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Note

"Nulichal" Barley Extract Suppresses Nitric Oxide and Pro-Inflammatory Cytokine Production by Lipopolysaccharides in RAW264.7 Macrophage Cell Line

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ABSTRACT: The cultivar "Nulichal," a type of naked waxy barley (*Hordeum vulgare* L.), was developed by the National Institute of Crop Science, Rural Development Administration, Korea, in 2010. In this study, we investigated the anti-inflammatory and antioxidant properties of the "Nulichal" ethanol extract (NRE) using various assays. The NRE exhibited a total phenolic content of 7.55±0.30 mg gallic acid equivalent/g and a flavonoid content of 1.74±0.08 mg rutin equivalent/g. Cell viability assays showed no toxicity of NRE on RAW264.7 macrophage cells up to concentrations of 500 μg/mL. The NRE (300 and 500 μg/mL) significantly reduced nitric oxide (NO) production induced by lipopolysaccharides (LPS). It also down-regulated the mRNA expression and protein levels of inducible NO synthase and cyclooxygenase-2 in a dose-dependent manner. Moreover, the NRE treatment significantly decreased the levels of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, and their mRNA expression compared to LPS treatment alone. The NRE demonstrated strong free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radicals in a dose-dependent manner. The ferric reducing antioxidant power assay also showed increased antioxidant activity with increasing NRE concentrations. These findings suggest that the NRE can be used as a functional food with anti-inflammatory and antioxidant properties.

Keywords: antioxidant, barley, inflammation, nitric oxide

INTRODUCTION

Inflammation is a vital human immune system response, serving as a physiological defense mechanism against infection, injury, and tissue stress (Zhong and Shi, 2019). It can be classified into acute and chronic responses, and an increasing body of evidence links chronic inflammation to various diseases, including diabetes, metabolic syndrome, cardiovascular disease, cancer, rheumatoid arthritis, inflammatory bowel disease, asthma, and chronic obstructive lung disease (Arulselvan et al., 2016). Consequently, chronic inflammation has become a focal point in current disease research, and exploring plant extracts and their natural compounds with anti-inflammatory properties holds significant promise for treating inflammatory diseases (Zhong and Shi, 2019).

Barley (*Hordeum vulgare* L.) is a valuable grain known for its therapeutic effects on conditions such as diabetes, obesity, hypertension, and inflammatory bowel disease (Min et al., 2021). These benefits can be primarily attributed to its abundant dietary fibers, particularly β-glucan, in barley (Goudar et al., 2020). Additionally, barley is an excellent source of starch, minerals, vitamins, and protein, making it an ideal food supplement. However, its sensory characteristics have hindered its widespread use, with only approximately 2% of global barley production being a food supplement (Baik and Ullrich, 2008). Consequently, ongoing research endeavors aim to develop new cultivars or explore novel processing methods to enhance barley's sensory attributes (Nevo, 2012).

One such cultivar is "Nulichal," developed by the National Institute of Crop Science (NICS), Rural Development Administration (RDA), Korea, in 2010. "Nulichal" possesses desirable traits such as excellent water absorption and expansion (Lee et al., 2018). Moreover, when compared to "Saechalssal," it exhibits higher peak viscosity and lower setback viscosity when cooked. Additionally, "Nulichal" demonstrates reduced hardness, elasticity,

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and adhesiveness, resulting in a softer texture when preparing barley rice (Matsumoto et al., 2005). Despite these attributes, no research currently investigates the physiological activity of "Nulichal." Therefore, the aim of this study was to evaluate the anti-inflammatory and antioxidant properties of the "Nulichal" ethanol extract (NRE) using the RAW264.7 macrophage cell line, along with 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, and ferric reducing antioxidant power (FRAP) assays.

