Bioconversion enhances anti-oxidant and anti-inflammation activities of different parts of the Mulberry Tree (*Morus alba* L.), especially the leaf (*Mori Folium*)

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Abstract The mulberry tree (*Morus alba* L.) has been traditionally used in Chinese medicine to treat inflammatory diseases. We investigated the effects of bioconversion on different components of the mulberry tree, and determined changes in the physiological activities. Ethyl acetate-soluble fractions of five different segments (fruit, *Mori Fructus*; leaf, *Mori Folium*; twig, *Mori Ramulus*; root, *Mori Cortex*; and mistletoe, *Loranthi Ramulus*) of the mulberry tree show enhanced anti-oxidant effects in the 2,2-diphenyl-1-picrylhydrazyl, and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assays, and enhanced anti-inflammatory effects of lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production in RAW 264.7 macrophages, after being treated with a crude enzyme extract from *Aspergillus kawachii*, in the following order of activity: *Mori Folium* > *Mori Cortex* > *Mori Ramulus* > *Mori Fructus* > *Loranthi Ramulus*. Ethyl acetate-soluble fraction of mulberry leaves (*Mori Folium*) that underwent bioconversion was most effective, and was devoid of any cytotoxicity. The fraction was also effective against mRNA expression of LPS-induced pro-inflammatory cytokines, such as inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor- α , interleukin-1 β , and interleukin-6. In addition, the fraction was effective in LPS-induced phosphorylation of mitogen-activated protein kinases and

IKK, and I κ B degradation, followed by translocation of the nuclear factor- κ B from the cytoplasm to the nucleus. Thus, bioconversion increased the anti-oxidative and anti-inflammatory activities of the mulberry leaf.

Keywords Anti-inflammation · Anti-oxidant · Bioconversion · Macrophages · *Mori Folium* · *Morus alba* L.

Introduction

Recently, bioconversion by food-processing techniques, such as enzyme treatment (fermentation) has been studied to enhance the beneficial effects of medicinal herbs by changing the content of active components [1,2]. In addition, enzyme mediated synthesis or degradation of glycosides has been examined to enhance biologically important compounds [3]. Thus, enzyme treatment techniques may be promising tools for improving the biological activities of medicinal herbs.

Antioxidants have been used as the most promising therapy for the prevention and treatment of several diseases such as cardiovascular disorders, cancer, neurodegenerative diseases, and inflammatory diseases [4]. Reactive oxygen species (ROS) induced oxidative stress, including damage to cell matrices, and antioxidant activity can suppress oxidative damage to organic molecules [5]. ROS have an important role in chronic inflammation as they activate pro-inflammatory cytokines, a major mechanism of inflammatory diseases [6]. Antioxidants can attenuate inflammation. Inflammation is a multi-step process, and several classic symptoms, such as pain, redness, swelling, heat, and loss of function [7]. Nowadays, prevalence of inflammatory diseases has become a public health concern. LPS, a complex glycolipid found in the outer membrane of gram-negative bacteria, plays an important

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role as a strong bacterial virulence factor that triggers inflammation [8–10]. Inflammatory stimuli by LPS lead to the activation of transcription factors such as Nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK), which promote the expression of several pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and other inflammatory mediators, such as NO synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [11–13].

The mulberry tree (*Morus alba* L.) is native to warm and subtropical regions of Asia, Africa, and the Americas. It is widely used as a functional food, herbal medicine, and tea [14]. In addition, it is used to treat and prevent various chronic diseases and as a general tonic to enhance health in traditional oriental medicine. Extract from the mulberry tree has potent antioxidant activity [15], antitumor activity [16], hypolipidemic effect [17,18], macrophage activating effect [19], and neuroprotective activity [20,21]. However, comparison of the effects of bioconversion on the anti-oxidant and anti-inflammatory activities of different parts of the mulberry tree (*Morus alba* L.) has not yet been performed. The aim of this study was to determine whether bioconversion changed the radical-scavenging effect and anti-inflammatory activities of the mulberry tree on LPS-treated RAW 264.7 cells. Moreover, the part of the mulberry tree that showed increased effects due to bioconversion was identified.

