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Polyphenolic compounds, antioxidant and anti-inflammatory effects of *Abeliophyllum distichum* Nakai extract

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Summary

The present study was conducted to evaluate the antioxidant and anti-inflammatory activities of crude methanolic extract of *Abeliophyllum distichum* Nakai, and those of its partitioned fractions, including hexane, ethyl acetate, n-butanol, and aqueous. The antioxidant activities were analyzed by DPPH free radical scavenging and oxygen radical antioxidant capacity assay. Results showed that the BuOH fraction possessed a strong antioxidant activity through a hydrogen atom transfer reaction-based mechanism and a single electron transfer reaction-based mechanism. In lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, the BuOH fraction of *A. distichum* methanol extract exhibited a strong inhibitory effect on the nitric oxide production and inhibited the expression of pro-inflammatory mediators, including COX-2, TNF-α, and IL-6, through the inhibition of the MEK/ERK signaling pathway. In addition, the BuOH fraction inhibited the LPS-induced ROS level through the NADPH oxidase-independent mechanism. Furthermore, HPLC analysis identified chlorogenic acid, caffeic acid, gentisic acid, rutin, ferulic acid, and quercetin, and suggested that the antioxidant and anti-inflammatory activities of the BuOH fraction should be mediated by the presence of higher amounts of caffeic acid, rutin, and ferulic acid than other fractions. Taken together, these results suggest that *A. distichum* extract is a source of antioxidant and anti-inflammatory compounds, and could be developed as a potential source for functional food and dietary health supplement.

Introduction

Inflammation is a pervasive form of defense process that evolved in organisms to protect themselves from injury, infection, and exposure to contaminants (Ashley et al., 2012). Inflammatory diseases such as asthma, arthritis, vascular diseases, and cancer caused by excessive inflammation have become one of the major health issues. Macrophages are large white blood cells that play a critical role in the initiation, maintenance, and resolution of inflammation (Fujiwara and Kobayashi, 2005) and can be activated by cytokines (interferon gamma, granulocyte-monocyte colony stimulating factor, and tumor necrosis factor alpha), extracellular matrix proteins, bacterial endotoxins such as lipopolysaccharide (LPS), and other chemical mediators (Fujiwara and Kobayashi, 2005). LPS, an endotoxin and a constituent of the outer membrane of gram-negative bacteria, stimulates innate immunity and results in the production of many inflammatory mediators, including nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), prostanoids, and leukotrienes (Gillespie et al., 2007; Lee et al., 2003). Inflammatory mediators were produced by the activation of the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) cascades in macrophages (Chun et al., 2012). Therefore, LPS-induced inflammatory responses are suggested to be mediated by the activation of the NF-κB and MAPK signaling pathway. LPS also increases the intracellular reactive oxygen species (ROS) through the activation of NADPH oxidases and mitochondria respiratory chain systems, thus indicating that ROS regulates inflammatory diseases (Emre et al., 2007).

ROS, the modulator of redox-sensitive signaling, contribute to the innate immune response to resist various pathogens. However, an excessive amount of ROS caused by an imbalance between their cellular generation and antioxidant mechanisms can induce progressive oxidative damage to cellular components (i.e., lipids, membranes, proteins, and nucleic acids), resulting in the induction of cell death (Schieber and Chandel, 2014). The most important source of ROS is the NADPH oxidase (NOX) family in the plasma membrane and mitochondria (Bedard and Krause, 2007). Although NOX-induced ROS are essential modulators of MAPK signaling pathways, which play a major role in cell physiology such as cell growth, proliferation, differentiation, and immune responses (Manea et al., 2015), the NOX inhibitor has been shown to prevent the NF-κB-dependent inducible NO synthase (iNOS) expression and NO production in LPS-stimulated RAW264.7 cells (Bai et al., 2005). In addition, the suppression of LPS-induced ROS production in mitochondria results in the reduction of LPS-induced MAPK activation and pro-inflammatory mediator production (Park et al., 2015). Therefore, the increased intracellular ROS activate ROS-sensitive signaling pathways that lead to the induction of inflammation (Liu et al., 2014b), suggesting that the therapeutic targeting of oxidative stress with antioxidant should be beneficial to improve inflammatory diseases.

Abeliophyllum distichum Nakai, commonly called white forsythia, is a monotypic genus with a single species of deciduous shrub in the olive family, Oleaceae, and is native to the south and central areas of Korea (Oh et al., 2003). Although *A. distichum* has been used as a landscape plant, the pharmaceutical properties of *A. distichum*, such as anti-cancer activity (Park et al., 2014), anti-diabetic activity through the inhibition of aldose reductase (Li et al., 2013), and antihypertensive activity (Oh et al., 2003), have been recently revealed. In addition, biologically active compounds, including acteoside, eutigoside B, isoacteoside, rutin, hirsutrin, and cornoside, have been isolated from this plant. These findings indicate that *A. distichum* has potential as a crude drug and a dietary health supplement. However, extensive biological studies and the elucidation of

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the underlying molecular mechanisms of action should be required for using this plant as a beneficial material for the food and pharmaceutical industries.

In this study, methanolic extract and its fractions were submitted to bioactivity evaluation by analyzing anti-oxidant and anti-inflammatory activities. In addition, the investigation of the underlying intracellular mechanisms of action suggested that the anti-inflammatory activity of *A. distichum* extract is mediated by the suppression of ROS-sensitive signaling pathways, including extracellular signal regulated kinase (ERK) in LPS-stimulated RAW 264.7 cells. Furthermore, phenolic compounds were analyzed using high-performance liquid chromatography (HPLC) to determine the existence of various active phenolic compounds.

