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Note

Anti-Inflammatory and Antioxidant *in Vitro* Activities of Magnoliae Flos Ethanol Extract

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ABSTRACT: This study evaluated Magnoliea Flos ethanol extract (MFE) as a potential natural anti-inflammatory and antioxidant in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages and *in vitro* antioxidant assays. MFE (10, 30, and 50 μg/mL) dose-dependently inhibited LSP-induced nitric oxide production, which is mediated by down-regulating gene and protein expression of inducible nitric oxide synthase and cyclooxygenase-2. MFE also down-regulated both gene and protein expression of nuclear factor-kappa B and its downstream genes, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), compared with vehicle-treated cells. As a result, MFE treatment of LPS-stimulated macrophages significantly suppressed release of pro-inflammatory cytokines, such as TNF-α and IL-6. The antioxidant *in vitro* test revealed 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activities of MFE (0.25～5 mg/mL) of 16.62% to 75.17% and 38.54% to 92.91%, respectively. The ferric reducing antioxidant ability of MFE was 0.54 mM to 2.14 mM. Overall, MFE exhibited antioxidant activity and an effective anti-inflammatory response in LPS-stimulated macrophages, which is potentially valuable for application as a natural functional material.

Keywords: anti-inflammatory, antioxidants, Magnoliae Flos, RAW 264.7 macrophage

INTRODUCTION

Inflammation is one of the complex physiological protective responses to foreign organisms, such as pathogens, irritants, dust particles, and viruses (Arulselvan et al., 2016). In particular, macrophages have important roles in inducing inflammatory responses through secretion of several cytokines (Dung et al., 2009, Lee et al., 2011). Macrophages are activated by lipopolysaccharide (LPS), endotoxin isolated from Gram-negative bacteria, and released inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Kanno et al., 2006). However, an excessive inflammatory response is associated with progression of various chronic diseases, including obesity, diabetes, inflammatory bowel disease, cancer, and cardiovascular disease (Esser et al., 2014). Therefore, inhibition of the inflammatory response is crucial for controlling various diseases, and efforts have been made to derive anti-inflammatory substances from natural sources (Van et al., 2009; Mueller et al., 2010).

Magnoliae Flos refers to the flower buds of *Magnolia denudata*, and is often known by its Chinese name "Xin-yi" (Shen et al., 2008). Magnoliae Flos is commonly used to treat allergic rhinitis, asthma, sinusitis, and headache in China and Korea (Kim et al., 2008). It contains various bioactive compounds, such as lignans (magosalin, magnosalicin, magnone A, B, magnolin, eudesmin), essential oils (α-pinene, cineole, citral, eugenol, and capric acid), and alkaloids [(−)-coclaurine, (−)-N-methylcoclaurine, (+)-reticuline] (Yun and Kim, 2021). A previous study showed that a Magnoliae Flos methanol extract protected against glutamate-mediated oxidative stress in mouse hippocampal neuronal cells (Jung et al., 2018). Recently, Chen et al. (2020) suggested that Magnoliae Flos essential oil can act as an immunosuppressant because it suppressed cytokine (TNF-α) production in LPS-stimulated dendritic cells. However, the anti-inflammatory impact of Magnoliae Flos ethanol extract (MFE) on LPS-stimulated macrophages is unclear. Therefore, this study evaluated the anti-inflammatory activity and underlying mechanism of MFE on LPS-stimulated RAW 264.7 macrophages, and measured the *in vitro* antioxidant activity of MFE.

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MATERIALS AND METHODS

Preparation of MFE

Magnoliae Flos was purchased from Dong-Bu Herbal Medicine (Suncheon, Korea). Magnoliae Flos (100 g) was mixed with 80% ethanol (1.5 L) and extracted three times at 55°C for 4 h. The extract was filtered with a 0.2 μm syringe filter (Sartorius, Göttingen, Germany) and concentrated using a rotary evaporator (EYELA Rotary Evaporator N-1000; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). To obtain a powder, the concentrate was dried in a freeze-dryer (Freeze Dryer PVTFD10R; ilShinBioBase Co., Ltd., Yangju, Korea), generating a powder yield of 11%. The total polyphenol and flavonoid contents of MFE were 54.33±1.10 mg gallic acid equivalents (GAE)/g and 16.84 ±1.83 mg rutin equivalents/g, respectively.

