esculetin 6-*O*-phosphate (ESP), at concentrations of 1.25, 2.5, and 5 µM inhibited the LPS-stimulated production of inflammation markers of nitric oxide synthase 2 and cyclooxygenase 2 as well as their respective enzymatic reaction products of nitric oxide and prostaglandin E2 in the order of increasing concentrations (1.25, 2.5, and 5 µM). Additionally, ESP treatment suppressed the LPS-stimulated secretion of pro-inflammatory cytokines of interleukin (IL)-1β, IL-6, and tumor necrosis factor- α. Furthermore, these anti-inflammatory effect of ESP was associated with the downregulation of mitogen-activated protein kinase signaling, that is, extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase signaling pathways. This study would therefore provide interesting insights into the biorenovation-assisted generation of a novel anti-inflammatory compound. ESP may be used to develop treatments for inflammatory disorders.

Keywords Anti-inflammatory activity · Esculetin (6,7-dihydroxy-coumarin) · Mitogen-activated protein kinase signaling · RAW 264.7 cells

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Introduction

Inflammation process is initiated after harmful substances and invading pathogens such as bacteria and during which symptoms such as pain and swelling appear [1-3]. As a component of Gram-negative bacteria, lipopolysaccharides (LPS) known to be present in the outer cell membrane trigger an inflammatory response, which secrete inflammation related cytokines such as interferon gamma and interleukin-1β to alert surrounding immune cells [4-6]. During inflammation, these immune cells such as neutrophils are recruited to the site of infection and produce pro-inflammatory cytokines and factors such as inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO), cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E2 (PGE_2), which in turn facilitate a series of wound healing processes from initiation of inflammation to its resolution [7-9]. However, dysregulated and/or prolonged production of inflammatory proteins and cytokines can compromise body immune system and contribute to the pathogenesis of autoimmune disorders, including rheumatoid arthritis and cancer [10-12].

When inflammatory stimulation occurs in RAW 264.7 macrophages, the mitogen-activated protein kinase (MAPK) pathway mediated by sequential phosphorylation events plays an essential role in the progression and increase of inflammation [13, 14]. The MAPK family signaling pathway is activated by LPS via Toll-like receptors on macrophages and includes extracellular

signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 [15-17]. In addition, signaling pathways in turn activate various transcription factors, including nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), which increase the production of genes encoding pro-inflammatory enzymes (iNOS, COX-2) and cytokines and receptors [18-20]. In more detail, LPS stimulation results in activation of a multi-subunit IκB kinase (IKK) complex which in turn phosphorylates IκBα triggering its ubiquitin-dependent degradation. The disassociation of IκBα from NF-κB causes rapid and transient nuclear translocation of NF-κB thus mediating transcription of target genes such as COX-2, the enzyme that play key roles in the biosynthesis of PGE_2, and causes vasodilation and tumorigenesis [21]. In addition, during the inflammatory response process, NO overexpressed from iNOS is known to be the cause of several diseases, including rheumatoid arthritis and cancer, and has been reported to induce gene expression of several inflammatory mediators, such as inflammatory cytokines, to deepen and increase inflammation [22,23]. Hence, NF-κB and AP-1 act as targets for the prevention and treatment of inflammatory diseases, and sub-stances that inhibit the activation of NF-κB and AP-1 can be considered as potential anti-inflammatory materials.

Coumarins are one of the most important classes of fluorescent molecules and are reported to possess diverse biological activities, and in particular, constitute an important class of anti-inflammatory molecules [24,25]. Esculetin (also known as 6,7-dihydroxycoumarin), a type of coumarin, found in the medicinal plant species of the genera of Cortex, Aesculus, Artemisia, and Citrus has been exhibited anti-inflammatory and anti-aging effects by down-regulating the hydrogen peroxide-induced metalloproteinase-1 protein expression in skin keratinocytes and LPS-elicited inflammatory markers in RAW 264.7 macrophages, respectively [26-30]. Even though various studies have shown these plant metabolites to have diverse bioactivity, these studies are only limited to in vitro studies and many of them fail to demonstrate similar effect in in vivo studies, with some even showing toxicity in test subjects. Therefore, current study aims to produce esculetin analogs having better bioavailability and safety, and thus better therapeutic index, which is possible through molecular manipulation of the compound [31].