MATERIALS AND METHODS

Preparation of the NRE

To obtain the NRE, "Nulichal" flour (100 g, NICS, RDA) was defatted using hexane (CAS no. 110-54-3, 1 L) for 24 h at room temperature. The defatted mixture was filtered through a Whatman filter paper. The remaining "Nulichal" residue was subjected to three extractions using 1 L of fermented ethanol (CAS no. 64-17-5). The extractions were filtered using a Buchner funnel lined with filter paper (Carl Roth, 111 A, Ø100 mm). The resulting extracts were concentrated using a rotary evaporator (EYELA Co., Ltd.). Finally, the NRE was freeze-dried using a freeze dryer (EYELA FDU-2100, EYELA Co., Ltd.). The total phenolic and flavonoid contents of the NRE were determined to be 7.55±0.30 mg gallic acid equivalent/g and 1.74±0.08 mg rutin equivalent/g, respectively.

Cell culture and cell viability assay

The murine macrophage cell line RAW264.7 (KCLB no. 40071; Korea Cell Line Bank) was cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco). The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

To evaluate the effects of NRE on cell viability, the RAW264.7 cells were seeded at a density of 5×10^4 cells per well in a 96-well plate and incubated at 37°C for 12 ~20 h. Before NRE treatment, the cells were pretreated with 1 μg/mL lipopolysaccharides (LPS) (Sigma-Aldrich Co.) for 1 h. Subsequently, the cells were treated with different concentrations of NRE (100, 300, or 500 μg/mL). After incubating for 24 h, the cell viability of RAW264.7 cells was assessed using an EZ-Cytox cell viability assay kit (DoGenBio Co., Ltd.) following the manufacturer's instructions. The absorbance was measured using a microplate reader (VersaMax, Molecular Devices).

Nitric oxide assays

Cells were seeded in a 96-well plate at a density of 5×10^4 cells per well and incubated at 37°C for 12~20 h. The cells were then treated with LPS at a concentration of 1 μg/mL and various concentrations of NRE (100, 300, or 500 μg/mL) for 24 h. After the incubation period, the cultured medium supernatants were mixed with the Griess reagent and incubated for 10 min at room temperature. The absorbance of the samples was measured at 550 nm using a microplate reader (VersaMax). The nitrate concentration was determined by comparing the absorbance values to a sodium nitrate standard curve.

Measurement of pro-inflammatory cytokines

Cells were seeded in a 96-well plate at a density of 5×10^4 cells per well and incubated at 37°C for 12~20 h. The cells were then treated with LPS (1 μg/mL) and various concentrations of NRE (100, 300, or 500 μg/mL) for 24 h. The cytokine content in the cell culture medium was quantified using a mouse Duoset enzyme-linked immunosorbent assay kit, following the manufacturer's instructions (R&D Systems Inc.).

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

Cells were seeded at a density of 1×10^6 cells per well in a 6-well plate and incubated for 12~20 h at 37°C. The cells were pretreated with LPS (1 μg/mL) for 1 h and incubated with NRE (300 or 500 μg/mL) for 24 h. Total RNA was extracted from the cells using the RiboEx kit (GeneAll Biotechnology Co., Ltd.). The absorbance of the isolated RNA was measured at 260 and 280 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific) for quantification. The cDNA was synthesized from the total RNA using the SuperiorScript III cDNA Synthesis Kit (Enzynomics). Real-time PCR was performed using the TOPreal SYBR Green qPCR PreMIX (Enzynomics) and a CFX RT-PCR system (Bio-Rad Laboratories Inc.). The mRNA expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the relative expression of target transcripts was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primer sequences used were as follows: GAPDH (forward 5'-AAGGTCATCCCAGAGCT GAA-3', reverse 5'-CTGCTTCACCACCTTCTTGA-3'), cyclooxygenase-2 (COX-2) (forward 5'-AGCCCATTGAACC TGGACTG-3', reverse 5'-ACCCAATCAGCGTTTCTCGT-3'), inducible nitric oxide synthase (iNOS) (forward 5'-AG AACGGAGAACGGAGAACG-3', reverse 5'-GAAGAGAA ACTTCCAGGGGCA-3'), tumor necrosis factor-α (TNF-α) (forward 5'-AAAGACACCATGAGCACAGAAAGC-3', reverse 5'-GCCACAAGCAGGAATGAGAAGAG-3'), interleukin-6 (IL-6) (forward 5'-AGTCCTTCCTACCCCAATT TCC-3', reverse 5'-TGGTCTTGGTCCTTAGCCAC-3').