Cell culture and cell viability assay

The murine macrophage cell line RAW 264.7 (KCLB no. 40071; Korea Cell Line Bank, Seoul, Korea) was cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) with 10% (v/v) fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco) at 37°C in a humified incubator with an atmosphere of 5% CO_2.

To determine the effects of MFE on cell proliferation, cells were seeded at a density of 5×10^4 cells per well in a 96-well plate and incubated at 37°C for 16~24 h. Cells were then treated with 1 μg/mL LPS (Sigma-Aldrich Co., St. Louis, MO, USA) and MFE (10, 30, or 50 μg/mL), and incubated for 24 h. Macrophage viability was determined by using a EZ-Cytox cell viability assay kit (DAEIL Lab Service Co., Ltd., Seoul, Korea) according to the manufacturer's instructions. Absorbance was measured using a microplate reader (VersaMax, Molecular Devices, San Jose, CA, USA).

Nitric oxide assays

Cells (5×10^4 cells/well) were plated in a 96-well plate and incubated at 37°C for 16~24 h, and treated with LPS (1 μg/mL) and MFE (10, 30, or 50 μg/mL) for 24 h. Culture medium supernatants (100 μL) were mixed with the same amount of Griess reagent [1% sulfanilamide : 0.1% naphtylenthylenediamine (1:1)] at room temperature for 10 min. The absorbance was measured using a microplate reader (VersaMax, Molecular Devices).

Pro-inflammatory cytokines

Cells (5×10^4 cells/well) were seeded in a 96-well plate and incubated at 37°C for 16~24 h, treated with LPS (1 μg/mL) and MFE (10, 30, or 50 μg/mL), and incubated for a further 24 h. Cytokine content in the cell culture medium was quantified using a mouse Duoset enzyme-linked immunosorbent assay, according to manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA).

RNA isolation and real-time polymerase chain reaction (PCR)

Cells were seeded at a density of 1×10^6 cells per well in a 6-well plate and incubated for 16~24 h at 37°C, treated with LPS (1 μg/mL) and MFE (10, 30, or 50 μg/mL), and incubated for a further 24 h. Total RNA was extracted from RAW 264.7 macrophage cells using Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). The absorbance of the isolated RNA was measured at 260 nm and 280 nm using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and quantified. The ReverTra Ace qPCR RT master mix (Toyobo Co., Ltd., Osaka, Japan) was used to reverse-transcribe total RNA into cDNA.

mRNA expression was quantified using SYBR green PCR kits (Qiagen, Hilden, Germany) and a CFX96TM real-time system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primers used for each gene were as follows: cyclooxygenase-2 (COX-2) (forward/reverse: 5'-AGCCCATTGAACCTGGACTG-3'/5'-ACCCAATCAGCGTTTCTCGT-3'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward/reverse: 5'-AAGGTCATCCCAGAGCTGAA-3'/5'-CTGCTTCACCACCTTCTTGA-3'); IL-6 (forward/reverse: 5'-AGTCCTTCCTACCCCAATTTCC-3'/5'-TGGTCTTGGTCCTTAGCCAC-3'); inducible nitric oxide synthase (iNOS) (forward/reverse: 5'-AGAACGGAGAACGGAGAACG-3'/5'-GAAGAGAAACTTCCAGGGGCA-3'); nuclear factor kappa-B (NF-κB) (forward/reverse: 5'-GAAGTGAGAGAGTGAGCGAGAGAG-3'/5'-CGGGTGGCGAAACCTCCTC-3'); TNF-α (forward/reverse: 5'-AAAGACACCATGAGCACAGAAAGC-3'/5'-GCCACAAGCAGGAATGAGAAGAG-3'). After normalizing to expression of GAPDH, relative expression of the target transcripts were calculated. mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Western blot analysis

Cells (1×10^6 cells/well) were seeded in a 6-well plate and incubated at 37°C for 16~24 h, treated with LPS (1 μg/mL) and MFE (10, 30, or 50 μg/mL), and incubated for a further 24 h. At the end of the incubation period, cells were washed with phosphate buffered saline and lysed in lysis buffer for 3 h. After lysis, cells were centrifuged at 13,000 rpm for 5 min, and the supernatants were assessed using Western blots. Total protein concentrations were measured using the Bradford method (Bradford, 1976). Protein samples (10 μg) were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were incubated overnight with antibodies against COX-2, iNOS, IL-6, and TNF-α (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and β-actin (1:2,000; Sigma-

