



## The inflammatory activity of purified-ferulic acid from *Tetragonia tetragonioides*

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**Abstract** In this study, an evaluation of the anti-inflammatory effect of ferulic acid isolated from *Tetragonia tetragonioides* in lipopolysaccharide (LPS) simulated RAW 264.7 cells was made. The chemical structure of the active compound was elucidated by <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, and FAB-MS, and was confirmed to be ferulic acid. Ferulic acid was purified via open column chromatography with Sephadex LH-20 and MCI gel CHP-20. To test the anti-inflammatory effect of ferulic acid, LPS-stimulated RAW 264.7 cells were treated in subsequent experiments with different concentrations of ferulic acid (5, 10, and 25 µg/mL) and the levels of inflammatory cytokines and enzymes were also measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Cell viability was above 95% at acid concentrations ranging from 5-25 µg/mL. The results showed that 30% of the production of nitric oxide and 66% of prostaglandin E<sub>2</sub> were inhibited by 25 µg/mL of ferulic acid, it also inhibited the protein expression of both inducible nitric oxide synthase and cyclooxygenase-2 by 70%. Additionally, it inhibited the production of the pro-inflammatory cytokines, tumor necrosis factor- $\alpha$ , interleukin-6, and interleukin-1 $\beta$  by 40, 75, and 77%, respectively. According to these results, the anti-inflammatory activity of ferulic acid was demonstrated via his implication in the inhibition of the expression and secretion of inflammatory substances in LPS-stimulated RAW 264.7 cells. Therefore, we concluded that ferulic acid can be used as a functional additive having anti-inflammatory activity.

**Keywords** Anti-inflammation · Cytokine · Ferulic acid · RAW 264.7 cell · *Tetragonia tetragonioides*

### Introduction

The inflammatory response is a set of reactions generated by a living organism in answer to an undergone aggression. This one can be of origin outside as a wound, a physical agent, a chemical agent, a bacterial infection, a trauma, or result from the inside of the living organism itself as in autoimmune pathologies. However, in spite the healing role of inflammation, severe or supported inflammation may cause chronic diseases such as degenerative arthropathy, allergic diseases, and hypertension [1,2]. When an inflammatory reaction occurs in the body, macrophages and mast cells at the site of injury secrete inflammatory mediators, which attract leukocytes and neutrophils to the damaged area. Neutrophils affect the permeability of vascular endothelial cells by secreting reactive oxygen species (ROS), proteinases, and elastases, which are activated to remove the invading pathogens [3]. Lipopolysaccharide (LPS), a major constituent in the external membrane of gram-negative bacteria, induces an immune response by enhancing the toll-like receptor 4 (TLR4) signaling in host macrophages [4]. LPS binds to LPS binding protein (LBP) and interacts with TLR4, which subsequently activates nuclear factor- $\kappa$ B (NF- $\kappa$ B) through myeloid differentiation primary response gene 88 (MyD88), an adaptor protein, leading to the secretion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-1 $\beta$  (IL-1 $\beta$ ) [5]. These inflammatory cytokines stimulate the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), leading to excessive production of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), respectively [6]. *Tetragonia tetragonioides*, known as New Zealand spinach, is a perennial plant distributed in Korea, China, Japan, Australia, and South Africa. It grows well in dry and barren places such as rock gaps and gravel fields [7,8]. The white sticky juice

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that exudes from the broken stem/leaves of this plant has a protective effect on the stomach wall and has been used as an herbal remedies to cure and prevent stomach diseases such as gastritis and gastric ulcer [9,10]. Steryl glucoside, oxalic acid, cannabisin B, cerebroside,  $\beta$ -carotene, and 4-hydroxybenzamide have been isolated from *T. tetragonioides* [11-14]. However, only few studies have focused on the separation and analysis of active ingredients from *T. tetragonioides*, and the beneficial effects of isolated single compounds have not been extensively investigated. In this study, we isolated a single compound, ferulic acid, from *T. tetragonioides* and evaluated its anti-inflammatory effect on LPS-stimulated RAW 264.7 cells. Our results supplied with precious data which can allow the development of a natural anti-inflammatory substance.

## Material and Methods

### Preparation of *T. tetragonioides* extract

The procurement of *T. tetragonioides* was made at the level of the local market. To desiccate the biological material, it was put down inside the dry oven (Jeiotech, Daejeon, Korea) at 45 °C. 1 g of *T. tetragonioides* powder, pulverized into 40 mesh, was put into 200 mL of distilled water and boiled until the volume decreases to 100 mL. Then, the extract was stir-extracted by shaker incubator (120 rpm) for 24 h at room temperature. The extract was filtered by using Whatman No. 1 filter paper (Whatman Inc, Piscataway, NJ, USA) and the filtrate was lyophilized and stored at -70 °C. If necessary, the extracts were concentrated in a rotary vacuum evaporator (Eyela NE, Tokyo, Japan) to the needful concentration.