Western blot analysis

Cells were prepared in a 6-well plate at a density of 1×

10^6 cells per well and incubated at 37°C for 12~20 h. The cells were pretreated with LPS (1 μg/mL) for 1 h and incubated with NRE (300 or 500 μg/mL) for 24 h. After treatment, the cells were washed with phosphate-buffered saline and lysed in a lysis buffer for 3 h. The cell lysates were centrifuged at 6,810 *g* for 5 min, and the supernatant was collected for protein analysis. The total protein concentration in the lysates was measured using the Pierce BCA Protein Assay kit following the manufacturer's instructions (Thermo Fisher Scientific).

Protein samples (15 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with specific primary antibodies against iNOS and COX-2 (diluted 1:2,000, Cell Signaling Technology Inc.) and β-actin (diluted 1:2,000, Santa Cruz Biotechnology Inc.). After primary antibody incubation, the membranes were incubated with anti-rabbit and anti-mouse immunoglobulin G secondary antibodies (diluted 1:2,500, Cell Signaling Technology Inc.) for 2 h. Protein bands were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific) and detected with a chemiluminescence image analyzer (Davinchi-K). The intensity of the protein bands was quantified using Image J software.

In vitro antioxidant activity assay

The DPPH and ABTS radical scavenging activities of NRE were determined. NRE was mixed with an equal volume of 200 μM DPPH solution or ABTS radical solution in the respective assay. After 30 min of incubation in the dark at room temperature, the absorbances were measured at 517 nm for DPPH and 734 nm for ABTS using a microplate reader (VersaMax). The radical scavenging activity was calculated using the formula: scavenging activity (%) $=[1-(\text{OD of the sample}/\text{OD of the blank})]\times 100$. The half-maximal inhibitory concentration (IC_{50}) was calculated to determine the effectiveness of NRE in inhibiting free radicals.

The FRAP assay was performed to assess the antioxidant capacity of NRE according to the modified methods of Alagumanivasagam et al. (2012). The FRAP reagent, consisting of 300 mM acetate buffer, 20 mM $FeCl_3 \cdot 6H_2O$, and 10 mM 2,4,6-tripyridyl-S-triazine (in 40 mM HCl), was prepared. NRE, $FeSO_4 \cdot 7H_2O$ (standard solution), or ascorbic acid (positive control) was mixed with the FRAP reagent and incubated for 30 min in the dark at 37°C. The absorbance was measured at 593 nm.

Statistical analysis

All experiments described were conducted in independent triplicates, and the results were expressed as mean±standard error values. Statistical analysis was performed using one-way analysis of variance, followed by Duncan's multiple post hoc tests to determine significant differences between groups. A *P*-value of less than 0.05 ($P<0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

NRE reduces inflammation by acting on iNOS and COX-2 in LPS-stimulated macrophages

The cytotoxicity test conducted on RAW264.7 macrophage cells using NRE at concentrations ranging from 100 to 500 μg/mL revealed no signs of cytotoxicity. As a result, further experiments were performed using NRE at 100, 300, and 500 μg/mL concentrations.

In this study, we discovered that NRE significantly reduced the levels of LPS-induced NO at concentrations of 300 and 500 μg/mL compared to cells treated only with LPS (Fig. 1). NO plays a crucial role in various physiological functions, including pathogen defense, blood pressure regulation, and neurotransmission in the central nervous system (Liew, 1994; Duncan and Heales, 2005; Naseem, 2005). It is synthesized from L-arginine by three isoforms of NO synthases (NOS): NOS1 (neuronal NOS or nNOS), NOS2 (inducible NOS or iNOS), and NOS3 (endothelial

