Fermentation enhances the antioxidant and anti-inflammatory effects of Bat Faeces (Ye Ming Sha) via the ERK, p38 MAPK and NF- κ B signaling pathways in RAW 264.7 cells

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Abstract The ethyl acetate fraction of Bat Faeces (Ye Ming Sha; natural products used in Chinese Medicine) after fermentation (EFBF-AF) showed enhanced anti-oxidative effects in 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt assays. Fermentation of the Bat Faeces by using the crude enzyme extract from *Aspergillus kawachii*, significantly increased the anti-inflammatory effects. Fermented Bat Faeces markedly inhibited nitric oxide production, inducible nitric oxide synthase, and cyclooxygenase-2 expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. The EFBF-AF reduced the nuclear translocation of nuclear factor kappa B (NF- κ B) via IKK α and I κ B α phosphorylation, and decreased the phosphorylated the extracellular signal-regulated kinases (ERK) and p38 expression in LPS-treated RAW 264.7 macrophages. In addition, the EFBF-AF suppressed the expression of pro-inflammatory genes, such as interleukin-1 β , interleukin-6, and tumor necrosis factor- α . These results suggest that fermented Bat Faeces may suppress pro-inflammatory responses in LPS-stimulated RAW 264.7 macrophages cells via ERK, p38 mitogen-activated protein kinase and NF- κ B signaling pathways.

Keywords Anti-inflammatory effect · Antioxidative effect ·

Aspergillus kawachii · Bat Faeces · Fermentation · *Vespertilio superans* Thomas

Introduction

Bat Faeces (*Vespertilio superans* Thomas), Ye Ming Sha, is one of the natural products used in Chinese medicine [1]. The other natural products used by Chinese medical practitioners include plants, minerals and animal substances [2]. Bat Faeces is a coarse powder, dark brown in color, resembles tea-dust in appearance, and includes debris of the Mylabris insect, Bat dung, dirt, and other extraneous substances. Bat dung is popularly referred to as night-bright sand. It is used in the treatment of chronic eye diseases in China [3].

Reactive oxygen species (ROS) is classified into two categories. The first category includes the oxygen radicals such as alkoxy (RO $\cdot$), superoxide anion (O $_2^{\cdot-}$), peroxy (ROO $\cdot$), hydroxy (HO $\cdot$), and hydroperoxy (HOO $\cdot$) radicals. Non-radical derivatives of oxygen including hydrogen peroxide (H $_2$ O $_2$), ozone (O $_3$), and singlet oxygen (1 O $_2$) from the second category [4]. Excess production of ROS in body results in many biological changes, such as ageing, DNA damage, heart disease and cancer [5]. Antioxidants help to protect the body against damage caused by ROS. Medicinal plants and other natural substances have gained much interest in the recent times as sources of biologically active substances such as antioxidants, antimutagens, and anticarcinogens [6]. Inflammation is one of the defensive reactions of living tissues to physical damage, toxic substances and external chemical stimuli [7]. In murine RAW 264.7 cells, an innate immune response may involve recognition of lipopolysaccharide (LPS) by the Toll like receptor 4 (TLR4) and activation of NF- κ B [8]. Exposure to LPS leads to increased expression of inducible nitric

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oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which in turn increases the levels of nitric oxide (NO) resulting in inflammation [9,10]. NF- κ B activation and signaling play a central role, releasing inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in LPS-induced inflammatory RAW 264.7 cells [11,12]. Phosphorylation of the mitogen-activated protein kinases (MAPKs) is also associated with inflammatory responses including mitosis, differentiation, and cell survival/apoptosis [13].

Fermentation is one of the commonly used food processing techniques for increasing the flavor or fragrance [14]. Further, it is used as a method for increasing the content of the physiologically active substances. For example, fermentation of licorice (crude enzyme treatment) increased the content of liquiritigenin which has anti-oxidant, anti-dementia, and anti-microbial activities [15]. Another example is the enhancement of the active form of quercetin in onion by enzyme treatment. Bioavailability and physiological activity of quercetin are very low in onion, because quercetin mostly exists in the glycoside form in combination with glucose. β -Glycosidase treatment enhances the antioxidant activity by increasing the active quercetin content in onion [16].