### Determination of the extract phenolic content

To calculate the phenolic content of each fraction of solvent, the Folin-Denis method was used. This method requires to take first 1 mL of the sample and a blank solution (distilled water), by adding to each of them 1 mL of 95% ethanol. Then, 5 mL of distilled water and 0.5 mL of 1 N Folin-Ciocalteu reagent (Junsei Chemical Co, Tokyo, Japan) were added as a color developing reagent. After, the mixture was left for 5 min for the color to develop, then 1 mL of Na<sub>2</sub>CO<sub>3</sub> was added and the optical density (OD) was measured within 1 h at 725 nm (Optizen 3220UV, Mecasys, Daejeon, Korea). Concentrations were calculated from a standard curve constructed using gallic acid as a standard.

### Purification of solvent fractions and isolation of the active compound

First, the extract was subjected to normal phase chromatography. Sephadex LH-20 (Pharmacia Co, Uppsala, Sweden), having the property to separate substances in function of their adsorbability, was added at the level of the column, and the mobile phase consisted of 60% EtOH. Components were eluted using EtOH  $\rightarrow$  H<sub>2</sub>O gradient system (100  $\rightarrow$  0%) at a flow rate of 20 mL/min,

and then concentrated and dried using TLC after identifying the phenolic compounds. Reverse phase chromatography was realized using MCI gel (Supelco, Bellefonte, PA, USA) as the stationary phase and gradient system of H<sub>2</sub>O  $\rightarrow$  MeOH (0  $\rightarrow$  100%) as the eluting solvent. Then, the identified phenolic compounds were separated using TLC to isolate the active compound.

### Identification of the chemical structure of the active compound

Melting point determination: The melting point was determined by using a micro-electrothermal actuator (Fisher-Jhons, Mexico City, Mexico) with sample of 1 mg. Infrared spectroscopy: One milligram of the pure sample was mixed with 100 mg of KBr powder (a halogenated alkali) under an appropriate pressure; then, IR spectroscopy was realized (Perkin-Elmer IR-1330, Perkin Elmer, Waltham, MA, USA). NMR: <sup>1</sup>H and <sup>13</sup>C-NMR spectral data were acquired using AVANCE III HD 500 MHz NMR spectrometer (Bruker, Germany) at KBSI Western Seoul center, using TMS [tetramethylsilane; (CH<sub>3</sub>)<sub>4</sub>Si] as a reference substance. 10 mg of purified compound was solved at a ratio of 5 to 20% (W/V) to give deuterated methanol (CD<sub>3</sub>OD), which was used as the solvent for NMR. FAB-MS: One milligram of the solid sample was analysed by using negative ion FAB-MS (Jeol JMS-PX 300, Tokyo, Japan) in the decompressed phase of 10<sup>-4</sup>-10<sup>-6</sup> mmHg. Thioglycerol was used as the matrix, and electric current was emitted at 22-28 eV at determination conditions. The sample was bombarded with an ion source accelerated to 6-7 kV. The results of these tests showed that the active compound is ferulic acid.

### Hyaluronidase (HAase)-inhibitory assay

HAase-inhibitory activity was determined according to the method of Reissig et al. [16]. The enzyme activity was determined by measuring the optimal density of glucoxazoline, induced and modified from N-acetylglucosamine, which is released from sodium-hyaluronic acid (HA). The color is expressed by adding p-dimethylaminobenzaldehyde (DMAB) (Sigma Chemical Co. Yongin, Korea). The assay was carried out as follows; 0.1 M acetate buffer (pH 3.5) was mixed with 7,900 U/mL dissolved HAase (Wako Pure Chemical Industries, Osaka, Japan). First, 0.05 mL HAase and 0.1 mL sample solutions were mixed and cultured for 20 mins at 37 °C then, 0.1 mL of 12.5 mM CaCl<sub>2</sub> was added to mixture and reacted again for 20 mins. Next, 12 mg/mL of melted hyaluronic acid (substrate) was added and cultured for 40 min then 0.1 mL of 0.4 N NaOH and 0.1 mL of 0.4 N K<sub>2</sub>B<sub>4</sub>O<sub>7</sub> were added to mixture. The mixture was boiled in a water bath for 3 min and completely cooled. After adding 3 mL of DMAB, as a color forming reagent, to the cooled reaction product were incubated at 37 °C for 20 min. Finally, the inhibitory activity was calculated by using the data of absorbance taken at 585 nm. The following equation was used to make the calculation: Inhibition ratio (%) = (1 - absorbance of sample group / absorbance of control group)  $\times$  100.