Materials and Methods

Chemicals

Extraction solvents, namely *n*-hexane, ethyl acetate (EtOAc), methanol (MeOH), and *n*-butanol (*n*-BuOH), were purchased from Duksan Chemical (Anseong, Korea). Ultra performance liquid chromatography (UPLC) grade acetonitrile (MeCN) and acetic acid (HOAc) were obtained from Merck (Darmstadt, Germany). Optical density (OD) was measured using a microplate reader (SPECTROstar Nano BMG Labtech). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), LPS, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin (Pen Strep) were obtained from Gibco (Invitrogen Life Technologies Corporation, NY, USA). Antibodies against iNOS, JNK, p-JNK, ERK, p-ERK, p38, p-p38, IKK, p-IKK, I κ B, p-I κ B, NF κ B p65, and p-NF κ B p65 were purchased from Cell Signaling (Boston, MA, USA) and COX-2 was purchased from Abcam (Cambridge, MA, USA). Antibodies against anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The nuclear extraction kit was purchased from Abcam.

Plant material and preparation of crude enzyme extract

Mori Fructus and *Mori Folium* were purchased from Omniherb (Young cheon, Korea) and *Mori Ramulus* and *Mori Cortex* were obtained from Humanherb in Daegu, Korea in March, 2015. *Loranthi Ramulus* was purchased from a local market in Daegu, Korea on the same day. A voucher specimen has been stored at the National Development Institute of Korean Medicine (NIKOM). Five different parts of mulberry trees (500 g) were refluxed twice with 70% methanol (3.5 L) for 3 h. The extracted solution was filtered and evaporated using a rotary evaporator. Crude enzyme extract of *Aspergillus kawachii* was prepared using a previously described method report [1]. This crude enzyme extract contained 0.260 U/mL (1 U is defined as the enzyme activity needed to produce 1 mmol of p-nitrobenzene from p-nitrophenyl- β -D-glucopyranoside per min) of β -glucosidase activity. First, 10 mL of 1% glucose solution and 10 g of wheat bran were mixed and sterilized at 121 °C for 30 min. *A. kawachii* was inoculated and incubated for 5 days at 30 °C and then, mixed with 30 mL of 100 mM sodium phosphate buffer (pH 7) for 5 h at 18 °C. The mixture was centrifuged for 15 min at 12000 rpm, and the supernatant was collected.

Bioconversion

Methanolic extracts (10 g, 70%) of five different parts of the mulberry tree were suspended in 100 mL of distilled water and treated with 100 mL of the crude enzyme extract for 12 h at 37 °C. Inactivated enzyme-treated extracts were used as the control. The inactivated crude enzyme extract was inactivated by autoclaving for 30 min at 121 °C. Each sample of the activated enzyme-treated extracts and inactivated enzyme-treated extracts (control) was consecutively partitioned with *n*-hexane, EtOAc, and *n*-BuOH and then evaporated.

UPLC conditions analyzing

Changes compounds during bioconversion were quantified using Waters UPLC (ACQUITY Ultra Performance LC systems H class) with the wavelength set at 280 nm. The samples were dissolved to 0.5 mg/mL in MeOH and filtered through a 0.22 μ m membrane filter, and 2 μ L of the filtrate was analyzed. UPLC analyses were performed using an ACQUITY UPLC CSH C18 (2.1 $\times$ 100 mm, 1.7 μ m; Waters, Milford, MA, USA) reverse phase column, and the mobile phase consisted of water (solvent A) and MeCN (solvent B), each containing 0.1% HOAc. After the sample was injected into the column, solvent B was increased to 100% in 10 min and then decreased to 0% in 1 min and held at 0% for 1min. The solvent flow rate was 0.3 mL/min.

