JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Protective Effect of Deferricoprogen Isolated from *Monascus purpureus* NTU 568 on Citrinin-Induced Apoptosis in HEK-293 Cells

Li-Chuan Hsu,[†,‡] Ya-Wen Hsu,[†,‡] Yu-Han Liang,[†,‡] Zhi-Hu Lin,[‡] Yao-Haur Kuo,*[,‡,§] and Tzu-Ming Pan*[,†]

[†]Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei 106, Taiwan, Republic of China

[‡]National Research Institute of Chinese Medicine, Taipei 112, Taiwan, Republic of China

[§]Graduate Institute of Integrated Medicine, China Medical University, Taichung 404, Taiwan, Republic of China

ABSTRACT: *Monascus* species have traditionally been used in Asian food, with rice as their fermentation substrate. Red mold rice (RMR) contains citrinin, a nephrotoxic agent capable of exerting oxidative stress and cellular apoptosis. We investigated the components in RMR that could minimize the adverse effects of citrinin. Combining chemical separations and bioactivity assays, we identified an antioxidative component called deferricoprogen (DFC) in the fermented rice of *Monascus purpureus* NTU 568. The DFC structure was confirmed by nuclear magnetic resonance (NMR) and mass spectra analysis. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical-scavenging activity of DFC was similar to that of vitamin E. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and flow cytometric analysis showed the effect of DFC and citrinin on cell viability and cell cycle. DFC was found to be protective against the cytotoxicity and cell death induced by citrinin on human embryonic kidney (HEK-293) cells. DFC also demonstrated anti-apoptotic property in preventing citrinin-induced apoptosis.

KEYWORDS: *Citrinin, HEK-293 cells, deferricoprogen, Monascus purpureus, nephrotoxicity*

INTRODUCTION

Monascus species have traditionally been used to make food more colorful and easy to preserve in Asian countries for thousands of years. Recently, *Monascus*-fermented rice, also known as red mold rice (RMR), has been reported to possess a variety of biological functions, such as hypolipidemic effects,[1] antifatigue activities,[2] neuroprotective properties against Alzheimer's disease,[3,4] prevention of obesity,[5] and carcinogenesis[6,7] or tumor progression.[8]

Several bioactive secondary metabolites from *Monascus* species have been identified and characterized. For example, a family of monacolins possessed HMG-CoA reductase inhibitory activity.[9] Other bioactive secondary metabolites, including γ-aminobutyric acid (GABA) and dimerumic acid, have been considered to possess hypotensive[10,11] and antioxidant activities.[12] In addition, yellow pigments in *Monascus* species were demonstrated to possess cytotoxic and anti-inflammatory effects.[13,14]

However, *Monascus* species produce a mycotoxin known as citrinin.[15,16] The kidney is the major target organ of citrinin toxicity.[17] In an animal study, the continuous exposure of male mice to 200 mg/kg of citrinin through feed for 70 weeks caused mild renal lesions.[18] *In vitro* studies also confirmed that oxidative stress was an important mechanism in citrinin-induced cytotoxicity and cell death.[19,20] Because of its nephrotoxicity, the contents of citrinin need to be considerably reduced in RMR and other fermented products of *Monascus* species.

Recently, resveratrol was reported to inhibit citrinin-induced apoptosis in human hepatoma G_2 cells by its antioxidative activity.[21] In this study, we investigated antioxidative components of RMR that could minimize the nephrotoxicity of citrinin. To carry out this study, we combined chemical separations and bioactivity assays to purify an antioxidative component called deferricoprogen (DFC) from the fermented rice of *Monascus purpureus* NTU 568. We also designed several experiments to investigate the protective effects of DFC against cell injuries because of citrinin on human embryonic kidney (HEK-293) cells.

MATERIALS AND METHODS

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were run on a Bruker NMR (Unity Plus 400 MHz; Bruker BioSpin, Rheinstetten, Germany) using d_4-methanol as the solvent. High-performance liquid chromatography (HPLC) separations were performed on a Shimadzu LC-6AD series apparatus with a SPD-6AV UV detector, equipped with a 250 × 20 mm inner diameter preparative Cosmosil AR-II column (Nacalai Tesque, Inc., Kyoto, Japan).