Aldrich Co.) at 4°C, followed by anti-rabbit IgG secondary antibody (1:10,000; Cell Signaling Technology Inc., Danvers, MA, USA) for 2 h. The protein bands were visualized by incubating membranes with enhanced chemiluminescence reagent (Santa Cruz Biotechnology Inc.) and assessed with a chemiluminescence image analyzer and quantified by densitometry analysis using the UVITEC Alliance Q9 advanced system (UVITEC, Cambridge, UK).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging activity was determined using the method of Anggraini et al. (2011), with modifications. Briefly, 200 mM DPPH was dissolved in ethanol and MFE (dilution of different concentrations) or ascorbic acid mixed in equal amounts. Mixtures were incubated at room temperature for 30 min in the dark, and then absorbances were measured at 517 nm using a microplate reader (VersaMax, Molecular Devices). Radical scavenging activity was calculated using the following equation: scavenging activity (%)=[1−(A_0/A_1)]×100, where A_0=optical density (OD) of the blank control and A_1=OD of the sample.

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

ABTS radical scavenging activity was determined using the $ABTS^+$ method described by Pawlak at al. (2010). The ABTS radical solution and MFE (dilution of different concentrations) or ascorbic acid mixed in equal amounts. Mixtures were incubated in the dark at room temperature for 30 min. After incubation, absorbances were measured at 734 nm in a microplate reader (VersaMax, Molecular Devices). Radical scavenging activity was calculated using the following equation: scavenging activity (%)=[1−(A_0/A_1)]×100, where A_0=OD of the blank control and A_1=OD of the sample.

Ferric reducing antioxidant power (FRAP) assay

Reducing power was determined using the method described by Thaipong et al. (2006). FRAP reagents were prepared by mixing 300 mM acetate buffer, 20 mM $FeCl_3 \cdot 6H_2O$ in 40 mM HCl and 10 mM 2,4,6-trippyl-*S* triazine at a ratio of 10:1:1. Mixtures were incubated at 37°C for 30 min, then FRAP solution and standard ($FeSO_4 \cdot 7H_2O$), MFE, or ascorbic acid were added and samples were incubated in the dark at 37°C for a further 30 min. After incubation, absorbance was measured at 593 nm using a microplate reader (VersaMax, Molecular Devices).

Statistical analysis

All experiments were performed in independent triplicates, and results were presented as mean±standard error values. The data were analyzed by performing one-way ANOVA followed by Tukey post hoc tests to determine significant differences between groups. Statistical significance was considered at $P<0.05$.

RESULTS AND DISCUSSION

Anti-inflammatory response of MFE in LPS-stimulated RAW 264.7 macrophages

In many clinical trials and experiments, infiltration of macrophage into sites of inflammation, including liver, adipose, pancreas, and muscle tissues, has been observed in subjects with obesity, diabetes, etc. (Esser et al., 2014). The present study examined the anti-inflammatory activity of MFE in LPS-stimulated macrophages. Herein, no cytotoxicity was detected at concentrations of up to 50 μg/mL of MFE in RAW 264.7 macrophages (Fig. 1A). As a result of treating LPS (1 μg/mL)-stimulated macrophage cells with MFE concentrations of 10, 30, or 50 μg/mL, nitric oxide (NO) production was significantly reduced by 19.2%, 50.5%, and 71.9%, respectively, compared to vehicle-treated cells (Fig. 1B). A previous study showed that a *Magnolia sieboldii* methanol extract (25 and 50 μg/mL) inhibited NO production in a dose-dependent manner in LPS-treatment RAW 264.7 cells by 16.6% and 66.1%, respectively (Oyungerel et al., 2013). This trend was similar to our results, although reduction of NO induced by MFE was more than that by the *Magnolia sieboldii* methanol extract at their low concentration.