Based on this background knowledge, we attempted to improve the anti-oxidant and anti-inflammatory activities of Bat Faeces by fermentation. In addition, we investigated whether the ethyl acetate fraction of Bat Faeces after fermentation (EFBF-AF) increase inhibition of the inflammatory response such as NF- κ B activation and phosphorylation of ERK and p38 in LPS-stimulated RAW 264.7 cells.

Materials and Methods

Chemicals

Extraction solvents, including methanol (MeOH), ethanol (EtOH), *n*-hexane (hexane), ethyl acetate (EtOAc) and *n*-butanol (BuOH) were purchased from Duksan Chemical Co. (Seoul, Korea). Chemical reagents such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies for iNOS, NF- κ B, β -actin, Lamin B, p-IKK α / β , IKK α , p-IkB α , IkB α , p38, p-p38, p-ERK, and ERK were purchased from Cell Signaling Technology Inc. (Boston, MA, USA) and COX-2 antibodies were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Bat Faeces and preparation of *Aspergillus kawachii* enzyme extract

Bat Faeces (500 g), purchased from Daegu Oriental Medicine

Market, in Korea was refluxed two times with 3.5 L of 70% MeOH for 3 h. The extract was filtered through the filter paper and concentrated using Rotary evaporator. Crude enzyme extract was prepared from the soybean paste fungi, *Aspergillus kawachii*, according to the previous report with some modification [16]. Ten grams of wheat bran in 10 mL of distilled water was sterilized at 121 °C for 30 min. *A. kawachii* grown in potato dextrose agar media (Difco Laboratories, Detroit, MI, USA) was inoculated into the sterilized wheat bran and incubated at 30°C for 3 days. Incubated wheat bran was suspended in 100 mL of 100 mM sodium phosphate buffer (pH 7.0) and kept at 4 °C for 18 h. The reaction mixture was then centrifuged at 10,000 rpm for 15 min. The supernatant was used for the fermentation of Bat Faeces. This solution contained 0.276 U/mL (1 U is defined as the enzyme activity needed to produce 1 μ mol of p-nitrophenol from p-nitrophenyl- β -D-glucopyranoside per min) of β -glucosidase activity.

Fermentation of Bat Faeces

The methanol extract of the Bat Faeces was re-suspended in 200 mL of distilled water and 100 mL aliquot of this suspension was treated with 100 mL of active or inactive crude enzyme extract. The mixture was incubated with shaking at 100 rpm at 30 °C [16]. After 18 h, the reaction mixture was partitioned sequentially in three different solvents, hexane, EtOAc and BuOH. Inactive crude enzyme was prepared by autoclaving at 121 °C for 30 min, served as the control.

UPLC conditions for fermentation of Bat Faeces

The UPLC was performed to compare changing compounds during the fermentation. The fermented Bat Faeces were quantified by Acquity™ Ultrapformance LC system (UPLC, Waters, Milford, MA, USA) with the wavelength set at 280 nm. The UPLC analyses were performed using a ACQUITY UPLC CSH C18 (2.1 $\times$ 100 mm, 1.7 μ m; Waters, Milford, USA) reverse phase column and the mobile phase consisted of water (solvent A) and acetonitrile (solvent B) each containing 0.1% formic acid. After 2 μ L of the sample was injected into the column, solvent B increased from 0% to 100% in 9 min, held at 100% for 1 min. The solvent flow rate was 0.3 mL/min.

DPPH radical scavenging assay

The DPPH assay was performed according to a method described by Yoshiki [17] with some modifications. DPPH (150 μ M in EtOH, 190 μ L) was mixed with samples in dimethyl sulfoxide (DMSO, 10 μ L) at different concentrations (10, 25, 50 and 100 μ g/mL). After 30 min in the dark room, the absorbance was measured at 517nm.

ABTS radical cation decolorization assay

In order to generate ABTS cation free radicals, ABTS (7 mM in H₂O) solution and potassium persulfate (2.45 mM in H₂O) solution were mixed in 2:1 ratio by volume and kept at 4 °C for

24 h in dark. The ABTS⁺ solution was diluted with 80% EtOH to an absorbance of 0.7 ± 0.02 at 734 nm [18]. The samples were dissolved in DMSO to various final concentrations. Antioxidant effect of these samples was evaluated by incubating 10 μ L of sample solution with 190 μ L of the ABTS solution in a 96-well plate at room temperature, and the optical density at 734 nm was read using microreader.