Biorenovation allows structurally diversified compounds from their natural synthetic substrate compounds with reduced cell toxicities and improved biological efficacies. In one study, genistein derivatives were produced through biorenovation, of which one derivative, 4-*O*-isopropyl genistein, tested for its antimicrobial activity against both methicillin resistant and methicillin-sensitive *Staphylococcus*, in which MIC values significantly lower than its substrate counterpart, genistein. Other examples include production of Formononetin 7-*O*-phosphate with enhanced anti-inflammatory activity and reduced cytotoxicity compared to formononetin. Therefore, with a similar approach, we carried out the study to produce relatively safer esculetin analog with higher bioactivity [32,33]. In this study, a novel anti-inflammatory compound, esculetin 6-*O*-phosphate (ESP), was synthesized via biorenovation. We hypothesized that esculetin derivatives may show anti-inflammatory potential. In particular, the role of ESP in the suppression of LPS-triggered inflammatory response was studied. The effect of ESP on the regulation of MAPK and NF-κB signaling pathways was further evaluated. We found that biorenovation represents a suitable approach for repurposing bioactive compounds, and ESP may be used to develop treatments for inflammatory disorders.

Materials and Methods

Chemicals and materials

Bacillus sp. JD3-7 was purchased from the Korean Collection for Type Cultures (designated number 92346P, KACC, Wanju, Korea). Beef extract (cat. no. LP0029) and peptone (cat. no. LP0037) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Nutrient broth comprised beef extract (3 g/L) and peptone (5 g/L) at pH 6.2. Phosphate glycerine (PG buffer) contained 2% glycerin in 50 mM sodium phosphate at pH 7.2. Dulbecco's modified Eagle's medium (DMEM) (cat. no. LM001-05), and fetal bovine serum (FBS) (cat. no. S001-01) were purchased from Welgene (Gyeongsan, Korea). Penicillin-streptomycin (P/S) (cat. no. 15140122) was purchased from Thermo Fisher Scientific. Esculetin (6,7-dihydroxycoumarin) (cat. no. 246573) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS (Escherichia coli O55:B4; cat. no. L6529), griess reagent (cat. no. G4410), and dimethyl sulfoxide (DMSO; cat. no. D4540) were purchased from Sigma-Aldrich. The IL-1β enzyme-linked immunosorbent assay (ELISA) kit (cat. no. MLB00C) and PGE_2 ELISA kit (cat. no. KGE004B) were purchased from R&D Systems (Minneapolis, MN, USA). A TNF-α ELISA kit (cat. no. BMS607-3) and IL-6 ELISA kit were purchased from Thermo Fisher Scientific and BD Biosciences (Franklin Lakes, NJ, USA), respectively. Antibodies against β-actin (cat. no. VMA00048) and iNOS (cat. no. AHP2399) were purchased from Bio-Rad (Hercules, CA, USA), and anti-COX-2 antibodies (cat. no. 100-401-226) were obtained from Rockland (Limerick, PA, USA). Antibodies against JNK (cat. no. 9252S), phosphorylated (p) JNK (cat. number 9251S), ERK (cat. no. 9102S), p-ERK (cat. no. 9101S), p38 MAPK (cat. no. 9212S), p-p38 MAPK (cat. no. 9211S), p-NF-κB (cat. no. 3033S), and inhibitor of NF-κB alpha (IκB-α) (cat. no. 4814S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Radio-immunoprecipitation acid (RIPA) buffer (cat. no. R2002) was purchased from Biosesang (Seongnam, Korea). Proteinase inhibitor cocktail was purchased from Quartett (Berlin, Germany). high-performance liquid chromatography (HPLC) grade acetonitrile and water were purchased from Thermo Fisher Scientific. Trifluoroacetic acid (TFA) (cat. no. T0672) was purchased from Samchun (Pyeongtaek, Korea).

Biorenovation

Biorenovation was performed as demonstrated in the papers [32,33]. Briefly, a single colony of *Bacillus* sp. JD3-7 was transferred into a 4 mL nutrient broth and further cultured. The culture broth was then scaled up by 25 fold. The culture broth was then centrifuged and rinsed in phosphate-glycerol (PG) buffer. The pellets were resuspended in PG buffer and inside to completely remove any remaining nutrient broth. The washing step was repeated twice. esculetin (0.8 mg/mL) was then added to a PG buffer. The resuspension was further incubated for 72 h on a shaker. The cell culture was centrifuged and the resulting supernatant was evaporated.

Purification of biorenovation product and HPLC analysis

Biorenovation product was purified and HPLC-analyzed as described in the papers[34-36]. Briefly, acetone-soluble compounds were extracted from the residues after evaporation. After spin-down, the supernatant was derived and re-suspended in 80% methanol. Gradient HPLC analysis of the crude sample was then performed. The mobile phase consisted of water (0.1% TFA) (A), and acetonitrile (B). Gradient elution procedure was set: 0 to 20 min, 10% to 100% B; 20 to 25 min, 100% B; 25 to 27 min, 10% B; and 27 to 32 min, 10% B. Other parameters included: flow rate, 1 mL/min; injection volume, 10 µL; 254 nm; and oven temperature, 40 ^oC. The esculetin derivatives were further purified in the same gradient elution procedure as described above but in a preparative HPLC.

