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Enhanced antioxidant activity of *Monascus pilosus* fermented products by addition of ginger to the medium

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ABSTRACT

The fermented products of *Monascus* sp. are known for their antihypercholesterolaemic effects, however, their antioxidant activities are different from those of many plant-derived foods. To evaluate the effect of ginger addition into the medium on the antioxidant activity of *Monascus pilosus* fermented products, we cultured uninoculated PDB medium (PDB), inoculated PDB medium (MP), uninoculated ginger-containing medium (PDBG), and inoculated ginger-containing medium (MPG). The broth and mycelia were collected, freeze-dried, and extracted to evaluate their free radical scavenging activities, inhibition of peroxidation, phenolic content, inhibition of DNA damage, cellular antioxidant activity, and expression of the antioxidant enzymes. The results showed that MPG had significantly higher antioxidant activity than PDB, MP, and PDBG at all fermentation time points. Moreover, the fermentation process significantly increased the antioxidant activities of MPG. After the inherent level of antioxidant capacity was increased, the modified *M. pilosus* fermented product demonstrated a higher anti-atherosclerotic value than the unmodified product.

1. Introduction

Atherosclerosis is the leading cause of death in modern societies. Oxidation of lipoproteins, especially low density lipoprotein (LDL) cholesterol, plays a crucial role in the initiation and progression of atherosclerosis (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). Antioxidants that prevent LDL oxidation *in vitro* also inhibit atherosclerosis in animals (Carew, Schwenke, & Steinberg, 1987). Therefore, reducing blood lipids and inhibiting lipid oxidation are both important for the prevention and treatment of atherosclerosis.

Monascus sp. fermented products have been developed as nutraceuticals for the prevention of hypercholesterolaemia. Monacolin K, commercially known as lovastatin, mevinolin, or cholestin, is a secondary metabolite obtained from *Monascus* sp. fermentation and was identified as a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reducase in cholesterol de novo synthesis (Endo, 1980). Long-term intake of foods containing monacolin K or similarly structured molecules significantly lower plasma cholesterol concentrations (Mangaloglu et al., 2002; Wang, Chen, & Lin, 2000). In addition to their cholesterol-lowering effect, *Monascus* sp. fermented products also have antioxidant activities (Aniya et al., 1999), however, these activities are different from those of many plant-derived foods (Liu & Ng, 2000). In order to increase the nutraceutical value of *Monascus* sp. fermented products, we previously increased the antioxidant activities of *Monascus pilosus* fermented products by supplementing the medium with garlic (Kuo, Wang, Yang, Jao, & Lin, 2006). An animal study indicated that *M. pilosus* fermented products collected from the garlic-containing medium lowered blood lipids significantly more than non-garlic-containing medium. Moreover, they not only inhibited HMG-CoA reductase expression but also downregulated the proteins involved in lipoprotein assembly and upregulated hepatic antioxidant enzymes (Kuo, Jao, & Yang, 2008).

Ginger (*Zingiber officinale*) has been consumed since antiquity and is known to play diverse biological roles including antioxidation, anti-inflammation, hypolipidemia, anti-carcinogenesis, anti-nausea, antithrombosis, and antibacterial process (Grzannar, Lindmark, & Frondoza, 2005; Kadnur & Goyal, 2005; Kikuzaki & Nakatani, 1993; Stoilova, Krastanov, Stoyanova, Denev, & Gargova, 2007). Both *Monascus* and ginger contain functional components, but liquid fermentation of ginger-containing medium by *Monascus* sp. is a new area of investigation. The objective of this study was to evaluate the antioxidant properties of *M. pilosus* fermentated products collected from ginger-containing medium and to compare

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with those collected from regular medium. The results of this study will facilitate the development of nutraceuticals with anti-atherosclerotic function.

