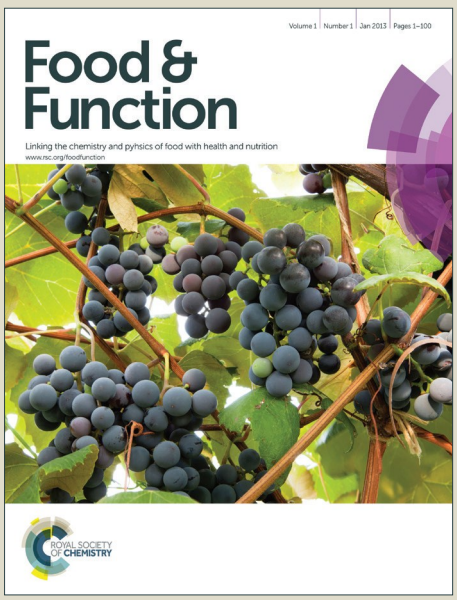
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Protective effect of *Tremella fuciformis* Berk extract on LPS-induced acute inflammation via inhibition of the NF-kB and MAPK pathways

Jangho Lee [1], Su Jeong Ha [2], Hye Jin Lee [2], Min Jung Kim [3], Jin Hee Kim [3], Yun Tai Kim [1,2], Kyung-Mo Song [2], Young-Jun Kim [4], Hyun Ku Kim [2], Sung Keun Jung [1, 2]*

[1] Food Biotechnology Program, Korea University of Science and Technology, Daejeon 305-350, Republic of Korea

[2] Division of Functional Food Research, Korea Food Research Institute, Gyeonggi-do, 463-746, Republic of Korea

[3] Division of Nutrition and Metabolism Research, Korea Food Research Institute, Gyeonggi-do, 463-746, Republic of Korea

[4] Food Safety Center, Ottogi Corp, Gyeonggi-do, 431-070, Republic of Korea

Note: J. Lee and S. J. Ha contributed equally to this work

Corresponding author: Hyun Ku Kim, Ph.D.: Division of Functional Food Research, Korea Food Research Institute, Gyeonggi-do, 463-746, Republic of Korea; E-mail: hyunku@kfri.re.kr Tel: +82 31 780 9134; Fax: +82 31 709 9876; Sung Keun Jung, Ph.D.: Division of Functional Food Research, Korea Food Research Institute, Gyeonggi-do, 463-746, Republic of Korea; E-mail: skjung@kfri.re.kr Tel: +82 31 780 9369; Fax: +82 31 709 9876

Running title: TFE inhibits inflammation via inhibition of NF-κB and MAPKs

Abbreviation: *Tremella fuciformis* Berk, TFB; *Tremella fuciformis* Berk extract, TFE; nitric oxide, NO; prostaglandin E_2, PGE_2; inducible nitric oxide synthase, iNOS; cyclooxygenase-2, COX-2; IkB kinase, IKK; mitogen-activated protein kinase, MAPKs; tumor necrosis factor-α, TNF-α

Abstract

Tremella fuciformis Berk (TFB) has long been used as a traditional medicine in Asia. Although TFB exhibits antioxidant and anti-inflammatory effects, the mechanisms of action responsible have remained unknown. We confirmed the anti-inflammatory effects of *Tremella fuciformis* Berk extract (TFE) in RAW 264.7 cells and observed significantly suppressed LPS-induced iNOS/NO and COX-2/PGE_2 production. TFE also suppressed LPS-induced IKK, IkB, and p65 phosphorylation, as well as LPS-induced translocation of p65 from the cytosol. Additionally, TFE inhibited LPS-induced phosphorylation of MAPKs. In an acute inflammation study, oral administration of TFE significantly inhibited LPS-induced IL-1β, IL-6 and TNF-α production and iNOS and COX-2 expression. The major bioactive compounds from TFB extract were identified as gentisic acid, protocatechuic acid, 4-hydroxygenzoic acid, and 4-coumaric acid. Among these compounds, protocatechuic acid showed the strongest inhibitory effects on LPS-induced NO production in RAW 264.7 cells. Overall, these results suggest that TFE is a promising anti-inflammatory agent that suppresses iNOS/NO and COX-2/PGE_2 expression, as well as the NF-κB and MAPK signaling pathways.

Keywords: *Tremella fuciformis* Berk extract (TFE); inflammation; nitric oxide (NO); nuclear factor κB (NF- κB); mitogen-activated protein kinase (MAPK)

1. Introduction

Edible mushrooms are a popular ingredient in Asian cuisine and herbal medicines [1]. Although *Tremella fuciformis* Berk (TFB) is less well-known as a functional food and nutraceutical than *Phellinus linteus* and *Lentinula edodes*, there has been recent interest in a number of bioactive properties of TFB including anti-cancer, anti-hypocholesterolemic, and immunomodulatory effects.[1-4] Advances in cultivation methods for TFB have contributed to more efficient methods of production.[5, 6] and increased its potential to become a high-value functional food ingredient. The biological mechanisms of action therefore need to be more thoroughly understood.

Chronic inflammation is a major cause of metabolic diseases including atherosclerosis, rheumatoid arthritis, inflammatory bowel disease and cancer.[7, 8] Inflammation is accompanied by activation of the immune system by immune cells in order to protect the host from pathogens.[9] Macrophages are one of the most important cells in the innate immune system.[10] Upon encountering antigens such as LPS (the outer cell wall component of gram-negative microorganisms), the cells are rapidly activated and consequently cause inflammation by upregulating the production of inflammatory mediators including nitric oxide (NO) and prostaglandin E_2 (PGE_2).[11] In addition, inflammatory cytokines such as IL-1β, TNF-α, and IL-6 are cell signaling proteins involved in inflammation, and are essential for acute macrophage inflammatory reactions.[12]

NF-κB plays a central role in inflammation.[9, 12, 13] In the inactive form, NF-κB dimers are sequestered in the cytoplasm and bound to their suppressor, IκB.[14] Upon phosphorylation, IκB is degraded after phosphorylation by IκB kinase (IKK), rendering NF-kB free to translocate from the cytoplasm to the nucleus where it can then bind to the response elements of NF-kB target genes.[14] Consequently,

activated NF-κB transcribes its inflammatory target genes including iNOS, COX-2, IL-1β, TNF-α, and IL-6.[12, 15] Recent studies have demonstrated that mitogen activated kinases (MAPKs) and activator protein-1 are heavily involved in inflammation via the associated production of NO and PGE_2.[16]

In the present study, we investigated the inhibitory effects of *Tremella fuciformis* Berk extract (TFE) on LPS-induced inflammation, and observed that TFE inhibits LPS-induced NO/iNOS and PGE_2/COX-2 expression via suppression of the NF-κB and MAPK signaling pathways. An *in vivo* acute inflammatory study revealed that oral administration of TFE significantly suppresses LPS-induced IL-1β, TNF-α, and IL-6 production in mouse blood, as well as iNOS and COX-2 expression in mouse liver. Overall, these findings suggest that TFE may be a potent anti-inflammatory agent that inhibits iNOS/NO and COX-2/PGE_2 production by suppressing the NF-κB and MAPK signaling pathways.

2. Materials and methods

2.1 Materials

All reagents were purchased from Sigma–Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), gentamicin, L-glutamine, penicillin–streptomycin and fetal bovine serum (FBS) were obtained from Thermo Scientific HyClone (Logan, UT, USA). The antibodies against iNOS, β-actin, α Tubulin were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The antibodies against COX-2, IKKα, IKKβ, phospho-IKKα/β (Ser176/180), NF-κB p65, phospho-NF-κB p65 (Ser536), IκBα, phospho-IκB-α (Ser32), p44/42 MAP Kinase, SAPK/JNK, p38 MAPK, phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), MKK3b, phospho-MKK3 (Ser189)/MKK6 (Ser 207), SEK1/MKK4,

phospho-SEK1/MKK4 (Ser257/Thr261), MEK1/2 and phospho-MEK1/2 (Ser217/221) were purchased from Cell Signaling Biotechnology (Beverly, MA, USA). The antibody against phosphorylated p38 MAPK (pT180/pY182) was purchased from BD Biosciences (Franklin Lakes, NJ, USA) and Lamin B2 was purchased from EMD Millipore Corporation (Temecula, CA, USA). Coumaric acid, gentisic acid, 4-hydroxybenzoic acid, and protocatechuic acid were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2 TFE preparation

Tremella fuciformis Berk was purchased from a rural market (Yeonam-gun, Jeollanam-do, Korea). Freeze-dried (FD 5512, Ilshin Lab Co., Ltd., Seoul, Korea) TFB was pulverized with a mixer (Hanil Electric Inc, Seoul, Korea), and homogenized using 500 μm mesh, and stored frozen in sealed 0.2 mm polyethylene film. The resultant powdered form of TFB was extracted in 70% ethanol with microwave assisted (MAP, Soxwave-100, ProLabo, Paris, France) extraction at 90 watts for 5 min.