Cell maintenance

RAW 264.7 macrophages were cultured as described in previously published papers [37-39]. RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS with 1% P/S and placed in a humidified incubator in a 5% CO_2 atmosphere at 37 ^oC and sub-cultured every two or three.

Cytotoxicity assay

The MTT assay was performed to assess the cytotoxicity of ESP. RAW 264.7 cells were plated at 8×10^4 cells/well in a 24-well plate and incubated for 18 h prior to 24 h of treatment with 1 µg/mL LPS alone or in combination with different doses of the test compound (1.25, 2.5, or 5 µM) at 37 ^oC, in 5% CO_2. The MTT reagent (0.5 mg/mL) was then added following incubation for 3 h. The medium was completely removed and dissolved in DMSO. Absorbance at 570 nm was measured using a microplate reader (Waltham, MA, USA). The average absorbance of each sample group was used to evaluate the cell viability.

Determination of NO production

The quantity of nitric oxide present in culture medium was measured using griess reagent as previously described [40,41]. RAW 264.7 cells were cultured and treated with 1 µg/mL LPS alone or in combination with different doses of the test compound. Equal volumes of supernatant from each well and reconstituted Griess reagent were mixed and incubated (21-23 ^oC) for 15 min, followed by measurement of the absorbance at 540 nm using a microplate reader.

ELISA for PGE_2, IL-1β, IL-6 and TNF-α

RAW 264.7 cells were cultured as described above and treated for 24 h with 450 µL of LPS (1 µg/mL) and 50 µL of ESP. The culture medium was then centrifuged at 12,000 rpm for 3 min, and the resulting supernatant was used to measure the levels of PGE_2, IL-1β, IL-6, and TNF-α. All samples were stored at −20 ^oC until quantification. The pro-inflammatory cytokines were quantified using ELISA kits (Mouse TNF alpha ELISA Kit; Invitrogen, Carlsbad, CA, USA; Mouse IL-6 ELISA Kit, BD Biosciences; Mouse IL-1β/IL-1F2, R&D Systems); the R-squared value for the standard curve was ≥0.99.

Western blotting

Immunoblotting was conducted as described in previously published papers [42-44]. Briefly, RAW 264.7 cells were cultured and treated with LPS and/or vary amounts of ESP. The cells were PBS-rinsed and lysed in proteinase inhibitors-added RIPA buffer. The cell lysate was on ice for 30 min with frequent vortex every 10 min. The amount of proteins in the cleared cell lysate was measured and equal amount of protein sample was loaded on acrylamide gel and subject to electro-transfer. The blocked membranes were incubated with primary antibodies. The protein bands on the blot were visualized using ECL Substrate and photographs were obtained using an Image Reader (LAS4000 mini, GE Healthcare Japan Corp., Tokyo, Japan). Western blot densitometry was performed using ImageJ software. The protein expression of iNOS and COX-2 was represented as the ratio of the band intensity of each protein to that of β-actin. The activation level of the MAPK and NF-κB signaling proteins was presented as the ratio of the band intensities of phosphorylated forms to non-phosphorylated forms.

Statistical analysis

Value difference between samples groups were calculated by one way analysis of variance (ANOVA)-Tukey-Kramer test. Statistical significance of P values of <0.05.

Results

Structural elucidation of the biorenovation product

Novel bioactive components of the biorenovation product were separated via HPLC. The two major peaks of F1 and F2 were eluted at retention times (tR) of 5.00 and 5.96 min and the largest peak F1 was used a reference to purify ESP from biorenovation products. According to time-of-flight mass spectrometry analysis,