### Cell culture

The culture of cells was made on purchased Mouse macrophage RAW 264.7 cells from the Korean Cell Line Bank (Seoul, Republic of Korea). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, GE Healthcare Life Sciences, Chicago, IL, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone) and 100 U/mL penicillin-streptomycin (HyClone) at 37 °C in a 5% CO<sub>2</sub> incubator.

### MTT assay

The method used by Carmichael et al. [17] was applied to determine the cytotoxic effect of ferulic acid by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were cultured at a concentration of  $5 \times 10^3$  cells/mL in 48-well plates. Samples (50 µL) of different concentrations of ferulic acid (5, 10, 25, 50, and 100 µg/mL) were added to the cells and incubated for 24 h at 37 °C and 5% CO<sub>2</sub>. Control group cells were not treated with ferulic acid. After added MTT solution (Sigma Chemical Co.) at 5 mg/mL and incubated for 4 h, then the medium was removed, dimethylsulfoxide was added to each well and left at room temperature for 30 min. Finally, the absorbance values were detected at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Spectrostar Nano, BMG Labtech, Ortenberg, Germany).

### Measurement of NO production

The NO concentration in cell supernatants was identified as nitrite and nitrate. The Griess reagent (Sigma Chemical Co.) was used to take measures because the oxidation of nitrite to nitrate is stabilized. Briefly,  $5 \times 10^4$  cells/mL of RAW 264.7 macrophages were sowed in 96-well plates and cultivated for 24 h at 37 °C in 5% CO<sub>2</sub>. Next, the cells, apart from the normal group, were stimulated with 1 µg/mL LPS then treated with different concentrations of ferulic acid (5, 10, and 25 µg/mL) for 24 h at 37 °C. In the control group, the ferulic acid was replaced by an equal amount of distilled water. After, 100 µL of the cell culture supernatants was incubated for 24 h in a 96 well plate and reacted with 100 µL of Griess reagent in the dark room for 10 mins. The NO production was determined by measuring the absorbance at 540 nm.

### Western blotting analysis

RAW 264.7 cells were seeded at a density of  $5 \times 10^5$  cells/well in a 6 well plate for 24 h. Then, the cells were pre-treated with 1 µg/mL of LPS and added to various concentrations (5, 10, and 25 µg/mL) of the ferulic acid, followed by incubation for 18 h. Cells were washed with cold PBS, lysed with 100 µL of radio-immunoprecipitation assay (RIPA) buffer (pH 7.4) and harvested by centrifugation (1730R, Gyrozen, Seoul, Korea) at 12,000 rpm and 4 °C for 20 min. Then, the supernatant was put into a new tube and the Bradford assay was realized to determine the concentration of The transferred membrane was incubated with 5% skimmed milk blocking buffer (in Tris-Buffered Saline Tween-20 (TBST))

for 1 h at room temperature. A total of 20 µL of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to semi-dry transfer cell machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using polyvinylidene difluoride membrane (Millipore Corp, Billerica, MA, USA). The blocked membrane was washed 3 times every 10 min with TBST and then reacted with the diluted primary antibodies, iNOS (1:1,000, BD Bioscience, Sanjose, CA), COX-2 (1:1,000, Cayman, Ann arbor, MI), and glyceraldehyde 3-phosphate dehydrogenase (1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), at 4 °C for overnight. Again, the membrane was washed 3 times every 10 min with TBST, and incubated with the diluted secondary antibodies, Mouse anti-rabbit IgG horseradish peroxidase (HRP) (1:1,000, Santa Cruz) and bovine anti-goat IgG HRP (1:1,000, Santa Cruz), for 2 h at room temperature. After being washed 3 times, the membranes were reacted with enhanced chemiluminescence (ECL) solution (Millipore Corp.) and the band was analyzed in a dark room by using Molecular Imager (Bio-Rad Laboratories, Inc.).

### PGE<sub>2</sub> and cytokine assay

$5 \times 10^5$  cells/mL of RAW 264.7 macrophages were seeded in 96-well plates and were stimulated with 1 µg/mL LPS. Next cell were treated with different concentrations of ferulic acid (5, 10, and 25 µg/mL) for 24 h at 37 °C then cell culture media were collected. Enzyme immunoassay kits (R&D systems Inc., Minneapolis, MN, USA) were used for the determination of PGE<sub>2</sub>, TNF-α, and IL-1β. The content of each cytokine, such as TNF-α, and IL-1β, was calculated by using the standard curve ensuing from the reaction of the standard material.