2. Materials and methods

2.1. Chemicals

Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from BD (Sparks, MD, USA). The 1,1-diphenyl-2-picrylhydrazyl (DPPH), Tris–HCl, nitroblue tetrazolium chloride (NBT), hypoxanthine, ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), xanthine oxidase, horseradish peroxidase (HRPase), phenol red, ferrozine, linoleic acid, Tween 20, ammonium thiocyanate, ferrous chloride, potassium ferricyanide, trichloroacetic acid, peroxidase, Folin–Ciocatlteu reagent, *tert*-butylhydroperoxide (*t*-BHP), sodium pyruvate, MEM non-essential amino acid solution, formic acid, ammonium formate, standard compounds 6-, 8-, 10-gingerol, and 6-shogaol were obtained from Sigma (St. Louis, MO, USA). Plasmid pGEM-7ZF(−) was obtained from Promega (Madison, WI, USA). Luria-Bertani medium was obtained from BD (Franklin, NJ, USA), and M199 medium, Minimum Essential Alpha Medium, penicillin, streptomycin, and foetal bovine serum were purchased from GIBCO (Grand Island, NY, USA). The 7′-dichlorodihydrofluorescein diacetate (DCHF-DA) was purchased from Invitrogen (Carlsbad, CA, USA). Nonidet P40 was obtained from Fluka (Buchs, Switzerland), and all polyclonal and monoclonal antibodies were purchased from NOVUS (Littleton, CO, USA).

2.2. Extract preparation

Ginger purchased at a farmer's market in Nanto County, Taiwan, was washed and ground by food preparing machine. The juice collected during grinding was filtered twice to make "ginger 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 a potato dextrose agar (PDA) slants and incubated at 28 °C. After a pure culture was obtained, the mycelia were re-inoculated into potato dextrose broth 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 1:25–2.4% fresh potato dextrose broth containing 20% ginger juice for either 4 or 8 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 two hours, and centrifuged at 100*g* for 10 min. After centrifugation, the supernatant was collected and referred to as "MPG". Inoculated regular PDB medium, uninoculated ginger-containing medium, and uninoculated regular PDB medium were cultured, collected, freeze-dried, and extracted using the same conditions used for MPG to obtain "MP", "PDBG", and "PDB", respectively.

2.3. Measurement of DPPH scavenging activity

The scavenging activity of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined using the method described by Chang et al. (2001) with slight modification. One millilitre 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, PDBG, or MPG 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.

2.4. Measurement of superoxide scavenging activity

Measurement of superoxide scavenging activity was carried out according to the method of Kirby and Schmidt (1997) with slight modifications. Fifty microlitres of 0.6 mM NBT (nitroblue tetrazolium chloride), 30 μl of 3 mM hypoxanthine, 20 μL of 15 mM Na_2EDTA, and 120 μl of buffer (50 mM KH_2PO_4/KOH, pH 7.4) were mixed in 96-well plate. Thirty microlitres of the extracts (PDB, MP, PDBG, or MPG) were added to the plate to yield a final concentration of 500 ppm before the reaction was initiated by addition of 50 μl of xanthine oxidase (1 unit/10 ml). The absorbance at 570 nm was measured every 20 s for 5 min. The inhibition ratio (%) was calculated according to the following equation: $[(\Delta A_0 - \Delta A_1/\Delta A_0) \times 100]$, where ΔA_0 is the change rate in the absorbance of control reaction and ΔA_1 is the change rate in the absorbance in the presence of sample extracts.

2.5. Measurement of ferrous ions chelating activity

Three hundred microlitres of 2 mM $FeSO_4 \cdot H_2O$ were mixed with 100 μl test samples (final concentration 500 ppm) before 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.

2.6. Measurement of reducing power

The reducing power was measured by the method of Amarowicz, Pegg, Rahimi-Moghaddam, Barl, and Weil (2004) 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 cooling the mixture, 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 indicates increased reducing power.

2.7. Measurement of inhibition on linoleic acid peroxidation

Inhibition on linoleic acid peroxidation was evaluated using the thiocyanate method (Haraguchi, Hashimoto, & Yagi, 1992). 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 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 reaction mixture was acquired at 500 nm. The inhibitory effect was calculated as: $[1\text{-}(A_1/A_0)] \times 100$, 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.

2.8. Determination of total phenolics

The total soluble phenolic content in the extracts were determined according to the method of Shahidi and Naczk (1995) 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 were 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 compounds present in the extracts was determined as gallic acid equivalents.