Cell culture and viability

Murine RAW 264.7 cells, a macrophage-like cell line, was purchased from Korean Cell Line Bank and cultured in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 100 U/mL of penicillin and 100 μ g/mL of streptomycin (P/S, Gibco BRL, Grand Island, NY, USA) and incubated at 37 °C in an atmosphere of 5% CO₂. For the cell viability analysis, RAW 264.7 cells were seeded at a density of 5×10^4 cells/well in 96-well plates. After 24 h, fermented and unfermented samples of different concentrations (10, 25, 50 and 100 μ g/mL) were added to the wells and incubated for additional 24 h. The culture medium was then removed and MTT in phenol red free DMEM (Welgene, Daegu, Korea) was added to a concentration of 0.5 mg/mL. After incubation at 37 °C for 4h, the supernatant was removed and the formazan blue formed was dissolved in 100 μ L of DMSO. Optical density at 575 nm was determined with a microplate reader.

Nitric oxide assay

Cells were seeded into 96-well plates at a density of 5×10^4 cells/well for 24 h. Cells were treated with each of the samples for 1 h, and then with LPS (final concentration, 1 μ g/mL) for 24 h. After 24 h of incubation, 100 μ L of the cell supernatant medium was used for NO determination. The NO produced in culture medium was measured by the method of the Griess reaction. Briefly, 100 μ L of the cell culture medium was mixed with 100 μ L of Griess reagent, in which N1 and N2 were mixed in a ratio of 1:1 by volume (N1 reagent: 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, N2 reagent: 0.1% (w/v) naphthylethylenediamide-HCl), incubated in dark at room temperature for 10 min, and then the absorbance was measured at 540 nm [19], using a microplate reader.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared with TRIzol reagent (Ambion, Austin, TX, USA) from LPS-treated RAW 264.7 cells, and stored at -70 °C until used. To synthesize cDNA from RNA, PrimeScriptTM 1st strand cDNA synthesis kit (Takara, Otsu, Japan) was used. The reverse transcription reaction cocktail (20 μ L) was incubated at 42 °C for 45 min and 95 °C for 5 min. The synthesized cDNA was diluted 20-fold in diethyl pyrocarbonate water. Conventional PCR for target gene amplification using specific gene primers, was

performed using Takara Ex Taq kit (Takara). The reaction comprised 30 cycles of 98 °C for 10 s (denaturing), 55 °C for 30 s (annealing) and 72 °C for 1 min (primer extension). The primers was designed as reported previously [20] and were purchased from Bioneer (Daejeon, Korea). The primer sequences were as follows: iNOS sense 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'; iNOS antisense 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'; COX-2 sense 5'-CACTACATCCTGACCCACTT-3'; COX-2 antisense 5'-ATGCTCCTGCTTGAGTATGT-3'; β -actin sense 5'-GTGGGCCGCCCTAGGCACCAG-3'; β -actin antisense 5'-GGA GGAAGAGGATGCGGCAGT-3'; IL-1 β sense 5'-CAGGATGAG GACATGAGCACC-3'; IL-1 β antisense 5'-CTCTGCAGACTC AAAGTCCAC-3'; IL-6 sense 5'-GTACTCCAGAAGACCAGA GG-3'; IL-6 antisense 5'-TGCTGGTGACAACCACGGCC-3'; TNF- α sense 5'-TTGACCTCAGCGCTGAGTTG-3'; TNF- α antisense 5'-CCTGTAGCCCCACGTCGTAGC-3'. PCR products of iNOS, COX-2, β -Actin, IL-1 β , IL-6 and TNF- α were electrophoresed in 1.2% agarose gels and stained with EcoDyeTM DNA staining solution (Biofact, Daejeon, Korea). Image J was used to quantify RT-PCR products.