### Statistical analysis

All data was presented as means ± SD from 3 independent experiments. The data were analyzed by SPSS 7.5 for window program (Statistical Package for Social Science, Chicago, USA) and used one-way ANOVA, followed by Duncan's multiple range tests. A *p*-value <0.05 was considered to indicate a statistically significant difference.

## Results and Discussion

### Purification and identification of the anti-inflammatory compound in *T. tetragonioide*

30 g of freeze-dried *T. tetragonioide* were extracted by the ethanol and then mixed with 200 mL of water/ethyl acetate/n-butanol, accommodating 3 solvent fractions. The Solvents from the three fractions were removed, diluted with distilled water to certain concentrations, and then their inhibitory activities against HAase were examined. The layer of water showed the strongest inhibitory activity. Consequently, it was eluted by Sephadex LH-20 and MCI gel CHP-20 column chromatography to separate the compound

**Table 1** Spectroscopic data of compound isolated from *T. tetragonioides*

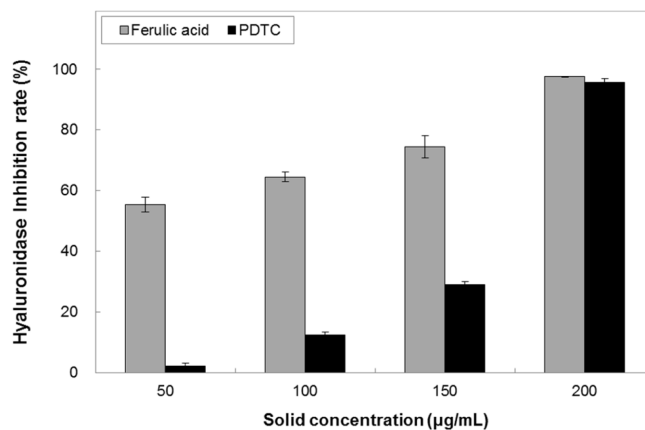
Melting point (°C)	174
FAB-MS ( <i>m/z</i> )	194.19
<sup>1</sup> H-NMR	δ: 3.88 (3H, s, H-OCH <sub>3</sub> ), 7.59 (1H, d, <i>J</i> =15.8 Hz, H-7), 6.31 (1H, d, <i>J</i> =15.8 Hz, H-8), 7.15 (1H, d, <i>J</i> =1.7 Hz, H-2), 7.04 (1H, dd, <i>J</i> =1.7 and 8.1 Hz, H-6), 6.81 (1H, d, <i>J</i> =8.1 Hz, H-5)
<sup>13</sup> C-NMR	δ: 49.3 (C-OCH <sub>3</sub> ), 171.1 (C-9), 147.1 (C-7), 116.0 (C-8), 116.6 (C-5), 111.8 (C-2), 149.4 (C-3), 124.1 (C-6), 127.9 (C-1), 150.5 (C-4)

responsible for the HAase inhibitory activity.

The melting point of the cleansed isolated compound turned out to be 174 °C and its molecular weight was 194.19 *m/z* according to FAB-MS. A summit of methoxy typical was shown by this compound at 3.88 ppm in spectre <sup>1</sup>H-NMR, what refers to a structure in which it has a bound of a methoxy group instead of an OH group in the position of C-3 of caffeic acid. The doublet signals at 7.59 ppm and 6.31 ppm, *J*=15.8 Hz, represented the peaks of H-7 and H-8, respectively. It can be concluded that they are coupled one to another in the trans form [18]. The doublet signal at 7.15 ppm, *J*=1.7 Hz, represented the peak of H-2; it was meta-coupled with H-6. The double doublet signal at 7.04 ppm, *J*=1.7 and 8.1 Hz, represented the peak of H-6. These results were coherent to those obtained by Kim and others [19]. Signal of the group methoxy and the group ketone C-9 were observed at 49.3 ppm and at 171.1 ppm respectively in C-NMR spectrum. C-7 and C-8 signals were observed at 147.1 at 116.0 ppm respectively, what refers to a double bound of a typical cinnamic acid compound. The C-5 signal was shifted up field to 116.6 ppm due to the attachment of the OH group to the adjacent carbon. A signal at 111.8 ppm represents the peak of C-2, it was shown similar to C-5 when the OH group is present at C-3, but because the methoxy group is substituted, it is shifted up field. Gentry et al. [20] showed that the C-5 and C-2 spectra of hycandinic acid ester were shifted up field by about 5 ppm, and the C-3 and C-4 signals were spaced by about 1 ppm. This indicates that the OH group at C-3 is substituted with a methoxy group. The signal of C-6 showed a characteristic peak at 124.1 ppm, while the signal of C-1 showed a characteristic peak at 127.9 ppm. Signals of C-4 and C-3 were influenced by the OH and methoxy groups, and were shifted downfield to 150.5 and 149.4 ppm, respectively. According to this information, this compound was identified as ferulic acid.