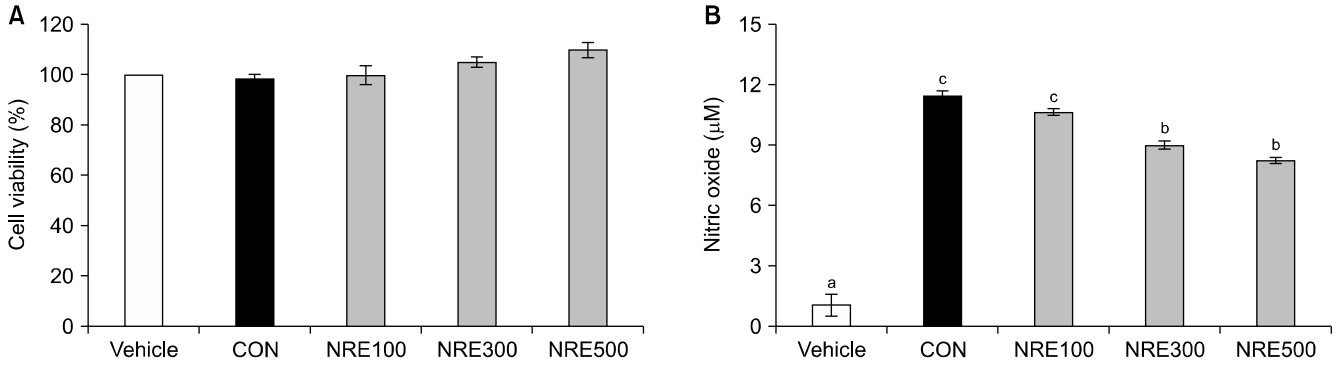

Fig. 1. Effects of the "Nulichal" ethanol extract (NRE) on cell viability and nitric oxide levels in lipopolysaccharides-induced RAW264.7 macrophages. Data are presented as mean±SE of three independent experiments. Values not sharing a common letter (a-c) indicate significant between-group differences ($P<0.05$). CON, control; NRE100, 100 μg/mL of NRE; NRE300, 300 μg/mL of NRE; NRE500, 500 μg/mL of NRE.

NOS or eNOS) (Ibrahim et al., 2018). Excessive and persistent production of NO-mediated by iNOS in macrophages, monocytes, and other cells under pathological conditions can be toxic and is considered a major contributor to inflammation in chronic diseases (Liew, 1994; Guzik et al., 2003). LPS is known to be a potent inducer of iNOS. Hence, we investigated the impact of NRE on modulating iNOS activity in LPS-stimulated macrophages. Our study demonstrated that LPS significantly increased the expression of iNOS mRNA by 21.7-fold. However, both doses of NRE (300 and 500 μg/mL) similarly down-regulated iNOS mRNA levels by 32.0% to 44.4% compared to cells treated only with LPS. Moreover, the expression of the iNOS protein was dose-dependently reduced in NRE-treated cells (Fig. 2).

LPS similarly induces COX-2 (Guzik et al., 2003). COX-2 is the key enzyme regulating the production of prostaglandins, the central mediators of inflammation (Tsatsanis et al., 2006). The excessive induction of COX-2 expression contributes to tissue injury in various cancers (Tu et al., 2014). Therefore, the discovery of a new COX-2 inhibitor could prove beneficial in lowering inflammation levels, thereby lowering the risk of developing chronic inflammatory disorders (Sakthivel and Guruvayoorappan, 2016). NRE (300 and 500 μg/mL) significantly inhibited the mRNA and protein expressions of COX-2 induced by LPS (Fig. 2). Previous studies have demonstrated that phytochemical compounds inhibit iNOS-mediated NO production as well as several enzymes—including COX, lipoxygenase, and xanthine oxidase—all of which are implicated in inflammatory pathologies (Lim et al., 2013; Ravishankar et al., 2013). Thus, the anti-inflammatory activity of NRE is mediated by inhibiting the iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells.