Reagents. Methanol, acetonitrile (HPLC grade), ethanol, and methanol (analytical grade) were purchased from ECHO (Miaoli, Taiwan). Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and trypan blue were purchased from Biological Industries (Kibbutz Beit-Haemek, North District, Israel). Other chemicals, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2,2-diphenel-1-picrylhydrazyl (DPPH), vitamin E, and propidium iodide, were obtained from Sigma (St. Louis, MO).

Received: May 2, 2012
Revised: July 19, 2012
Accepted: July 26, 2012
Published: July 26, 2012

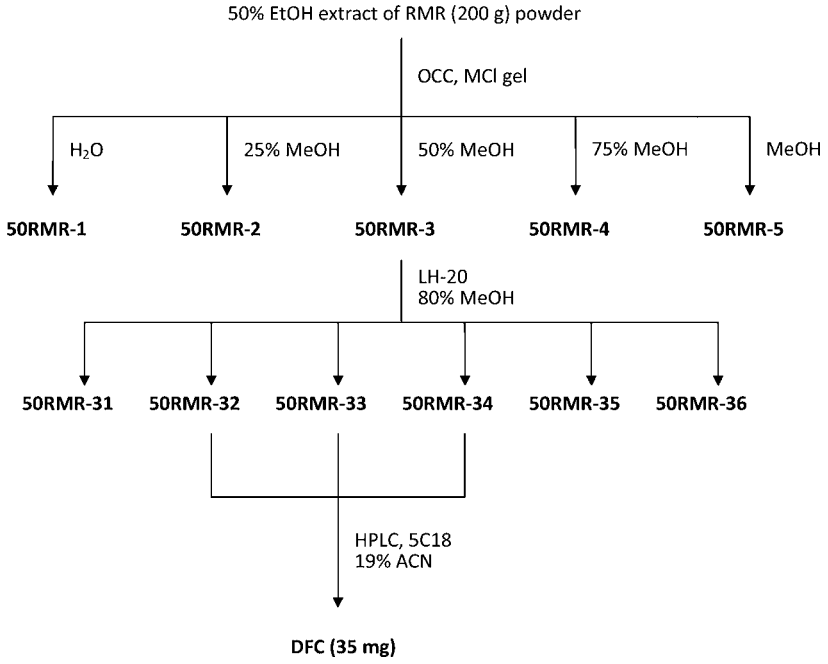

Figure 1. Flowchart for the isolation of DFC from RMR extracted in 50% ethanol.

Extraction and Purification. Isolations of DFC were performed using an activity-directed procedure; the isolation flowchart is shown in Figure 1. Briefly, the dried RMR was extracted with 50% ethanol. After filtration and concentration, the 50RMR (defined as the dried residue of 50% ethanol extract) was subject to chromatography on a MCI gel (CHP 20P, 75−150 μm; Mitsubishi Chemical Industries, Tokyo, Japan) to generate five fractions labeled 50RMR-1, 50RMR-2, 50RMR-3, 50RMR-4, and 50RMR-5. The antioxidative activities of these fractions were tested by a standard DPPH method. The fraction with the highest DPPH scavenging activity (50RMR-3) was further subjected to chromatography on a Sephadex LH-20 gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) to generate six fractions. Finally, the three fractions (50RMR-32, 50RMR-33, and 50RMR-34) that possessed the highest DPPH scavenging activities were purified by preparative HPLC to obtain purified DFC.

DPPH Radical Scavenging Assay. The antioxidative activity to scavenge DPPH free radicals was quantitated using a modified antioxidant assay.[22] Briefly, 0, 5, 10, 20, 50, 100, and 200 μg/mL of the test agents were mixed with 0.004% DPPH in methanol for 30 min. The mixture was then analyzed by spectrophotometry with an absorbance at 517 nm, which provided a relative estimate of the DPPH radical-scavenging activity.

Cell Lines and Culture. The human kidney cancer cell line HEK-293 was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were maintained in MEM (including 5% FBS) in a humidified incubator with 5% CO_2 at 37 °C.

