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Enhanced Antioxidant and Anti-inflammatory Activities of *Monascus pilosus* Fermented Products by Addition of Turmeric to the Medium

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Monascus sp. fermented products are known for their antihypercholesterolemic effects; however, their antioxidant and anti-inflammatory activities are different from those of many plant-derived foods. To evaluate the effect of turmeric addition into the medium on the antioxidant and anti-inflammatory activities of *Monascus pilosus* fermented products, we cultured uninoculated PDB medium (PDB), inoculated PDB medium (MP), uninoculated turmeric-containing PDB medium (PDBT), and inoculated turmeric-containing PDB medium (MPT). The broth and mycelia were collected, freeze-dried, and extracted to evaluate their free radical scavenging and iron-chelating activities, inhibition of peroxidation, phenolic and curcuminoid contents, and cellular antioxidant activity. The effects of the extracts on cell viability, cytokines and nitric oxide (NO) production, and expression of enzymes that regulate antioxidation and inflammation were also evaluated. The results showed that MPT had a significantly higher antioxidant activity than PDB, MP, and PDBT at all fermentation time points; moreover, the fermentation process significantly increased the phenolic and curcuminoid contents of MPT. As compared with MP, MPT had a more significant effect on down-regulating the production of NO and TNF-α as well as the expression of inducible nitric oxide synthase, cyclooxygenase-2, glutathione peroxidase, superoxidase dismutase, and catalase. After the inherent levels of antioxidant and anti-inflammatory capacities were increased, the modified *M. pilosus* fermented product demonstrated a higher antiatherosclerotic value than the unmodified product.

KEYWORDS: *Monascus pilosus*; turmeric; antioxidant; anti-inflammation

INTRODUCTION

Atherosclerosis is one of the leading causes of death in modern societies. Hypercholesterolemia is the major factor involved in the initiation of the atherogenic process. When low-density lipoprotein (LDL) particles are trapped in an artery, they can undergo progressive oxidation and be internalized by macrophages. The internalization leads to the formation of lipid peroxide, facilitating the accumulation of cholesterol ester and the formation of foam cells, which can be further converted into complex plaque and thrombus if the atherogenic process is not inhibited. Antioxidants that prevent LDL oxidation in vitro also inhibit atherosclerosis in animals (*1*).

In addition to the ability to injure endothelial and smooth muscle cells, modified LDL is chemotactic for monocytes and upregulates the expression of cytokines and proteins that expand the inflammatory responses (*2*). The release of inflammatory mediators such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) increases the binding of LDL to the arterial endothelium, leading to cycles of inflammation, lipoproteins oxidation, and further inflammation. Atherosclerosis has been considered an inflammatory disease (*3*).

Monascus belongs to the class Ascomycetes and the family Monascaceae. The pigments produced during *Monascus* spp. fermentation have been applied in the processing of rice (red mold rice, also known as "anka"), alcohol, and vegetables in Asia for hundreds of years (*4*). Monacolin K, commercially known as lovastatin, mevinolin, or cholestin, is a secondary metabolite obtained from *Monascus* spp. fermentation and has been identified as a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in cholesterol de novo synthesis (*5*). Long-term intake of foods containing monacolin K or similarly structured molecules significantly lowers plasma cholesterol concentrations (*6*, *7*). In addition to their cholesterol-lowering effects, *Monascus* spp. fermented products also have antioxidatant activities (*8*); however, these activities are different from those of many plant-derived foods.

Recently, the intake of commercial *Monascus* spp. fermented products for the prevention of hypercholesterolemia has become more common in some Asian counties. Because oxidation and

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inflammation are two major factors involved in the progression of atherosclerosis, it is of interest to increase antioxidant and anti-inflammatory activities of *Monascus* spp. fermented products. Because the growth of mold is affected by the composition of medium, it was hypothesized that the addition of nature products, which possess high antioxidant and anti-inflammatory activities into the medium, will modify the nutraceutical properties of *Monascus* spp. fermented products. In previous studies, we increased the antioxidant activities of *Monascus pilosus* fermented products by supplementing the medium with garlic (*9*) and ginger (*10*). An animal study indicated that *M. pilosus* fermented products collected from the garlic-containing medium lowered blood lipids more significantly than those from a nongarlic-containing medium (*11*).

Since curcumin was reported for its antioxidant, anti-inflammatory, and anticarcinogenic activities (*12*−*14*), it has become the focus of pharmaceutical and nutraceutical development. Curcumin, a polyphenolic compound, is the major active ingredient in turmeric (*Curcuma longa* Linn.) and is responsible for the yellow color of turmeric. In Ayurvedic medicine, turmeric has been used in the treatment of trauma and gastric diseases (*15*). In addition to medical applications, turmeric is also widely used in Indian cuisine, especially the well-known curry.

Although turmeric has been extensively studied, it has never been applied to the fermentation of *Monascus* spp. Therefore, we were interested in evaluating the antioxidant and anti-inflammatory properties of *M. pilosus* fermented products collected from a turmeric-containing medium and comparing them with those of products collected from a regular medium.

MATERIALS AND METHODS

Chemicals. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from BD (Sparks, MD). The 1,1-diphenyl-2-picrylhydrazyl (DPPH), horseradish peroxidase (HRPase), phenol red, ferrozine, linoleic acid, ammonium thiocyanate, potassium ferricyanide, trichloroacetic acid, peroxidase, Folin−Ciocatlteu reagent, *tert*-butylhydroperoxide (t-BHP), MEM nonessential amino acid solution, formic acid, ammonium formate, lipopolysaccharide (LPS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Luria−Bertani medium was obtained from BD (Franklin, NJ), and M199 medium, minimum essential α-medium (MEM), Dulbecco's modified Eagle's medium-high glucose (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from GIBCO (Grand Island, NY). The 7′-dichlorodihydrofluorescein diacetate (DCHF-DA) was purchased from Invitrogen (Carlsbad, CA). All polyclonal and monoclonal antibodies were purchased from NOVUS (Littleton, CO), except for the anticyclooxygenase (COX)-2 polyclonal antibody and the anti-inducible NO synthase (iNOS) polyclonal antibody, which were obtained from Cayman (Ann Arbor, MI).