2.9. HPLC/MS analysis of the gingerol-related compounds

The separation and identification procedures for gingerol-related compounds including 6-, 8-, 10-gingerol, and 6-shogaol were according to the method described by Jiang, Solyom, Timmermann, and Gang (2005) with slight modification. In brief, fermented ginger extracts were performed on an HPLC system consisted of a Finnigan Surveyor module separation system and a photodiodearray detector (DAD) (Thermo Electron Co., MA, USA). The chromatographic separation of the compounds was achived using an analytical column (Luna 3μ C18(2), 150 × 2.0 mm) with a guard column (SceurityGuard C18(ODS) 4 × 3.0 mm ID, Phenomenex Inc., Torrance, CA, USA) at an elution flow rate of 0.2 ml/min. Solvent A was ammonium formate (10 mM, pH 4.0) and solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 0.2 ml/min. The entire course of programmed gradient elution was carried out as follows: 0–30 min, with 30–95% B; 30–40 min, with 95% B isocratic; 40–50 min, with 95–5% B. Absorption spectra of eluted compounds were recorded from 220 to 500 nm with the in-line DAD monitored at 230 (signal A), 280 (signal B), and 425 nm (signal C).

The separated peaks were further identified with a Finnigan LCQ Advantage MAX ion trap mass spectrometer and were operated in Electrospray Ionisation (ESI) with negative ionisation mode. Samples of 20 μl of extracts were directly injected into the column using a Rheodyne (model 7725i) injection valve. The ion trap instrument was operated at the following setting: capillary voltage, −23 V; tube lens offset, −45 V; source voltage, 4.5KV; ion transfer capillary temperature, 275 °C; nitrogen sheath gas, 50; and auxiliary gas, 5 (arbitrary units). Mass spectra were acquired in a *m/z* range of 150–800, with five microscans and a maximum ion injection time of 200 ms. These were the optimised parameters for the maximum transmission of the 6-gingerol-derived ions. Selective ion monitor (SIM) analysis was a narrow scan event that monitored the m/z value of the selected ion, in a range of 1.0 mass centered on the peak for the molecular ions of 6-, 8- and 10-gingerol, and 6-shogoal.

2.10. Measurement of Inhibition on DNA damage

Measurement of DNA strand breakage was carried out according to the methods previously reported (Hong & Wang, 2005). *E. coli* were transformed with plasmid pGEM-7ZF(−) and grown in Luria-Bertani medium. Plasmid DNA was purified using Qiagen plasmid purification kit (Valencia, CA, USA). The reagents were added in the following order: phosphate buffer (pH 7.3), supercoiled plasmid DNA (3 μg/ml), test samples (100 ppm), $FeCl_2$ (10 μM), and H_2O_2 (1 mM). The mixtures were incubated at room temperature for one hour prior to the electrophoresis in a 1% agarose gel and ethidium bromide staining for analysis. Quantification of the DNA conformation was performed using Uniphoto Band Tool software (EZlab, Taipei, Taiwan).

2.11. Measurement of cellular antioxidant activity (CAA)

Inhibition on the production of reactive oxygen species (ROS) was determined according to the cellular antioxidant activity assay (Wolfe & Liu, 2007) with modifications. The human umbilical vein endothelial cell line ECV304 (Biosource Collection and Research Center of Food Industry Research and Development Institute, Shinchu City, Taiwan) was maintained in M199 medium supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% foetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Then, 10^6 cells were incubated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) at 37 °C for 30 min and challenged with 15 mM *tert*-butylhydroperoxide (*t*-BHP) either with or without the extracts for an additional 90 min. After incubation, emission at 538 nm was measured with excitation at 485 nm using a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan).

2.12. Cell culture and immunoblot analysis

The human hepatoblastoma cell line HepG2 (Biosource Collection and Research Center of Food Industry Research and Development Institute, Shinchu City, Taiwan.) were maintained in minimum essential alpha medium supplemented with 10% foetal bovine serum, 0.15% sodium bicarbonate, 0.011% sodium pyruvate, 1% MEM non-essential amino acid solution, 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 PDB, MP, PDBG, or MPG extracts (final concentration of 500 ppm) for 24 h. 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, pH7.5. The cell suspension was centrifuged at 10 000*g* for 30 min and the supernatant was collected and subjected to further centrifugation at 105 000*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, USA).

Equal amount of proteins were denatured and separated by gel electrophoresis before being transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Pittsburg, PA, USA). The membrane was 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 hour and then incubated for 2 h at room temperature with a 1:500 dilution of anti-glutathione peroxidase (GPx) polyclonal antibody, 1:1000 dilution of anti-catalase (CAT) polyclonal antibody, 1:2500 anti-copper/zinc superxide dismutase (SOD) polyclonal antibody, or 1:5000 anti-β-actin monoclonal antibody. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (ECL, PerkinElmer, Waltham, MA, USA) and the film were analysed using ImageQuant software (Molecular Dymatics, Sunnyvale, CA, USA).