Cytotoxicity Assay. The cytotoxicity assay was a modified version of the reported methods.[23,24] HEK-293 cells (3×10^3 per well) were seeded with 180 μL of MEM in 96-well plates. After 4 h, 20 μL of the test agents dissolved in PBS solution was added and incubated in a 37 °C incubator with 5% CO_2. After culturing for 24 h, 20 μL of the MTT solution (2 mg/mL) was added to each well and incubated for 4 h for the cellular conversion of a tetrazolium salt into a formazan product. Next, the supernatant was removed, and 200 μL of DMSO was added to dissolve the formazan. Finally, the formazan was now detectable at an absorbance of 570 nm and provided a relative estimate of the cell viability.

Assay of DNA Contents by Flow Cytometry. HEK-293 cells (5×10^4 per well) were seeded with MEM (2 mL) in 6-well plates. After 12 h, test agents dissolved in MEM solution (2 mL) were added. After 24 h of incubation, the cells were harvested and fixed with 80% ethanol for 30 min. Next, the cell pellets were washed 3 times with PBS and co-incubated with propidium iodide (4 μg/mL), Triton X-100 (1%), and RNase (0.1 μg/mL) in the dark for 30 min. Finally, the cells were analyzed by flow cytometry (FACSCalibur, Ser. No. E1577; BD Biosciences, Franklin Lakes, NJ) equipped with the CellQuest software to provide a relative estimate of the DNA content.

Caspase Activity Assay. Cells (5×10^5) were seeded with 10 mL of media in a 75 cm^2 flask. After 12 h, 10 mL of the test agent media was added to cells in the flask. After 24 h of incubation, the cells were harvested and tested for caspase-3 and caspase-9 activities using a colorimetric assay kit (BioVision, Linda Vista Avenue, CA). Caspase activity was determined according to the protocol of the manufacturer.

Statistical Analysis. Data were presented as the mean ± standard deviation ($n = 3$). The statistical comparisons were performed by one-way analysis of variance (ANOVA) with Duncan's test. The significant differences were indicated as $p < 0.05$.

RESULTS

Bioactivity-Directed Isolation of Antioxidative Components from RMR. Combining chemical separations and bioactivity assays, isolation of antioxidative components from RMR was performed as in the flowchart in Figure 1. Each fraction was examined and investigated for the antioxidative components by a standard DPPH method. Finally, the DPPH free-radical-scavenging activities of each fraction or pure compound were screened, as listed in Table 1. Briefly, the antioxidative components of RMR were rich in the 50% ethanol extracts or the fraction eluted with 50% methanol (50RMR-3). Because 50RMR-3 possessed the highest DPPH scavenging activity (90.1% at 200 μg/mL), it was further separated into additional fractions. Three of these fractions (50RMR-32, 50RMR-33, and 50RMR-34) that showed the highest DPPH scavenging activities (ranging from 90.6 to 96.7%) were purified by HPLC to obtain the antioxidative compound, DFC.

Identification of DFC from RMR. We purified DFC by preparative HPLC in the final step and characterized it according to the following MS and NMR data. ESIMS m/z: 791.5 $[M + Na]^+$ and 767.5 $[M - H]^-$; $C_{35}H_{56}N_6O_{13}$. ^1H NMR (CD_3OD, 400 MHz) δ: 6.30 (brs, H-7, 7′, 7″), 4.38 (t, J = 8.0 Hz, H-2″), 4.31 (m, H-10′), 4.01 (brs, H-2, 2′), 3.70 (t, J

dx.doi.org/10.1021/jf301889q | J. Agric. Food Chem. 2012, 60, 7880−7885

Table 1. DPPH Radical Scavenging Activities on RMR Extracts or Fractions at 200 μg/mL

sample	scavenging activity (%)
RMR	17.1 ± 0.5
50RMR	28.6 ± 0.3
50RMR-1	7.2 ± 0.6
50RMR-2	83.7 ± 1.2
50RMR-3	90.1 ± 1.4
50RMR-4	79.1 ± 1.2
50RMR-5	5.0 ± 0.4
50RMR-31	47.1 ± 1.1
50RMR-32	95.2 ± 1.5
50RMR-33	96.7 ± 1.3
50RMR-34	90.6 ± 1.4
50RMR-35	57.7 ± 0.9
50RMR-36	32.7 ± 0.5
vitamin E	100.0 ± 1.6