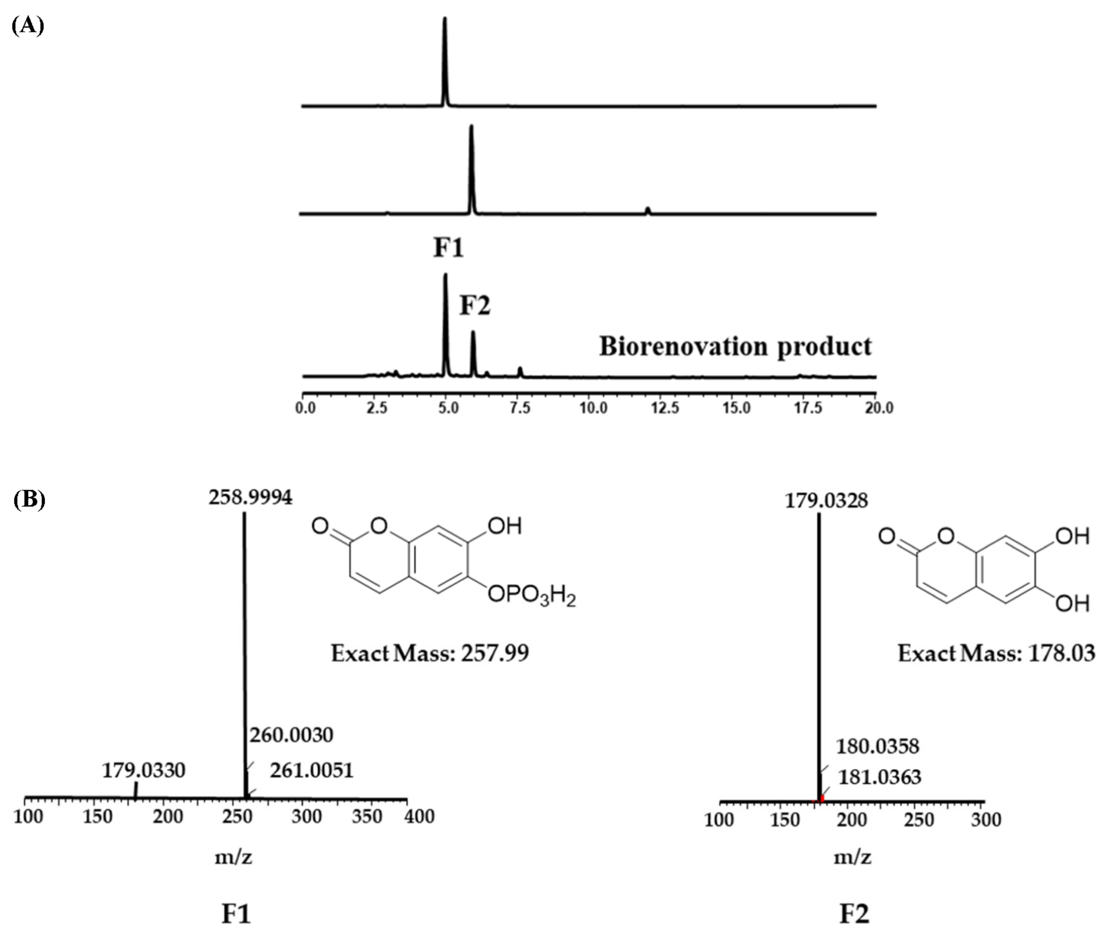

Fig. 1 HPLC chromatograms coupled with flight electrospray mass spectrometry analyses. (A) HPLC chromatograms for esculetin biorenovation product. F1, Esculetin; F2, ESP. (B) Structure and mass spectra of ESP and its exact mass, $[M+H]^+$. HPLC, high-performance liquid chromatography; ESP, esculetin 6-*O*-phosphate

peak F1 exhibited a mass ion at m/z 259.0011 $[M+H]^+$ and m/z 179.0329 $[M+H-H_2PO_3]^+$, corresponding to the protonated and dephosphorylated forms of F1, respectively. Based on this result, the molecular composition for the peak F1 was assumed to be $C_9H_7O_7P$, indicating a phosphorylated form of esculetin.

Nuclear magnetic resonance (NMR) results

The position of phosphorylation in the ESP structure was determined via one-dimensional and two-dimensional NMR experiments. The chemical shifts of H-5 and carbon-5 (δH 7.45 ppm and δC-5 119.49 ppm) were downfield-shifted compared to those of esculetin. In addition, carbon-6 was observed at 137.41 ppm in the ^{13}C NMR spectrum based on shielding by phosphorylation. These results indicate the phosphorylation at carbon-6 of esculetin. Furthermore, the presence of phosphorous at F1 was confirmed based on the ^{31}P NMR spectrum (supplementary data), and the split carbon signals of the 5, 6, and 7 of carbon due to carbon-phosphorous coupling (JC-H 3.4-6.9 Hz) were also observed in the ^{13}C NMR spectrum. Based on these results, the structure of F1 was elucidated as esculetin 6-*O*-phosphate (ESP).

Esculetin 6-*O*-phosphate (ESP, 1): ^{1}H NMR (DMSO-d6, 400 MHz): δ 7.96 (1H, d, J=9.5 Hz, H-4), 7.45 (1H, br d, H-5), 6.82 (1H, s, H-8), 6.23 (1H, d, J=9.5 Hz, H-3) (Fig. S1).

^{13}C NMR (DMSO-d6, 100 MHz): δ 160.40 (Carbon-2), 153.29 (Carbon-7, d, J=6.9 Hz), 151.20 (Carbon-8a), 144.27 (Carbon-4), 137.41 (Carbon-6, d, J=6.7 Hz), 119.49 (Carbon-5, d, J=3.4 Hz), 111.96 (Carbon-3), 110.45 (Carbon-4a), 103.91 (Carbon-8) (Figs. S2-S3).

^{31}P NMR (DMSO-d6, 162 MHz): δ -4.26 (Fig. S4).