2.3 Cell culture and viability assay

RAW 264.7 mouse macrophage cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in DMEM containing 5% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2 humidified incubator. Cell viability was assessed using the 3-(4, 5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA). Cells were seeded (2 x 10^6 cells/mL) in 96-well plates and incubated at 37°C in a 5% CO_2 incubator. After the cells were treated with TFE for

24 hrs, 20 μL of MTS reagent was added to each well. After 1 hr of incubation, the absorbance at 490 nm was measured with a microplate reader (Bio-Rad Inc., Hercules, CA, USA).

2.4 Nitrite assay

RAW 264.7 mouse macrophage cells were seeded (2 x 10^6 cells/mL) in 96-well plates and incubated at 37°C for 2 hrs in a 5% CO_2 incubator. The medium was changed with TFE for 1 hr. Cells were then treated with LPS (1 μg/mL) and incubated for 20 hrs. To determine the concentration of nitrite in the culture media, Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 5% phosphoric acid) was added to 50 μL of supernatant at each treatment condition and the absorbance at 550 nm was measured with a microplate reader (Bio-Rad Inc., Hercules, CA, USA).

2.5 PGE_2 assay

RAW 264.7 mouse macrophage cells were seeded (2 x 10^6 cells/mL) in 6-well plates and incubated at 37°C for 24 hrs in a 5% CO_2 incubator. The medium was changed with TFE for 1 hr. Cells were then treated with LPS (1 μg/mL) and incubated for 20 hrs. The quantity of PGE_2 released into the medium was measured using a PGE_2 enzyme immunoassay kit (Cayman Chemical Company, Michigan, USA).

2.6 Western blot assay

For *in vitro* Western blot assays, RAW 264.7 mouse macrophage cells were seeded (1.5 × 10^6 cells/mL) in 6-cm dishes and incubated at 37°C for 24 hrs in a 5%

CO_2 incubator. The medium was changed with TFE for 1 hr. Cells were then treated with LPS (1 μg/mL) and incubated for the specified time periods. After incubation, cells were collected and washed twice with cold PBS. Cells were lysed in Cell Lysis Buffer (Cell Signaling Biotechnology, Beverly, MA, USA) and maintained on ice for 30 min. For the *in vivo* Western blots, mice were injected with LPS/GalN. In the TFE-treated groups, mice were administered 40 or 200 mg/kg of TFE orally 1h before the LPS/GalN treatment, while other groups received an equivalent volume of vehicle. To isolate protein from mouse liver, tissue was added to 2 mL microcentrifuge tubes containing lysis buffer and stainless steel bead, and subsequently homogenized twice for 2 min at 20 Hz in a TissueLyser II (Qiagen, Valencia, CA, USA). Liver lysates were centrifuged at 12,000 rpm for 20 min. Protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories) following the manufacturer's manual. The lysate protein was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Immobilon®-P transfer membrane). After transferring, the membranes were incubated with specific primary antibodies at 4°C overnight. Protein bands were visualized using a chemiluminescence detection kit (ATTO, Tokyo, Japan) after hybridization with a horseradish peroxidase (HRP)-conjugated secondary antibody.

2.7 Cytoplasmic and Nuclear Fractionation

RAW 264.7 mouse macrophage cells were seeded (2×10^6 cells/mL) in 10-cm dishes and incubated at 37°C for 24 hrs in a 5% CO_2 incubator. The medium was changed with TFE for 1 hr. Cells were then treated with LPS (1 μg/mL) and incubated for 30 min. After incubation, cells were collected and washed twice with

cold PBS. Nuclear protein extractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, Illinois, USA) according to the manufacturer's protocol.

2.8 Immunofluorescence

RAW 264.7 mouse macrophage cells were seeded (2×10^4 cells/mL) in Nunc™ 8-well Lab-Tek™ chamber slides (Thermo Fisher Scientific) and incubated at 37°C for 24 hrs in a 5% CO_2 incubator. The medium was changed with TFE for 1 hr. Cells were then treated with LPS (1 μg/mL) and incubated for 15 min. Cells were fixed, permeabilized with 1% Triton X-100 and stained with VECTASHIELD® (Vector Laboratories, Burlingame, CA), anti-p65 antibody, then visualized with goat anti-rabbit IgG-h+l conjugated to DyLight® 488 conjugated labeled secondary antibodies (Bethyl Laboratories, TX, USA). The nuclear translocation of nuclear factor-kappaB (NF-κB) p65 was visualized using a fluorescent microscope (Nikon Eclipse Ti-S, Tokyo, Japan) and images were analyzed using Metamorph (Molecular Devices, Danville, PA) software.

2.9 Animals

Six-week-old male ICR mice, weighing approximately 26–28 g, were purchased from OrientBio Inc. (Gyeonggi-do, Korea). The mice were housed in an air-conditioned room (23 ± 2°C) with a 12-h light/dark cycle. They were allowed free access to food and tap water. All animals received humane care, and the study protocol (KFRI-M-14013) was approved and performed in accordance with the guidelines for animal care and use at Korea Food Research Institute. Animals were randomly assigned to the following groups (each group, n=9-10): (a) vehicle-treated

control, (b) vehicle-treated LPS/GalN, (c) 40 mg/kg TFE-treated LPS/GalN and (d) 200 mg/kg TFE-treated LPS/GalN. Mice were injected with GalN (0.8 g/kg; Santa Cruz Biotech) and LPS (40 μg/kg; Sigma-Aldrich) dissolved in phosphate-buffered saline. In the TFE-treated group, mice were orally administered 40 and 200 mg/kg of TFE 1 h prior to the LPS/GalN treatment, while other groups received an equivalent volume of vehicle. After 6 hrs, the mice were euthanized and blood and liver were aseptically collected for further studies.

2.10 ELISA assay

In blood levels of interleukin (IL)-6, IL-1β and TNF-α were quantified at 6 hrs after LPS/GalN injection using commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA, USA). All ELISAs were conducted in accordance with the manufacturer's instructions.

2.11 Immunohistochemistry

Sections (5-μm thick) of 10% neutral formalin solution-fixed, paraffin-embedded liver tissues were cut on silane-coated glass slides. Deparaffinized sections were heated for 15 min in 10 mM citrate buffer (pH 6.0) in a microwave oven for antigen retrieval. For the detection of target proteins, slides were incubated with affinity-purified primary antibody in a refrigerator overnight in 1% BSA solution and then developed using the SignalStain® Boost IHC Detection Reagent (HPR, rabbit) antibodies (Cell Signaling Biotechnology). Peroxidase-binding sites were detected by staining with SignalStain® DAB Substrate Kit (Cell Signaling Biotechnology). Finally, counterstaining was performed using Harris hematoxylin solution (Sigma–Aldrich). The expression of iNOS and COX-2 was visualized using a fluorescent microscope

(Nikon Eclipse Ti-S, Tokyo, Japan) and images were analyzed using Metamorph (Molecular Devices, Danville, PA) software.

2.12 UPLC-MS/MS analysis

The analyses were performed using an Acquity UPLC system (Waters, Milford, MA, USA) with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm). The raw data were processed using MassLynx 4.1 (Waters) software. The mobile phase included 0.1% formic acid aqueous solution (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a gradient elution program was performed: 0-10 min, 99-70% solvent A; 10-12 min, 70-5% solvent A; 12-14 min, 5-99 % solvent A; 14-20 min, 99% solvent A. The flow rate was set at 0.65 mL/min. The column temperature was kept at 40°C and the total run time was 5 min. The auto-sampler was conditioned at 4°C and the injection volume was 5 uL, Identification and quantification of flavonoids was carried out on a Waters Xevo TQ triple-quadrupole mass spectrometer (Waters, Miliford, MA, USA) equipped with an electrospray ionization (ESI) mode. The ESI source was operated by switching between positive and negative ion mode. The data were acquired with a multiple reaction monitoring (MRM) mode with a cone voltage of 20 V, a capillary voltage of 2.5 kV, and a cone gas flow of 50 L/h. The source temperature was set at 150°C, while the desolvation flow was set at 800 L/h; the desolvation gas temperature was set at 400°C.