#### HAase-inhibitory activity of ferulic acid

Upon inflammation, activated HAase hydrolyzes high-molecular-weight HA, which fills the intercellular spaces, into low-molecular-weight HA. Due to extravasation of neutrophils and lymphocytes from blood vessels, HAase contributes to inflammatory and allergic reactions [21]. Accordingly, inhibition of HAase is necessary to suppress inflammation by maintaining the polymeric form of HA. As shown in Fig. 1, ferulic acid isolated from *T. tetragonioides* extract was found to inhibit HAase activity by 97.6% at a concentration of 200 µg/mL, and showed a stronger inhibitory activity than that of pyrrolidine dithiocarbonate (PDTC), a known



**Fig. 1** Inhibitory activity of ferulic acid isolated from *T. tetragonioides* extract on HAase enzyme. The presented results are means ± SD of three independent experiments. Means with different letters (a-e) above the bars for the same material are significantly different at the threshold of 5% of probability (*p*<0.05) according to the test of multiple range of Duncan

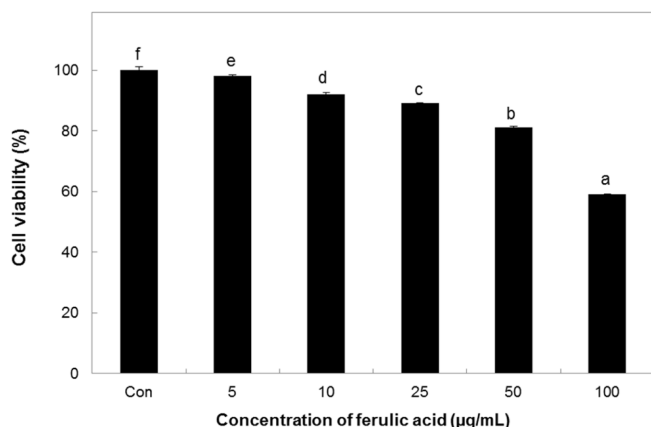
NF-κB inhibitor. In the study carried out by Park [22], 1,000 µM of each vanillic acid, caffeic acid, and quercetin, obtained from *Rubus coreanus* fruit, exhibited HAase-inhibitory activities of 27.4, 29.3, and 50.8%, respectively. These results showed that ferulic acid isolated from *T. tetragonioides* has a superior HAase-inhibitory effect. Therefore, additional experiments were conducted to confirm its anti-inflammatory effect in LPS-stimulated RAW 264.7 cells.

#### Effect of ferulic acid on cell viability

The viability of RAW 264.7 cells treated with ferulic acid was measured via an MTT assay (Fig. 2). Cell viability decreased significantly, as ferulic acid concentrations increased (5, 10, 25, 50, and 100 µg/mL). For example, from 50 to 100 µg/mL of concentrations, cell viability passed from 80 to 59%. This demonstrates that ferulic acid has an inhibitory effect on cell viability at high concentrations. Since ferulic acid concentrations ranging from 5-25 µg/mL were associated with cell viability values above 95%, concentrations of 5, 10, and 25 µg/mL were used in all ulterior experiments.

#### Inhibitory effect of ferulic acid on NO and PGE<sub>2</sub> production

Nitric oxide synthase (NOS) catalyzes the production of NO from L-arginine. In the body, NO can easily pass through cell membranes



**Fig. 2** The cell viability of RAW 264.7 cells treated with increasing concentrations of ferulic acid. During a period of 24 h a treatment of cells was made with different concentrations of ferulic acid (5, 10, 25, 50 and 100 µg/mL). Cell viability was measured using MTT assay. The presented results are means ± SD of three independent experiments. Means with different letters (a-e) above the bars for the same material are significantly different at the threshold of 5% of probability ( $p < 0.05$ ) according to the test of multiple range of Duncan