Cell viability and NO secretion

RAW 264.7 cells were treated as described in the material and method section. The changes in cell viabilities and the amount of NO present in the culture medium were evaluated on the data of MTT and griess assay, respectively. ANOVA was applied to resolve the positive relationship of ESP concentration and cell viability/NO production. Cell viability was above 90% in all tested ESP concentrations (1.25, 2.5 and 5 μM) (Fig. 2A). Furthermore, the suppressive effect of ESP on NO secretion

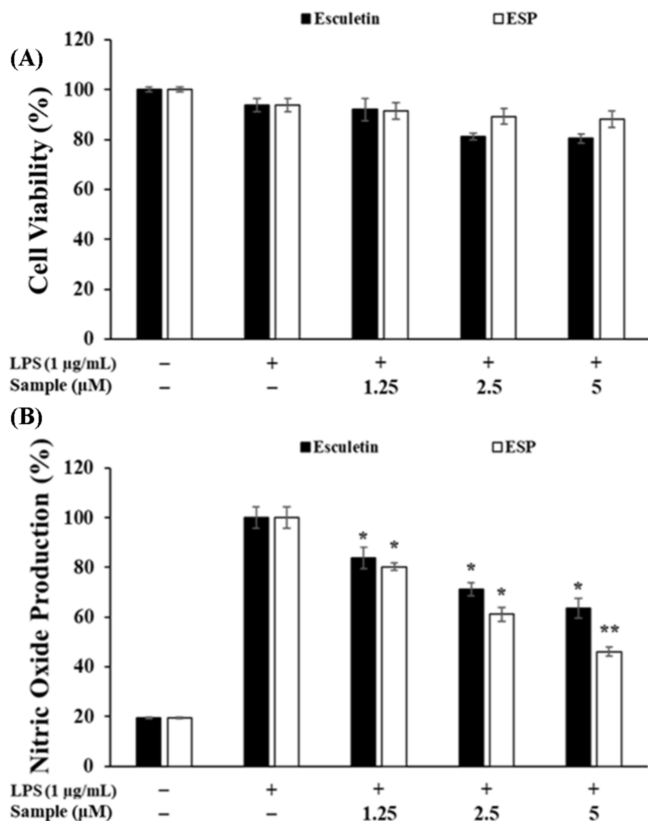

Fig. 2 Effect of esculetin and ESP on (A) cell viability, and (B) nitric oxide production. Cells were co-treated with LPS and three concentrations of ESP. The results are presented as the relative percentage of the control (untreated cells). Data points are expressed as the mean ± standard deviation. The mean of each sample group was compared to the mean of an LPS-treated group, *p <0.05, **p <0.01. LPS, lipopolysaccharide; ESP, esculetin 6-*O*-phosphate

relative to LPS only-treated sample was statistically significant and positively correlated (Fig. 2B). In addition, it was confirmed that the improved NO inhibitory activity compared to esculetin.

Effect of ESP treatment on LPS-induced iNOS and COX-2 protein expression

The increased biosynthesis of iNOS and COX-2 during the early phase of inflammation cause the marked upregulation of their respective catalysis product NO and PGE_2 level. ELISA and western blots confirmed that the secretion of NO and PGE_2 relative its LPS only-treated sample was simultaneously down-regulated in the order of ESP concentrations (1.25, 2.5, 5 μM). ESP at 5 μM inhibited the LPS-induced generation of NO and PGE_2 by 54.2 and 60.3%, respectively (Fig. 3A). Concomitantly, the protein expression of iNOS and COX-2 was respectively downregulated by 60.2% and 85.5% (Fig. 3B, C).