2.13 Statistical analysis

Where appropriate, data are expressed as the mean ± S.E.M., and significant differences between LPS and TFE groups were calculated with Student's t-test with

two-tailed distributions and two-sample equal variance. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. TFE inhibits LPS-induced nitrites and PGE_2 production, as well as iNOS and COX-2 expression in RAW 264.7 cells.

The upregulated production of NO and PGE_2 plays a critical role in inflammatory processes.[12-14] Using a Griess assay, we screened 15 mushroom species to evaluate anti-inflammatory properties (data not shown). We then confirmed the effect of TFE on LPS-induced production of nitrites and PGE_2 in RAW 264.7 cells using Griess and ELISA assays, and observed that TFE significantly suppressed LPS-induced production (Fig. 1A and B). iNOS and COX-2 are representative enzymes that regulate inflammation via the production of nitrites and PGE_2, respectively.[13, 17] We next sought to investigate whether TFE inhibits LPS-induced iNOS and COX-2 expression. Western blot analysis results showed that TFE strongly suppresses LPS-induced iNOS and COX-2 expression in RAW 264.7 cells (Fig. 1C and D), at concentrations that did not affect cell viability (Fig. S1).

3.2. TFE inhibits LPS-induced NF-κB and MAPKs signaling pathways in RAW 264.7 cells.

Previous studies have reported that iNOS and COX-2 expression are regulated by NF-κB, and their activity is mediated through IKK and IkB phosphorylation.[14] To further confirm whether NF-κB signaling is affected by TFE, we examined the effects of treatment on LPS-induced phosphorylation of IKK, IκB, and p65. TFE was found to inhibit LPS-induced phosphorylation of IKKα/β and IκBα in RAW 264.7 cells (Fig.

2A). TFE also inhibited LPS-induced p65 phosphorylation (Fig. 2B).

A Previous study has reported that members of the MAPK family activated by LPS also play a critical role in the production of inflammatory mediators including NO and PGE_2.[16] We thus investigated the effect of TFE on LPS-induced MAPKs phosphorylation, and observed that LPS-induced all three MAPK family members including JNK1/2, p38, and ERK1/2, which were strongly suppressed by TFE treatment (Fig. 2C). Additionally, TFE suppressed LPS-induced MEK and MKK3/6 phosphorylation in RAW264.7 cells (Fig. 2D).

3.3. TFE inhibits LPS-induced p65 translocation from the cytosol to the nucleus in RAW 264.7 cells.

Phosphorylation of p65 at Ser536 is critical for NF-kB activity, as it regulates p65 translocation from the cytosol to the nucleus.[18] In order to determine whether TFE inhibits LPS-induced phosphorylation of p65, we investigated the effect of TFE on LPS-induced p65 translocation. Nuclear fractionation results showed that LPS-induced nuclear translocation of p65 was suppressed by TFE (Fig. 3A). Immunofluorescence assay results further confirmed that TFE inhibits LPS-induced p65 translocation from the cytosol to the nucleus in the same cell line (Fig. 3B).

3.4. TFE inhibits LPS-induced IL-1β, IL-6, and TNF-α production, as well as iNOS and COX-2 expression in vivo.

Based on the above results, we further investigated the effect of TFE on LPS-induced inflammation using an acute animal model of inflammation. A single injection of LPS and GalN mixture induced abnormal upregulation of IL-1β, IL-6 and TNF-α production, while oral administration of TFE significantly inhibited LPS-induced IL-1β,

TNF-α, and IL-6 production in mouse blood (Fig. 4A). Immunohistochemistry results showed that TFE inhibited LPS-induced iNOS and COX-2 expression *in vivo* (Fig. 5).

3.5. Identification and quantification of phenolic compounds by ESI-MS/MS analysis.

We next investigated the compound composition of TFE. The major phenolic compounds were listed by comparison of their ESI quadrupole MS/MS spectra with the standards (Fig. 6). 4-hydroxybenzoic acid, gentisic acid, coumaric acid, and protocatechuic acid were subsequently identified as the major phenolic compounds in TFE (Fig. 6). Moreover, total levels of phenolic compounds in the TFE were calculated to be 2.38 ± 0.05, 8.39 ± 0.3, 2.99 ± 0.1, and 1.02 ± 0.10 μg/g (gentisic acid, 4-hydroxybenzoic acid, 4-coumaric acid, and protocatechuic acid, respectively) (Table 1).

3.6. Protocatechuic acid exhibits the strongest inhibitory effect among TFE compounds on LPS-induced nitrites production in RAW 264.7 cells.

To determine which compounds present in TFE exert the strongest inhibitory effects on LPS-induced inflammation, we conducted nitrite assay in RAW 264.7 cells. Of the four major compounds, protocatechuic acid showed the strongest inhibitory effects on LPS-induced nitrites production in RAW 264.7 cells. Protocatechuic acid inhibited 19.7 ± 8.3, 23.6 ± 6.5, and 37.6 ± 4.9% of the total nitrites production at 25, 50, and 100 μM concentration compared to LPS-treated RAW 264.7 cells. These results suggest that protocatechuic acid is major compounds responsible for the inhibition of LPS-induced inflammation by TFE.

4. Discussion

The regular consumption of natural and unprocessed foods has been correlated with the prevention of a number of chronic diseases.[19] In 2013, the World Health Organization released guidelines recommending that people increase their intake of natural food sources to prevent the occurrence of chronic diseases, including cardiovascular diseases and various cancers.[20] Although many clinical trials have been undertaken to develop novel functional food materials, effective and safe commercial functional ingredients require further investigation.

TFB has a long history of use as an ingredient in traditional medicines as well as Asian cuisine. One of the major bioactive properties of TFB is its immune-stimulatory effect via the increased production of IL-1, IL-6 and TNF-α in human monocytes.[2, 3, 21] Accumulated evidence suggests that popular mushrooms such as *Phellinus linteus* and *Lentinula edodes* are also effective in eliciting the same immunostimulatory effects.[22, 23] However, recent studies have begun to investigate the hypothesis that mushrooms also have strong anti-inflammatory effects.[13, 24]

Using a Griess assay, we screened 15 cultivars of commercially-available mushrooms, and selected TFE for further investigation as an anti-inflammatory functional food material, based on NO production results (data not shown). A previous study has demonstrated that *Tremella fuciformis* MeOH extract exerts antioxidant and anti-inflammatory effects in Raw264.7 cells,[25] although the mechanisms of action responsible and *in vivo* confirmation were left unaddressed. We first confirmed the anti-inflammatory effect of *Tremella fuciformis* and sought to investigate whether TFE exerts anti-inflammatory effects *in vitro* and *in vivo* as a potential value-added property for use as a functional food ingredient.

TFE significantly suppressed NO and PGE_2 production in RAW 264.7 cells, and because such production is dependent on iNOS and COX-2 expression

(respectively),[17] we further investigated whether TFE could inhibit LPS-induced iNOS and COX-2 expression. Our Western blot assay results showed that TFE significantly suppresses LPS-induced iNOS and COX-2 expression in these cells. Previous studies have reported that TFE enhances immune responses by stimulating cytokine production,[2, 3, 21] while our results demonstrated that TFE strongly suppresses inflammatory mediators including NO and PGE_2.

Since the representative target genes of NF-κB, iNOS and COX-2 play central roles in inflammation by producing NO and PGE_2 respectively,[13, 14] we investigated whether TFE could inhibit LPS-induced NF-κB signaling. Our results clearly showed that TFE inhibits LPS-induced IKK/IκB/p65 signaling. Ubiquitination and proteasomal degradation of IκB directly activates dimeric NF-κB, and as a result it translocates from the cytoplasm to the nucleus, where it can transcribe target genes.[14] Based on these findings, we determined by fractionation of cytoplasmic and nuclear cell lysates, as well as immunofluorescence, that LPS-induced p65 translocation is strongly inhibited by TFE treatment. Previous studies have reported that MAPKs also play crucial roles in regulating LPS-induced iNOS and COX-2 expression.[16, 26] LPS can mediate the activation of transcriptional factor AP-1 via the MAPK pathway as well as the NF-kB pathway.[27] Additionally, our recent study results showed that AP-1 regulate LPS-induced NO and PGE_2 production without affecting NF-kB signaling pathways.[28] These accumulate results suggest that AP-1 also plays a critical role in inflammation associated with the abnormal expression of iNOS and COX-2.[29, 30]

In this study, TFE also inhibited LPS-induced expression of all three MAPKs. Although several mushroom cultivars and their components have been demonstrated to exhibit anti-inflammatory effects via the inhibition of NF-κB signaling,[13, 24] to our knowledge this is the first report to show that TFE inhibits LPS-

induced inflammatory mediators by suppressing NF-κB signaling and MAPK phosphorylation.