Extract Preparation. Turmeric purchased at a farmer's market in Nanto County, Taiwan, was washed and ground with a food-preparing machine. The juice collected during grinding was filtered twice to make "turmeric juice", which was then used for the experiments. *M. pilosus* (BCRC 31527) was obtained from the Biosource Collection and Research Center of Food Industry Research and Development Institute (Shinchu City, Taiwan). The *M. pilosus* fungi were inoculated onto PDA slants and incubated at 28 °C. After a pure culture was obtained, the mycelia were reinoculated into PDB at 28 °C for 7 days. The mycelia and broth from the submerged culture were blended together, and the mixture was added at a ratio of 1:25 to 2.4% fresh PDB containing 20% turmeric juice for either 5 or 7 days of further submerged cultivation at 28 °C. At each time point, the mycelia and broth were collected, blended, and then freeze-dried. The dried powder was dissolved in ethanol (1/20, w/v), sonicated for 2 h, and centrifuged at 100*g* for 10 min. After centrifugation, the supernatant was collected and referred to as "MPT". Inoculated regular PDB medium, uninoculated turmeric-containing medium, and uninoculated regular PDB medium were cultured, collected, freeze-dried, and extracted using the same conditions used for MPG to obtain "MP", "PDBT", and "PDB", respectively.

High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography/Electrospray Ionization/Tandem Mass Spectrometry (LC/ESI/MS/MS) Analysis. The identification procedure for curcumin-related compounds was performed according to the method described by Jiang et al. (*16*) with slight modification. In brief, fermented turmeric extracts were performed on a HPLC system (Hitachi, Tokyo, Japan) equipped with a Hitachi pump (model L-2130) and a UV−vis detector (Hitachi, model L-2400). The chromatographic separation of the compounds was achieved using an analytical column (Luna 3 μm C18(2), 150 mm × 2.0 mm) with a guard column [SceurityGuard C18 (ODS) 4 mm× 3.0 mm i.d., Phenomenex Inc., Torrance, CA] at an elution flow rate of 0.2 mL/min. Solvent A was H_2O, and solvent B was acetonitrile. The flow rate was 0.2 mL/min. The entire course of programmed gradient elution was carried out as follows: 2 min isocratic of 5% B; 38 min linear gradient from 5 to 95% B, followed by 5 min isocratic with 95% B, 5 min linear gradient from 95 to 5% B, and 5 min isocratic with 5% B. Absorption spectra of eluted compounds were recorded at 425 nm. For all experiments, 20 μL of standards and sample extract was injected. The flow generated by chromatographic separation was directly injected into the electrospray ion source. The negative ion mode for MS and MS/MS analysis was selected, working under the condition described in the ESI-MS paragraph. For fragmentation pattern study, two scan events were prescribed to run simultaneously in the LCQ mass spectrometer (Thermo Electron Co., MA). Five scan events were prescribed to run simultaneously in the LCQ mass spectrometer. The first event was a full scan MS to acquire data on ions in the range 160−1000 m/z. The second event was simultaneously on selected mass ions m/z 307.0, m/z 337.0, and m/z 367.0, respectively. The scan events 3−5 were of the MS/MS products scan event of the above selected ions at a collision energy of 35%.

Measurement of DPPH Scavenging Activity. To determine the appropriate extract concentration for the assays, various concentrations (0, 100, 250, 500, and 1000 ppm) of fermented product extracts collected at day 0, 5, 7, 9 were applied to the DPPH scavenging activity assay. The results indicated that the extracts from day 7 showed higher antioxidant activities than those from the other time points; moreover, 500 ppm of extracts possessed higher antioxidant activity than 100 and 250 ppm but did not differ from 1000 ppm (data not shown). Therefore, the extracts of fermented products collected at day 0, 5, and 7 were selected for further assays at a final concentration of 500 ppm.

The scavenging activity of the DPPH free radical was determined using the method described by Chang et al. (*17*) with slight modification. One milliliter of 0.1 mM DPPH solution and 300 μL of 50 mM Tris-HCl buffer (pH 7.4) were mixed with 150 μL of PDB, MP, PDBT, or MPT solution to yield a final concentration of 500 ppm. Ethanol was used as a control. After a 30 min incubation at room temperature, the absorbance at 517 nm was taken. The ability to scavenge the DPPH radical (% inhibition) was calculated as $[(A_0 - A_1/A_0) \times 100]$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of sample extracts.

Measurement of Hydrogen Peroxide Scavenging Activity. During cellular oxidation, superoxide radicals are initially formed and then converted to hydrogen peroxides, which subsequently generate reactive hydroxyl radicals through the Fenton reaction (*18*). Hydrogen peroxide scavenging activity was measured by the method of Yen and Chung (*19*) with slight modification. One milliliter of extract was mixed with 400 μL of 4 mM H_2O_2 and incubated at room temperature for 20 min. A 600 μL of HRPase-phenol red solution (HRPase 300 μg/mL and phenol red 4.5 mM) was added and incubated for another 10 min. The reaction was stopped by leaving on ice for 10 min before the absorbance was read at 610 nm. The scavenging activity was calculated as $[(1 - A_1/A_0) \times 100]$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of sample extracts.

Measurement of Ferrous Ion Chelating Activity. Ferrous ions(II) catalyze the production of hydroxyl radicals and hydroxyl anions from hydrogen peroxide (*18*); therefore, agents capable of exerting a Fe^{2+}-chelating effect can decrease the cellular damage caused by the Fenton reaction. Three hundred microliters of 2 mM $FeSO_4 \cdot H_2O$ was mixed with 100 μL test samples (final concentration 500 ppm) before the addition of

600 μL of 5 mM ferrozine. After the incubation at room temperature for 10 min, the absorbance was obtained at 562 nm. The metal binding capacity was calculated as $[(A_0 - A_1/A_0) \times 100]$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of sample extracts (*9*).