Material and methods

Materials

A. distichum methanolic extract (GNEP-AD-001; AD01) and its fractions [hexane fraction (GNEP-AD-002; AD02), ethyl acetate fraction (GNEP-AD-003; AD03), n-butanol fraction (GNEP-AD-004; AD04), and aqueous fraction (GNEP-AD-005; AD05)] were obtained from the Nature Environment Research Park of Gangwon Province, South Korea. Bacterial LPS from *Escherichia coli* 0127, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Griess reagent, and sodium nitrite were purchased from Sigma-Aldrich. Antibodies including MEK1/2, phospho-MEK1/2 (Ser217/221), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), p38 MAPK, and phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signalling Technology. iNOS and β-actin were obtained from Santa Cruz Biotechnology. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from GE Healthcare Life Sciences. All other chemicals were obtained from Sigma-Aldrich.

DPPH radical scavenging activity

The free radical scavenging activity of methanolic extract and its fractions was analyzed using 1,1-diphenyl-2-picrylhydrazyl (DPPH). A total of 180 µl of freshly prepared DPPH solution (0.4 mM in 80% MeOH) was plated in 96-well plates, and 20 µl of the sample (10 mg/ml) was added, followed by serial dilution to each well. Then, the plate was incubated for 10 min at room temperature in the dark condition. After 10 min, the absorbance values were measured at 520 nm using an iMark^{TM} microplate reader (Bio-Rad). The RC50 (50% reduction of DPPH radicals) was obtained from a graph of radical scavenging activity versus extract concentration. Butylated hydroxytoluene (BHT) was used as the standard.

Oxygen radical antioxidant capacity (ORAC) assay

A total of 150 µl of 0.08 µM fluorescein was mixed with 25 µl of phosphate buffer (blank), trolox (TE) standard (6.25 µM to 50 µM), or each concentration of extract in each well of a microplate. After incubation at 37 °C for 10 min, 25 µl of fresh 2,2'-azobis(isobutyramidine) dihydrochloride (0.12 g/ml) added to each well. Fluorescence intensity was measured every 1 min for 90 min using the Synergy^{TM} HTX Multi-mode Microplate Reader (BioTek, Vermont, USA) with fluorescent filters (485 nm excitation and 530 nm emission). The net area under the fluorescein decay curve (AUC) was analyzed, and the ORAC values were calculated by comparing the AUC with the TE as a standard.

Cell culture

The RAW 264.7 macrophage cell line was purchased from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium and supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/ml streptomycin in an incubator containing humidified CO_2 (5%) at 37 °C.

Cell viability assay and determination of the NO level

Cell viability was measured with MTT reduction assay. RAW 264.7 cells were plated at a density of 1.5×10^5 cells/ml in 96-well plates and incubated at 37 °C for 4 h. Then, the cells were treated in each concentration of extracts and stimulated with LPS (1 µg/ml). After incubation for 24 h, 20 µl of MTT (5 mg/ml in PBS) solution was added to each well. Incubation was continued for 4 h more, the formazan crystals were dissolved in DMSO, and the optical density was measured at 520 nm using an iMARK microplate reader (Bio-RAD).

To analyze the NO level in the cultured medium, RAW264.7 cells were treated with or without LPS in the presence of extracts. After 24 h, 100 µl of Griess reagent [1% sulfanilamide (w/v) and 0.1% (w/v) naphtylethylenediamine in 2.5% phosphoric acid (v/v)] was mixed with an equal volume of cell supernatant and incubated at room temperature for 10 min. The optical density at 540 nm was measured, and the NO concentration was calculated with reference to the standard curve generated using sodium nitrite.

Intracellular ROS measurement

Intracellular oxidative stress was detected using 2',7'-dichlorofluorescein diacetate (DCF-DA), as described by Warleta et al. (2011). RAW 264.7 cells treated with or without LPS in the presence of extracts for 24 h were washed twice with Hank's buffered salt solution (HBSS) and incubated with 20 µM of fresh DCFH-DA in HBSS for 30 min at 37 °C in a 5% CO_2 incubator. Following washing with PBS, DCF fluorescence was measured at an excitation wavelength of 485 nm and emission at 525 nm. The intracellular ROS level percentage was calculated as follows:

$$F = [(A30 - A0)/A0] \times 100,$$

where A0 is the fluorescence before incubation, and A30 is the fluorescence after incubation for 30 min.

Western blotting

After treatment with extracts in the presence or absence of 1 µg/ml of LPS for 24 h, RAW 264.7 cells were analyzed by immunoblotting. The cells were washed three times with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxylcholate, 1 mM EDTA, and 10 mM NaF). Then, 10 µg protein of each sample was separated by 10% SDS-polyacrylamide gels with electrophoresis and transferred to a PVDF membrane. After blocking nonspecific sites with 5% nonfat dried milk, the membranes were hybridized with specific antibodies. The signal was detected and visualized using a chemiluminescence system according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RAW 264.7 cells treated with extracts in the presence or absence of 1 µg/ml of LPS using a TRI reagent (Molecular Research Center, Cincinnati, USA) and was reverse-transcribed into cDNA using the ReverTra Ace® qPCR RT Master Mix with qDNA Remover (TOYOBO, Co., Ltd, Osaka, Japan) in accordance with the manufacturer's recommendations. qRT-PCR was performed using the SYBR® Green Real-time PCR Master Mix (TOYOBO, Co., Ltd, Osaka, Japan) in the CFX96^{TM} Real-time system (BIO-RAD) with default parameters. The expression level of each gene was normalized to β-actin. The specific primer pairs used in qRT-PCR are listed in Tab. S1.