2.12.1. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) and Duncan's multiple range test were used to determine significant difference among means ($\alpha = 0.05$).

3. Results and discussion

To determine the appropriate extract concentration for the assays, various concentrations (0, 100, 250, 500, and 1000 ppm) of fermentation product extracts collected at different time points were applied to DPPH scavenging and cell viability assays. The

results indicated that 500 ppm possessed better antioxidant activity than 100 and 250 ppm, but did not differ from 1000 ppm (data not shown). Moreover, 500 ppm of extracts killed less than 50% of the cultured cells after 24 h of treatment. Therefore, 500 ppm was selected for further assays.

3.1. DPPH scavenging activity

DPPH has been widely used to evaluate the free radical scavenging activities of various compounds. According to the results of Fig. 1A, DPPH scavenging activities of both MP and MPG increased with fermentation time, and the activity of MPG was significantly higher than that of MP at each time point ($P < 0.05$). Although the scavenging activity of PDBG did not change with fermentation time, its DPPH scavenging activity was higher than that of MP at all time points. Similar to the results of PDBG, the scavenging activity of PDB did not change with time. The significantly higher activities of PDBG and MPG at day 0 indicated that the active agents in ginger contributed to the observed scavenging activities.

3.2. Superoxide scavenging activity

During cellular oxidation, superoxide radicals are initially formed; therefore, agents that are able to scavenge superoxides can efficiently alleviate cellular damage. Beginning at day 0, superoxide scavenging activities of PDBG and MPG were significantly ($P < 0.05$) higher than that of PDB and MP (Fig. 1B). From day 0 to day 8, the superoxide scavenging activity of MP and MPG increased 42% and 46.7%, respectively; however, the scavenging activity of PDB and PDBG did not change with time. This result implies that the fermentation process promoted the production of metabolites capable of scavenging superoxide.

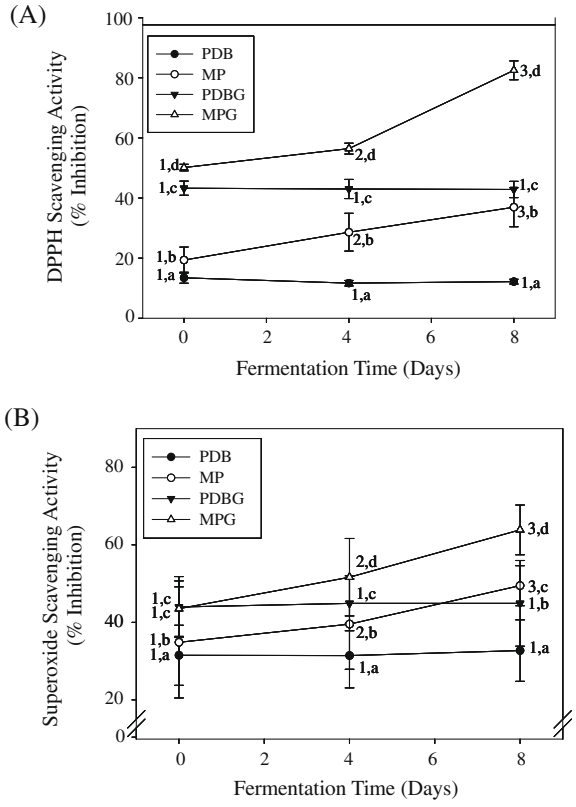

Fig. 1. Antioxidant activities of PDB, MP, PDBG, and MPG. (A) DPPH scavenging activity, (B) superoxide scavenging activity. Values indicate mean ± SE. In a group, means having the same number are not significantly different at various time points. At a time point, means having the same letter are not significantly different amongst groups ($\alpha = 0.05$).

3.3. Ferrous ions chelating activity

Ferrous ions (II) play an important role in the Fenton reaction, catalysing the production of hydroxyl radicals and hydroxyl anions from hydrogen peroxide (Fenton, 1894). Agents capable of exerting a Fe^{2+}-chelating effect can decrease the cellular damages caused by the Fenton reaction. There were no significant differences in Fe^{2+} chelation between PDB, MP, and PDBG at day 4 (Fig. 2A), although the chelating ability of MP was higher than that of PDB and PDBG at day 8. At day 4, the chelating activity of MPG (28.92%) was higher than that of MP, and the difference became more significant after 8 days of fermentation (74.4% vs. 23.35%). These results implied that the ginger-containing medium promoted the formation of Fe^{2+} chelators during fermentation.