Measurement of Reducing Power. The reducing power was measured by the method of Amarowicz et al. (*20*) with slight modifications. Test samples diluted in 1 mL of distilled water (final concentration of 500 ppm) were mixed with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50 °C for 20 min. After the mixture was cooled, 1 mL of 10% trichloroacetic acid was added, followed by centrifugation at 1000*g* for 10 min. The upper layer (1 mL) of the solution was mixed with 1 mL of distilled water and 1 mL of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Increased absorbance indicated increased reducing power.

Measurement of Inhibition on Linoleic Acid Peroxidation. Linoleic acid emulsions (0.02 M) were prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween 20, and 50 mL of 0.02 M phosphate buffer (pH 7.0). A 2.5 mL aliquot of this emulsion was mixed with 0.5 mL of extracts (final concentration of 500 ppm) and 2 mL of 0.2 M phosphate buffer (pH 7.0). A mixture prepared with ethanol was used as the control. After the mixture was incubated for 72 h at 50 °C in the dark, 0.1 mL of the mixture was sampled and combined with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride (in 3.5% HCl). Precisely 3 min after the addition of ferrous chloride, the absorbance of the reaction mixture was acquired at 500 nm (*21*). The inhibitory effect was calculated as: $[1 - (A_1/A_0)] \times 100$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the mixture with extracts. A larger percentage indicates a greater inhibitory effect.

Determination of Total Phenolics. The total soluble phenolic content in the extracts was determined according to the method of Shahidi and Naczk (*22*) with modifications. Briefly, 600 μL of 50% Folin−Ciocatlteu reagent was thoroughly mixed with 500 μL of the extracts (final concentration of 500 ppm). Five minutes later, 1.2 mL of 20% Na_2CO_3 was added to the mixture. The reaction mixture was incubated for 10 min at room temperature with shaking, followed by centrifugation at 150*g* for 10 min. The absorbance was obtained at 730 nm, and the total amount of phenolic compound present in the extracts was determined as gallic acid equivalents.

Measurement of Cellular Antioxidant Activity (CAA). The inhibition of the production of reactive oxygen species (ROS) was determined according to the CAA assay (*23*) with modifications. The human umbilical vein endothelial cell line ECV304 (Biosource Collection and Research Center of Food Industry Research and Development Institute) was maintained in M199 medium supplemented with 100 units/mL penicillin, 100 units/mL streptomycin, and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Then, 10^6 cells were incubated with 5 μM DCHF-DA at 37 °C for 30 min and challenged with 250 μM t-BHP either with or without the 7 day extracts for an additional 30 min. *N*-Acetylcystine (10 mM) was used as a positive control. After incubation, emission at 529 nm was measured with excitation at 504 nm using a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan). DCHF-DA is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped within cells. ROS produced by cells oxidize DCHF to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Therefore, the fluorescence intensity is proportional to the amount of ROS produced by cells. The ability to inhibit the production of ROS (% inhibition) was calculated as: $\{[(A_{BHP} - A_0) - (A_{BHP+E} - A_E)]/(A_{BHP} - A_0)\} \times 100$, where A_{BHP} is the absorbance in the presence of t-BHP, A_0 is the absorbance of the control, A_{BHP+E} is the absorbance in the presence of both t-BHP and extracts, and A_E is the absorbance when cells were treated with extracts only.

Cell Culture. The human hepatoblastoma cell line HepG2 and mouse BALB/c macrophage cell line RAW 264.7 were obtained from Biosource Collection and Research Center of Food Industry Research and Development Institute. HepG2 cells were maintained in MEM supplemented with 10% fetal bovine serum, 0.15% sodium bicarbonate, 0.011% sodium pyruvate, 1% MEM nonessential amino acid solution, 100 units/mL penicillin, and 100 units/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. RAW 264.7 macrophages were maintained in DMEM supplemented with 10% fetal bovine serum, 0.22% sodium bicarbonate,100 units/mL penicillin, and 100 units/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. When 60% confluence was attained, the cells were treated with 7 day PDB, MP, PDBT, or MPT extracts (final concentration of 500 ppm). After 24 h, the medium was collected for cytokine assays. The cells were washed twice with cold PBS, harvested, and then resuspended in lysis buffer containing 1% Nonidet P 40, 150 mM sodium chloride, and 50 mM Tris-HCl, pH 7.5. The cell suspension was centrifuged at 10000*g* for 30 min, and the supernatant was collected and subjected to further centrifugation at 105000*g* for 60 min. The supernatant was collected and referred to as the "cytosol", which was used for immunoblot analysis of antioxidant enzymes. The protein content in cytosol samples was determined by Dc Protein Assay Kit (Bio-Rad, Hercules, CA).