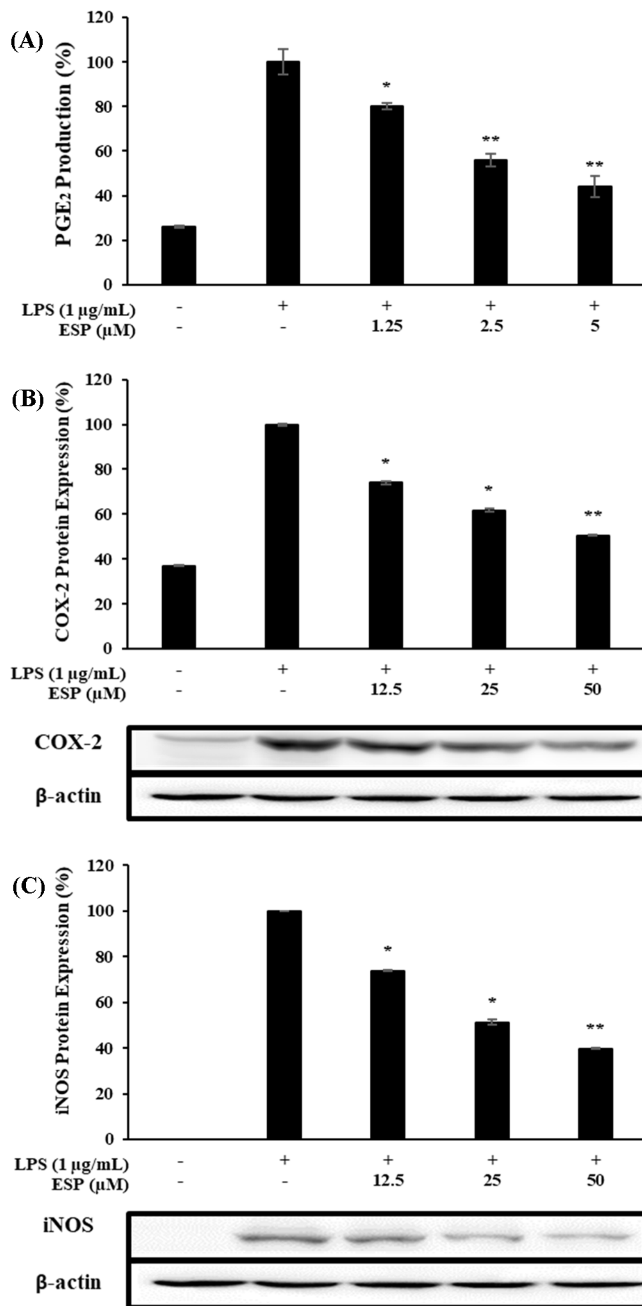

Fig. 3 The inhibitory effect of ESP on the LPS-induced production of PGE_2 and on the levels of iNOS and COX-2. (A) PGE_2 production, (B, C) protein levels of iNOS and COX-2 were determined via western blotting. Cells were treated with 1 μg/mL LPS alone or in combination with 1.25, 2.5, and 5 μM ESP for 24 h. The results are presented as the relative percentage of the control (untreated cells). Data points are expressed as the mean ± standard deviation. The mean of each sample group was compared to the mean of the LPS-treated group, *p <0.05, **p <0.01

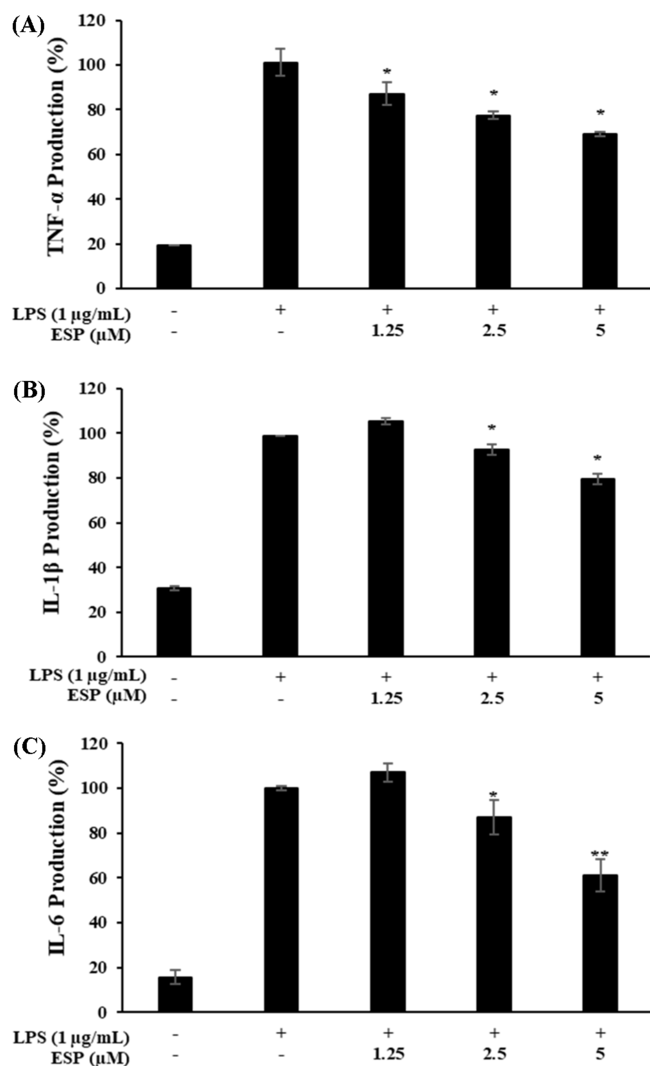

Fig. 4 Effect of ESP on the LPS-triggered secretion of (A) TNF-α, (B) IL-1β, (C) and IL-6. Cells were treated with LPS and/or 1.25, 2.5, and 5 μM ESP. The results are presented as the relative percentage of the control (untreated cells). Data are expressed as the mean and standard error. The mean of an LPS-treated group was used a reference to express any statistically significant difference between the means of each samples, $*p < 0.05$, $**p < 0.01$