Measurement of anti-oxidative activities

DPPH radical scavenging assay

The DPPH assay was based on the method reported by Blois et al [22]. A solution of 190 μ L of 150 μ M DPPH and 2 μ L of the

sample was mixed and incubated at 25 °C in the dark. Absorbance of the samples and blanks at 517 nm was determined after 30 min. The result was calculated using Eq. (1):

$$\text{Radical scavenging assay (\%)} = \frac{C-S}{C} \times 100 \quad (1)$$

where C and S are absorbances values of the blank and tested samples, respectively. Three measurements were performed for each tested sample.

ABTS^{•+} radical scavenging assay

The ABTS assay was performed according to the modified method used by Re et al. [23]. Briefly, 7 mM ABTS was mixed with 2.45 mM potassium persulfate (2:1, v/v) and maintained for 12–16 h in the dark. Then, the ABTS^{•+} radical solution was diluted in 5 mM phosphate buffer (pH 7.4) until the absorbance of the solution was 0.7±0.02 at 734 nm; 2 µL of the sample and 198 µL of the solution were incubated for 1 min and detected at 734 nm. The result was also estimated using Eq. (1).

Determination of anti-inflammatory activities

Cell culture

Murine RAW 264.7 macrophage cell line was purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in DMEM containing 10% FBS and penicillin (100 U/mL)/streptomycin (100 µg/mL) at 37 °C in a 5% CO₂ humidified incubator. The medium was changed once every 24 h. Stock cells were passaged 2 to 3 times per week. In all experiments, treated samples were dissolved in dimethyl sulfoxide (DMSO), and cells grown to 80% confluence were pretreated with samples at various concentrations for 1 h and then treated with LPS (1 µg/mL).

Cell viability assay and NO measurement

Cell viability was determined using the MTT assay. Briefly, cells were plated at a density of 5×10⁴ cells/well in 96-well plates and incubated for 24 h. The cells were treated with samples at different concentrations (0–50 µg/mL) for 1 h, followed by stimulation with LPS. After 24 h, cell-free supernatant was collected for measuring NO production, and rest of the media was suctioned and, 100 µL MTT (0.5 mg/mL) was added and maintained for 4 h at 37 °C. The supernatant was removed and formazan was dissolved with 100 µL/well of DMSO. Absorbance of formazan was measured at 570 nm, and the amount of formazan was calculated as the percentage of cells. NO production was measured using the collected supernatant before the MTT assay; 100 µL of the supernatant was mixed with an equal volume of Griess reagent and allowed to react at room temperature for 10 min in the dark. Then, absorbance was measured at 550 nm, and nitrite content was calculated on the basis of the standard a NaNO₂ curve.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

RAW 264.7 cells were seeded at a density of 1×10⁶ cells/well in 6well plates and treated with samples for 1 h before stimulation with LPS. After 24 h, total cellular RNA was isolated with TRIzol (TRI Reagent), according to the manufacturer's instructions. From each sample, 1 µg of RNA was reverse-transcribed (RT) using TaKaRa Ex Taq, 1 mM deoxyribonucleotide triphosphate (dNTP), and 0.5 µg/µL of oligo dT Primer. Complementary DNA fragment was amplified for 30 cycles in a volume of 50 µL containing 1 unit of Taq DNA polymerase, 10X reaction buffer, 0.2 mM dNTP, and 100 pmol of 5' and 3'primers. Cycling conditions were 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Primers sequences used in this study were purchased from MACRO GEN (Seoul, Korea) and are listed below: for iNOS sense 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3', antisense 5'-GGCTGTCAGAGCCTCGT GGCTTTGG-3', COX-2 sense 5'-CACTACATCCTGACCCACTT-3', antisense 5'-ATGCTCCTGC TTGAGTATGT-3', TNF-α sense 5'-TTGACCTCAGCGCTGAGT TG-3', antisense 5'-CCTGTAGCCACGTCGTAGC-3', IL-6 sense 5'-GTACTCCAGAAGACCAGAGG-3', antisense 5'-TGCTGGT GACAACCACGGCC-3', IL-1β sense 5'-CAGGATGAGGACAT GAGCACC-3', antisense 5'-CTCTGCAGACTCAAACCTCCAC-3', β-actin sense 5'-GTGGGCCGCCCTAGGCACCAG-3', and antisense 5'-GGAGGAAGAGGATGCGGCAGT-3' [24]. After amplification, the PCR products were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide. Quantitative analysis of PCR bands was performed using the Fusion Solo 5 gel documentation system (Vilber Lourmat, France).