HPLC analysis

The HPLC system used was a Shimadzu liquid chromatography system (LC-10ADvp) using an ultraviolet-visible (UV-vis) detector (SPD-10A, Shimadzu, Japan) equipped with a UV-vis detector (SPD-10A, Shimadzu, Japan) and Luna 5 μ C18(2) 100 A column (4.6 mm × 250 mm, particle size 5 μm). The mobile phase consisted of solvents A and B. Solvent A was 0.1% trifluoroacetic acid in distilled water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient was 0-0.01 min, 90% A; 0.01-28 min, 60% A; 28-39 min, 40% A; 39-50 min, 10% A; 50-55 min, 10% A; 55-56 min, 90% A; and 56-65 min, 90% A. Run time was 65 min using a flow rate of 0.7 ml/min. The phenolic compounds were identified by the retention time of the standard measured from the peak area at 254 nm (chlorogenic acid and rutin), 280 nm (gentisic acid and quercetin), and 360 nm (caffeic acid and ferulic acid). The concentration was calculated by comparing the peak areas of the samples with the calibration curve of the standards.

Statistical analysis

All the experiments were conducted for three independent replicates, and data were expressed as mean ± standard deviation. The significance of the between-group differences was determined by ANOVA and Duncan's multiple range test. Differences at $p < 0.05$ were considered significant.

Results

Antioxidant activities of *A. distichum* methanolic extract and its fractions

To investigate the antioxidant activity of *A. distichum*, we initially analyzed the free radical scavenging activities of crude methanolic extract (AD01) and its fractions (AD02 to AD05). As shown in Fig. 1A, the highest free radical scavenging activity was recorded in AD04 (RC50 = 29.98 μg/ml), followed by the AD01 (RC50 = 61.82 μg/ml), AD05 (RC50 = 106.64 μg/ml), AD03 (RC50 = 131.03 μg/ml), and AD02 (RC50 = 183.73 μg/ml) fractions. In addition, AD04 exhibited similar level of free radical scavenging activity to the positive control, BHT (RC50 = 33.81 μg/ml).

The assays used to determine the total antioxidant activity can be divided into hydrogen atom transfer reactions (HAT)-based assays and single electron transfer reactions (SET)-based assays. The DPPH assay is based on both HAT and SET, and the ORAC assay is based on HAT (Liang and Kitts, 2014). The ORAC assay is relevant in *in vivo* conditions because it provides peroxyl radicals, which are the most common free radical in human biology (Prior, 2015). Therefore, the ORAC assay is broadly applied in the nutraceutical, pharmaceutical, and food industries. To further characterize the antioxidant property of *A. distichum*, we analyzed the antioxidant capacity using the ORAC assay. As shown in Fig. 1B, the hydrophilic oxygen radical scavenging activities of crude methanolic extract and its fractions were concentration dependent. AD01 and AD04 at 50 μg/ml exhibited an ORAC value of 137.45 μM TE and 180.17 μM TE, respectively, and AD02 at 50 μg/ml displayed an ORAC value of 13.85 μM TE. Taken together, these results indicate that AD04 possesses a stronger antioxidant activity than other fractions and that AD04 may be used as a suppressor of ROS-sensitive inflammatory signaling pathways.

Effects of *A. distichum* methanolic extract and its fractions on NO production in LPS-stimulated RAW264.7 cells

NO is an intercellular messenger that has many physiological functions, including roles in the regulation of vascular relaxation, platelet aggregation, neurotransmission, cellular respiration, and immune

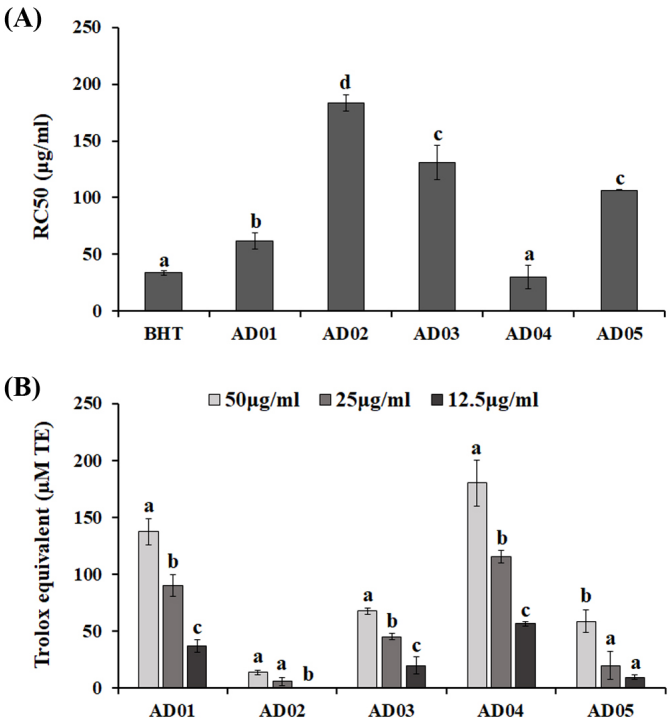

Fig. 1: Antioxidant activities of *Abeliophyllum distichum* extract. **(A)** DPPH radical scavenging activity of *A. distichum* methanol crude extract and its fractions. Bar graph showing the RC50 values for each extract from the DPPH assay. **(B)** ORAC values of *A. distichum* methanol crude extract and its fractions. Data are expressed as μM of Trolox (TE) equivalents. Data are expressed as the mean ± standard deviation of three independent experiments. Values in the same column with different superscripted letters are significantly different ($p < 0.05$). MeOH extract; AD01, hexane fraction; AD02, ethyl acetate fraction; AD03, n-butanol fraction; AD04, aqueous fraction; AD05.

responses (Ibiza and Serrador, 2008). Although NO has been recognized as an important component of the immune system, overproduction of NO under pathological conditions induces host tissue damage associated with acute and chronic inflammations (Nagy et al., 2007; Tripathi et al., 2007). Therefore, the inhibition of NO production under inflammatory stimuli is relevant in the development of new drugs for the treatment of chronic inflammatory diseases. The ability of *A. distichum* methanolic extract and its fractions to modulate the production of NO was determined in LPS-stimulated RAW 264.7 cells. As shown in Fig. 2A, the cells treated with LPS alone exhibited a marked increase in NO release (52.9 μM nitrite) compared with the non-treated cells, whereas all tested samples exhibited the inhibitory effect on LPS-induced NO production in a dose-dependent manner. AD03 at 100 μg/ml inhibited NO production (19 μM nitrite) by 35% over the level generated by LPS (Fig. 2A). Although AD04 exhibited a lower inhibitory effect on LPS-induced NO production than AD03, 100 μg/ml of AD04 significantly inhibited LPS-induced NO production (29.1 μM nitrite).