Western blot analysis

EtOAc fraction of fermented and unfermented Bat Faeces was added to LPS-treated RAW 264.7 cells as described above. After washing with 1X PBS, the cells were lysed using Pro-Prep protein extraction solution (iNtRON Biotechnology Inc., Seoul, Korea) to obtain whole cell lysate. Nuclear proteins were extracted using Nuclear Extraction Kit (Abcam, MA, USA). The amount of protein was measured using the BCA protein assay kit (Thermo Fisher Scientific, Waltman, MA, USA). Twenty micrograms of proteins from each sample was mixed with 5X loading buffer (Biosesang, Sungnam, Korea), boiled for 10 min and electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-Buffered Saline and Tween 20 solution, followed by incubation with primary antibodies against iNOS, COX-2, β -actin, Lamin B, IKK α , p-IKK α / β , I κ B, p-I κ B, ERK, p-ERK, p38, p-p38 and NF- κ B. After washing, the membranes were treated with HRP-conjugated secondary antibodies (1:1000) for 40 min. β -Actin was used as protein loading controls. Bands were detected using ECL kit (Thermo Fisher Scientific, Waltman, MA, USA) and measured with LAS instrument. Image J was used to quantify western blot signals.

Statistical analysis

All experiments were performed in triplicate. The results were presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's tests. P values less than 0.05 were considered to be significant.

Results and Discussion

UPLC patterns of fermented Bat Faeces fractions

The UPLC profile of activated enzyme-treated Bat Faeces fractions were compared with those from inactivated enzyme-treated Bat Faeces fractions. The UPLC chromatogram of each fraction had changing peaks due to the fermentation. Especially, the EtOAc fractions of Bat Faeces had significant new peaks after fermentation (black arrows) (Fig. 1).

Antioxidant effects of EFBF-AF

Endogenous ROS is generated in the course of metabolic activities as well as on exposure to cytokines, chemotherapeutic agents, ultraviolet radiation, and hyperthermia. ROS can perturb the normal redox balance and shift the cells into a state of oxidative stress [21]. In a recent study, a wide range of spectrophotometric assays have evaluated the antioxidant capacity of substances, using methods such as DPPH and ABTS radical scavenging assays [22]. We investigated the antioxidant effect of EFBF-AF using both of these methods. DPPH in EtOH solvent is a stable, free radical, and the solution is violet in color with strong absorbance at 517 nm. When an antioxidant substance scavenges the DPPH radicals, the color of the solution changes to yellow, and the consequent change in absorbance is a measure of the antioxidant activity of the substance under study [23]. The DPPH radical scavenging activity of EFBF-AF was $60.96 \pm 6.72\%$ at a concentration of $100 \mu\text{g/mL}$, while the control (autoclaved enzyme-treated Bat Faeces) showed only a negligible effect at the concentration ($10.77 \pm 4.08\%$; Fig. 2B), thus indicating that fermentation of Bat Faeces increased the DPPH radical scavenging activity by about 5.7 fold. EFBF-AF also showed higher ABTS radical scavenging effect than the ethyl acetate fraction of Bat Faeces before fermentation (EFBF-BF). In particular, at $100 \mu\text{g/mL}$, the

ABTS radical scavenging activity of EFBF-AF was $95.40 \pm 0.17\%$, whereas that of the control (EFBF-BF) was $10.73 \pm 3.79\%$ (Fig. 2F). There was no difference between the antioxidant activities of hexane and BuOH fractions of the fermented Bat Faeces. This data clearly suggests that fermentation enhanced both the DPPH and the ABTS radical scavenging activity of Bat Faeces.

Effects of EFBF-AF on LPS-stimulated NO production in RAW 264.7 cells

Excess NO results in apoptosis of some cell types, including macrophages [24]. Activated macrophages produce large amounts of NO from L-arginine by iNOS. NO is an important intracellular and intercellular regulatory molecule, involved in multiple biological functions such as macrophage-mediated cytotoxicity, smooth muscle relaxation, and neurotransmission [19]. In the present study, we compared the inhibitory effects of the EtOAc fraction of Bat Faeces before and after fermentation, on the LPS-induced pro-inflammatory molecules, including NO production [25]. The effect on cell viability of the EtOAc fraction from pre- and post-fermentation Bat Faeces at different concentrations was investigated using the MTT assay. As shown in Fig. 3A and 3B, EFBF-BF and EFBF-AF did not affect the cell viability at concentrations up to $100 \mu\text{g/mL}$. Therefore, we used samples between 10 – $100 \mu\text{g/mL}$ concentrations in all subsequent experiments. The result for NO production is presented in Fig. 2C. When the RAW 264.7 cells were exposed to LPS, NO levels increased significantly, by 100% over the control. The EFBF-AF decreased the NO production in a dose-dependent manner, whereas the NO production inhibition activity of EFBF-BF was negligible. EFBF-AF suppressed the NO production by 83.5% at $50 \mu\text{g/mL}$, and by 50% at $100 \mu\text{g/mL}$, in LPS-stimulated RAW 264.7 macrophages. Based on these data, EFBF-BF and EFBF-AF were used at concentrations of 50 and $100 \mu\text{g/mL}$, respectively, in subsequent experiments.