Western blot

Pellets of RAW264.7 cells were prepared described in RNA extraction and RT-PCR and lysed using PRO-PREP buffer (iNtRON Biotechnology, Seoul, Korea) at 4 °C for 30 min and centrifuged at 12000 rpm for 10 min for extracting cytosolic proteins. Nuclear proteins were extracted using the Nuclear Protein Extraction Kit (Abcam). Proteins in the cell lysates or nuclear extracts were quantified with the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), and concentration was fixed for each sample. The proteins were separated using 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked using TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20) containing 5% skim milk at room temperature for 1 h on a rotary shaker. Then, specific primary antibodies and the membranes were incubated at room temperature for 1 h and washed with TBST. Subsequently, the membranes were incubated with secondary antibodies. The blots were visualized using Fusion Solo 5 (Vilber Lourmat, France) with the

enhanced chemiluminescence kit solution (DOGEN, Seoul, Korea). Relative amounts of each band were analyzed using Image J software.

Statistical analysis

All experiments were performed in triplicate, and data were expressed as mean $\pm$ SD values. Statistical significance was determined

Fig. 1 Comparison of UPLC chromatograms of each fraction before and after bioconversion by *A. kawachii* treatment. Arrows represent new peaks. (A) *Mori Folium*, (B) *Mori Cortex*, (C) *Mori Ramulus*, (D) *Mori Fructus*, (E) *Loranthe Ramulus*

Fig. 2 DPPH radical scavenging activity of each fraction at 100 ppm. Bioconversion significantly enhanced DPPH radical-scavenging activity of each fraction, especially the EtOAc soluble fraction. M.Fo.; *Mori Folium*, M.C.; *Mori Cortex*, M.R.; *Mori Ramulus*, M.Fr.; *Mori Fructus*, L.R.; *Loranthe Ramulus*. Values represents mean $\pm$ SD of relative OD obtained from three independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 when compared with the control. # p < 0.05, ## p < 0.01 and ### p < 0.001 when compared with bioconversion of the EtOAc soluble fraction

Fig. 3 DPPH radical scavenging activity of EtOAc fractions. Bioconversion enhanced DPPH radical scavenging activity in a dose-dependent manner in EtOAc fractions. *Mori Folium* was increased to the greatest extent to $37.55 \pm 1.67\%$ than before bioconversion at 200 ppm. (A) *Mori Folium*, (B) *Mori Cortex*, (C) *Mori Ramulus*, (D) *Mori Fructus*, (E) *Loranthe Ramulus*. Values represents mean $\pm$ SD of relative OD obtained from three independent experiments performed. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the control

Fig. 4 Bioconversion enhanced ABTS radical scavenging activity in a dose-dependent manner in EtOAc fractions. *Mori Folium* and *Mori Fructus* were increased to $37.60 \pm 1.87\%$ and $39.50 \pm 0.65\%$ than before bioconversion at 50 ppm. (A) *Mori Folium*, (B) *Mori Cortex*, (C) *Mori Ramulus*, (D) *Mori Fructus*, (E) *Loranthe Ramulus*. Values represents mean $\pm$ SD of relative OD obtained from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the control