To further confirm the effect of TFE on LPS-induced inflammation, we conducted an *in vivo* acute inflammation study and investigated whether TFE inhibits LPS-induced production of inflammatory mediators, including IL-1β, IL-6, and TNF-α. ELISA assay results showed that oral administration of TFE significantly inhibits LPS-induced production of these inflammatory mediators in mouse blood. Additionally, the Western blot assay and immunohistochemistry results showed that oral administration of TFE strongly suppresses LPS and galactosamine-induced iNOS and COX-2 expression *in vivo*.

Although our results overall reveal that TFE has preventive effects against LPS-induced inflammation, it remains to be determined which component(s) are primarily responsible for this anti-inflammatory effect. We identified the active components gentisic acid, protocatechuic acid, 4-hydroxybenzoic acid, and 4-coumaric acid in TFE by using ESI-MS/MS. In agreement with previous reports, we identified protocatechuic acid as a prime candidate for the anti-inflammatory effect of TFE. Additionally, our NO assay with the major compounds present in TFE showed that protocatechuic acid has the strongest inhibitory effect on LPS-induced nitrites production. Previous study results also showed that protocatechuic acid has anti-inflammatory effects.[31, 32] However, the exact anti-inflammatory mechanisms of protocatechuic acid such as the inhibition of iNOS, COX-2 gene expression, as well as AP-1, NF-kB transcription activity need further elucidation. In this study, we have shown that the anti-inflammatory effect of TFE arises due to suppression of the NF-κB and MAPK signaling pathways. With further characterization of TFE and the

active components that directly regulate the inflammatory effects will need to be identified.

6. Conclusions

Collectively, our results show that TFE significantly inhibits LPS and galactosamine-induced IL-1β, IL-6 and TNF-α production, as well as iNOS and COX-2 expression *in vivo*. This inhibition occurs primarily via the blocking of NF-κB translocation through the inhibition of IKK and IκB phosphorylation and MAPK signaling pathways. We identified 4 types of phenolic acids (gentisic acid, protocatechuic acid, 4-hydroxybenzoic acid, and coumaric acid) in TFE, and protocatechuic acid is likely to be the major active compounds in TFE. This represents the report elucidating anti-inflammatory factors present TFE and sheds light on its mechanism of action.

Acknowledgement

This research was supported by a grant from the Korea Food Research Institute, Republic of Korea.

Conflict of Interest Statement

The authors declare no conflicts of interest.

References

1. H. H. Cheng, W. C. Hou and M. L. Lu, Interactions of lipid metabolism and intestinal physiology with Tremella fuciformis berk edible mushroom in rats fed a high-cholesterol diet with or without nebacitin, *J Agr Food Chem*, 2002, **50**, 7438-7443.

2. Q. Gao, M. K. Killie, H. Chen, R. Jiang and R. Seljelid, Characterization and cytokine-stimulating activities of acidic heteroglycans from Tremella fuciformis, *Planta medica*, 1997, **63**, 457-460.
3. Q. P. Gao, R. Z. Jiang, H. Q. Chen, E. Jensen and R. Seljelid, Characterization and cytokine stimulating activities of heteroglycans from Tremella fuciformis, *Planta medica*, 1996, **62**, 297-302.
4. S. Ukai, K. Hirose, T. Kiho, C. Hara and T. Irikura, Antitumor activity on sarcoma 180 of the polysaccharides from Tremella fuciformis Berk, *Chemical & pharmaceutical bulletin*, 1972, **20**, 2293-2294.
5. J. K. Gusman, C. Y. Lin and Y. C. Shih, The optimum submerged culture condition of the culinary-medicinal white jelly mushroom (Tremellomycetes) and its antioxidant properties, *International journal of medicinal mushrooms*, 2014, **16**, 293-302.
6. H. Zhu, B. Tian, W. Liu, S. Zhang, C. Cao, Y. Zhang and W. Zou, A three-stage culture process for improved exopolysaccharide production by Tremella fuciformis, *Bioresource technology*, 2012, **116**, 526-528.
7. V. Chiurchiu and M. Maccarrone, Chronic Inflammatory Disorders and Their Redox Control: From Molecular Mechanisms to Therapeutic Opportunities, *Antioxid Redox Sign*, 2011, **15**, 2605-2641.
8. R. Ranjha and J. Paul, Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer, *Inflamm Res*, 2013, **62**, 343-355.
9. A. Kauppinen, T. Suuronen, J. Ojala, K. Kaarniranta and A. Salminen, Antagonistic crosstalk between NF-kappa B and SIRT1 in the regulation of inflammation and metabolic disorders, *Cell Signal*, 2013, **25**, 1939-1948.
10. S. Hallam, M. Escorcio-Correia, R. Soper, A. Schultheiss and T. Hagemann, Activated macrophages in the tumour microenvironment-dancing to the tune of TLR and NF-kappaB, *The Journal of pathology*, 2009, **219**, 143-152.
11. T. H. Kao, C. W. Huang and B. H. Chen, Functional components in Luffa cylindrica and their effects on anti-inflammation of macrophage cells, *Food Chem*, 2012, **135**, 386-395.
12. Y. Z. Yang, Y. Z. Tang and Y. H. Liu, Wogonoside displays anti-inflammatory effects through modulating inflammatory mediator expression using RAW264.7 cells, *J Ethnopharmacol*, 2013, **148**, 271-276.

13. G. J. Huang, S. S. Huang and J. S. Deng, Anti-Inflammatory Activities of Inotilone from Phellinus linteus through the Inhibition of MMP-9, NF-kappa B, and MAPK Activation In Vitro and In Vivo, *Plos One*, 2012, **7**.
14. Q. Li and I. M. Verma, NF-kappaB regulation in the immune system, *Nature reviews. Immunology*, 2002, **2**, 725-734.
15. D. W. S. Cheung, C. M. Koon, E. Wat, C. H. Ko, J. Y. W. Chan, D. T. W. Yew, P. C. Leung, W. Y. Chan, C. B. S. Lau and K. P. Fung, A herbal formula containing roots of Salvia miltiorrhiza (Danshen) and Pueraria lobata (Gegen) inhibits inflammatory mediators in LPS-stimulated RAW 264.7 macrophages through inhibition of nuclear factor kappa B (NF kappa B) pathway, *J Ethnopharmacol*, 2013, **145**, 776-783.
16. W. Liu, X. Ouyang, J. Yang, J. Liu, Q. Li, Y. Gu, M. Fukata, T. Lin, J. C. He, M. Abreu, J. C. Unkeless, L. Mayer and H. Xiong, AP-1 activated by toll-like receptors regulates expression of IL-23 p19, *The Journal of biological chemistry*, 2009, **284**, 24006-24016.
17. K. N. Kim, Y. J. Ko, M. C. Kang, H. M. Yang, S. W. Roh, T. Oda, Y. J. Jeon, W. K. Jung, S. J. Heo, W. J. Yoon and D. Kim, Anti-inflammatory effects of trans-1,3-diphenyl-2,3-epoxypropane-1-one mediated by suppression of inflammatory mediators in LPS-stimulated RAW 264.7 macrophages, *Food Chem Toxicol*, 2013, **53**, 371-375.
18. C. Y. Hsieh, M. J. Hsu, G. Hsiao, Y. H. Wang, C. W. Huang, S. W. Chen, T. Jayakumar, P. T. Chiu, Y. H. Chiu and J. R. Sheu, Andrographolide enhances nuclear factor-kappaB subunit p65 Ser536 dephosphorylation through activation of protein phosphatase 2A in vascular smooth muscle cells, *The Journal of biological chemistry*, 2011, **286**, 5942-5955.
19. P. R. Conlin, The dietary approaches to stop hypertension (DASH) clinical trial: implications for lifestyle modifications in the treatment of hypertensive patients, *Cardiology in review*, 1999, **7**, 284-288.
20. R. Rekhy and R. McConchie, Promoting consumption of fruit and vegetables for better health. Have campaigns delivered on the goals?, *Appetite*, 2014, **79**, 113-123.
21. Q. Gao, R. Seljelid, H. Chen and R. Jiang, Characterisation of acidic heteroglycans from Tremella fuciformis Berk with cytokine stimulating activity,

Carbohydrate research, 1996, **288**, 135-142.