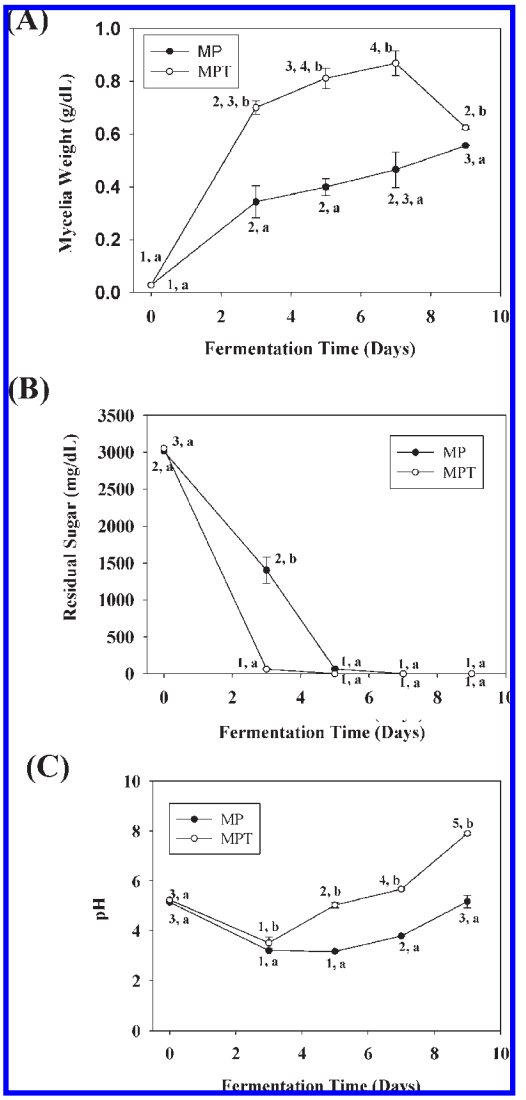

Figure 1. Growth curves of MP and MPT. (**A**) Mycelia weight, (**B**) residual sugar, and (**C**) pH of medium. MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Values indicate means ± SEs. In a group, means having the same number are not significantly different at various time points. At a time point, means having the same letter are not significantly different among groups ($\alpha = 0.05$).

Immunoblot Analysis. An equal amount of proteins was denatured and separated by gel electrophoresis before being transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween (TBST), 20 mM Tris-HCl, pH 8.3, 137 mM NaCl, and 0.1% Tween-20 for 1 h and then incubated sequentially with primary

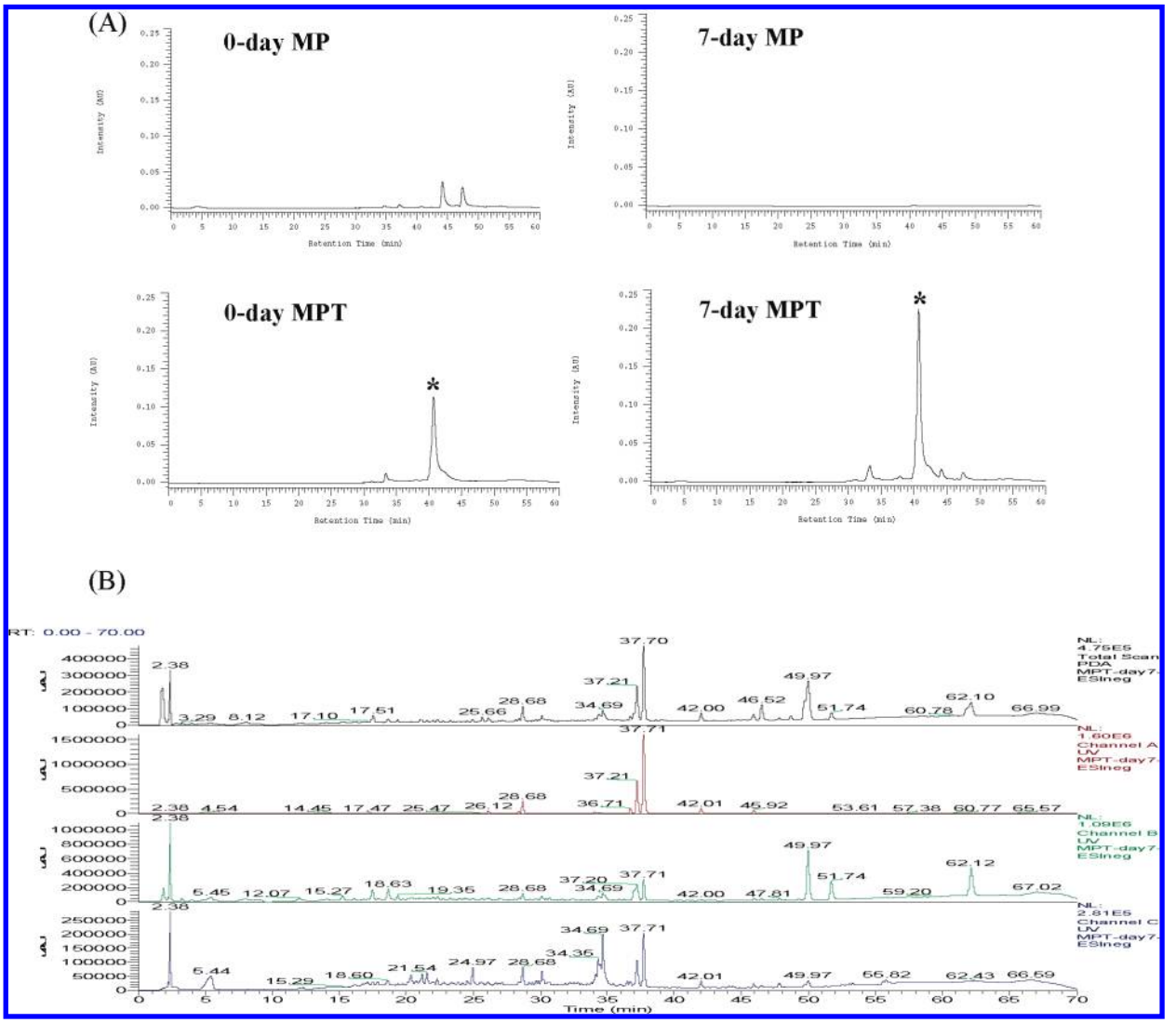

Figure 2. (**A**) Comparison of HPLC analysis for different extracts using a normal-bore column (4.6 mm × 250 mm); *curcumin-related compounds. (**B**) HPLC chromatograms from narrow-bore column (2.0 mm × 150 mm) analysis for 7 day MPT extracts detected with a photodiode array detector (PDA, 220−400 nm) and selected UV wavelength of 425 (channel A), 280 (channel B), and 320 nm (channel C). MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*.

antibody for 2 h and HRPase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (ECL, PerkinElmer, Waltham, MA), and the film was analyzed using ImageQuant software (Molecular Dymatics, Sunnyvale, CA).