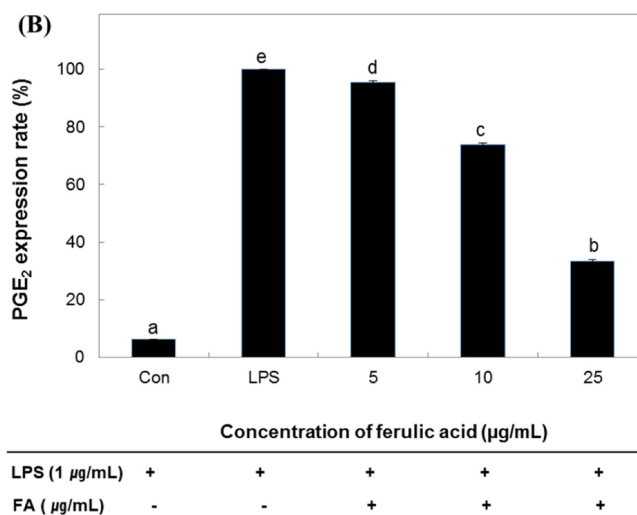
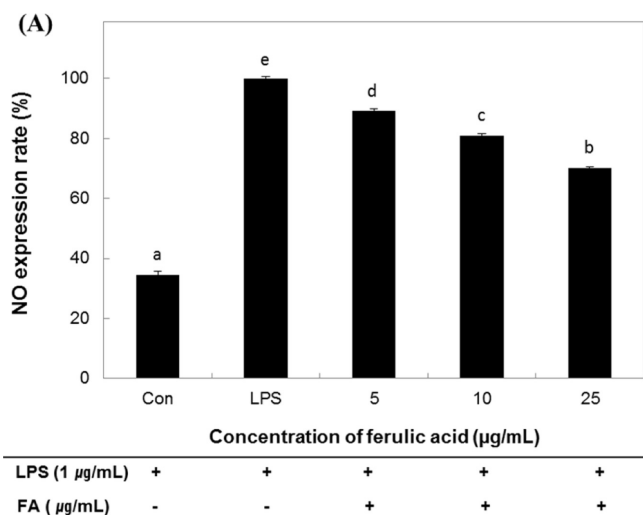
and transmit signals between cells in the immune system and the nervous system [23]. To confirm the inhibitory effect of ferulic acid on NO production, LPS-induced RAW 264.7 cells were treated with different concentrations of ferulic acid (5, 10, and 25 µg/mL). As shown in Fig. 3A, NO production in the LPS (1 µg/mL)-stimulated cells increased nearly 3 times comparing to that in the unstimulated cells. However, NO production was found to decrease significantly in the cells treated with ferulic acid in a concentration-proportional manner, as compared with that in the

LPS-stimulated cells.

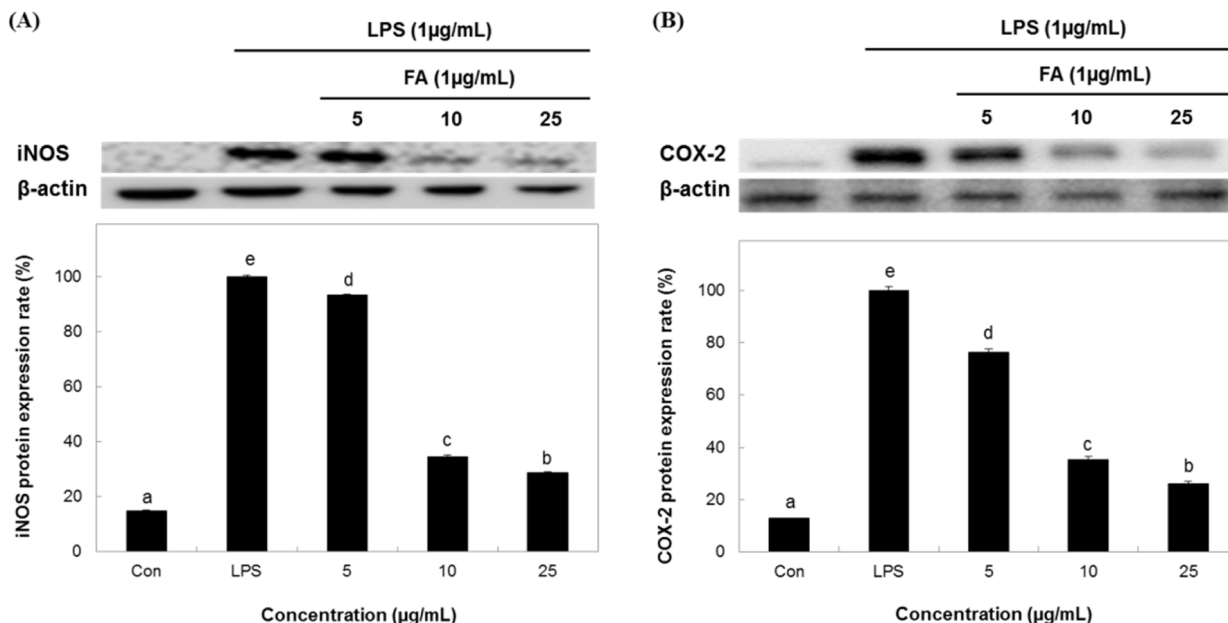
PGE<sub>2</sub>, an inflammatory mediator that induces pain and fever, is biosynthesized from arachidonic acid by the action of COX enzyme [24]. To confirm the inhibitory effect of ferulic acid on PGE<sub>2</sub> production, LPS-stimulated RAW 264.7 cells were treated with different concentrations (5, 10, 25 µg/mL) of ferulic acid and PGE<sub>2</sub> levels were measured (Fig. 3B). PGE<sub>2</sub> production in the cells stimulated with 1 µg/mL LPS was found to be nearly 10 times higher than that in the unstimulated cells. However, it decreased significantly in the cells treated with ferulic acid in a concentration-dependent manner, as compared with the values in the LPS-stimulated cells.