NRE inhibits the production of LPS-induced pro-inflammatory cytokines in macrophages

Oxidative stress-induced inflammation is known to involve the activation of COX-2, iNOS, and the up-regulation of inflammatory cytokines, including TNF-α, IL-6, and chemokines such as CXC chemokine receptor 4 (Federico et al., 2007; Hussain and Harris, 2007). In mammals, TNF-α and IL-6 are recognized as major mediators of inflammatory responses originating from macrophages (Zhang and An, 2007). When stimulated by LPS, monocytes and macrophages produce pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (Beutler and Rietschel, 2003), contributing to vascular injury and dysfunction of multiple organs (Hawiger, 2001). In our study, LPS significantly increased the levels of inflammatory factors, including TNF-α and IL-6. However, NRE exhibited a dose-dependent decrease in TNF-α (at doses of 300 and 500 μg/mL) and IL-6 (at doses of 100, 300, and 500 μg/mL) levels compared to cells treated only with LPS (Fig. 3). Furthermore, NRE (300 and 500 μg/mL) significantly down-regulated the expression of TNF-α and IL-6 genes that were up-regulated by LPS (Fig. 3). Therefore, NRE demonstrated the ability to reduce the production of pro-inflammatory cytokines, such as TNF-α and IL-6, which were increased by LPS, by modulating their mRNA transcription levels.

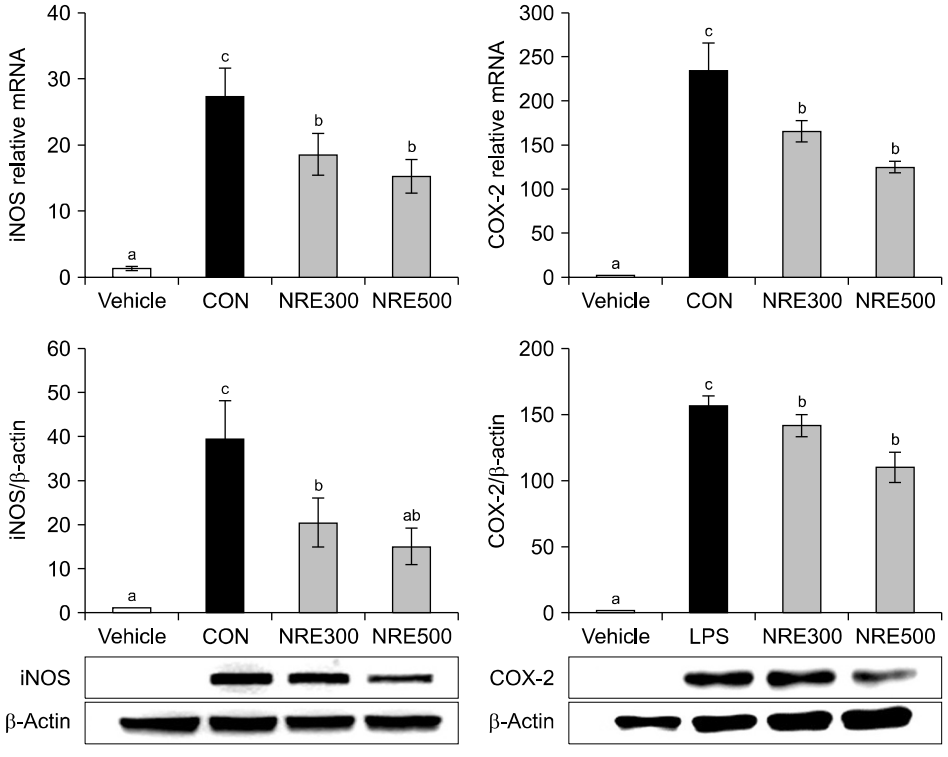

Fig. 2. Effects of the "Nulichal" ethanol extract (NRE) on inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression and their protein expression in lipopolysaccharides (LPS)-induced RAW264.7 macrophages. Data are presented as mean±SE of three independent experiments. The protein expression data were normalized with β-actin and calculated as a ratio to the vehicle value. Values not sharing a common letter (a-c) indicate significant between-group differences (P<0.05). CON, control; NRE300, 300 μg/mL of NRE; NRE500, 500 μg/mL of NRE.