Measurement of NO and Cytokines Production. Inflammation is a key process in atherosclerosis. Biomarkers of inflammation have been adopted for risk prediction of atherosclerosis. After the macrophages were treated with LPS and various extracts for 24 h, the culture medium was collected for the analysis of NO, TNF-α, and IL-1β. The production and release of NO were determined by commercial kit (Cayman). The concentration of TNF-α and IL-1β was assayed by ELISA kits (eBioscience, San Diego, CA).

Statistical Analysis. Statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC). Two-way analysis of variance (two-way ANOVA) was used to investigate the effects of main factors and their interaction. When significant effects of factors were detected, lease square means were applied to determine significant difference among means ($\alpha = 0.05$).

RESULTS

Growth Curve. According to **Figure 1A**, *M. pilosus* inoculated in turmeric-containing medium (MPT) grew faster than that inoculated in regular medium (MP); therefore, the depletion of sugar was faster in the turmeric-containing medium (**Figure 1B**). After the sugar was depleted, the pH of medium started to rise (**Figure 1C**).

HPLC Analysis. Curcumin-related compounds (curcuminoids) were not detected in PDB (data not shown) and MP (**Figure 2A**) but were present in 0 day PDBT (data not shown) and MPT extracts. After 7 days of fermentation, the content of curcumin-related compounds was not changed in PDBT extracts; however, it was increased in MPT extracts. According to LX/ESI-MS/MS analysis (**Figure 2B**), the major peak observed in HPLC chromatography includes three of the major curcuminoids: bisdemethoxycurcumin (retention time, 36.71 min), demethoxycurcumin (retention time, 37.21 min), and curcumin (retention time, 37.71 min).

Antioxidant Activities of Extracts. Two-way ANOVA indicated that significant effects of time, treatment, and their interaction were observed in all antioxidant assays. According to the results of **Figure 3A**, DPPH scavenging activities of both MPT and MP increased with fermentation time, and the activity of MPT was significantly higher than that of MP at each time point ($P < 0.05$). Although the scavenging activity of PDBT did not

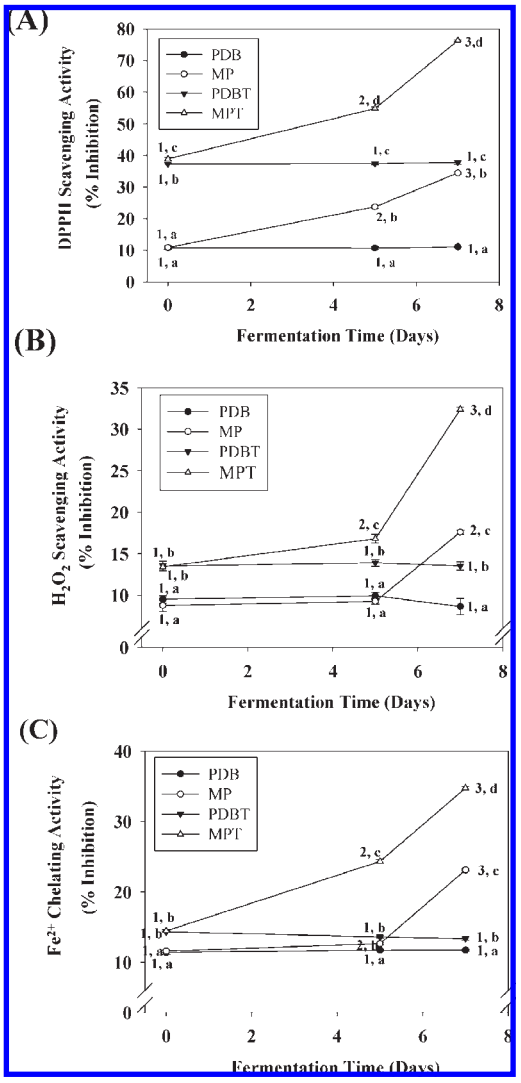

Figure 3. Antioxidant activities of PDB, MP, PDBT, and MPT. (**A**) DPPH scavenging activity, (**B**) hydrogen peroxide scavenging activity, and (**C**) ferrous ion chelating activity. PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBT, fermented products collected from uninoculated turmeric-containing PDB medium; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Values indicate means ± SEs. In a group, means having the same number are not significantly different at various time points. At a time point, means having the same letter are not significantly different among groups ($\alpha = 0.05$).

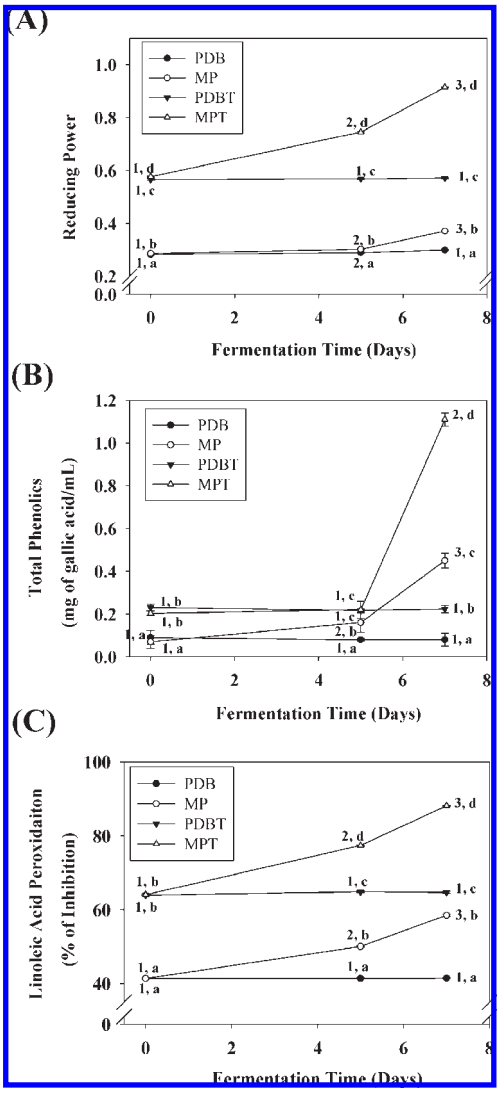

Figure 4. Antioxidant activities of PDB, MP, PDBT, and MPT. (**A**) Reducing power, (**B**) total phenolic contents, and (**C**) inhibition of linoleic acid peroxidation. PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBT, fermented products collected from uninoculated turmeric-containing PDB medium; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Values indicate means ± SEs. In a group, means having the same number are not significantly different at various time points. At a time point, means having the same letter are not significantly different among groups ($\alpha = 0.05$).

change with fermentation time, its DPPH scavenging activity was higher than that of MP at all time points. Similar to the results of PDBT, scavenging activity of PDB did not change with time.