Fig. 1 Comparison of UPLC chromatogram of Bat Faeces before and after crude enzyme extract from *A. kawachii* treatment. BF, Bat Faeces; B.F, before fermentation (inactivated crude enzyme extract from *A. kawachii*); A.F, after fermentation (activated crude enzyme extract from *A. kawachii*). Black arrows represent increased peaks

Fig. 2 Antioxidant activity of fractions of Bat Faeces treated with inactivated crude enzyme extract from *A. kawachii* or activated crude enzyme extract from *A. kawachii*. DPPH radical scavenging effect of hexane (A), EtOAc (B), and BuOH (C) fractions. ABTS radical scavenging effect of hexane (D), EtOAc (E), and BuOH (F) fractions. CA, crude enzyme extract from *A. kawachii*; BF, before fermentation (inactivated crude enzyme extract from *A. kawachii*); AF, after fermentation (activated crude enzyme extract from *A. kawachii*). Values represent mean±SD of relative OD obtained from three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to control. ### $p < 0.001$ when compared with fermentation of the EtOAc soluble fraction

Fig. 3 Effect of EFBF-AF on the viability of RAW 264.7 cells and suppression of LPS-induced NO production. Cultured cells were treated with different concentrations of Bat Faeces EtOAc fraction before (A) and after (B) fermentation to confirm cell cytotoxicity. Cells were pre-treated with 5–100 µg/mL of EFBF-BF and EFBF-AF prior to LPS (1 µg/mL) treatment. Supernatants were collected following 24 h in culture to determine their NO production (C). EFBF-BF, ethyl acetate fraction of Bat Faeces before fermentation; EFBF-AF, ethyl acetate fraction of Bat Faeces after fermentation. ** $p < 0.01$ and *** $p < 0.001$ compared to LPS alone. ## $p < 0.01$ and ### $p < 0.001$ when compared with fermentation of the EtOAc-soluble fraction

Effects of EFBF-AF on LPS-stimulated iNOS and COX-2 expression in RAW 264.7 cells

iNOS and COX-2 are important enzymes that mediate inflammatory processes. Overproduction of iNOS and/or COX-2 has been associated with pathophysiology of certain types of human cancers as well as inflammatory disorders [26]. Expression of iNOS and COX-2 genes increases in response to pro-inflammatory and inflammatory mediators [27]. To investigate the inhibitory effect of EFBF-AF on the mRNA and protein expression of iNOS and COX-2, we performed RT-PCR and western blotting experiments. RAW 264.7 cells exposed to 1 µg/mL of LPS, both with and without pretreatment with the EFBF-BF, showed markedly increased expression of both iNOS and COX-2 expression. However, The EFBF-AF pretreatment significantly suppressed the mRNA and protein levels of iNOS in a dose-dependent manner in LPS-stimulated RAW 264.7 cells, indicating that EFBF-AF reduced the LPS-stimulated NO production by inhibiting the iNOS gene expression at the mRNA and protein levels. Pretreatment with

EFBF-AF also significantly repressed mRNA and protein expression of COX-2 in LPS-stimulated RAW 264.7 macrophage cells (Fig. 4).