NO is synthesized from oxygen and L-arginine by the action of iNOS. Prostaglandins generated by COX-2 is also an important inflammatory mediator (Moita et al., 2013). Increased iNOS and COX-2 levels are associated with various metabolic diseases (Moita et al., 2013). The results of the present study show that MFE dose-dependently down-regulates gene and protein expression of iNOS and COX-2, which were increased by LPS treatment (Fig. 1C and 1D). Previous studies have shown that phenolic compounds in traditional medicinal plants can ameliorate diseases caused by inflammation and oxidative stress (Wadsworth and Koop, 1999; Luthria, 2006). The MFE used in this study contained 54.33±mg GAE/g of polyphenol.

Both iNOS and COX-2 are tightly regulated by pro-inflammatory transcription factors, including NF-κB (Moita et al., 2013; Arulselvan et al., 2016). Therefore, we measured gene expression of NF-κB and found that MFE inhibited LPS-induced increases in NF-κB expression in a concentration-dependent manner (Fig. 2A). Furthermore, the current study showed that gene and protein expression of other factors downstream of NF-κB, TNF-α, and IL-6, were also significantly down-regulated by MFE treatment compared with vehicle treatment (Fig. 2B and

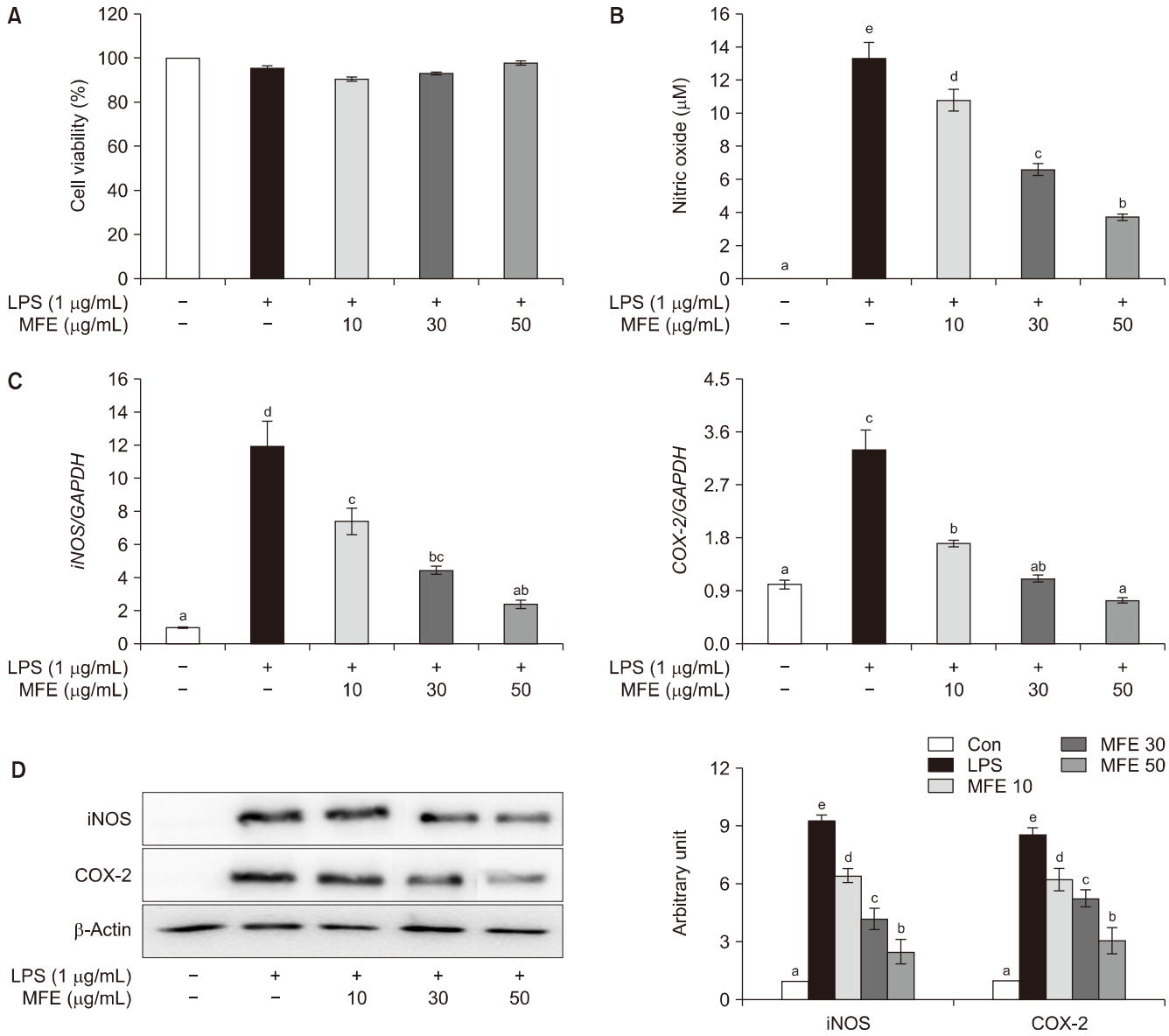

Fig. 1. Effects of MFE on cell viability (A), nitric oxide production (B), and iNOS and COX-2 gene (C), and protein expression (D) in LPS-stimulated RAW 264.7 macrophages. Data are presented as mean±standard error of three independent experiments. Values not sharing a common letter (a-e) significantly differ between groups ($P<0.05$). Con, control; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MFE, Magnoliae Flos ethanol extract.