Fig. 5 Inhibitory effect of EtOAc-fractions on nitric oxide production in RAW 264.7 cells. RAW 264.7 cells were treated with EtOAc-soluble fractions (5, 10 and 25 ppm) for 24 h, and cell viability was determined using the MTT assay. RAW 264.7 cells were pretreated with EtOAc-soluble fractions (5, 10 and 25 ppm) for 1 h, followed by stimulation with LPS (1 µg/mL). After 24 h of incubation, NO levels in the culture supernatants were determined. *Mori Folium* showed remarkable inhibition of NO production in LPS-stimulated RAW 264.7 cells at 25 ppm without cytotoxicity. M.Fo.; *Mori Folium*, M.C.; *Mori Cortex*, M.R.; *Mori Ramulus*, M.Fr.; *Mori Fructus*, L.R.; *Loranthi Ramulus*. (A) Cell viability of RAW 264.7 cells. (B) Nitric oxide production (%). Values represent mean $\pm$ SD of relative OD obtained from three independent experiments performed. * $p < 0.05$ and ** $p < 0.01$ when compared with LPS-treated cells. # $p < 0.05$ and ## $p < 0.01$ when compared with bioconversion of the EtOAc-soluble fraction

using one-way analysis of variance (ANOVA), followed by Tukey's tests. P values < 0.05 were considered to be significant.

Results

Effects of bioconversion on changes UPLC patterns

To compare the UPLC patterns of the activated enzyme-treated group and inactivated enzyme-treated group (control group), we performed UPLC analysis. Each fraction showed modifications in the composition of compounds due to bioconversion. EtOAc soluble fractions of *Mori Folium* were significantly changed, and showed a new peak (t_R , 3.7 min., Fig. 1).

Fractions exhibit free radical-scavenging activity

To investigate the effects of antioxidants by free-radical scavenging, we used DPPH and ABTS assays. Effect of each fraction of the activated enzyme-treated group and inactivated enzyme-treated

group (control group) on DPPH radical scavenging activity was evaluated at 100 µg/mL, and the EtOAc soluble fractions were found to be most effective after bioconversion (Fig. 2). *Mori Folium* was significantly increased activity in a dose-dependent manner, and about 2-3 fold increase in activity was observed by bioconversion at 50, 100, and 200 ppm (Fig. 3).

The other evaluation by ABTS assay also showed that *Mori Folium* had significantly increased activity in a dose-dependent manner, and about 2-3 fold increase in activity was observed by bioconversion (Fig. 4). These results suggest that bioconversion enhanced the DPPH and ABTS radical scavenging effects of mulberry trees, especially *Mori Folium*.

Effects of EtOAc-soluble fractions of mulberry trees that underwent bioconversion, on LPS-induced NO production, and expression of iNOS and COX-2 in RAW 264.7 cells

We first examined the effects of bioconverted EtOAc-soluble fractions of mulberry trees, on the LPS-induced NO production

Fig. 6 Effect of EtOAc soluble fractions on iNOS and COX-2 protein expression levels in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of EtOAc soluble fractions at 25 ppm for 1 h and then stimulated with LPS (1 µg/mL) for 24 h. Cellular proteins were used for the detection of iNOS and COX-2 by western blotting. β-actin was used as the loading control. M.Fo.; *Mori Folium*, M.C.; *Mori Cortex*, M.R.; *Mori Ramulus*, M.Fr.; *Mori Fructus*, L.R.; *Loranthi Ramulus*. B.B; before bioconversion (Inactivated crude enzyme + extract), A.B; after bioconversion (Crude enzyme + extract). * $p < 0.05$ and ** $p < 0.01$ when compared with treatment with LPS only. ## $p < 0.01$ when compared with bioconversion of the EtOAc-soluble fraction

Fig. 7 Effect of EtOAc fractions of *Mori Folium* that underwent bioconversion (EMB) on LPS-induced mRNA expression levels of iNOS and COX-2 in RAW 264.7 cells. For mRNA expression, RAW 264.7 cells were pretreated with EMB at 25 ppm for 1 h and then stimulated with LPS (1 µg/mL) for 24 h. Total RNA was isolated, and mRNA levels of iNOS and COX-2 were then measured using RT-PCR. β-actin was used as the internal control. Quantification of relative band intensities from three independent experiments was performed. B.B; before bioconversion (Inactivated crude enzyme + extract), A.B; after bioconversion (Crude enzyme + extract). * $p < 0.05$ and ** $p < 0.01$ when compared with treatment with LPS only. ## $p < 0.01$ when compared with before bioconversion