**Inhibitory effect of ferulic acid on iNOS and COX-2 protein expression**

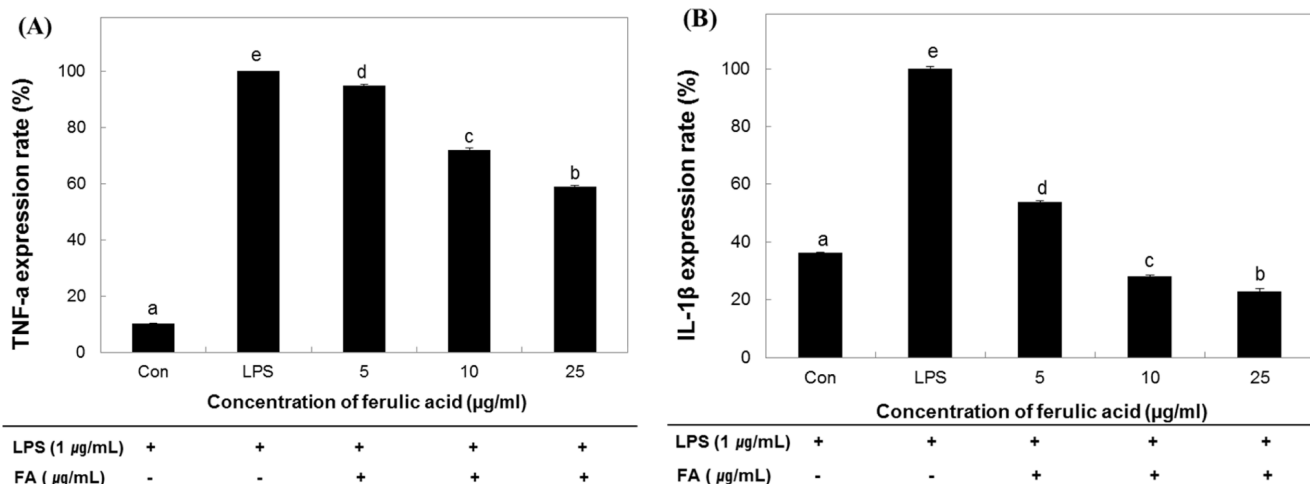
Unlike endothelial NOS (eNOS), which is constantly expressed to maintain the homeostasis of the human body, iNOS is expressed only in macrophages activated by different stimuli such as LPS; iNOS promotes excessive NO production and induces inflammation [25]. To corroborate the inhibitory effect of ferulic acid on iNOS protein expression, LPS-stimulated RAW 264.7 cells were treated with different concentrations of ferulic acid (5, 10, and 25 µg/mL) (Fig. 4A). The expression level of iNOS in the cells stimulated with 1 µg/mL LPS was nearly 5 times higher than that in the unstimulated cells. In addition, iNOS expression levels were revealed to decrease significantly in cells treated with ferulic acid in a concentration-dependent manner, as compared with that in the LPS-stimulated cells. In particular, 25 µg/mL ferulic acid exhibited 70% inhibition rate depending on the band density ratio of β-actin. Since ferulic acid was revealed to inhibit both NO production and iNOS protein expression, it was concluded that ferulic acid inhibits



**Fig. 3** NO (A) and PGE<sub>2</sub> (B) production in RAW 264.7 cells treated with different concentrations ferulic acid. During a period of 24 h a treatment of cells was made with different concentrations of ferulic acid (5, 10, and 25 µg/mL) and LPS (1 µg/mL). The totals of nitrate and PGE<sub>2</sub> in the supernatant were evaluated by Griess reagent and ELISA, respectively. The presented results are means ± SD of three independent experiments. Means with different letters (a-e) above the bars for the same material are significantly different at the threshold of 5% of probability ( $p < 0.05$ ) according to the test of multiple range of Duncan