2C). These results led to decreasing TNF-α and IL-6 levels from LPS-stimulated macrophages (Fig. 2D), since macrophages activated by LPS release pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β (Arango Duque and Descoteaux, 2014). Thus, MFE effectively inhibits LPS-induced inflammation via suppression of iNOS, COX-2, TNF-α, and IL-6 protein expression.

In vitro antioxidant activity of MFE

Overproduction of reactive oxygen species in an inflammatory site results in oxidative stress, which is related to various chronic and degenerative diseases, including cancer, diabetes, and coronary heart disease (Miliauskas et al., 2004; Whitton, 2007; Li et al., 2008). Previous studies have reported that phenolic and flavonoid compounds in plants exhibit antioxidant effects that can prevent many inflammatory diseases (Arulselvan et al., 2016). Therefore, we evaluated the antioxidant capacity of MFE by examining DPPH and ABTS radical scavenging activities and by performing FRAP assays. Free radical scavenging ability has an important role in preventing free radical-related damage in many diseases (Rahman et al., 2015). The present study showed that the DPPH and ABTS radical scavenging activities of MFE (0.25～5 mg/mL) were 16.6% to 75.2% and 38.5% to 92.9%, respectively (Fig. 3A and 3B). DPPH radical scavenging activity assays are widely used to assess the hydrogen donation ability of bioactive compounds (Ajila et al., 2007), and