3.4. Reducing power

From day 0 to day 8, the reducing powers of MP and MPG increased by 36% and 52.5%, respectively. However, the reducing power of PDB and PDBG remained unchanged (Fig. 2B). Yang, Mau, Ko, and Huang (2000) reported that fermented soybean broth exhibited higher reducing power than that of non-fermented soybean broth. Therefore, MP and MPG might contain reducing compounds that are formed during fermentation; moreover, the formation of these compounds was enhanced by the addition of ginger.

3.5. Inhibition on linoleic acid peroxidation

Polyunsaturated fatty acids, such as linoleic acid, are easily oxidised by oxygen, leading to chain reactions and the formation of coupled double bonds, aldehydes, ketones, and alcohols at later stages. A comparison of the antioxidant activities of four crude extracts was also evaluated by the inhibitory effect of these extracts on linoleic acid peroxidation, as measured by the thiocyanate method. Similar to the results obtained for Fe^{2+}-chelating activity, inhibitory effect of MPG on linoleic acid increased 40.99% with fermentation time (Fig. 2C), but the effects of PDB, MP, and PDBG did not significantly change. This assay used Fe^{2+} as the metal ion catalyst, therefore, the ability of the test extracts to inhibit linoleic acid peroxidation might be exerted by complexing iron.

3.6. Total phenolics

Phenolic constituents of plants have been suggested to play a role in their antioxidant functions (Velioglu, Mazza, Gao, & Oomah, 1998). The antioxidant properties of phenolic compounds result from their ability to donate electrons and act as free radical scavengers via formation of stable phenoxyl radicals. As shown in Fig. 2D, the phenolic compounds present in MP and MPG increased with fermentation time whilst the phenolics in PDB and PDBG remained unchanged with fermentation time. The total phenolics concentration of MPG increased 88% from day 0 to day 8, but the increase of that in MP was only 38%. At day 8, the phenolic concentration of MPG was 1.51 mg gallic acid/ml, significantly higher than the 0.79 mg gallic acid/ml detected in MP.

3.7. HPLC/MS analysis of the gingerol-related compounds

The pungency of fresh ginger is due primarily to the gingerols; the most abundant of which is [6]-gingerol. Because the 8-day

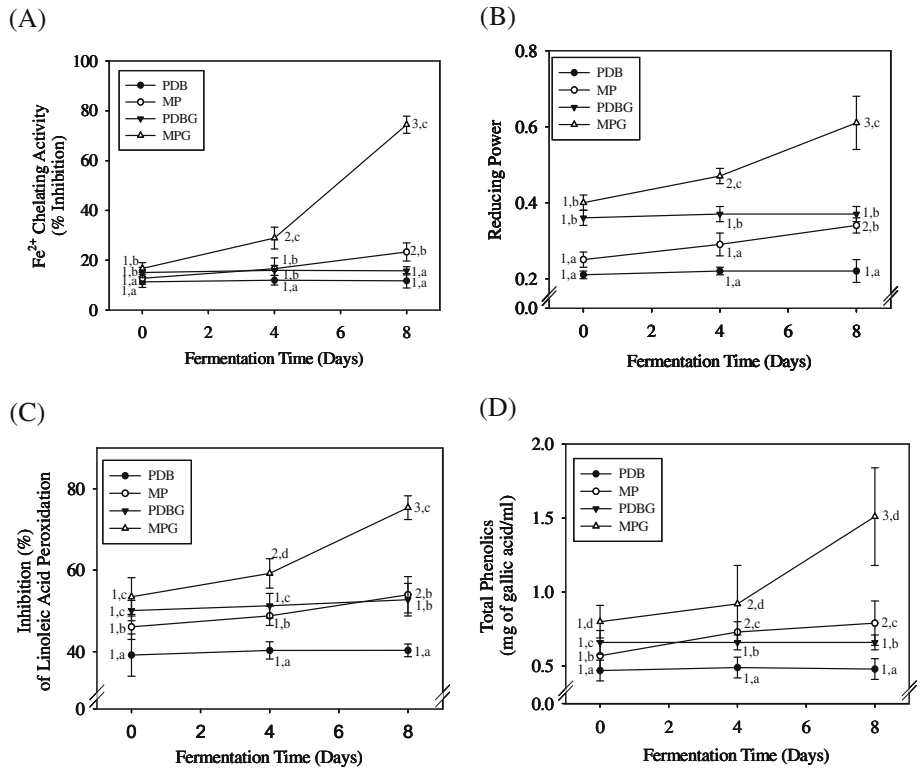

Fig. 2. Antioxidant activities of PDB, MP, PDBG, and MPG. (A) Fe^{2+}-chelating activity, (B) reducing power, (C) inhibition of linoleic acid peroxidation, (D) total phenolics content. Values indicate mean ± SE. In a group, means having the same number are not significantly different at various time points. At a time point, means having the same letter are not significantly different amongst groups ($\alpha = 0.05$).