To investigate whether these inhibitory effects on LPS-induced NO production were mediated by cell viability, the cytotoxic effect of *A. distichum* methanolic extract and its fractions on macrophages was determined by MTT assay (Fig. 2B). Incubation with AD03 significantly inhibited the proliferation of LPS-stimulated RAW 264.7 cells, and AD04 did not exhibit cytotoxic activity. This finding indicates that the inhibitory effect of AD03 on NO production is attributed to cytotoxicity. Therefore, AD04 was used to further investigate the mechanisms underlying the anti-inflammatory effect of *A. distichum*.

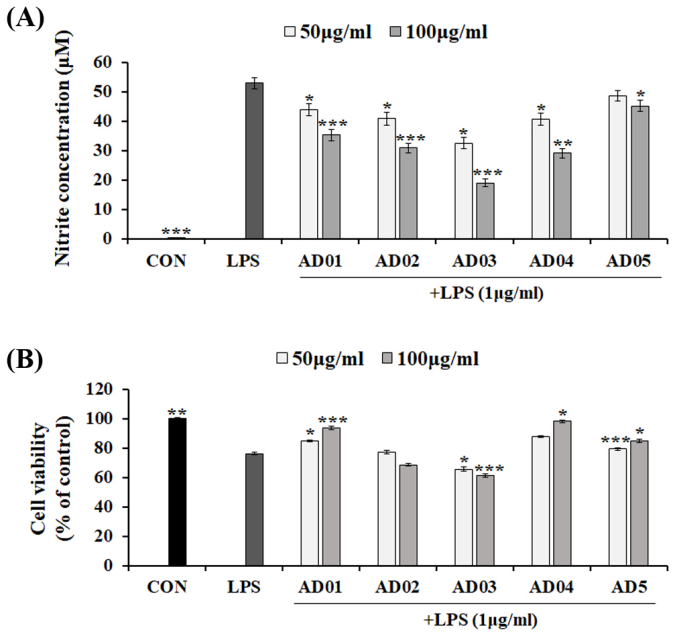

Fig. 2: Effects of *A. distichum* methanol crude extract and its fractions on NO production **(A)** and cell viability **(B)** in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were treated with or without LPS in the presence of *A. distichum* methanol crude extract and its fractions for 24 h. The amounts of NO were determined using the Griess reagent in the culture medium, and cytotoxicity was determined by MTT assay. Values are the mean ± SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ represent the significant differences in comparison with the cells treated with LPS alone. MeOH extract; AD01, hexane fraction; AD02, ethyl acetate fraction; AD03, n-butanol fraction; AD04, aqueous fraction; AD05.

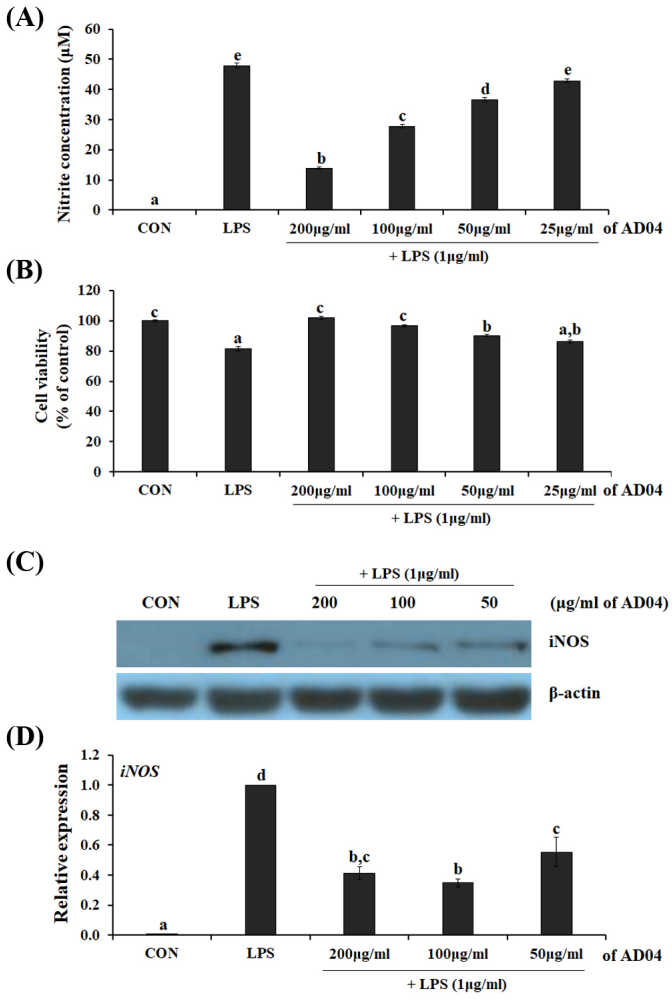

Fig. 3: Anti-inflammatory effect of BuOH fraction (AD04). The effect of AD04 on NO production **(A)** and cell viability **(B)** in LPS-stimulated RAW 264.7 cells were analyzed. NO contents were analyzed by the Griess assay, and cytotoxicity was determined by MTT assay. LPS-stimulated RAW 264.7 cells were treated with different concentrations of AD04 for 24 h and subjected to Western blotting **(C)** and qRT-PCR **(D)** for *iNOS*. The transcript level of *iNOS* was normalized to the constitutive expression level of *β-actin* and expressed relative to the values of LPS alone. β-actin was used as an internal control for Western blotting. Values in the same column with different superscripted letters are significantly different ($p < 0.05$). Values are the mean ± SD of triplicate experiments.