22. A. T. Borchers, C. L. Keen and M. E. Gershwin, Mushrooms, tumors, and immunity: An update, *Exp Biol Med*, 2004, **229**, 393-406.
23. S. P. Kim, S. O. Park, S. J. Lee, S. H. Nam and M. Friedman, A Polysaccharide Isolated from the Liquid Culture of Lentinus edodes (Shiitake) Mushroom Mycelia Containing Black Rice Bran Protects Mice against Salmonellosis through Upregulation of the Th1 Immune Reaction, *J Agr Food Chem*, 2014, **62**, 2384-2391.
24. Y. Nishitani, L. Zhang, M. Yoshida, T. Azuma, K. Kanazawa, T. Hashimoto and M. Mizuno, Intestinal Anti-Inflammatory Activity of Lentinan: Influence on IL-8 and TNFR1 Expression in Intestinal Epithelial Cells, *Plos One*, 2013, **8**.
25. H. Li, H. S. Lee, S. H. Kim, B. Moon and C. Lee, Antioxidant and anti-inflammatory activities of methanol extracts of Tremella fuciformis and its major phenolic acids, *Journal of food science*, 2014, **79**, C460-468.
26. E. Kefaloyianni, C. Gaitanaki and I. Beis, ERK1/2 and p38-MAPK signalling pathways, through MSK1, are involved in NF-kappaB transactivation during oxidative stress in skeletal myoblasts, *Cell Signal*, 2006, **18**, 2238-2251.
27. L. Kong and B.-X. Ge, MyD88-independent activation of a novel actin-Cdc42/Rac pathway is required for Toll-like receptor-stimulated phagocytosis, *Cell research*, 2008, **18**, 745-755.
28. K.-M. Song, S. J. Ha, J.-E. Lee, S.-H. Kim, Y. H. Kim, Y. Kim, S. P. Hong, S. K. Jung and N. H. Lee, High yield ultrasonication extraction method for Undaria pinnatifida sporophyll and its anti-inflammatory properties associated with AP-1 pathway suppression, *LWT-Food Science and Technology*, 2015, **64**, 1315-1322.
29. A. von Knethen, D. Callsen and B. Brüne, NF-κB and AP-1 activation by nitric oxide attenuated apoptotic cell death in RAW 264.7 macrophages, *Molecular biology of the cell*, 1999, **10**, 361-372.
30. F. Aktan, iNOS-mediated nitric oxide production and its regulation, *Life sciences*, 2004, **75**, 639-653.
31. M. Wei, X. Chu, L. Jiang, X. Yang, Q. Cai, C. Zheng, X. Ci, M. Guan, J. Liu and X. Deng, Protocatechuic acid attenuates lipolysaccharide-induced acute lung injury, *Inflammation*, 2012, **35**, 1169-1178.

32. S.-W. Min, S.-N. Ryu and D.-H. Kim, Anti-inflammatory effects of black rice, cyanidin-3-O-β-D-glycoside, and its metabolites, cyanidin and protocatechuic acid, *International immunopharmacology*, 2010, **10**, 959-966.

Table

Table 1. Four major phenolic compounds identified in TFE.

Compound	RT[a] (min)	Content (μg/g)[b]	MRM[c] transition (*m/z*)
Gentisic acid	1.65	2.38 ± 0.05	353 → 93
Protocatechuic acid	1.75	1.02± 0.10	153 → 108
4-hydroxybenzoic acid	2.38	8.39 ± 0.30	137 → 93
Coumaric acid	3.52	2.99 ± 0.10	163 → 119

[a] Retention time; [b] Contents based on the dry weight of TFE (n=3); [c] Multiple reaction monitoring

Figure Legends

Fig. 1 Effects of TFE on LPS-induced production of nitrites and PGE_2, as well as iNOS and COX-2 expression in RAW 264.7 mouse macrophage cells. The cells were treated with the indicated concentrations of TFE in the presence or absence of LPS (1 μg/mL) for 20 hrs. **(A)** TFE inhibits LPS-induced nitrites production in RAW 264.7 cells. Nitrite levels were assayed using the Griess reaction. **(B)** TFE inhibits LPS-induced PGE_2 generation in RAW 264.7 cells. Expression levels of PGE_2 were determined using a Prostaglandin E_2 Express EIA kit. **(C)** TFE significantly inhibits LPS-induced iNOS expression in RAW 264.7 cells. **(D)** TFE inhibits LPS-induced COX-2 expression in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 20 hrs. Expression levels of iNOS, COX-2, and β-actin were determined by Western blot assay. Each band was quantified using software-based densitometry. ** and ***

indicate significant differences [($p < 0.01$) and ($p < 0.001$), respectively] between groups treated with LPS and TFE.

Fig. 2 Effects of TFE on LPS-induced NF-κB and MAPK signaling pathways in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 15 min. **(A)** TFE inhibits LPS-induced phosphorylation of IKKα/β and IκBα phosphorylation, and increases LPS-induced IκBα expression in RAW 264.7 cells. **(B)** TFE inhibits LPS-induced p65 phosphorylation in RAW 264.7 cells. Phosphorylation and expression were detected by Western blotting with specific antibodies. * and *** indicate significant differences [($p < 0.05$) and ($p < 0.001$), respectively] between groups treated with LPS and TFE. **(C)** TFE inhibits LPS-induced phosphorylation of JNK1/2, p38, and ERK1/2 in RAW 264.7 cells. **(D)** TFE inhibits LPS-induced phosphorylation of MEK1/2, but does not affect phosphorylation of MKK3/6 in RAW 264.7 cells. Phosphorylation and expression were detected by Western blotting with specific antibodies. *, ** and *** indicate significant differences [($p < 0.05$), ($p < 0.01$) and ($p < 0.001$), respectively] between groups treated with LPS and TFE.

Fig. 3 Effects of TFE on LPS-induced p65 nuclear translocation in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 30 min. **(A)** TFE inhibits LPS-induced p65 nuclear expression in RAW 264.7 cells. Protein expression was detected by Western blotting with specific antibodies. **(B)** TFE inhibits LPS-induced p65 translocation from the cytosol to the nucleus in RAW 264.7 cells. Nuclear p65 translocation was determined by immunofluorescence. Data are representative of three independent experiments

that gave similar results. C, cytosol; N, nuclear.

Fig. 4 TFE significantly suppresses LPS/GalN-induced **(A)** IL-1β, **(B)** IL-6 and **(C)** TNF-α production in mouse blood. Mice were pre-treated with TFE (40 and 200 mg/kg) for 1 hr before intraperitoneal injection with LPS/GalN challenge for 6 hrs. To determine ELISA outcomes, serum samples from each group were obtained by collecting blood from the orbital venous plexus. * and ** indicate significant differences [($p < 0.05$) and ($p < 0.01$), respectively] between the groups treated with LPS and TFE.

Fig. 5 Effects of TFE on LPS/GalN-induced iNOS and COX-2 expressions *in vivo.* Mice were pre-treated with TFE (40 and 200 mg/kg) 1 hr before intraperitoneal injection with LPS/GalN challenge for 6 hrs. **(A)** TFE inhibits LPS/GalN-induced iNOS and COX-2 expression in the liver. Recovered livers were lysed as described in the Materials and Methods. Expression levels of iNOS, COX-2 and β-actin were determined by Western blot. Each band was densitometrically quantified by software image analysis. Results are shown as the means ± SEM, ($n = 5$). * and ** indicate significant differences [($p < 0.05$) and ($p < 0.01$), respectively] between the groups treated with LPS and TFE. **(B)** Immunohistochemistry of iNOS and COX-2 expressions in the liver. iNOS and COX-2 are stained brown. Representative photographs of overall immunohistochemical staining patterns from each group are shown.

Fig. 6 LC-MS/MS chromatograms of **(A)** gentisic acid, **(B)** 4-hydroxybenzoic acid and **(C)** coumaric acid **(D)** protocatechuic acid in 50 mg/L standard solution (left) and

TFE sample (right).