**Fig. 4** Expression levels of iNOS (A) and COX-2 (B) in RAW 264.7 cells treated with different concentrations of ferulic acid. During a period of 24 h a treatment of cells was made with different concentrations of ferulic acid (5, 10, and 25 µg/mL) and LPS (1 µg/mL). Control cells were incubated with vehicle alone. The presented results are means ± SD of three independent experiments. Means with different letters (a-e) above the bars for the same material are significantly different at the threshold of 5% of probability ( $p < 0.05$ ) according to the test of multiple range of Duncan



**Fig. 5** Production of pro-inflammatory cytokines in RAW 264.7 cells treated with different concentrations of ferulic acid. (A): TNF- $\alpha$ , (B): IL-1 $\beta$ . During a period of 24 h a treatment of cells was made with different concentrations of ferulic acid (5, 10, and 25 µg/mL) and LPS (1 µg/mL). The total of cytokines in the supernatant was evaluated by ELISA. The presented results are means ± SD of three independent experiments. Means with different letters (a-e) above the bars for the same material are significantly different at the threshold of 5% of probability ( $p < 0.05$ ) according to the test of multiple range of Duncan

NO production by suppressing iNOS protein expression. Similarly, COX-2 is expressed in a short period due to inflammatory stimulation and is known to be involved in the biosynthesis of prostaglandins (PGs), which are inflammatory and pain mediators [26,27]. To confirm the inhibitory effect of ferulic acid on the expression of COX-2, a treatment of LPS-stimulated RAW 264.7 cells was made with different concentrations of ferulic acid (5, 10, and 25 µg/mL) (Fig. 4B). The expression level of COX-2 in cells

stimulated with 1 µg/mL LPS was nearly 6 times higher than that in the un-stimulated cells. In addition, the expression levels of COX-2 were found to be canceled in cells treated with ferulic acid in a concentration-dependent manner, as compared with that in the LPS-stimulated cells. Since a reduction of the production of PGE<sub>2</sub> and the expression of COX-2 by ferulic acid were revealed, it was concluded that ferulic acid inhibits PGE<sub>2</sub> production by suppressing COX-2 protein expression.

### Inhibitory effect of ferulic acid on the production of pro-inflammatory cytokines

The effect of the ferulic acid on the inhibition of pro-inflammatory cytokines production by processing RAW 264.7 cell stimulated by LPS with the various concentrations of ferulic acid (5, 10 and 25  $\mu\text{g/mL}$ ) could be confirmed. TNF- $\alpha$  is a pro-inflammatory cytokine secreted by activated macrophages [28,29]. Our results confirmed that ferulic acid inhibits TNF- $\alpha$  production (Fig 5A). With 1  $\mu\text{g/mL}$  LPS, stimulated cells showed a TNF- $\alpha$  production nearly 10 times higher than that in un-stimulated cells. Nevertheless, it was suppressed in cells treated with ferulic acid in a concentration-proportional manner, as compared with the values in the LPS-stimulated untreated cells.

Similarly, IL-1 $\beta$  is a pro-inflammatory cytokine secreted from activated macrophages, which is involved in immune reactions such as cell formation and apoptosis. As a pro-inflammatory cytokine, it is engaged in the formation of PGs and degrading enzymes such as collagenase. Thus, it is known to activate T-cells and B-cells and to induce inflammatory responses [30,31]. Our results confirmed that ferulic acid inhibits IL-1 $\beta$  production (Fig 5B). IL-1 $\beta$  production in cells stimulated with 1  $\mu\text{g/mL}$  LPS is nearly 3 times higher than that in the un-stimulated cells. However, it significantly decreased in cells treated with ferulic acid in a concentration-proportional manner, as compared with the values in the LPS-stimulated untreated cells.

Activated macrophages secrete IL-6, the third pro-inflammatory cytokine, which is associated with inflammatory reactions and tissue damage. It is also known to be secreted upon B-cell differentiation and T-cell proliferation [32,33]. It seems that ferulic acid suppresses the production of TNF- $\alpha$ , IL-1 $\beta$  and then inhibits IL-6 secretion. In conclusion, our results demonstrated that ferulic acid suppresses the generation of TNF- $\alpha$  and IL-1 $\beta$ , which are the initial pro-inflammatory factors, in a concentration-dependent manner. Therefore, ferulic acid could be used as a functional material for inhibiting inflammatory responses and promoting immunity.

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