Inhibition of LPS-induced iNOS by AD04

To confirm the inhibitory effect on LPS-induced NO production, the inhibitory effect of different concentrations (25, 50, 100, and 200 μg/ml) of AD04 was analyzed. As expected, AD04 inhibited the LPS-induced NO production in a dose-dependent manner (Fig. 3A). In addition, all tested concentrations of AD04 did not affect cell viability regardless of the presence of LPS for 24 h (Fig. 3B). This result indicates that AD04 has the most potent anti-inflammatory property against LPS stimulation.

NO is produced in endothelial cells by three distinct isoforms of nitric oxide synthases (NOS), namely, endothelium NO synthase (eNOS), neural NO synthase (nNOS), and iNOS (Wink et al., 2011). *eNOS* and *nNOS* are constitutively expressed, and *iNOS* is expressed only after cellular activation (Mcadam et al., 2012). Although NO is produced more rapidly by the eNOS or nNOS than by the iNOS pathway, iNOS is considered to be capable of generating larger quantities of NO than eNOS or nNOS (Nagy et al., 2007). The large quantity of NO generated by iNOS is used as a defense mechanism. Therefore, the induction of iNOS is the direct consequence of the inflammatory process (Suschek et al., 2004). Based on the findings above, we investigated whether the inhibitory effect of AD04 on LPS-induced NO production was related to the down-regulation of iNOS. As shown in Fig. 3C, stimulation with LPS strongly induced the accumulation of iNOS protein in RAW 264.7 cells compared with the unstimulated cells. In addition, the treatment of cells with AD04 suppressed the LPS-induced iNOS protein. The level of iNOS is known to be regulated in both transcriptional and post-transcriptional (*e.g.*, total protein synthesis and degradation of iNOS protein) mechanisms (Chiou et al., 2000). In RAW 264.7 cells, LPS increased the endogenous mRNA level of *iNOS*, whereas AD04 significantly suppressed the LPS-induced *iNOS* expression (Fig. 3D). These results indicate that AD04 inhibits the LPS-induced NO production through the suppression of *iNOS* expression.

AD04 suppresses *TNF-α*, *IL-6*, and *COX-2* expression through the inhibition of MAPK cascade activation in LPS-induced RAW 264.7 cells

LPS is known as a strong inducer of proinflammatory cytokines, including IL-6, IL-1, and TNF-α involved in the up-regulation of inflammatory reactions (Frost et al., 2002). In addition, proinflammatory cytokines can induce cyclooxygenase-2 (COX-2), which is the important source of prostanoid formation in inflammation (Ricciotti and Fitzgerald, 2011). Therefore, proinflammatory cytokines have been used as predictive biomarkers of inflammatory diseases. To further investigate the anti-inflammatory effect of AD04

on LPS-stimulated RAW 264.7 cells, we analyzed the transcription levels of *TNF-α*, *IL-6*, and *COX-2*. The significantly increased levels of *TNF-α*, *IL-6*, and *COX-2* were observed in LPS-stimulated RAW 264.7 cells, and these increases were inhibited by the treatment of AD04 (Fig. 4). Therefore, the anti-inflammatory property of *A. distichum* is mediated by the down-regulation of LPS-induced proinflammatory cytokine production.

The MAPK signaling pathway is responsible for important intracellular signaling cascades, which regulate cell proliferation, differentiation, development, inflammatory responses, and apoptosis (KAMINSKA, 2005). In LPS-induced proinflammatory responses, the MAPK signaling pathway plays an important role in the production of proinflammatory cytokines and NO (TURNER et al., 2014), thus suggesting that the MAPK signaling pathway is a potential molecular target for anti-inflammatory therapy. To investigate the inhibitory effect of AD04 on the MAPK signaling pathway in LPS-stimulated RAW264.7 cells, the activation of MEK1/2, ERK1/2, and p38 was analyzed. As shown in Fig. 5, LPS stimulation strongly induced the activation of MAPK pathways, whereas AD04 inhibited the LPS-induced phosphorylation of MEK1/2 and ERK1/2. In case of p38, LPS-induced activation was slightly or not inhibited by AD04, thereby indicating that the suppression of LPS-induced *TNF-α*, *IL-6*, and *COX-2* transcriptions was due to the inhibition of the MEK/ERK signaling pathway by AD04.