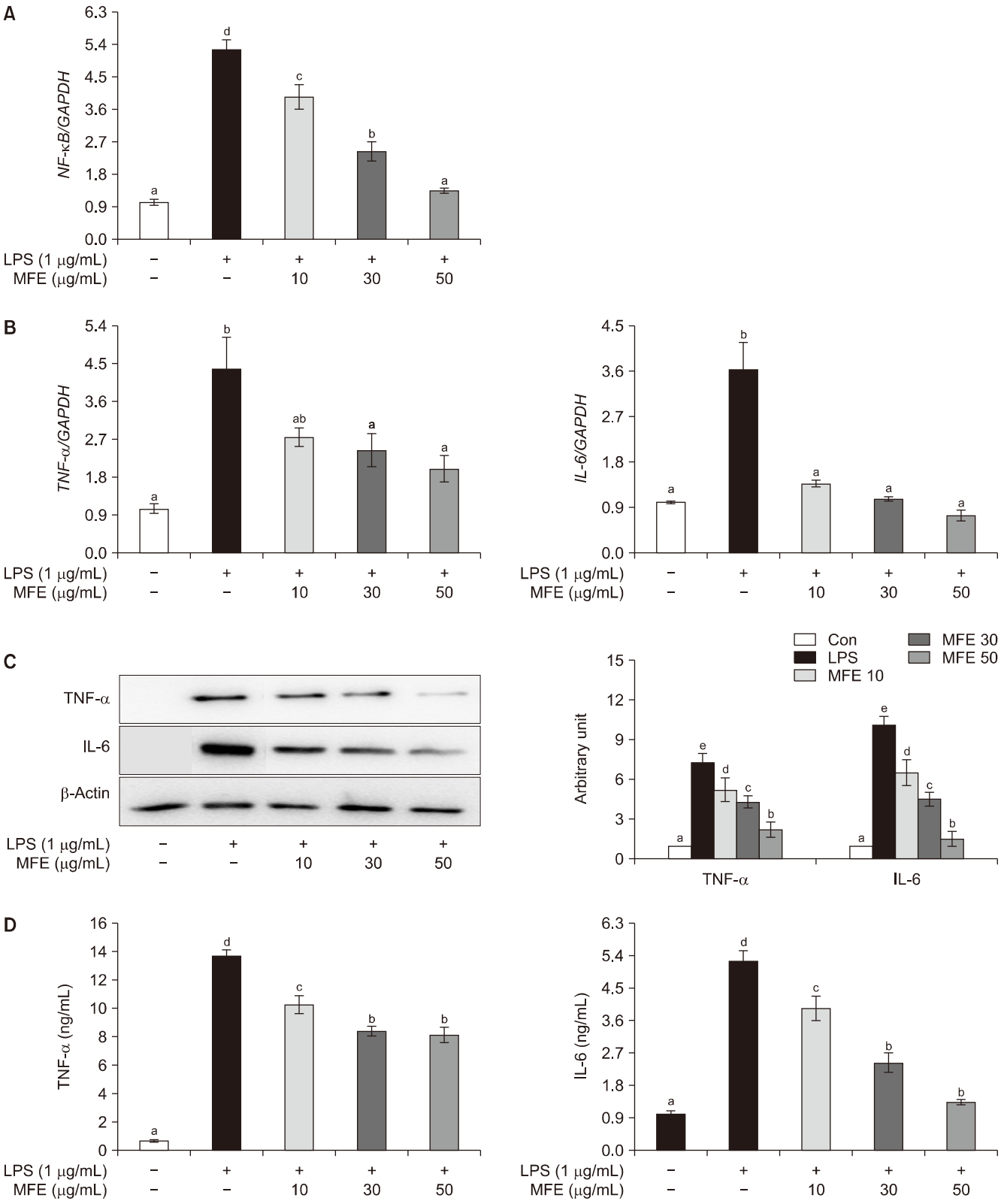

Fig. 2. Effects of MFE on NF-κB gene expression (A), TNF-α and IL-6 genes (B) and protein expression (C), and levels of pro-inflammatory cytokines (D) in LPS-stimulated RAW 264.7 macrophages. Data are presented as mean±standard error of three independent experiments. Values not sharing a common letter (a-e) significantly differ between groups (P<0.05). Con, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; LPS, lipopolysaccharide; MFE, Magnoliae Flos ethanol extract; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-α.

ABTS radical cation-based assays are one of the most commonly used antioxidant capacity assays (Fang, 2015). FRAP assay is also used to determine antioxidant activity, and their results positively correlate with those of DPPH and ABTS radical scavenging activity assays (Tian et al., 2021). In this study, FRAP activity of MFE was 0.55 mM