Beginning at day 0, hydrogen peroxide scavenging activities of MPT and PDBT were significantly ($P < 0.05$) higher than those of MP and PDB (**Figure 3B**). The scavenging activities of four extracts did not change significantly from day 0 to day 5; however, the activities of MP and MPT increased by 90.06 and 92.74% from day 5 to day 7, respectively. The scavenging activities of PDB and PDBT did not change with time.

The Fe^{2+}-chelating activity of MPT significantly increased during fermentation; however, the activity of MP did not change during first 5 days (**Figure 3C**). The chelating activity of MPT was significantly higher than that of MP at day 5 and day 7 (24.33 vs 12.63% and 34.75 vs 23.12%, respectively).

Similar to the results of Fe^{2+}-chelating activity, the reducing power of MPT significantly increased during fermentation, but that of MP did not change before day 5. From day 0 to day 7, the reducing powers of MPT and MP increased by 58.41 and 29.72%, respectively. However, the reducing power of PDB and PDBG remained unchanged (**Figure 4A**). At day 0, the reducing power of PDBT and MPT was already significantly greater than that of PDB and MP, which indicated that the active agents in turmeric contributed to the observed activity.

As shown in **Figure 4B**, the phenolic compounds in PDB and PDBT remained unchanged with fermentation time, but the phenolics present in MP and MPT significantly increased from day 5 to day 7 by 1.18- and 4.05-fold, respectively. At day 7, the phenolic concentration of MPT was 1.10 mg gallic acid/mL, significantly higher than the 0.459 mg gallic acid/mL detected in MP.

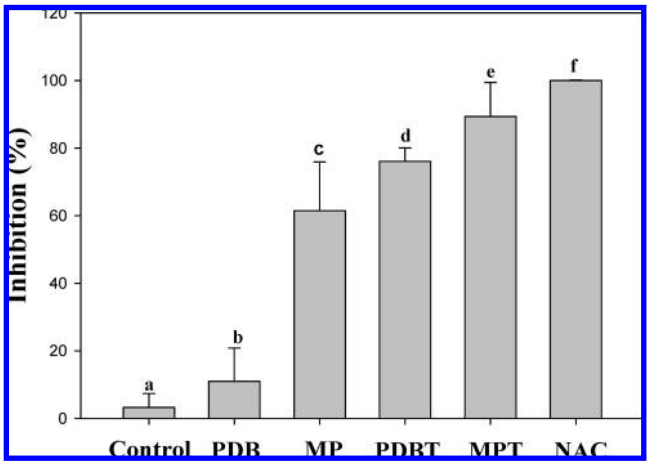

Figure 5. Protective effect of extracts on *t*-BHP-induced ROS generation in ECV304 cells. PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBT, fermented products collected from uninoculated turmeric-containing PDB medium; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Means having the same letter are not significantly different ($\alpha = 0.05$).

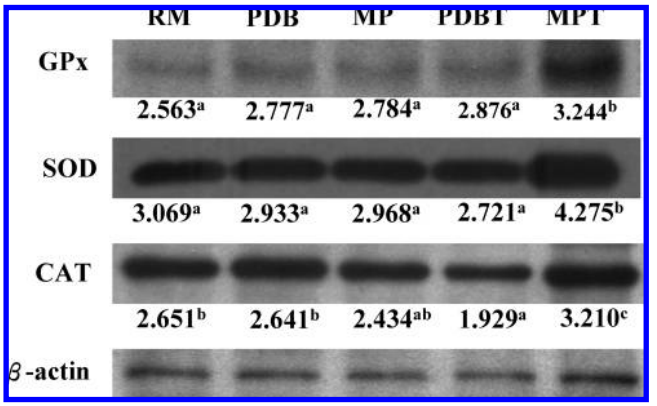

Figure 6. Expression of GPx, SOD, and CAT in HepG2 cells treated with PDB, MP, PDBT, or MPT for 24 h. GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; and R, regular medium. PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBT, fermented products collected from uninoculated turmeric-containing PDB medium; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Numbers under the bands indicate the ratios to the corresponding β-actin bands. Numbers having the same letter are not significantly different ($\alpha = 0.05$).

Polyunsaturated fatty acids, such as linoleic acid, are easily oxidized by oxygen. Similar to the results obtained for reducing power, the inhibitory effect of MPT on linoleic acid increased significantly with fermentation time, but that of MP did not change before day 5 (**Figure 4C**). From day 0 to day 7, the inhibitory effects of MPT and MP increased by 37.61 and 41.44%, respectively. However, those of PDB and PDBT remained unchanged.

CAA of Extracts. Fluorescence intensity in ECV304 challenged with t-BHP was significantly decreased when the cells were coincubated with extracts (**Figure 5**). The inhibitory effect of MPT on ROS production was greater than that of MP but smaller than that of *N*-acetylcysteine (NAC), an antioxidant that can scavenge ROS and regenerate intracellular glutathione stores. Because the active ingredients of extracts had to pass through the cell membrane before they involved the intracellular antioxidation, it is noticed that the antioxidant effect of MPT measured in cellular assay is weaker than those measured in chemical assays.