Fig. 7. Effects of 4 major compounds in TFE on LPS-induced nitrite production in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 20 hours. *, **, and *** indicate significant differences [($p < 0.05$), ($p < 0.01$), and ($p < 0.001$), respectively] between groups treated with LPS and each compound. CA, coumaric acid; 4-HB, 4-hydroxybenzoic acid; PCA, protocatechuic acid; GA, gentisic acid

Figure 1

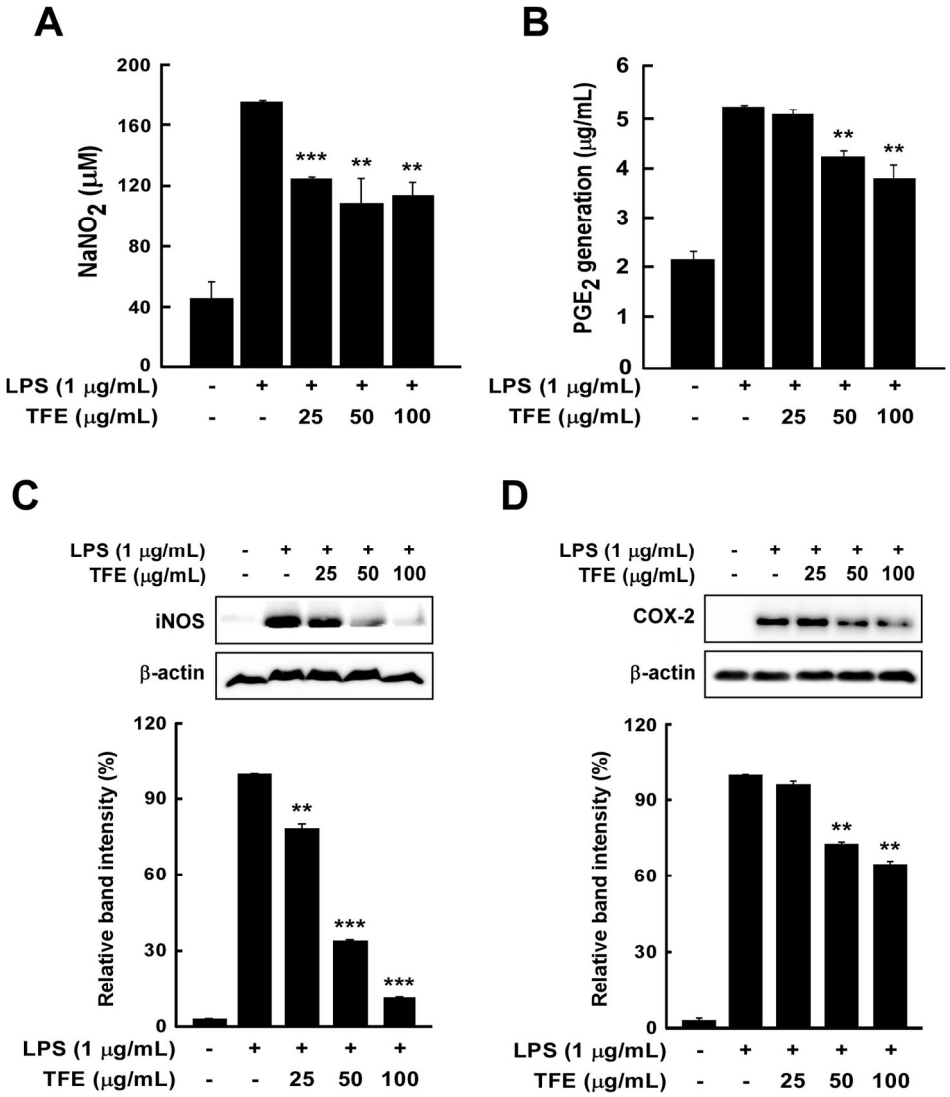


Fig. 1 Effects of TFE on LPS-induced production of nitrites and PGE2, as well as iNOS and COX-2 expression in RAW 264.7 mouse macrophage cells. The cells were treated with the indicated concentrations of TFE in the presence or absence of LPS (1 μg/mL) for 20 hrs. (A) TFE inhibits LPS-induced nitrites production in RAW 264.7 cells. Nitrite levels were assayed using the Griess reaction. (B) TFE inhibits LPS-induced PGE2 generation in RAW 264.7 cells. Expression levels of PGE2 were determined using a Prostaglandin E2 Express EIA kit. (C) TFE significantly inhibits LPS-induced iNOS expression in RAW 264.7 cells. (D) TFE inhibits LPS-induced COX-2 expression in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 20 hrs. Expression levels of iNOS, COX-2, and β-actin were determined by Western blot assay. Each band was quantified using software-based densitometry. ** and *** indicate significant differences [($p < 0.01$) and ($p < 0.001$), respectively] between groups treated with LPS and TFE.
196x250mm (214 x 214 DPI)

Figure 2

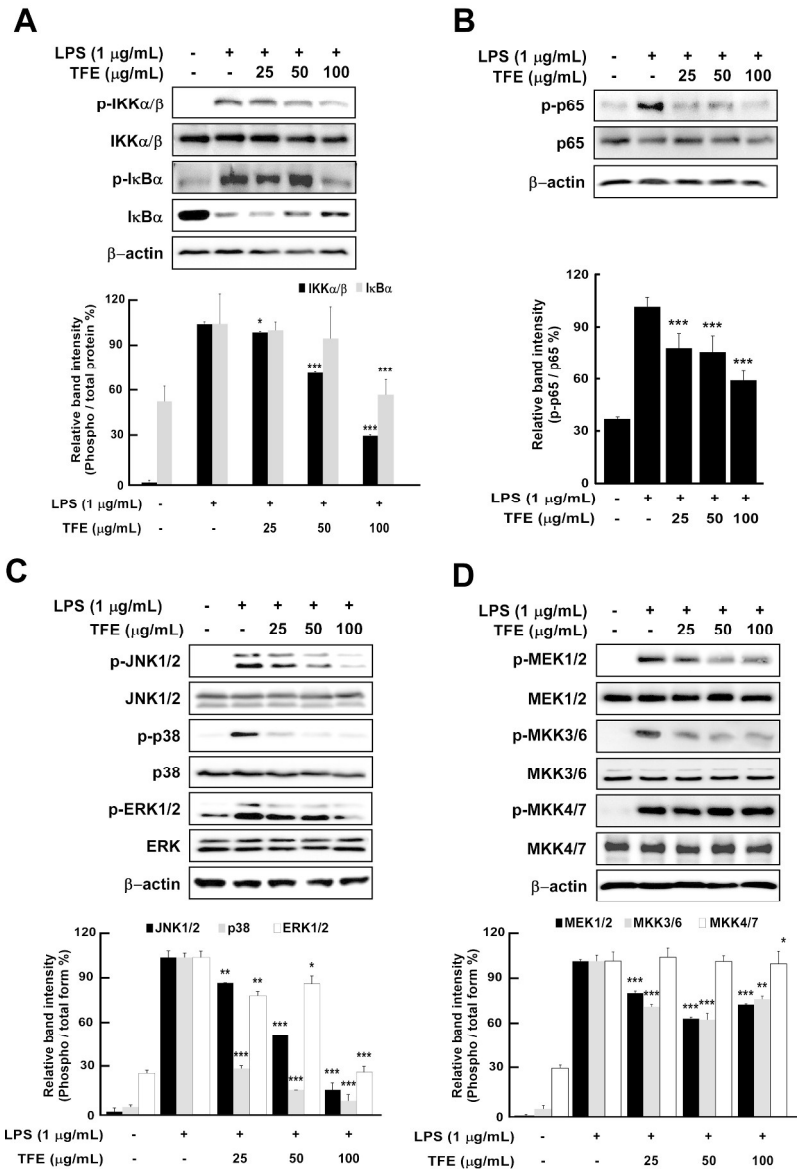


Fig. 2 Effects of TFE on LPS-induced NF-κB and MAPK signaling pathways in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 15 min. (A) TFE inhibits LPS-induced phosphorylation of IKKα/β and IκBα phosphorylation, and increases LPS-induced IκBα expression in RAW 264.7 cells. (B) TFE inhibits LPS-induced p65 phosphorylation in RAW 264.7 cells. Phosphorylation and expression were detected by Western blotting with specific antibodies. * and *** indicate significant differences [($p < 0.05$) and ($p < 0.001$), respectively] between groups treated with LPS and TFE. (C) TFE inhibits LPS-induced phosphorylation of JNK1/2, p38, and ERK1/2 in RAW 264.7 cells. (D) TFE inhibits LPS-induced phosphorylation of MEK1/2, but does not affect phosphorylation of MKK3/6 in RAW 264.7 cells. Phosphorylation and expression were detected by Western blotting with specific antibodies. *, ** and *** indicate significant differences [($p < 0.05$), ($p < 0.01$) and ($p < 0.001$), respectively] between groups treated with LPS and TFE.
311x472mm (300 x 300 DPI)