Effect of ESP on the attenuation of LPS-induced pro-inflammatory cytokine production

We evaluated the concentration-dependent inhibitory effect of ESP on the three representative pro-inflammatory cytokines, that is, TNF-α, IL-1β, and IL-6. The higher ESP treatment (1.25, 2.5, and 5 μM), the more decrease in the LPS-triggered release of the pro-inflammatory cytokines. Notably, ESP treatment at 5 μM suppressed the LPS-induced secretion of TNF-α, and IL-1β 26%, 28%, IL-6 by 41%, respectively (Fig. 4A, B, C)

Effect of ESP on LPS-induced activation of MAPK and NF-κB signaling pathways

MAPK proteins are phosphorylated in response to various stresses, such as LPS, which in turn the stress signals are passed to the NF-κB signaling pathway. Western blotting analysis showed that LPS-induced phosphorylation of MAPKs (ERK, JNK, and p-38) was decreased by increasing ESP concentration (1.25, 2.5, and μM) and ESP at 5 μM inhibited the LPS-triggered phosphorylation of ERK, JNK, and p-38 by 20, 40, and 65%, respectively (Fig. 5A, B, C). Similarly, LPS-stimulated phosphorylation of NF-κB was inhibited in the order of increasing concentration of ESP and the degradation of IκB-α was simultaneously prevented (Fig. 5D, E). These results indicate that ESP exerted anti-inflammatory activity via the downregulation of MAPK and NF-κB signaling pathways.

Discussion

Our research was conducted to discover new compounds by applying biorenovation and to explore their potential as effective functional agents for anti-inflammatory action through efficacy verification. Esculetin, used in this study, has been exhibited anti-inflammatory and anti-aging efficacies. Such compounds can be modified via biorenovation to obtain derivatives that exhibit biological activity. In the present study, biorenovation of esculetin produced ESP which contained a phosphate group attached to the C6-OH site of esculetin. Therefore, this study aimed to investigate the effect of ESP on LPS-triggered inflammatory response of RAW 264.7 and the activation of MAPK and NF-kB signaling pathways and the release of inflammatory cytokines. LPS triggers inflammation by synthesizing and secreting inflammatory substances which also act as signaling molecules. Protein expression of iNOS and COX-2 in LPS-treated macrophages increases drastically during inflammation and the unregulated production of NO and PGE_2 could cause serious inflammatory decease such as septic shock Thus, the inhibition of inflammation-related mediators and cytokines may have therapeutic use in the treatment of inflammatory conditions. In this study, ESP treatment inhibited the LPS-induced protein expression of iNOS and COX-2 as well as their respective enzymatic product NO and PGE_2 in the order of increasing ESP concentration. Based on these results, ESP treatment showed its effectiveness in inhibiting the increased production of iNOS, and COX-2 in RAW 264.7 cells and their reduced protein expression led to the inhibition of NO and PGE_2.

Upon inflammation, macrophages secrete pro-inflammatory cytokines in addition to inflammatory cytokines to warn other immune cells and restore body homeostasis. Pro-inflammatory cytokines produced in response to inflammatory stimuli can