Expression of Antioxidant Enzymes. In present study, treatment of MPT increased the expression of GPx, CAT, and SOD in HepG2 cells (**Figure 6**). The effect of MP on the expression of these antioxidant enzymes was not different from that of PDB or PDBT. The detection of β-actin was also performed as the loading control and noted as a consistent band.

Cytokines and Nitric Oxide Production. Exposure to 1 μg/mL of LPS for 24 h significantly increased the release of TNF-α and IL-1β into medium by 19- and 2.5-fold, respectively (**Figure 7A**,**B**). Both MPT and MP had inhibitory effects on the production of TNF-α, but MPT had a more significant effect than MP (75.79 vs 18.47%). The ability to suppress the production of IL-1β was not different among MP, PDBT, and MPT. Treatment with LPS for 24 h significantly increased the production of NO by 10.8-fold; however, cotreatment with the extracts ameliorated this stimulatory effect. MPT and MP decreased the amount of NO released into medium by 93.49 and 71.65%, respectively (**Figure 7C**).

Expression of iNOS and COX-2. As shown in **Figure 8**, exposure to 1 μg/mL of LPS for 24 h significantly enhanced the expression of iNOS and COX-2. Co-treatment with MPT down-regulated the expression of iNOS and COX-2 by 15.76- and 7.66-fold, respectively, more significant than the down-regulation exerted by MP.

DISCUSSION

Before fermentation (day 0), PDBT and MPT already demonstrated higher antioxidant activities than their counterparts, PDB and MP. This is probably due to the antioxidant properties of turmeric, which was added to the medium. After fermentation, the content of curcumin-related compounds in MPT increased while that in PDBT did not change. The increased amount of curcuminoids accounts for the difference between antioxidant activities of MPT and PDBT. Curcumin, the major curcuminoid in turmeric, was metabolized to six new metabolites during fermentation with *Monascus pururеus* (*24*); moreover, these metabolites possess higher antioxidant activity than curcumin. Therefore, it is reasonable to infer that curcumin-related metabolites were produced in MPT, and these metabolites account for the increased curcuminoids observed.

Because some secondary metabolites of *Monascus* spp. have antioxidant properties (*8*), the antioxidant activities of MP did increase with fermentation time; however, the change was not as significant as that observed in MPT. This observation may result from the increased amount of curcumin-related compounds in MPT during fermentation. It is also presumed that the observed enhancement of the antioxidant activity of MPT may result from a stress response. Because curcumin is antifungal (*25*), the turmeric-containing medium is considered a stressful environment for *M. pilolsus*. Studies have shown that expression of related genes was up-regulated when fungi were cultivated in stress-inducing treatments (*26*, *27*); therefore, stress of growing in the medium containing antifungal turmeric may lead to increased expression of enzymes that regulate antioxidation. Moreover, because the growth of *M. pilosus* grown in turmeric-containing medium was faster than that grown in regular medium, the enhanced activity of MPT may also result from the increased production of secondary metabolites that possess antioxidant properties.

To test whether the observed enhancement of antioxidant activity of MPT can be achieved by mixing the *M. pilosus*

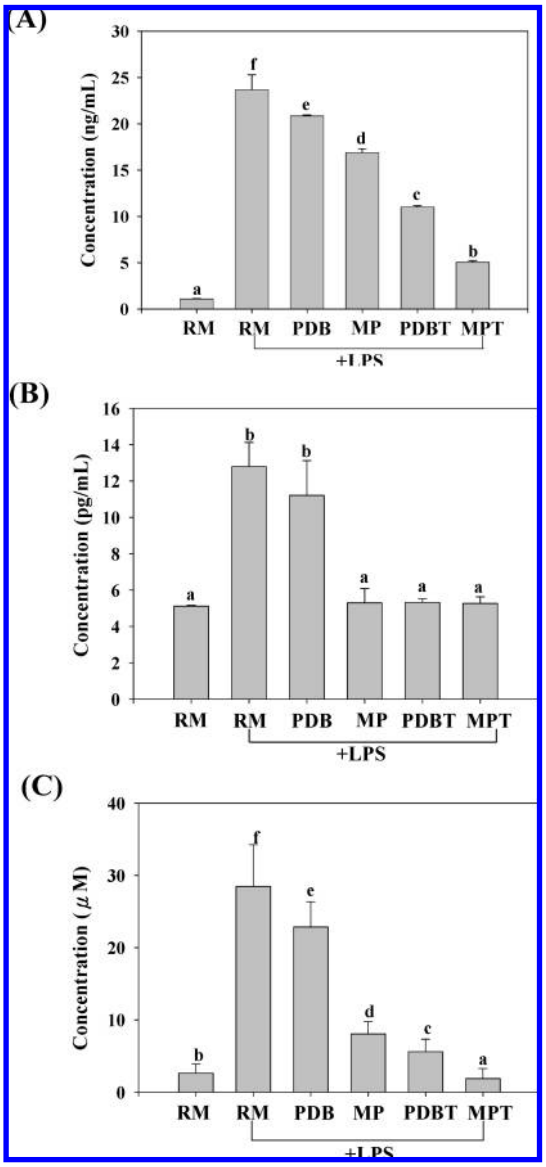

Figure 7. Effects of extracts on the production of (**A**) TNF-α, (**B**) IL-1β, and (**C**) NO of RAW 264.7 macrophages treated with PDB, MP, PDBT, or MPT for 24 h. IL-1β, interleukin-1β; +LPS indicates the stimulation by 1 μg/mL of LPS; and RM, regular medium. PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBT, fermented products collected from uninoculated turmeric-containing PDB medium; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Means having the same letter are not significantly different (α = 0.05).

fermented products collected from regular PDB medium with the turmeric-containing PDB medium, the 7 day MP extract and PDBT extract were mixed at a ratio of 1:1 to test the DPPH scavenging activity and compared with that of the 7 day MPT extract alone. The result revealed that the DPPH scavenging activity of 7 day MPT extract was greater than that of the mixture (data not shown). This result underscores the importance of modifications of the fermentation conditions for augmented antioxidant activities.