Fig. 8 Effect of EMB on LPS-induced TNF- α , IL-1 β , and IL-6 release. RAW 264.7 cells were pretreated with EMB at 25 ppm for 1 h and then stimulated with LPS (1 μ g/mL) for 24 h. Total RNA was isolated, and mRNA levels of TNF- α , IL-1 β , and IL-6 were then measured using RT-PCR. β -actin expression was used as the internal control. Quantification of relative band intensities from three independent experiments was performed. B.B; before bioconversion (Inactivated crude enzyme + extract), A.B; after bioconversion (Crude enzyme + extract). * p < 0.05 and ** p < 0.01 when compared with treatment with LPS only. ### p < 0.01 when compared with before bioconversion

Fig. 9 Effect of EMB on iNOS and COX-2 protein expression levels in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with EMB at 25 ppm for 1 h and then stimulated with LPS (1 μ g/mL) for 24 h. Cellular proteins were used for the detection of iNOS and COX-2 by western blotting. β -actin was used as the loading control. B.B; before bioconversion (Inactivated crude enzyme + extract), A.B; after bioconversion (Crude enzyme + extract). ** p < 0.01 when compared with treatment with LPS only. ### p < 0.01 and #### p < 0.001 when compared with before bioconversion

and potential cytotoxicity in RAW 264.7 cells. LPS stimulation greatly increased the NO production, but pretreatment of EtOAc-soluble fractions at 5–25 μ g/mL significantly inhibited LPS-

induced NO production (Fig. 5). Especially, at 25 μ g/mL, EtOAc-soluble fractions of *Mori Folium* that underwent bioconversion, inhibited the NO production by about 28.11 $\pm$ 0.29%.

Fig. 10 Effect of EMB on IKK, IκB, and NF-κB p65 phosphorylation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with EMB at 25 ppm for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Cellular proteins were used for the detection of phosphorylated forms of IKK, IκB (A), and NF-κB p65 (B) by western blotting. To compare NF-κB p65 levels in the nuclear and cytoplasm, nuclear proteins were extracted using the Nuclear Protein Extraction Kit. β-actin was used as the loading control. B.B; before bioconversion (Inactivated crude enzyme + extract), A.B; after bioconversion (Crude enzyme + extract). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with treatment with LPS only. ## $p < 0.01$ when compared with before bioconversion

We further determined the expression of iNOS and COX-2 to understand their anti-inflammatory mechanism. Western blot analysis showed that iNOS and COX-2 protein concentrations were elevated by LPS stimulation. However, treatment of EtOAc soluble fractions that underwent bioconversion, attenuated the iNOS and COX-2 protein levels at 25 μg/mL (Fig. 6). Of these, *Mori Folium* significantly inhibited the expression of iNOS protein level. Accordingly, these data suggest that EtOAc-soluble fractions of *Mori Folium* that underwent bioconversion have higher inhibitory activities at 25 μg/mL, against LPS-induced NO production, and iNOS and COX-2 protein expression, than other segments of the mulberry tree.

Effects of EtOAc soluble fraction of *Mori Folium* that underwent bioconversion (EMB) on LPS-induced mRNA expression levels of iNOS, COX-2, TNF-α, IL-1β and IL-6 in RAW 264.7 cells

Immoderate inflammatory cytokines, such as TNF-α, IL-1β, and

IL-6 are important mediators that regulate responses to the inflammatory molecular mechanism. Therefore, we investigated whether EMB can inhibit the production of LPS-induced pro-inflammatory cytokines. To confirm the expression of inflammatory cytokines by treating EMB in RAW 264.7 cells with LPS, total cellular mRNA was extracted to perform the RT-PCR assay. Results indicate that the LPS-stimulated RAW 264.7 cells show an increase in the iNOS, COX-2, TNF-α, IL-1β and IL-6 mRNA levels. However, treatment of EMB significantly decreased the mRNA expressions of inflammatory cytokines, when compared with the control group of *Mori Folium* (Figs. 7 and 8). These results show that EMB can mediate the inhibition of these inflammatory cytokines by probably regulating the protein levels.