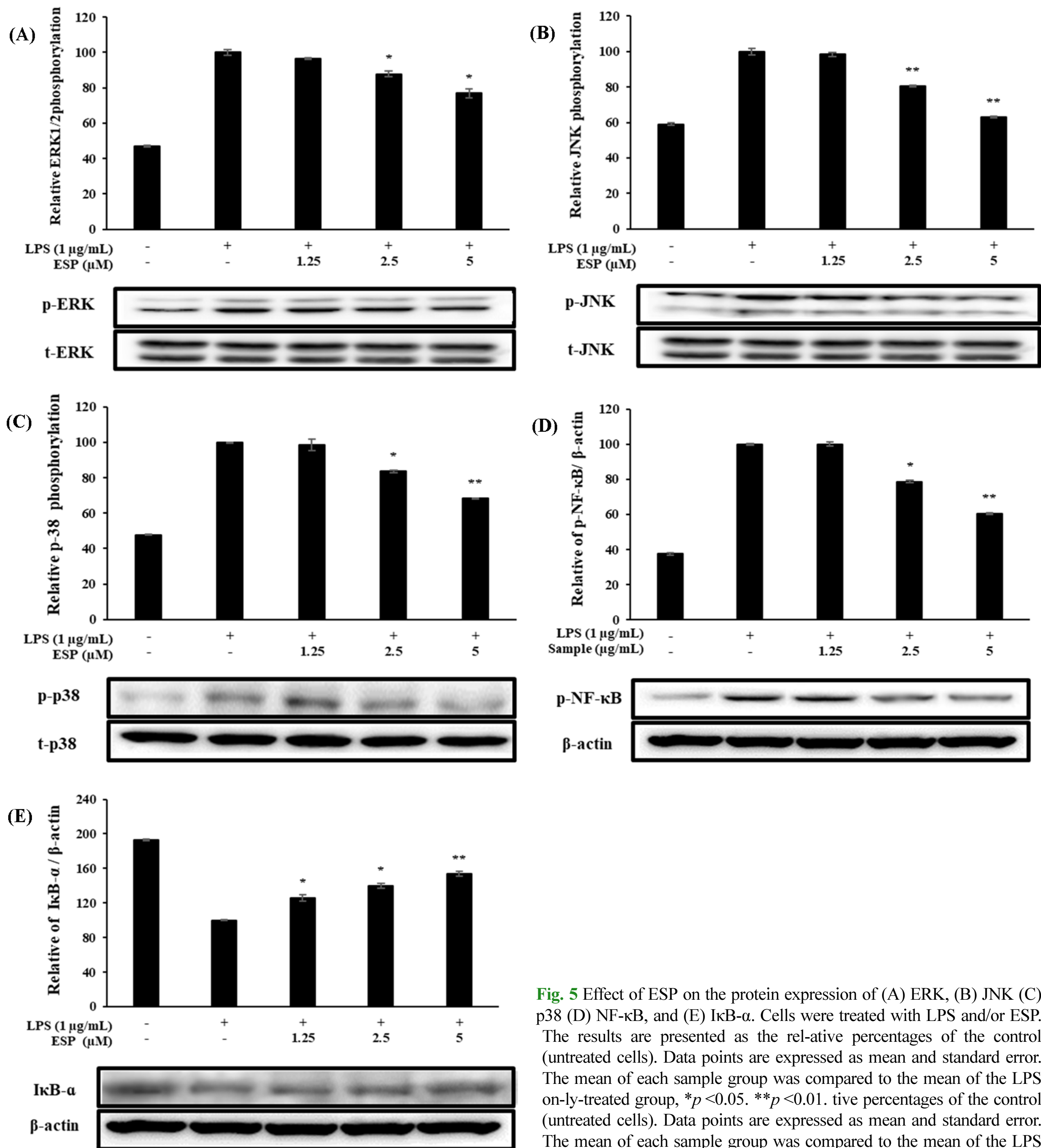

Fig. 5 Effect of ESP on the protein expression of (A) ERK, (B) JNK (C) p38 (D) NF-κB, and (E) IκB-α. Cells were treated with LPS and/or ESP. The results are presented as the rel-ative percentages of the control (untreated cells). Data points are expressed as mean and standard error. The mean of each sample group was compared to the mean of the LPS on-ly-treated group, **p* <0.05. ***p* <0.01. tive percentages of the control (untreated cells). Data points are expressed as mean and standard error. The mean of each sample group was compared to the mean of the LPS on-ly-treated group, **p* <0.05, ***p* <0.01

facilitate the production of other cytokine. And since it further increases the production of NO and PGE_2, it must be precisely controlled in the early stages of the inflammatory response. Our results, ESP inhibited the generation of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α and the production of NO and PGE_2 through the down-regulation of iNOS and COX-2.

In LPS-induced macrophages, the expression of inflammatory mediators and cytokines is regulated mainly through MAP Kinase and NF-κB signaling pathways. Therefore, we investigated the effect of ESP treatment on the expression of p38, JNK, ERK 1/2 phosphorylation, and IκB-α signaling pathways. Western blot results confirmed that the phosphorylation of p38, JNK, and ERK

1/2 in MAPK proteins was downregulated in the order of increasing ESP concentration compared to the LPS only treatment group. And ESP also inhibited the phosphorylation of NF-κB while promoting degradation of IκB-α. In conclusion, ESP showed anti-inflammatory activity by effectively inhibiting p38, ERK1/2, JNK, and NF-κB phosphorylation. Therefore, we suggest that ESP can be applied as an anti-inflammatory agent targeting inflammatory factors expressed through MAP Kinase and NF-κB signaling pathways.

Poor solubility in drug development is one of the major problems facing researchers. It is well known that decrease in bioavailability of poorly water-soluble drugs is often related to its poor solubility and low dissolution rate in an aqueous environment and there is a huge struggle in the pharmaceutical industry for improvement, with scientists and researchers spending lots of time and money in developing new techniques. The synthesis of phosphate derivatives is used in many cases to improve the water solubility of the parent compound [45], and according to our previous study, naringenin 7-*O*-phosphate synthesized through biorenovation showed an approximately 45-fold increase in water solubility compared to naringenin [46]. Therefore, phosphate derivatives can be used as a strategy to modify molecules to optimize the physicochemical and pharmacological properties of drugs. In conclusion, our study suggests that ESP, a novel compound, could be used as an effective anti-inflammatory agent with good water solubility, achieved through structural modification of esculetin by biorenovation.

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