fermented MPG extract exhibited the greatest antioxidant activity in all assays, 8-day MPG and its 8-day PDBG counterpart were used to detect gingerol-related compounds. As reported by He, Bernart, Lian, and Lin (1998), the HPLC chromatograms with in-line diode array detection set at 425, 280, and 230 nm were helpful in providing structural confirmation of gingerol-related compounds. The study by Jiang et al. (2005) indicated that gingerols show a characteristic UV absorption maximum at 280 nm and a shoulder at 230 nm; moreover, compounds were observed with absorption maxima at 425 nm, suggesting the presence of an extended conjugation system. In addition to UV absorption, retention time (RT) in reversed-phase (RP) – HPLC were also found to be useful in the structure confirmation of compounds belonging to homologous series (He et al., 1998). In this study, ESI – negative mass spectrometry were also used to determine the target compounds.

Compare MPG with PDBG (Fig. 3), the relative percentage of 6-gingerol increased whilst that of 8-gingerol, 6-shogoal, and 10-gingerol decreased. A major peak named unknown A in PDBG (UV absorption: 283, 294, and 223 nm) significantly increased in MPG, however, another major peak named unknown B in PDBG (M^+:312; UV absorption: 362, 371, 385, and 258 nm) significantly decreased in MPG. Due to the weak signal, molecular weight of unknown A was not determined by mass spectrometry. Because antioxidant activity of 6-gingerol is significantly higher than that of 8-gingerol, 6-shogoal, and 10-gingerol (Masuda, Kikuzaki, Hisamoto, & Nakatani, 2004), it is reasonable to infer that the increased antioxidant activity and phenolic content in the MPG may partially result from the relatively higher percentage of 6-gingerol in the extract. The study in metabolic pathway of 8-gingerol, 6-shogoal, and 10-gingerol during fermentation. Positive identification of unknown A and unknown B using NMR analysis following their purification remains to be carried out in the future.

3.8. Inhibition on DNA damage

Cleavage of plasma DNA converts the supercoiled circular form (SC) of DNA into the relaxed open circular form (OC). The larger percentage of OC indicates the greater DNA damage caused by ROS. Because a final extract concentration of 100 ppm provided significant protection against DNA damage in previous study, this concentration was used in the present study. Incubation with $FeSO_4$–H_2O_2 converted 48% of the supercoiled DNA into open circular form (Fig. 4). Co-incubation with PDB did not diminish the DNA damage; however, treatment with MP, PDBG, or MPG decreased the percentage of open circular DNA to 38%, 37%, and 10%, respectively. The results indicated that MPG showed more significant protection against DNA damage than MP and PDBG. The protective effect of MPG on DNA damage may result from its Fe^{2+}-chelating and H_2O_2-scavenging ability.

3.9. Cellular antioxidant activity

DCHF-DA is a stable compound that readily diffuses into cells and is hydrolysed by intracellular esterase to yield DCHF, which is trapped within cells. Hydrogen peroxide or low-molecular-weight reactive oxygen species (ROS) produced by cells oxidise DCHF to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Therefore, the fluorescence intensity is proportional to the amount of ROS produced by cells. Challenge of ECV304 with *t*-BHP significantly increased the production of ROS, but this effect was diminished by the addition of *N*-acetylcysteine ($P < 0.05$)