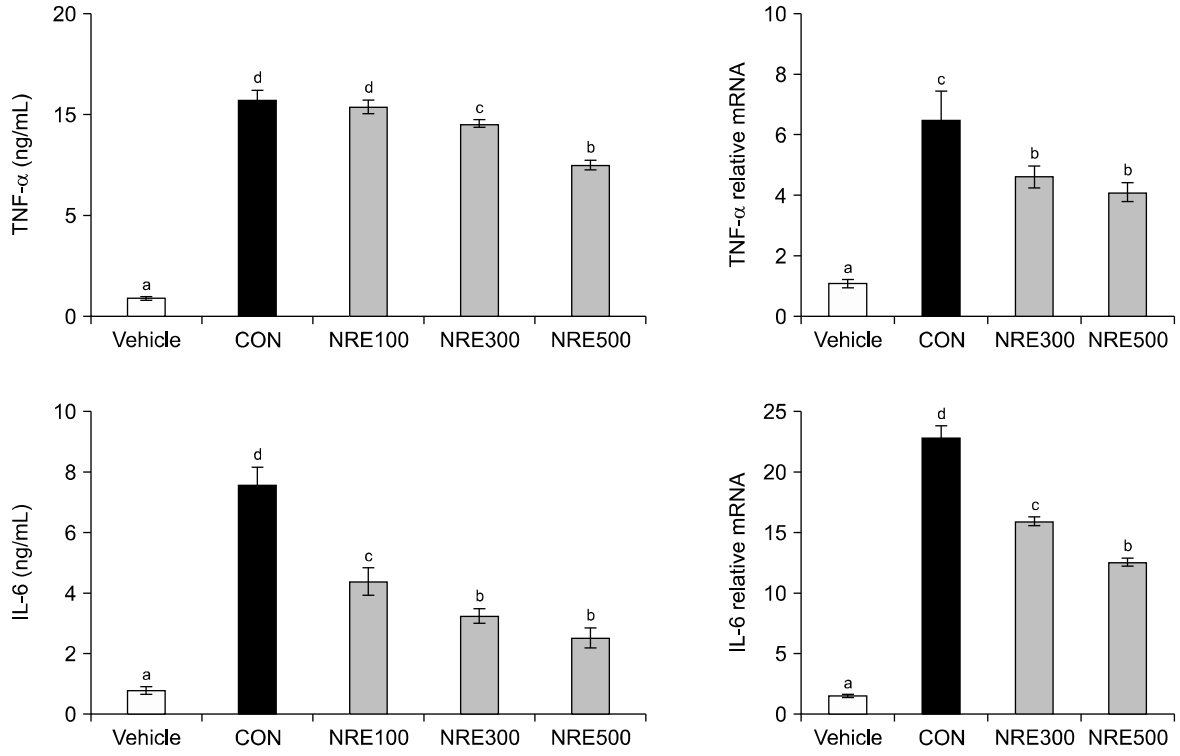

Fig. 3. Effects of the "Nulichal" ethanol extract (NRE) on tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels and their mRNA expression in lipopolysaccharides (LPS)-induced RAW264.7 macrophages. Data are presented as mean±SE of three independent experiments. Values not sharing a common letter (a-d) indicate significant between-group differences (P<0.05). CON, control; NRE100, 100 μg/mL of NRE; NRE300, 300 μg/mL of NRE; NRE500, 500 μg/mL of NRE.

In vitro antioxidant activity of the NRE

Oxidative stress causes chronic inflammation, leading to chronic diseases such as cancer, diabetes, and cardiovascular and neurological diseases (Reuter et al., 2010). The free radical scavenging activity of the NRE could be one of the possible mechanisms for its anti-inflammatory effect (Hussain et al., 2016). DPPH and ABTS radical scavenging assays were conducted to evaluate the antioxidant activity of the NRE. NRE significantly scavenged DPPH and ABTS radicals in a dose-dependent manner, with an IC_{50} of 2.45 mg/mL and 223.80 μg/mL, respectively (Fig. 4). Barley is a good source of antioxidants, including polyphenols, flavonoids, and polysaccharides (Na et al., 2011). Specifically, β-glucan is a dietary fiber abundant in barley and has been known to prevent/treat digestive disorders and support the immune system (Ciecierska et al., 2019). It also exhibits antioxidant activity by scavenging reactive oxygen species and can reduce the risk of various chronic diseases such as diabetes, cardiovascular diseases, cancer, and degenerative diseases (Ciecierska et al., 2019). In addition, the FRAP activity of the NRE was dose-dependently increased (Fig. 5), which showed that NRE has potential as a reducing agent. A previous study has also shown that FRAP increases with an increase in the total phenolic content of barley (Zhu et al., 2015). The NRE contains total phenolic (7.55±