Figure 3

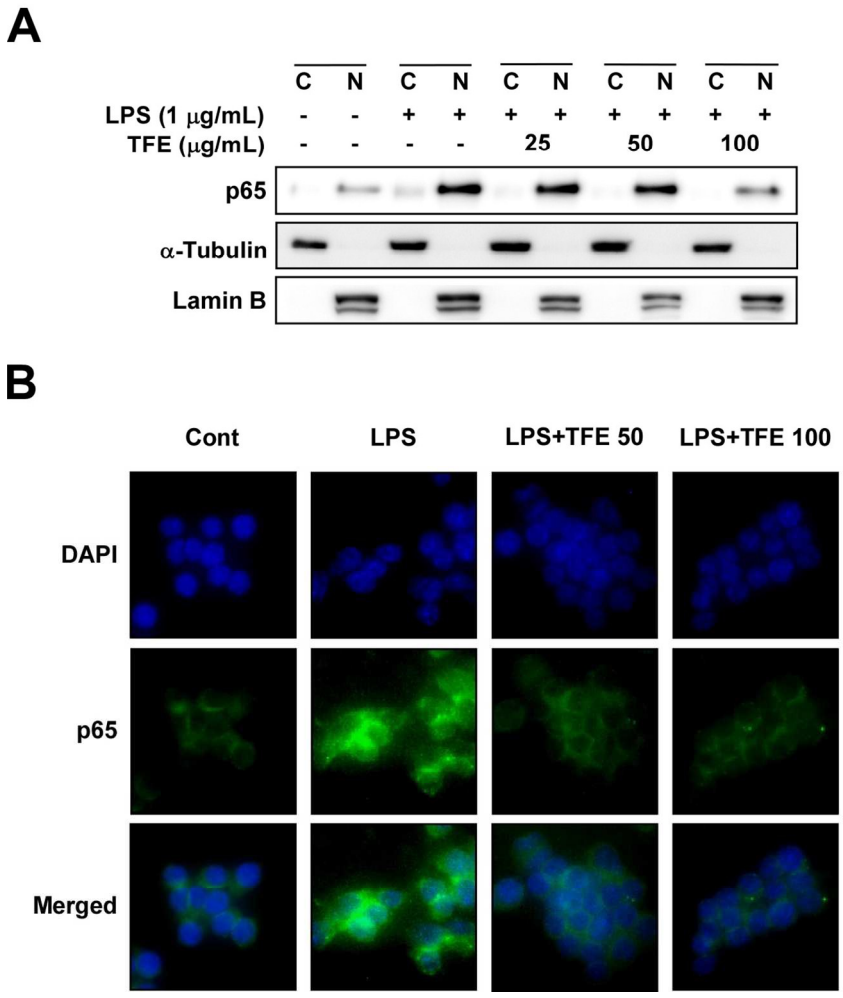


Fig. 3 Effects of TFE on LPS-induced p65 nuclear translocation in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 30 min. (A) TFE inhibits LPS-induced p65 nuclear expression in RAW 264.7 cells. Protein expression was detected by Western blotting with specific antibodies. (B) TFE inhibits LPS-induced p65 translocation from the cytosol to the nucleus in RAW 264.7 cells. Nuclear p65 translocation was determined by immunofluorescence. Data are representative of three independent experiments that gave similar results. C, cytosol; N, nuclear.
178x206mm (214 x 214 DPI)

Figure 4

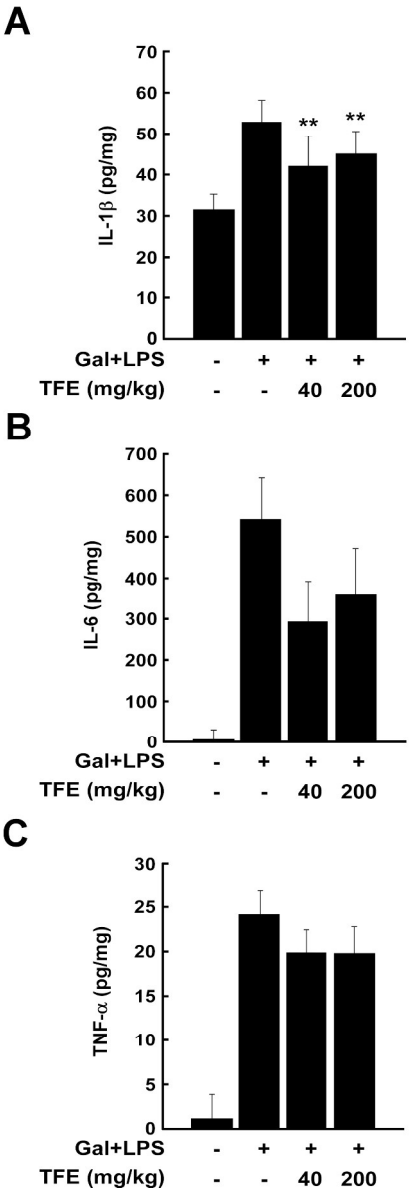


Fig. 4 TFE significantly suppresses LPS/GalN-induced (A) IL-1β, (B) IL-6 and (C) TNF-α production in mouse blood. Mice were pre-treated with TFE (40 and 200 mg/kg) for 1 hr before intraperitoneal injection with LPS/GalN challenge for 6 hrs. To determine ELISA outcomes, serum samples from each group were obtained by collecting blood from the orbital venous plexus. * and ** indicate significant differences [($p < 0.05$) and ($p < 0.01$), respectively] between the groups treated with LPS and TFE.
264x476mm (300 x 300 DPI)