Effects of EMB on iNOS and COX-2 protein expressions in LPS-induced RAW 264.7 cells

Although we had already determined that treatment of EMB decreased expressions of iNOS and COX-2 mRNA level, we

Fig. 11 Effect of EMB on MAPK phosphorylation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with EMB at 25 ppm for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Cellular proteins were used for the detection of phosphorylated or total forms of ERK1/2 (B), p38 (C) and JNK (D) by western blotting. B.B; before bioconversion (Inactivated crude enzyme + extract), A.B; after bioconversion (Crude enzyme + extract). ** $p < 0.01$ and *** $p < 0.001$ when compared with treatment with LPS only. ## $p < 0.01$ and ### $p < 0.001$ when compared with before bioconversion

further evaluated these signals at the protein level by using western blot to confirm their anti-inflammatory signaling. As shown in Fig. 9, iNOS and COX-2 protein expressions were increased up to 1.00 ± 0.08 -fold by LPS, while those treated by EMB was significantly reduced to 0.40 ± 0.01 -fold and 0.36 ± 0.07 -fold, respectively. These results therefore show that EMB was highly effective in reducing the iNOS and COX-2 protein levels.

Effects of EMB on LPS-induced NF-κB nuclear translocation, and IKK and IκB phosphorylation in RAW 264.7 cells

NF-κB is an important factor in the inflammatory response in RAW 264.7 cells. Hyperphosphorylation of IKK and IκB, and subsequent phosphorylation, are steps in NF-κB activation. When NF-κB is activated, it induces p65 nuclear localization and expression of several inflammatory cytokine genes. Therefore, we evaluated whether inhibition of the inflammatory effects by EMB is mediated via the NF-κB pathway. IKK, IκB and NF-κB proteins were evaluated using the western blot assay. The results indicated that EMB treatment significantly inhibited the phosphorylation of IKK and IκB in *Mori Folium* (Fig. 10A). Furthermore, levels of NF-κB

Fig. 12 Mechanism of inflammation in LPS-stimulated RAW 264.7 cells [30]

p65 in the nucleus were remarkably increased by exposure to LPS alone, but EMB reduced the LPS-mediated nuclear translocation of NF- κ B p65 in *Mori Folium* (Fig. 10B).

These results implied that EMB might block LPS-stimulated NF- κ B activation by regulating the IKK and I κ B phosphorylation.

Effects of EMB on LPS-induced MAPK phosphorylation in RAW 264.7 cells

Several stimuli that activate NF- κ B also activate MAPKs, which induce the expression of other proinflammatory genes. Three major subfamilies of MAPKs have been defined, hence we confirmed whether the MAPK pathways are included in the inhibitory effects of EMB on the release of proinflammatory mediators, by examining the phosphorylation of ERK1/2, p38, and JNK. However, co-treatment with EMB significantly blocked phosphorylation in *Mori Folium*. These results suggest that the MAPK signaling pathway, which plays critical roles in inflammation, is inhibited by bioconversion.

Discussion

The mulberry tree is known to have anti-cancer, anti-obesity, anti-oxidant, as well as anti-inflammatory activities [25]. However, bioconversion of the mulberry tree with the crude enzyme extract of *A. kawachii* has not yet been reported. Furthermore, effects of different parts of the mulberry tree (*Morus alba* L.) that underwent bioconversion are unknown.