Effects of EFBF-AF on LPS-stimulated cytokine expression in RAW 264.7 cells

Pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α , are up-regulated in LPS-stimulated RAW 264.7 macrophage cells [28]. Cytokines are an important cellular defense mechanism, because cytokine production is the first cellular response to a microbial infection [7,29]. We investigated whether EFBF-AF has an effect on the production of pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α . The expression and secretion of these cytokines in RAW 264.7 macrophage cells were measured using RT-PCR. As depicted in Fig. 4, RAW 264.7 cells incubated with LPS (1 µg/mL) for 24 h exhibited significantly increased expression of IL-1 β , IL-6, and TNF- α as evaluated by RT-PCR. However, pretreatment with EFBF-AF inhibited the LPS-induced secretion of IL-1 β , IL-6, and TNF- α in a dose-dependent manner. Conversely,

Fig. 4 Inhibition of LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expression by the addition of EtOAc fraction of fermented Bat Faeces to RAW 264.7 macrophage cells. RAW 264.7 cells were pre-treated with EFBF-BF and EFBF-AF (50, 100 µg/mL respectively) for 24 h prior to LPS (1 µg/mL) treatment. Cellular proteins were used for the detection of iNOS and COX2 by western blotting. mRNA levels of iNOS and COX-2 were then measured using RT-PCR. β -Actin expression was used as the internal control. CNT, control; LPS, 1 µg/mL of LPS; BF, EtOAc fraction of Bat Faeces before fermentation; AF, EtOAc fraction of Bat Faeces after fermentation. * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to LPS alone. # p < 0.05, ## p < 0.01 and ### p < 0.001 when compared with fermentation of the EtOAc-soluble fraction

EFBF-BF showed only a negligible inhibitory effect at the same concentration. These data indicate that EFBF-AF exerts its anti-inflammatory effect by the down-regulation IL-1 β , IL-6, and TNF- α expression (Fig. 5).

Effects of EFBF-AF on LPS-stimulated NF- κ B translation in RAW 264.7 cells

NF- κ B is a transcription factor that controls various genes, including iNOS, COX-2, IL-1 β , IL-6, and TNF- α . The role of

NF- κ B signaling pathways in immunity and inflammation in LPS-stimulated RAW 264.7 macrophage cells is well documented [30]. In unstimulated cells, NF- κ B is comprised mainly of proteins p50 and p65, and is combined with I kappa B (I κ B) in the cytoplasm. However, when the cells are treated with LPS, I κ B is phosphorylated and detaches from NF- κ B, resulting in translocation of NF- κ B to the cell nucleus [31]. To evaluate whether EFBF-AF was related to the NF- κ B signaling pathway, the cells were pre-treated with EFBF-AF for 1 h, followed by LPS treatment for 1.5 h. NF- κ B

Fig. 5 Inhibitory effect of EFBF-AF on LPS-induced IL-1 β , IL-6, and TNF- α production in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with EFBF-BF and EFBF-AF (50, 100 μ g/mL respectively) for 1 h and then stimulated with LPS (1 μ g/mL) for 24 h. The levels of IL-1 β (B), IL-6 (C), and TNF- α (D) mRNA were determined by RT-PCR. Each value is mean $\pm$ SE from three independent experiments. * p < 0.05, ** p < 0.01, and *** p < 0.001. ## p < 0.01 when compared with fermentation of the EtOAc-soluble fraction

Fig. 6 Effect of the EFBF-AF on the regulation of NF- κ B (A) via IKK α/β and I κ B α phosphorylation (B). Nuclear fraction and total cell lysates were prepared for Western blot analysis. RAW 264.7 cells were pre-treated with EFBF-BF and EFBF-AF (50, 100 μ g/mL respectively) for 1 h followed by treatment with LPS for 1.5 h to detect NF- κ B translocation. Cellular proteins were used for the detection of phosphorylated forms of IKK α/β and I κ B α by western blotting. β -Actin and Lamin B were used as loading controls.; LPS treatment for 30 min to confirm phosphorylation of IKK α and I κ B α/β

translocation was observed by western blot analysis. EFBF-AF treatment decreased the nuclear levels of NF- κ B p65, relative to those in the LPS only treatment and EFBF-BF-treated samples. Translocation of NF- κ B is associated with p-IKK and p-I κ B as TLR4 pathway [32]. As shown in Fig. 6, when EFBF-AF treatment preceded the LPS treatment, the phosphorylation of IKK and I κ B levels were significantly reduced. Pretreatment of the cells with EFBF-BF showed negligible effect on the phosphorylation of

Fig. 7 Effect of the EFBF-AF on phosphorylation of p38 and ERK in LPS-stimulated RAW 264.7 cells. Total protein levels were analyzed by western blot. The cells were incubated with EFBF-BF or EFBF-AF for 1 h and then exposed to 1 μ g/mL of LPS for 24 h

these two proteins. These data suggest that fermentation of Bat Faeces extract inhibits NF- κ B translocation by regulation of the NF- κ B signaling pathway. These results also suggest that the transcriptional down-regulation of the iNOS, COX-2 and several pro-inflammatory mediators, such as TNF- α , IL-6 and IL-1 β , by EFBF-AF results from an inhibition of the NF- κ B signaling pathway.