Figure 5

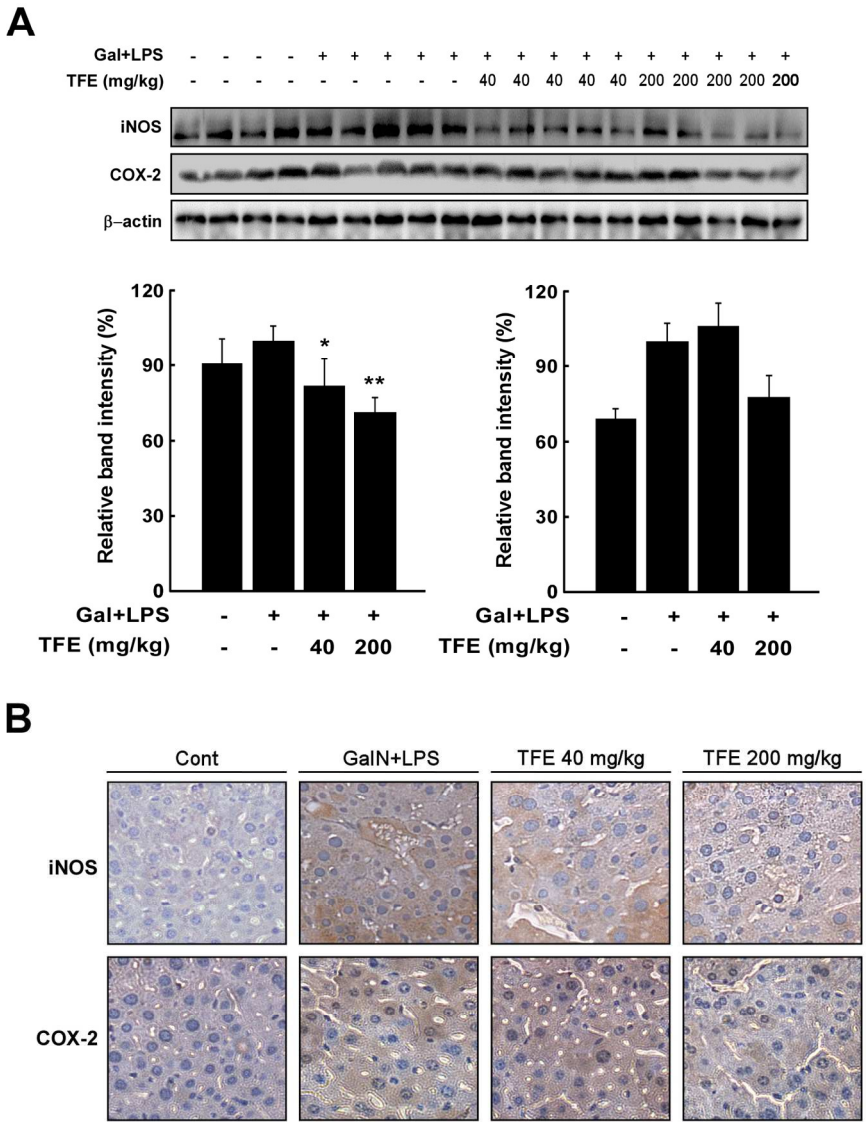


Fig. 5 Effects of TFE on LPS/GalN-induced iNOS and COX-2 expressions in vivo. Mice were pre-treated with TFE (40 and 200 mg/kg) 1 hr before intraperitoneal injection with LPS/GalN challenge for 6 hrs. (A) TFE inhibits LPS/GalN-induced iNOS and COX-2 expression in the liver. Recovered livers were lysed as described in the Materials and Methods. Expression levels of iNOS, COX-2 and β-actin were determined by Western blot. Each band was densitometrically quantified by software image analysis. Results are shown as the means ± SEM, (n = 5). * and ** indicate significant differences [($p < 0.05$) and ($p < 0.01$), respectively] between the groups treated with LPS and TFE. (B) Immunohistochemistry of iNOS and COX-2 expressions in the liver. iNOS and COX-2 are stained brown. Representative photographs of overall immunohistochemical staining patterns from each group are shown.
190x251mm (220 x 220 DPI)

Figure 6

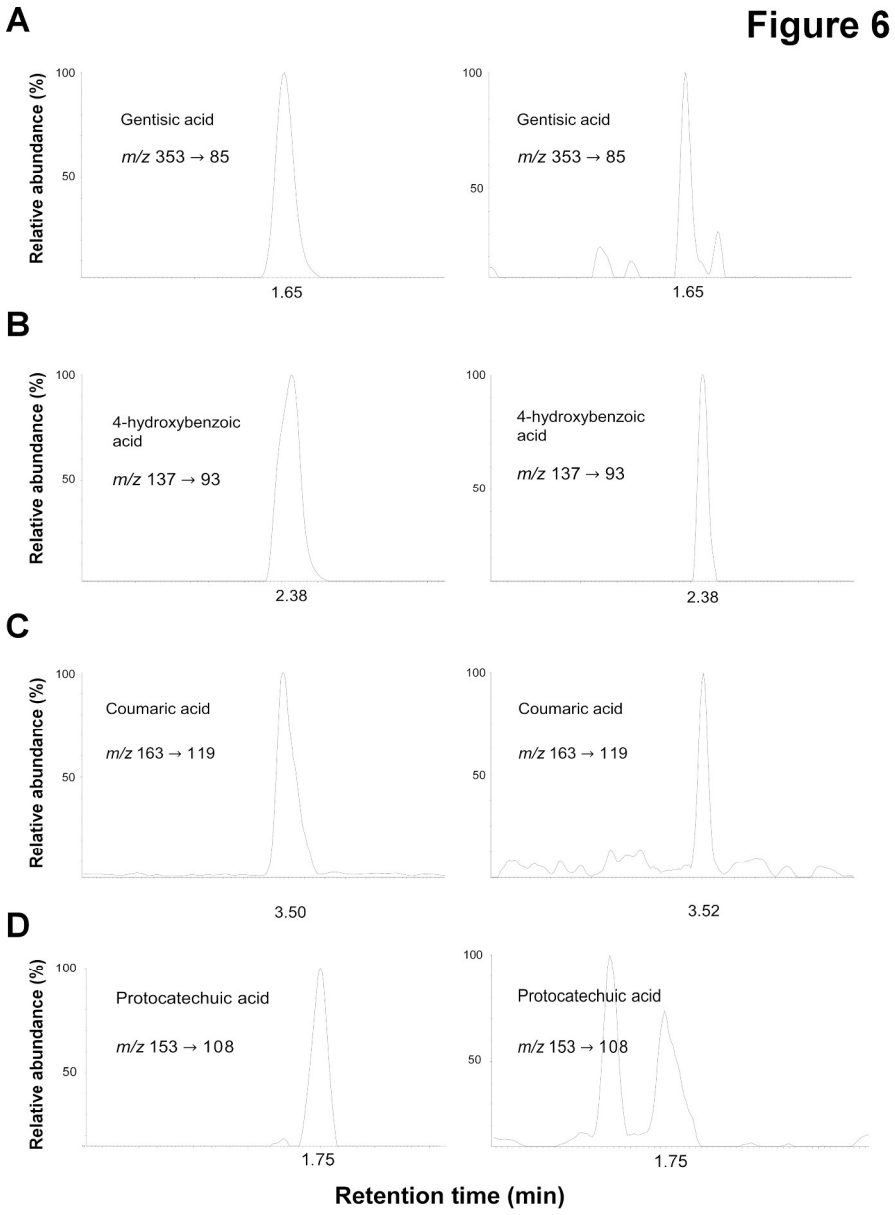


Fig. 6 LC-MS/MS chromatograms of (A) gentisic acid, (B) 4-hydroxybenzoic acid and (C) coumaric acid (D) protocatechuic acid in 50 mg/L standard solution (left) and TFE sample (right).
272x370mm (300 x 300 DPI)

Figure 7

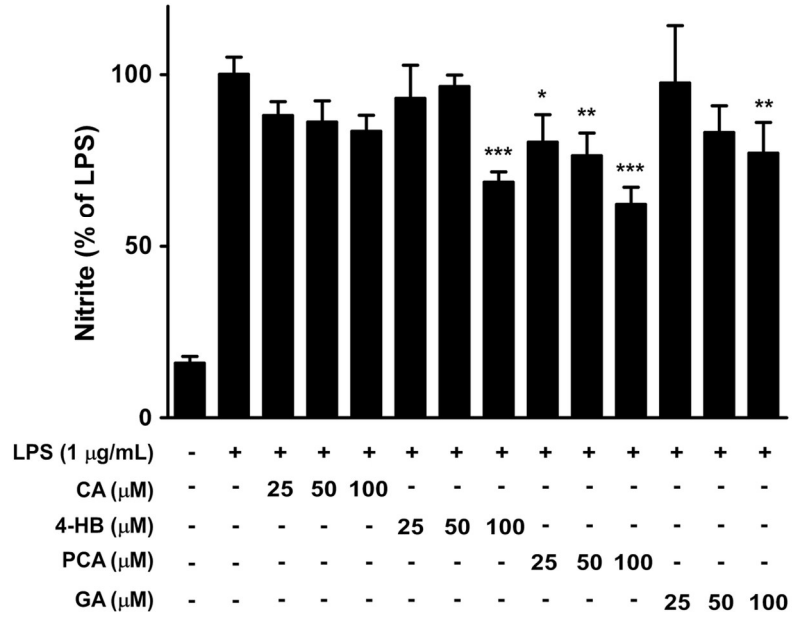
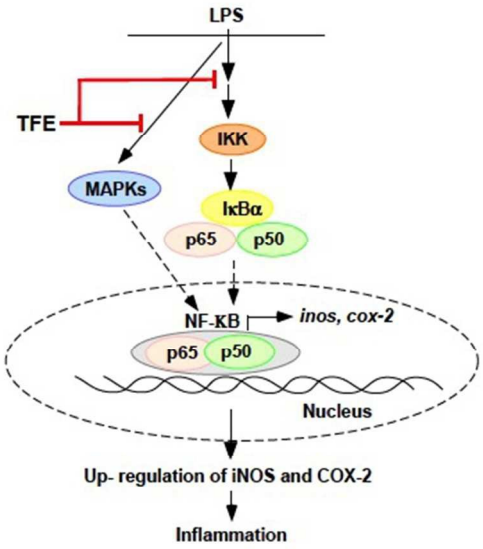


Fig. 7. Effects of 4 major compounds in TFE on LPS-induced nitrite production in RAW 264.7 cells. Cells were treated with the indicated concentrations of TFE for 1 hr and then stimulated with LPS (1 μg/mL) for 20 hours. *, **, and *** indicate significant differences [($p < 0.05$), ($p < 0.01$), and ($p < 0.001$), respectively] between groups treated with LPS and each compound. CA, coumaric acid; 4-HB, 4-hydroxybenzoic acid; PCA, protocatechuic acid; GA, gentisic acid
128x88mm (300 x 300 DPI)



254x190mm (72 x 72 DPI)