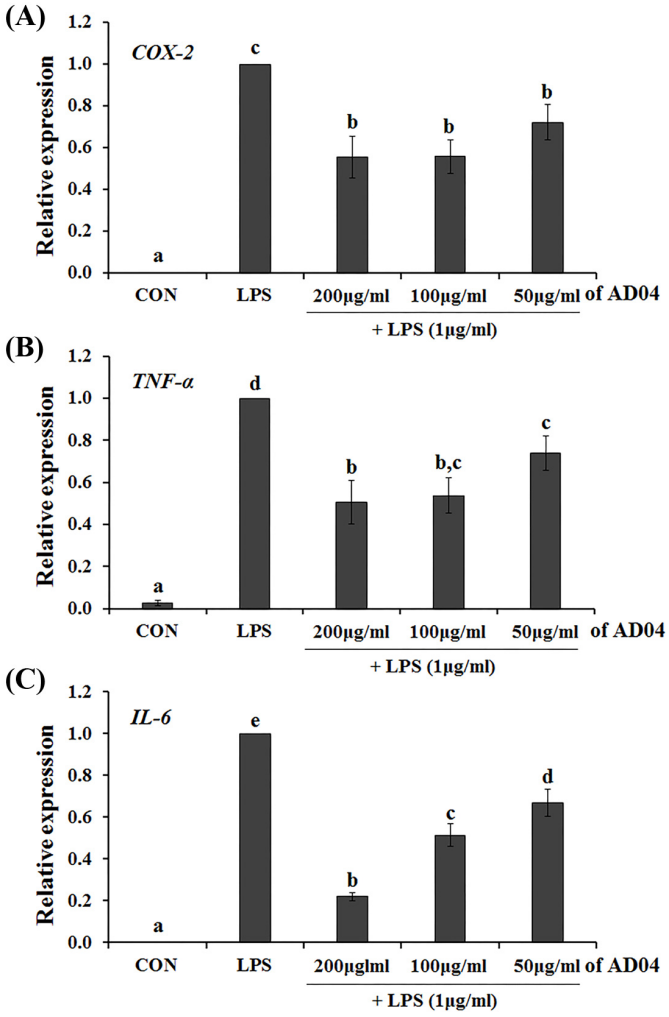

Fig. 4: Effect of BuOH fraction (AD04) on the expression of LPS-induced *COX-2*, *TNF-α*, and *IL-6*. Total RNA was isolated from RAW264.7 cells treated with or without LPS in the presence of AD04. The expression levels for each gene in different samples were calculated relative to its expression in LPS-stimulated RAW 264.7 cells. Data are expressed as the mean ± SD of three independent experiments. Mean separation within columns by Duncan's multiple range test is at the 0.05% level.

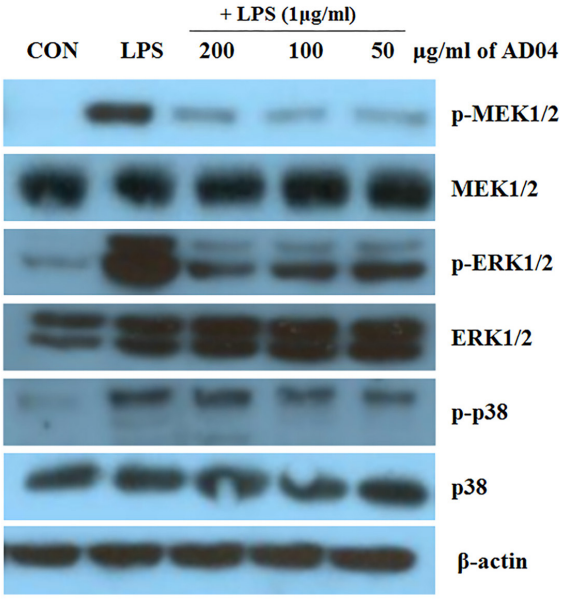

Fig. 5: Effects of BuOH fraction (AD04) on the activation of the LPS-induced MAPK signaling pathway. Immunoblotting was performed using the lysate from RAW264.7 cells treated with or without LPS in the presence of AD04. Blots were incubated with antibody anti-phospho-MEK1/2 (p-MEK1/2), anti-MEK1/2, anti-phospho-ERK1/2 (p-ERK1/2), anti-ERK1/2, anti-phospho-p38 (p-p38), and p38. β-actin was used as a loading control.

AD04 inhibits the LPS-induced ROS in RAW 264.7 cells

LPS has been characterized to induce the generation of ROS, which activates ROS-sensitive signaling pathways leading to the induction of inflammation. On the basis of this finding, we hypothesized that the inhibition of the LPS-induced MEK/ERK signaling pathway is due to the antioxidant activity of AD04, as shown in Fig. 1. To test this hypothesis, we analyzed the level of intracellular ROS using the fluorescent probe DCF-DA. When RAW 264.7 cells were treated with AD04, LPS-induced ROS was significantly suppressed in a dose-dependent manner (Fig. 6A). In addition, the expression of LPS-induced NOX-1 and NOX-2, which are known as major resources of ROS in immune cells (BEDARD and KRAUSE, 2007), was inhibited by AD04 in a dose-independent manner (Fig. 6B and 6C). However, the Pearson correlation analysis revealed that the intracellular ROS level was not correlated with the expression patterns of *NOX-1*(correlation coefficient $R^2 = 0.575$) and *NOX-2* ($R^2 = 0.584$). We also analyzed whether AD04 affected the expression of gene involved in the antioxidant system, such as copper/zinc superoxide dismutase (Cu/Zn SOD) and catalase, in LPS-stimulated RAW 264.7 cells. As shown in Fig. 6D and 6E, AD04 did not significantly induce the expression of catalase compared to that in the LPS-treated control group. Similarly, the expression of *Cu/Zn SOD*, an important enzyme in cellular oxygen metabolism, was not changed by the treatment of AD04 in LPS-stimulated RAW264.7 cells. These findings suggest that the suppression of LPS-induced ROS by *A. distichum* is most likely mediated through its ability to scavenge LPS-induced ROS rather than either the inhibition of ROS production or the enhancement of the antioxidant system.