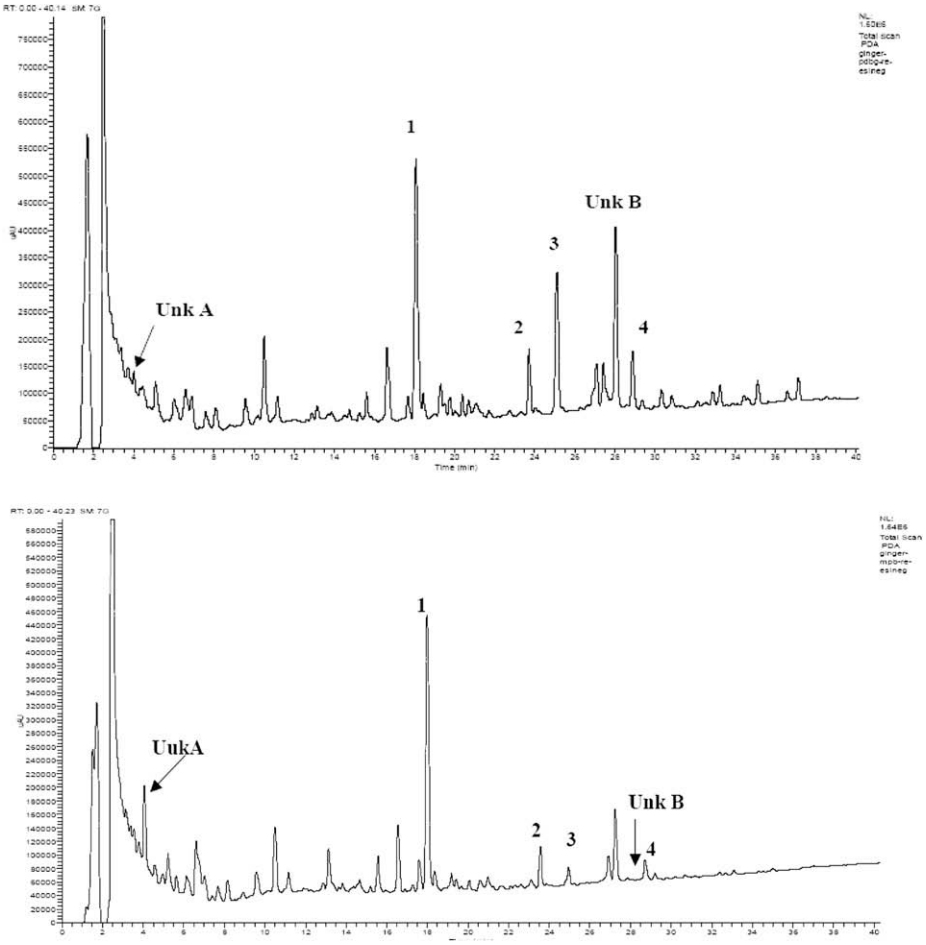

Fig. 3. HPLC chromatograms of 8-day PDBG (top) and 8-day MPG (bottom) by photodiode array detector at wavelength of 220–500 nm. 1: [6]-gingerol; 2: [8]-gingerol; 3: [6]-shogaol; 4: [10]-gingerol; UnkA: unknown compound A; UnkB: unknown compound B.

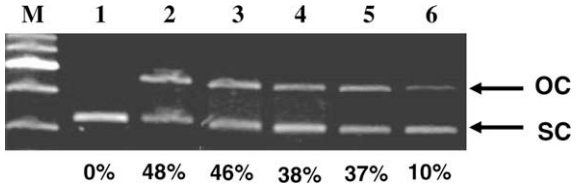

Fig. 4. Electrophoretic profile of DNA damage induced by Fe(II) and H_2O_2. Plasmid DNA pGEM-7ZF(−) was incubated for 1 h at room temperature (1) without $FeSO_4$–H_2O_2, (2) with $FeSO_4$–H_2O_2, (3) with $FeSO_4$–H_2O_2 plus PDB, (4) plus MP, (5) plus PDBG, or (6) plus MPG. SC: supercoiled circular form, OC: open circular form, M: marker. Numbers under the bands indicate the percentage of OC present in the total DNA.

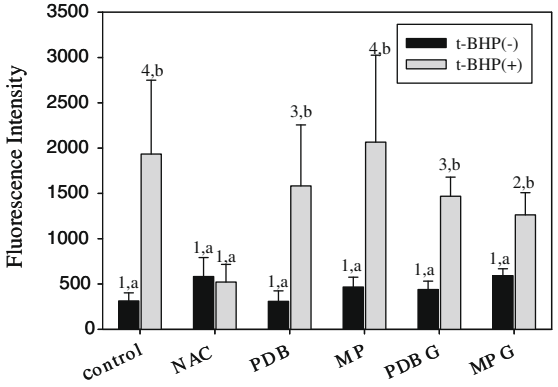

Fig. 5. Protective effect of extracts on *t*-BHP-induced ROS generation in ECV304 cells. NAC, *N*-acetylcysteine. In a group, means having the same letter are not significantly different between *t*-BHP (−) and *t*-BHP (+). In the cells treated with or without *t*-BHP, means having the same number are not significantly different amongst groups. ($\alpha = 0.05$).