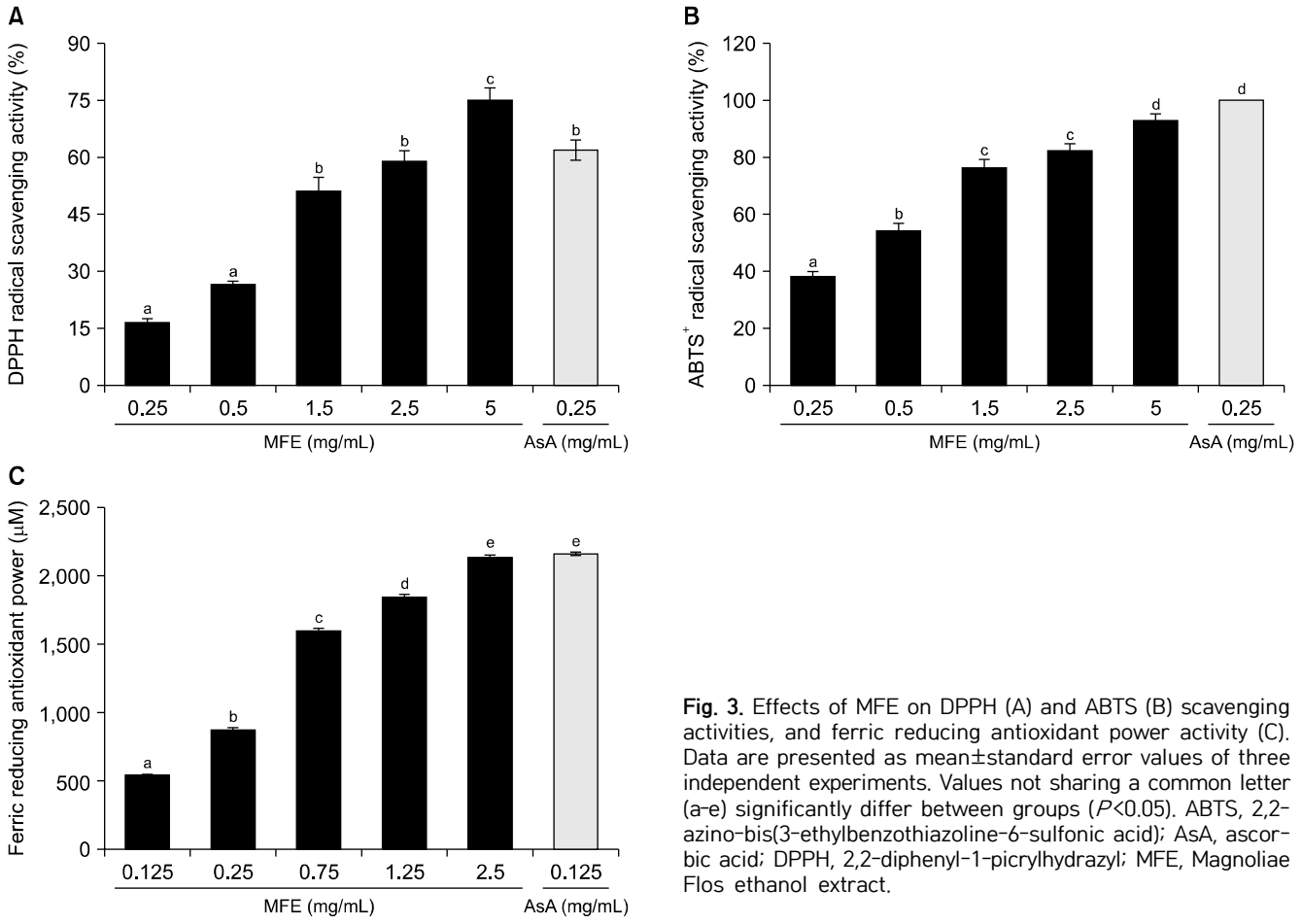

Fig. 3. Effects of MFE on DPPH (A) and ABTS (B) scavenging activities, and ferric reducing antioxidant power activity (C). Data are presented as mean±standard error values of three independent experiments. Values not sharing a common letter (a–e) significantly differ between groups (P<0.05). ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AsA, ascorbic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MFE, Magnoliae Flos ethanol extract.

to 2.14 mM (Fig. 3C), with higher concentrations of MFE having a higher antioxidant capacity, as evidenced by the DPPH and ABTS radical scavenging assays. A previous study showed that the DPPH radical scavenging activity of a fermented Magnoliae Flos extract was higher than that of a non-fermented Magnolia extract, a result which is related to the increasing polyphenol and flavonoid contents of the fermented extract (Park et al., 2015). In this study, the total polyphenol and flavonoid contents of MFE were 54.33±1.10 mg GAE/g and 16.84±1.83 mg rutin equivalents/g, respectively. Seo (2010) isolated several bioactive compounds, such as eudesmin, magnolin, epimagnolin A, fargesin, and lignin from Magnoliea Flos. However, the major constituents of MFE are not reported. In future studies, it would be helpful to identify the important active ingredients of MFE and their related mechanisms *in vitro* and *in vivo*.

In conclusion, MFE exhibited anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophages via suppressing production of NO and release of pro-inflammatory cytokines (TNF-α and IL-6). Furthermore, MFE treatment effectively removed DPPH and ABTS radicals and had a high FRAP value. These results suggest that MFE could be used as a functional anti-inflammatory and antioxidant.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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