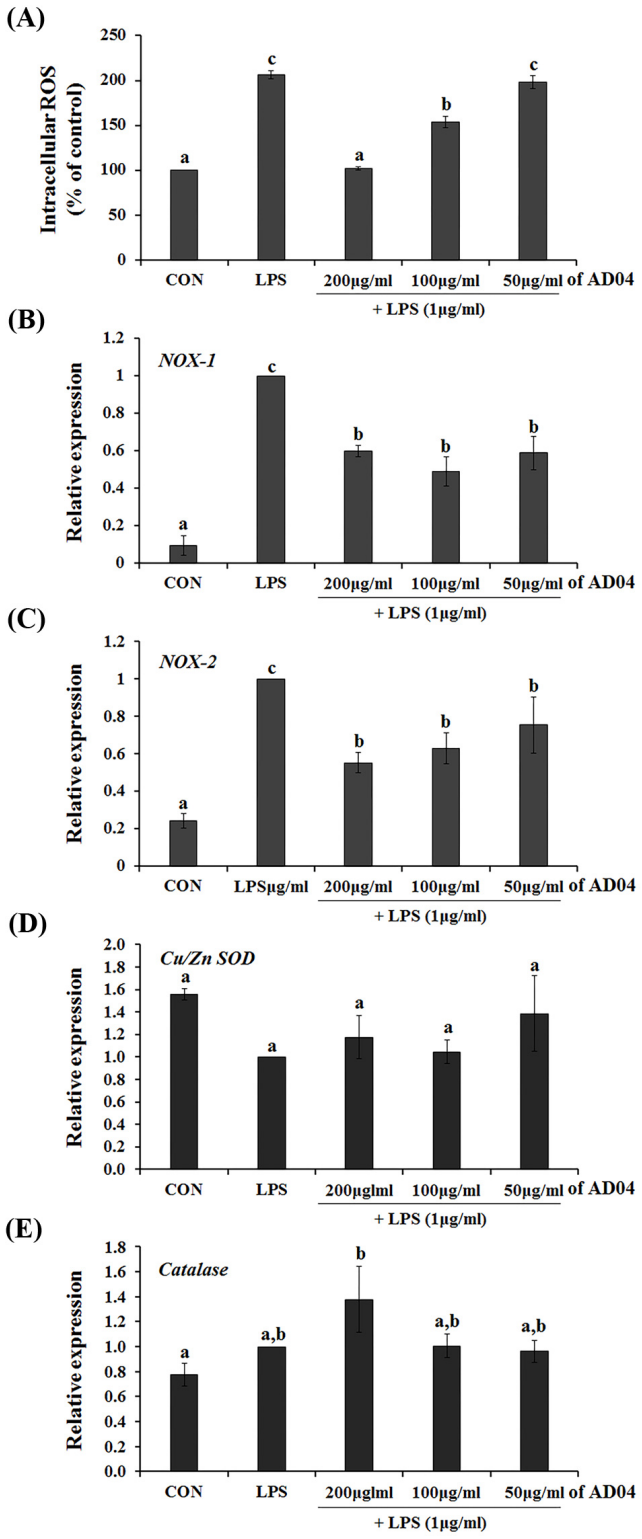

Fig. 6: Effects of BuOH fraction (AD04) on the production of LPS-induced ROS in RAW264.7 cells. **(A)** The LPS-induced intracellular ROS level was analyzed using the fluorescent probe DCF-DA. LPS-stimulated RAW 264.7 cells in the presence of AD04 were used to measure the mRNA levels of *NOX-1* **(B)** and *-2* **(C)** and those of *Cu/Zn SOD* **(D)** and catalase **(E)** by qRT-PCR. The relative amounts of transcripts were normalized to the levels of *β-actin* within the same sample. The value for the LPS-stimulated cells was set to 1.0. The average of three independent experiments is shown, and the error bars indicate the SD. Mean separation within columns by Duncan's multiple range test is $p < 0.05$.

Quantification of polyphenolic compounds in *A. distichum* extract by HPLC

Polyphenolic compounds including flavonoids are widely distributed in the plant kingdom. As more than several thousand polyphenolic compounds have been identified, flavonoids have been demonstrated to be beneficial in human healthcare, such as protecting against oxidative stress, cancer, cardiovascular diseases, nervous system-related syndromes, and inflammatory disorders (Pandey and Rizvi, 2009; Pérez-Cano and Castell, 2016). This finding suggests that polyphenolic compounds are potential active compounds in *A. distichum* extract. To identify the active compound that exhibits anti-inflammatory effects mediated by the inhibition of ROS-sensitive signaling pathways, polyphenolic compounds were identified and quantified in *A. distichum* methanolic extract and its fractions using HPLC analysis. Six polyphenolic compounds, including chlorogenic acid, caffeic acid, gentisic acid, rutin, ferulic acid, and quercetin, were identified in *A. distichum* extract (Tab. 1). The major compound was gentisic acid in various samples, but hexane contained chlorogenic acid as a major compound. Although most of the samples contained these six compounds, AD04 had the highest content of caffeic acid (13.54 μg/g of extract), gentisic acid (231.09 μg/g of extract), rutin (101.31 μg/g of extract), and ferulic acid (48.96 μg/g of extract). Among these compounds, caffeic acid, rutin, and ferulic acid have been reported to show multiple biological effects, such as antibacterial, antioxidant, anti-inflammatory, and anticancer activities (Srinivasan et al., 2007; Liu et al., 2014a; Al-Dhabi et al., 2015). Therefore, the antioxidant and anti-inflammatory activities of AD04 should be mediated by these active polyphenolic compounds together with phytochemicals, which were not identified in this study.

Discussion

As natural plants are used not only as food but also as sources of traditional medicine since ancient times, the raw materials of plants have become a rich source of structurally diverse substances, which potentially have a wide range of pharmacological activities. Therefore, the screening and the investigation of the pharmaceutical properties of natural plants have opened a new era of developing alternative or complementary therapies. In this study, we investigated the biological activities of *A. distichum*, which is a monotypic taxon of Oleaceae, and is endemic to Korea.

Plant-derived antioxidants with reducing or radical-scavenging capacity have been used because of growing concerns about the long-term safety of synthetic antioxidant agents (Atawodi, 2005) and additional health benefits, including reducing the risk of developing cancer and neurodegenerative diseases, anti-inflammatory actions, and anti-ageing effect (Alfadda and Sallam, 2012; Roleira et al., 2015; Szymanska et al., 2016). In the case of *A. distichum*, AD04 exhibited a strong antioxidant activity in both the HAT and SET mechanisms (Fig. 1). In addition, the free radical scavenging effect of AD04 (RC50 = 29.98 μg/ml) appeared to be similar to that of BHT (RC50 = 33.81 μg/ml), thus suggesting that AD04 could be a useful source of natural antioxidant agents. Furthermore, the presence of higher amounts of caffeic acid, rutin, and ferulic acid than other fraction (Tab. 1) indicates that the strong antioxidant property of AD04 is due to these polyphenolic compounds.