In this study, we demonstrated that bioconversion enhanced the anti-oxidant and anti-inflammatory effects of five different parts of the mulberry tree (*Morus alba* L.), and the most effective part was the mulberry leaf (*Mori Folium*).

We first evaluated the changes in the chemical composition after bioconversion, using UPLC analysis. We observed that EtOAc-soluble fractions of the five different segments of the mulberry tree that underwent bioconversion, displayed new peaks when compared with the inactivated enzyme treated group. We expected that bioconversion with *A. kawachii* crude enzyme would change the anti-oxidant activity, since the crude enzyme extract prepared from *A. kawachii* contains beta-glucosidase [1]. Several studies suggest that beta-glucosidase enhances the antioxidant effects by hydrolyzing β -glucosidic bonds of the conjugated form to sugar residues linked to hydroxyl groups [26]. We determined the antioxidant activity using two different tests, namely DPPH and ABTS assays. The DPPH assay is a convenient method for measuring antioxidant capacity by scavenging of free radicals. The ABTS assay is used to evaluate the hydrophilic and lipophilic antioxidant capacity by scavenging cation radicals, because of its solubility in aqueous and organic solvents [23]. The results reveal that the DPPH radical-scavenging effects of each fraction of the five different parts of the mulberry tree were increased by bioconversion, especially the EtOAc-soluble fraction

at 100 μ g/mL. Therefore, we evaluated the DPPH radical-scavenging effects of EtOAc-soluble fractions at various concentrations, and determined the ABTS radical-scavenging activity in a similar manner. Bioconversion enhanced the DPPH and ABTS radical-scavenging activities in a dose-dependent manner: leaf > fruit > root > twig > mistletoe of the mulberry tree. We expected these samples have anti-inflammatory effects because antioxidants inhibit the ROS generation by activating pro-inflammatory cytokines [6]. ROS especially stimulates NO [27]. Thus, we determined the anti-inflammatory effects of five different parts of the mulberry tree in LPS-stimulated RAW 264.7 cells by evaluating the NO production, and expression of inflammatory proteins such as iNOS and COX-2. The results show that bioconversion enhanced the inhibition of NO production in *Mori Folium* at a concentration of 25 μ g/mL. Furthermore, *Mori Folium* that underwent bioconversion, attenuated the iNOS and COX-2 protein levels, which are important enzymes mediating the inflammatory processes. Overall, bioconversion enhanced the radical-scavenging effects and anti-inflammatory properties of *Mori Folium*, thus confirming the anti-inflammatory mechanism of *Mori Folium* that had undergone bioconversion. As shown in Fig. 12, the inflammation mechanism in RAW 264.7 cells is initiated with LPS-stimulation. LPS activates the pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, which induce the expression of iNOS and COX-2 through the activation of NF- κ B and MAPK pathway, and thereby increase NO production [28,29]. In this study, we found that bioconversion significantly enhanced the effect of *Mori Folium* by inhibiting the production of TNF- α , IL-1 β , IL-6, iNOS, and COX-2. Furthermore, it inhibited the phosphorylation of IKK and I κ B, and blocked the NF- κ B p65 translocation into the nucleus. MAPK signaling proteins, such as ERK1/2, p38, and JNK, were also markedly inhibited by bioconversion. Taken together, our results demonstrate that bioconversion enhanced the anti-oxidant and anti-inflammatory effects of *Mori Folium*. Therefore, it may be useful as a potential anti-oxidant and anti-inflammatory agent.

In summary, bioconversion enhanced the anti-oxidant effects of *Mori Folium* by improving the DPPH and ABTS radical scavenging and anti-inflammatory signaling, such as decreased NO production. Moreover, *Mori Folium* that underwent bioconversion inhibited the production of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6), iNOS and COX-2, by regulating the phosphorylation of IKK and I κ B. Thus, *Mori Folium* that underwent bioconversion may have preventive and therapeutic functions against various diseases related to oxidative stress and inflammation. Further *in vivo* studies are required to identify the mechanism in the target organs.

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