Effects of EFBF-AF on LPS-stimulated ERK and p38 phosphorylation in RAW 264.7 cells

The activation and phosphorylation of ERK is an important signaling pathway of inflammation. The MAPKs are also involved in the modulation of iNOS as well as the expression of various cytokines in LPS-induced RAW 264.7 cells [33–35]. The inhibitory effects of the EFBF-AF on LPS-induced ERK and p38 phosphorylation were studied using a western blot assay. The phosphorylation levels of ERK and p38 significantly increased in the LPS-treated RAW 264.7 cells, and the phosphorylation of ERK and p38 were clearly attenuated by the EFBF-AF. These results indicate that the EFBF-AF might partially suppress pro-inflammatory mediator cytokine expression via ERK and p38 signaling pathways (Fig. 7).

References

- Li SX, Deng NS (2003) Speciation analysis of iron in traditional Chinese medicine by flame atomic absorption spectrometry. *J Pharmaceut Biomed* 32: 51–57
- Guo Y, Zou X, Chen Y, Wang D, Wang S (1997) Sustainability of wildlife use in traditional Chinese medicine. *Conserving China's Biodiversity* 190–220
- Riccucci M (2012) Bats as materia medica: an ethnomedical review and implications for conservation. *Vespertilio* 16: 249–270
- Choe E, Min DB (2005) Chemistry and reactions of reactive oxygen species in foods. *J Food Sci* 70: R142–R159
- Suja KP, Jayalekshmy A, Arumughan C (2005) Antioxidant activity of sesame cake extract. *Food Chem* 91: 213–219
- Reddy V, Urooj A, Kumar A (2005) Evaluation of antioxidant activity of some plant extracts and their application in biscuits. *Food Chem* 90: 317–321
- Rhule A, Navarro S, Smith JR, Shepherd DM (2006) Panax notoginseng attenuates LPS-induced pro-inflammatory mediators in RAW264.7 cells. *J Ethnopharmacol* 106: 121–128
- Guha M, Mackman N (2001) LPS induction of gene expression in human monocytes. *Cell. Signal* 13: 85–94
- Kim JB, Han AR, Park EY, Kim JY, Cho W, Lee J, Lee KT (2007) Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF- κ B inactivation in RAW 264.7 macrophage cells. *Biol Pharm Bull* 30:2345–2351
- Liu SF, Malik AB (2006) NF- κ B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol-Lung C* 290: L622–L645
- Cha BJ, Park JH, Shrestha S, Baek NI, Lee SM, Lee TH, Lee DY (2015) Glycosyl glycerides from hydroponic Panax ginseng inhibited NO production in lipopolysaccharide-stimulated RAW264.7 cells. *J Ginseng Res* 39: 162–168
- Cho W, Nam JW, Kang HJ, Windono T, Seo EK, Lee KT (2009) Zedoarondiol isolated from the rhizoma of *Curcuma heyneana* is involved in the inhibition of iNOS, COX-2 and pro-inflammatory cytokines via the downregulation of NF- κ B pathway in LPS-stimulated murine macrophages. *Int Immunopharmacol* 9: 1049–1057
- Kim KN, Heo SJ, Yoon WJ, Kang SM, Ahn G, Yi TH, Jeon YJ (2010) Fucoxanthin inhibits the inflammatory response by suppressing the activation of NF- κ B and MAPKs in lipopolysaccharide-induced RAW 264.7 macrophages. *Eur J pharmacol* 649: 369–375
- McFeeters RF (2004) Fermentation microorganisms and flavor changes in fermented foods. *J Food Sci* 69: 35–37
- Kim SI, Kim JE, So JH, Rhee IK, Chung SK, Lee KB, Song KS (2004) Changes in chemical composition and biological activities of oriental crude drugs by food processing techniques (I)-changes in liquiritigenin contents in licorice extract treated by crude enzyme extract from *Aspergillus kawachii*. *Kor J Pharmacogn* 35: 309–314