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are a group of enzymes that coordinate with other antioxidants to protect cells from oxidative damage. SOD catalyzes the conversion of $O_2^{\bullet -}$ to H_2O_2, and H_2O_2 is further

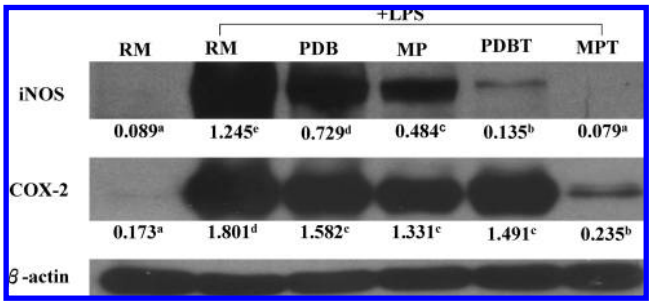

Figure 8. Expression of iNOS and COX-2 in RAW 264.7 cells treated with PDB, MP, PDBT, or MPT for 24 h. +LPS indicates the stimulation by 1 μg/mL of LPS. PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBT, fermented products collected from uninoculated turmeric-containing PDB medium; and MPT, fermented products collected from turmeric-containing PDB medium inoculated with *M. pilosus*. Numbers under the bands indicate the ratios to the corresponding β-actin bands. Numbers having the same letter are not significantly different (α = 0.05).

reduced to H_2O by the activity of GPx or CAT (*28*). In a previous study (*11*), *M. pilosus* fermented products collected from garlic-containing medium significantly up-regulated the expression of hepatic GPx by 5-fold in rats, while the fermented products collected from regular medium increased GPx expression by only 2-fold. *M. pilosus* fermented products collected from ginger-containing medium significantly increased the expression of GPx by 138%, whereas fermented products collected from regular medium showed no effect. In the present study, up-regulation of GPx, SOD, and CAT was observed in the HepG2 cells treated with MPT but not in the cells treated with MP. The results of these studies indicate that the modified *M. pilosus* fermented products had more significant effects on the expression of antioxidant enzymes both in vivo and in vitro.

Inflammation is associated with a wide variety of mediators that initiate the inflammatory response by recruiting and activating immune cells. Cytokines are regulatory polypeptides produced by virtually all immune cells. In the presence of stimuli, such as LPS, increased production of cytokines will occur, leading to initiation of an inflammatory response (*29*). TNF-α and IL-1β are two major cytokines involved in the initiation of inflammation. In the present study, the release of TNF-α and IL-1β significantly increased when RAW 264.7 macrophages were challenged with LPS; however, cotreatment with the extracts of *M. pilosus* fermented products inhibited the production TNF-α and IL-1β. Studies have shown that curcumin and turmeric extracts inhibited the LPS-induced production of proinflammatory cytokines TNF-α and IL-1 (*30*, *31*). Although the inhibitory effect on IL-1β production was not different between MPT and MP, MPT did show a more significant inhibition of TNF-α production than MP. This implies that the modified *M. pilosus* fermented product was more efficient in repressing inflammation.

Nitric oxide is an inorganic free radical synthesized from L-arginine by nitric oxide synthase in various tissues (*32*). On the basis of the location and catalytic mechanism, three NOSs have been identified, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are constitutively expressed, producing low levels of NO in the neurons and endothelium for the maintenance of physiological functions such as cardiovascular tone, central nervous signaling, and smooth muscle relaxation. iNOS is expressed in macrophages following exposure to cytokines, such as TNF-α and IL-1, and/or microbial products, such as LPS. Excessive NO production

catalyzed by iNOS causes oxidative damage to endothelial tissues, especially of the vascular system. Curcumin has been reported to inhibit induction of nitric oxide synthase and the consequent NO production in activated macrophages (*33*, *34*). In the present study, both MPT and MP showed an inhibitory effect on the production of NO; however, the repression was more significant in the cells treated with MPT. Consistent with the results of NO production, expression of iNOS in macrophages was down-regulated more significantly by MPT than by MP. TNF-α has been shown to stimulate the up-regulation of iNOS and NO production in human coronary microvascular endothelial cells (*35*), and repression of TNF-α production by MPT contributed to the observed down-regulation of iNOS and NO. Significant inhibition of the production of proinflammatory TNF-α, IL-1*β*, and NO by MPT allows the modified *M. pilosus* fermented product to possess an antiatherosclerotic function.

In addition to iNOS, the expression of COX-2 was also significantly repressed by MPT. COX catalyzes the conversion of arachidonic acids to eicosanoids. COX-1 is constitutively expressed in many cell types, whereas the expression of COX-2 is restricted and frequently observed after stimulation of cells with proinflammatory cytokines (*36*). COX-2 enhances the adhesion of monocytes to activated endothelial cells in the presence of oxidized-LDL. The proatherogenic role of monocytic COX-2 in the early stage of atherogenesis is suggested by a study in LDL-deficient mice, in which the formation of vascular lesion was reduced by the use of highly selective COX-2 inhibitor (*37*). Our study indicated that cotreatment with MPT down-regulated the LPS-induced COX-2 expression. The repressive effect of MPT on COX-2 was more significant than that of MP. Curcumin has been reported to down-modulate the transcription of iNOS and COX-2 by reducing the activation of MAPK p38 (*38*); therefore, the curcumin present in the medium may contribute to the observed down-regulation of iNOS and COX-2 by MPT.

In conclusion, the present study showed that modification of culture medium by addition of turmeric juice significantly enhanced the antioxidant and anti-inflammatory activities of *M. pilosus* fermented products. By increasing the inherent levels of the antioxidant and anti-inflammatory capacity of *Monascus* sp. fermented product, we created a nutraceutical that has the right prospects for the prevention of atherogenesis.

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