(Fig. 5), an antioxidant that can scavenge ROS and regenerate intracellular glutathione stores (De Flora, Izzoti, D'Agostini, & Cesarone, 1991). A shown in Fig. 5, none of the extracts exhibited the same scavenging ability as NAC, but, amongst the four extracts, MPG had the greatest protective effect. Compared with the control, MP had no inhibitory effect on the production of ROS.

3.10. Expression of antioxidant enzymes

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 catalyse the conversion of $O_2^{\cdot-}$ to H_2O_2, and H_2O_2, is further reduced to H_2O by the activity of GPx or CAT (Michiels, Raes, Toussaint, & Remacle, 1994). Compared with MP, MPG increased the expression of GPx, CAT, and SOD in HepG2 cells by 138%, 18%, and 28%, respectively (Fig. 6). The expression of CAT and SOD did not differ amongst PDB, MP, and PDBG, but PDBG increased the expression of GPx by 49% and 62% compared with PDB and MP, respectively. The detection of β-actin was also performed as the loading control and noted as a consistent band.

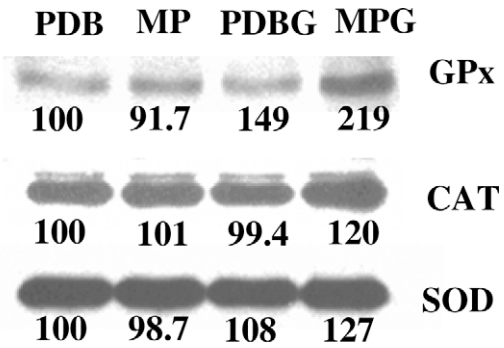

Fig. 6. Expression of GPx, CAT, and SOD in HepG2 cells treated with PDB, MP, PDBG, or MPG for 24 h. Numbers under the bands indicate relative the density compared with the PDB group at the same molecular weight location. GPx, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase.

In a previous study (Kuo et al., 2008), *M. pilosus* fermented products collected from garlic-containing medium significantly upregulated the expression of hepatic GPx by 5-fold in rats, whilst the fermented products collected from regular medium increased GPx expression by only 2-fold; however, the effects on the expression of SOD and CAT were not different. The results of both studies indicate that the modified *M. pilosus* fermented products had more significant effects on the expression of GPx than that of SOD and CAT both *in vivo* and *in vitro*.

Consumption of ginger is associated with reduced macrophage-mediated oxidation of LDL, reduced uptake of oxidised LDL by macrophages, a reduced oxidative state of LDL, and reduced LDL aggregation (Fuhrman, Rosenblat, Hayek, Coleman, & Aviram, 2000). In this study, the addition of ginger to the regular PDB medium significantly enhanced the antioxidant activities of *M. pilosus* fermented products. When the ginger juice extract and 8-day MP extract were mixed at ratios of 10:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:10 to test the DPPH scavenging activities and compared with the 8-day MPG extract alone, the results revealed that the DPPH scavenging activity of 8-day MPG extract was greater than that of the seven mixtures (data not shown). This underscores the importance of modifications of the fermentation conditions for augmented antioxidant activities.

Before fermentation (day 0), PDBG and MPG already demonstrated greater antioxidant activities than their counterparts, PDB and MP. This observation may be due to the antioxidant components of the ginger. During fermentation, the antioxidant activities of MPG were gradually increased and the increase became more significant from day 4 to day 8. This implies that the fermentation process promoted the formation of metabolites that exert superior antioxidant activities.

To estimate the physiological safety of the 8-day MPG extract for use as a supplement, 6-week old Spraque-Dawley rats were fed the MPG extract at a dosage of 2 g/kg BW for 7 days. Histological and biochemical analyses indicated that the 8-day MPG extract had no toxic effects on the liver or the kidney (data not shown).

4. Conclusions

The present study showed that modification of culture medium by addition of ginger juice significantly enhanced the antioxidant activities of *M. pilosus* fermented products. By increasing the inherent levels of the antioxidant capacity of *Monascus* sp. fermented product, we created a nutraceutical with greater anti-atherosclerotic value.

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