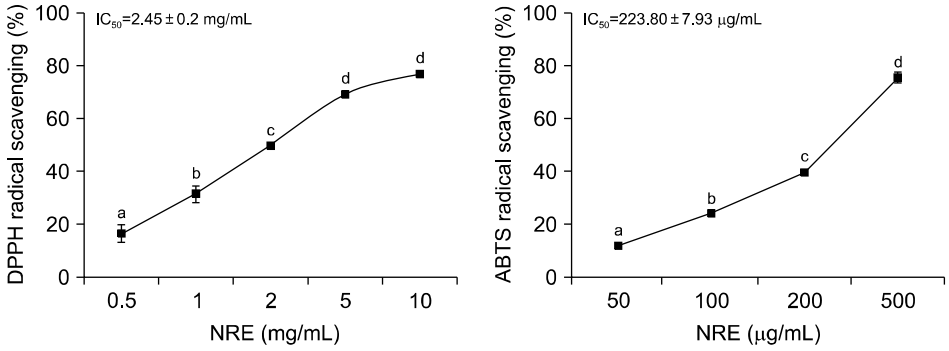

Fig. 4. Effects of the "Nulichal" ethanol extract (NRE) on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals scavenging activities and half-maximal inhibitory concentration (IC_{50}) values. Data are presented as mean±SE of three independent experiments. Values not sharing a common letter (a-d) indicate significant between-group differences (P<0.05).

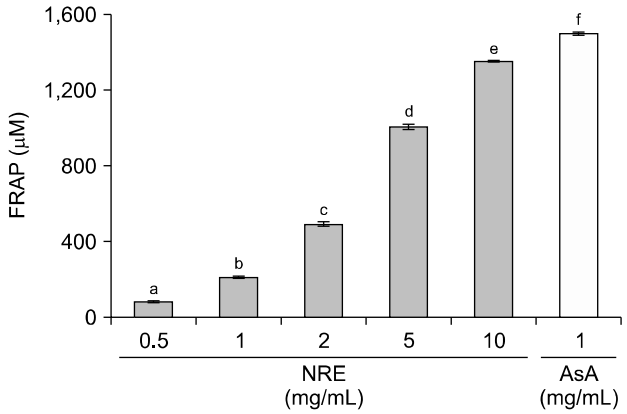

Fig. 5. Effects of the "Nulichal" ethanol extract (NRE) on ferric reducing antioxidant power (FRAP) activity. Data are presented as mean±SE of three independent experiments. Values not sharing a common letter (a–f) indicate significant between-group differences (P<0.05). AsA, ascorbic acid.

0.30 mg gallic acid equivalent/g) and flavonoid (1.74± 0.08 mg rutin equivalent/g) contents, which could contribute to its antioxidant capacity. These results showed higher total phenolic contents compared to the ethanol extracts of various other barley cultivars (ranging from 5.1 to 8.2 mg/g) but lower flavonoid contents (other cultivars ranged from 5.29 to 8.27 mg/g) (Yang et al., 2021), which may be due to differences in the standard materials used and the concentration of the extraction solvent.

In conclusion, the findings of this study demonstrate the anti-inflammatory effects of NRE, including the suppression of iNOS-mediated NO production and reduction of pro-inflammatory cytokines (TNF-α and IL-6) in LPS-stimulated macrophages. Additionally, NRE exhibited antioxidant activity through its ability to scavenge free radicals. To gain further insights, future investigations should focus on identifying the specific active components within NRE and elucidating the underlying target mechanisms involved in its beneficial effects. Such studies would contribute to a deeper understanding of the therapeutic potential of NRE in managing inflammation-related conditions.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: MKL, YJS. Analysis and interpretation: MJL, MKL. Data collection: JH, JRH, HJL. Writing the article: JH, MKL. Critical revision of the article: MJL. Final approval of the article: all authors. Statistical analysis: JH. Obtained funding: YJS, MKL. Overall responsibility: MKL.

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