Several plant-derived antioxidants, including polyphenolic compounds, have been demonstrated to suppress inflammation through their antioxidant activities, the regulation of cellular activities of inflammatory related cells, and the modulation of the production of proinflammatory molecules by controlling gene expression and enzyme activities (García-Lafuente et al., 2009). In LPS-stimulated RAW 264.7 cells, AD04 inhibited the activation of the MEK/ERK signaling pathway (Fig. 5), thus resulting in the decreased NO production (Fig. 3A) and the suppression of *COX-2*, *TNF-α*, and *IL-6*

Tab. 1: Polyphenolic compounds in *Abeliophyllum distichum* methanol crude extract and its fractions.

	MeOH extract (AD01)	Hexane fraction (AD02)	EtOAc fraction (AD03)	BuOH fraction (AD04)	Aqueous fraction (AD05)
Chlorogenic acid[1)]	11.28 ± 0.03	15.48 ± 0.05	n.d	8.46 ± 0.09	8.02 ± 0.02
Caffeic acid[1)]	10.17 ± 0.03	n.d	2.09 ± 0.03	13.54 ± 0.14	3.01 ± 0.02
Gentisic acid[1)]	224.11 ± 9.04	3.96 ± 0.16	165.21 ± 1.50	231.09 ± 0.38	100.26 ± 2.98
Rutin[1)]	61.75 ± 1.01	2.53 ± 0.02	11.91 ± 0.08	101.31 ± 1.33	6.46 ± 0.07
Ferulic acid[1)]	23.45 ± 0.05	0.54 ± 0.11	19.29 ± 0.24	48.96 ± 0.12	1.52 ± 0.30
Quercetin[1)]	2.90 ± 0.23	2.66 ± 0.00	6.51 ± 0.00	3.55 ± 0.17	n.d

[1)] μg/mg of extract values are the average of triplicate experiments.
n.d = Not detectable.

expressions (Fig. 4). Polyphenolic compounds, such as myricetin, quercetin, and procyanidin B2, have been shown to directly bind to MEK1 to inhibit MEK/ERK signaling (Nguyen et al., 2004; Lee et al., 2007; Kang et al., 2008). Therefore, one possible explanation for the anti-inflammatory property of AD04 may be that the polyphenolic compounds in AD04 directly regulate LPS-induced MEK/ERK signaling in macrophages. In addition, LPS-induced intracellular ROS production was inhibited by the treatment of AD04 (Fig. 6A). In response to LPS, ROS were produced by multiple mechanisms, including the induction and activation of NOX and mitochondria respiratory chain systems as well as the suppression of anti-oxidative enzymes (Wang et al., 2004; Emre et al., 2007). NOX-dependent ROS generation contributes to the activation of ERK1/2 and c-Jun-N-terminal kinase (Hsieh et al., 2010), which are involved in the excessive production of LPS-induced proinflammatory cytokines (Zhang et al., 2016). The treatment of AD04 caused the suppression of LPS-induced *NOX-1* and *-2* (Fig. 6B and 6C), thereby indicating that AD04 might be able to inhibit NOX-dependent ROS generation through the inhibition of *NOX* transcription. However, the *NOX* expression did not correlate with the level of intracellular ROS in AD04-treated RAW 264.7 cells (Fig. 6). Thus, other possible explanation is that the anti-inflammatory effect of AD04 is mediated by the suppression of LPS-induced intracellular ROS level through its antioxidant activities (Fig. 1), although analyzing the effect of AD04 on the release of LPS-induced mitochondrial ROS is required. Taken together, these findings indicate that the inhibition of ROS-sensitive signaling, that is the MEK/ERK signaling, by *A. distichum* extract results in the suppression of the LPS-induced inflammatory response.

In conclusion, the *in vitro* data obtained from this study demonstrated the antioxidant and anti-inflammatory activities of *A. distichum*. In LPS-stimulated RAW264.7 cells, AD04 attenuated the production of NO and modulated the expression of pro-inflammatory mediators, such as *COX-2*, *TNF-α*, and *IL-6*. In addition, the results clearly indicate that the inhibitory action of AD04 is mediated by the inhibition of the MEK/ERK signaling pathway. Although further studies are required to identify the active compounds as potential anti-inflammatory compounds, the presence of notable antioxidant and anti-inflammatory activities in AD04 indicates that *A. distichum* has the potential to become a crude drug and a dietary health supplement.

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Supplementary data

Tab. S1. Primer sequences for qReal-time PCR analysis.

Primer	Sequences (5'-3')
iNOS-F	TCCTACACCACACCAAAC
iNOS-Rev	CTCCAATCTCTGCCTATCC
COX-2-F	CCTCTGCGATGCTCTTCC
COX-2-Rev	TCACACTTATACTGGTCAAATCC
TNF-α-F	AGCACAGAAAGCATGATCCG
TNF-α-Rev	CTGATGAGAGGGAGGCCATT
IL-6-F	CCACTTCACAAGTCGGAGGCTTA
IL-6-Rev	GTGCATCATCGCTGTTCATACAATC
NOX-1-F	AAGTGGCTGTACTGGTTGG
NOX-1-Rev	GTGAGGAAGAGTCGGTAGTT
NOX-2-F	ACTTCTTGGGTCAGCACTGG
NOX-2-Rev	ATTCCTGTCCAGTTGTCTTCG
Cu/Zn SOD-F	CAGCATGGGTTCCACGTCCA
Cu/Zn SOD-Rev	CACATTGGCCACACCGTCCT
Catalase-F	AAGACAATGTCACTCAGGTGCGGA
Catalase-Rev	GGCAATGTTCTCACACAGGCGTTT
β-actin-F	CCCACTCCTAAGAGGAGGATG
β-actin-Rev	AGGGAGACCAAAGCCTTCAT