- Yang EJ, Kim SI, Park SY, Bang HY, Jeong JH, So JH, Song KS (2012) Fermentation enhances the in vitro antioxidative effect of onion (*Allium cepa*) via an increase in quercetin content. *Food Chem Toxicol* 50: 2042–2048
- Yoshiki Y, Kahara T, Okubo K, Sakabe T, Yamasaki T (2001) Superoxide- and 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activities of soyasaponin β g related to gallic acid. *Biosci Biotech Bioch* 65: 2162–2165
- Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio Med* 26: 1231–1237
- Ranneh Y, Ali F, Al-Qubaisi M, Esa NM, Ismail A (2016) The inhibitory activity of cocoa phenolic extract against pro-inflammatory mediators secretion induced by lipopolysaccharide in RAW 264.7 cells. *SpringerPlus* 5: 547
- Heo SJ, Yoon WJ, Kim KN, Ahn GN, Kang SM, Kang DH, Jeon YJ (2010) Evaluation of anti-inflammatory effect of fucoxanthin isolated from brown algae in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Food Chem Toxicol* 48: 2045–2051
- Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247
- Floegel A, Kim DO, Chung SJ, Koo SI, Chun OK (2011) Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *J Food Compos Anal* 24: 1043–1048
- Brand-Williams W, Cuvelier ME, Berset CLWT (1995) Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci Technol* 28: 25–30
- Gotoh T, Mori M (1999) Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J Cell Biol* 144: 427–434
- Kim JW, Kim C (2005) Inhibition of LPS-induced NO production by taurine chloramine in macrophages is mediated through Ras-ERK-NF- κ B. *Biochem pharmacol* 70: 1352–1360
- Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF- κ B activation. *Mutat Res-Fund Mol M* 480: 243–268
- Han M, Wen JK, Zheng B, Zhang DQ (2004) Acetylbritannilaton suppresses NO and PGE 2 synthesis in RAW 264.7 macrophages through the inhibition of iNOS and COX-2 gene expression. *Life Sci* 75: 675–684
- Hanada T, Yoshimura A (2002) Regulation of cytokine signaling and inflammation. *Cytokine Growth F R* 13: 413–421
- Håversen L, Ohlsson BG, Hahn-Zoric M, Hanson LÅ, Mattsby-Baltzer I (2002) Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF- κ B. *Cell Immunol* 220: 83–95
- Yun KJ, Kim JY, Kim JB, Lee KW, Jeong SY, Park HJ, Lee KT (2008) Inhibition of LPS-induced NO and PGE 2 production by asiatic acid via NF- κ B inactivation in RAW 264.7 macrophages: possible involvement of the IKK and MAPK pathways. *Int. Immunopharmacol* 8: 431–441

31. Sung MJ, Davaatseren M, Kim W, Park SK, Kim SH, Hur HJ, Kwon DY (2009) Vitisin A suppresses LPS-induced NO production by inhibiting ERK, p38, and NF- κ B activation in RAW 264.7 cells. *Int Immunopharmacol* 9: 319–323
32. Oh WJ, Jung U, Eom HS, Shin HJ, Park HR (2013) Inhibition of lipopolysaccharide-induced proinflammatory responses by *Buddleja officinalis* extract in BV-2 microglial cells via negative regulation of NF- κ B and ERK1/2 signaling. *Molecules* 18: 9195–9206
33. An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, Qin Z (2002) Involvement of ERK, p38 and NF κ B signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. *Immunology* 106: 38–45
34. Chen JC, Huang KC, Lin WW (2006) HMG-CoA reductase inhibitors upregulate heme oxygenase-1 expression in murine RAW264.7 macrophages via ERK, p38 MAPK and protein kinase G pathways. *Cellular signaling* 18: 32–39
35. Kim YS, Ahn CB, Je JY (2016) Anti-inflammatory action of high molecular weight *Mytilus edulis* hydrolysates fraction in LPS-induced RAW264.7 macrophage via NF- κ B and MAPK pathways. *Food Chem* 202: 9–14