= 6.4 Hz, H-10, 10″), 3.66 (brs, H-5, 5′, 5″), 2.49 (t, 7.6, H-9′), 2.36 (brt, J = 6.4 Hz, H-9, 9″), 2.07 (brs, H_3-11, 11′, 11″), 1.97 (s, H_3-13), 1.83 (m, H-3, 3′, 3″, 4, 4′), 1.75 (m, H-4, 4′), 1.68 (m, H-3″, 4″). ^{13}C NMR (CD_3OD, 100 MHz) δ: 173.5 (C-1″, C-12), 170.4 (C-1, C-1′), 169.6 (C-6, C-6″), 169.3 (C-6′), 152.7 (C-8, C-8″), 151.3 (C-8), 118.4 (C-7′), 117.8 (C-7, C-7″), 63.7 (C-10′), 60.9 (C-10, C-10″), 55.7 (C-2, C-2′), 53.8 (C-2″), 48.4 (C-5, C-5′, C-5″), 44.8 (C-9, C-9″), 40.5 (C-9′), 32.4 (C-3, C-3′), 29.6 (C-3″), 24.5 (C-4″), 23.5 (C-4, C-4′), 22.3 (C-13), 18.9 (C-11, C-11′, C-11″). In the above notations, brs represents a broad singlet and brt represents a broad triplet. Because these MS and NMR data were matching the published report,[25] we confirmed that the compound was DFC. The structure of DFC was similar to dimerumic acid, which was a known antioxidant in *Monascus* species (Figure 2).

DPPH Free-Radical-Scavenging Activities of DFC. To evaluate the antioxidative effects of DFC, we used the DPPH assay and selected vitamin E as a positive control (Figure 3). The DPPH free-radical-scavenging activity of DFC (IC_{50} = 15.37 μg/mL) was similar to that of vitamin E (IC_{50} = 11.86 μg/mL). The findings implied that DFC was a suitable agent for chemoprevention studies against reactive oxygen species (ROS)-related damages in the subsequent step. For example, we established a cell model to explore the cytotoxicity of citrinin on the kidney HEK-293 cell line.

Protective Effects of DFC against Citrinin-Induced Cytotoxicity on HEK-293 Cells. To study the protective effects of DFC, we treated HEK-293 cells with citrinin (50 or 100 μM), DFC (50 or 100 μg/mL), and both of them for 24 h. After the treatments, we used a MTT assay to analyze cell viability (Figure 4). Treatment of HEK-293 cells with citrinin (50 and 100 μM) resulted in cytotoxic activity against HEK-293 cells in a dose-dependent manner. However, treatment with sufficient DFC (100 μg/mL) resulted in significant reductions of citrinin-induced cytotoxicity. The decrease in cytotoxicity possibly resulted from stimulation of cellular proliferation or prevention of cell deaths. Therefore, we investigated the changes in the cell cycle.

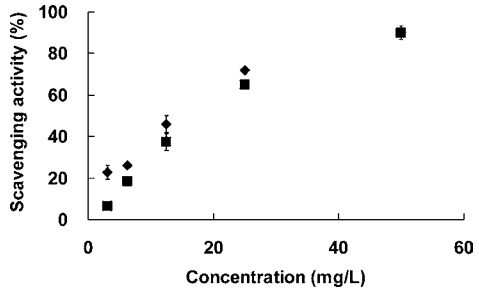

Figure 3. Free-radical-scavenging activities of DFC and vitamin E evaluated by the DPPH assay. The DPPH free radical was incubated with 3.13, 6.25, 12.5, 25, and 50 μg/mL DFC and vitamin E in the dark for 30 min. Data were expressed as the mean ± standard deviation (SD) (n = 3): (◆) vitamin E and (■) DFC.

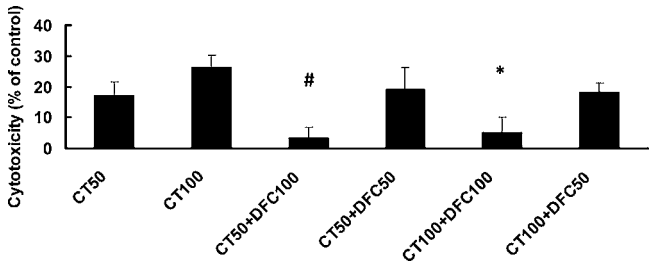

Figure 4. Protective effects of DFC against citrinin-induced cytotoxicity on HEK-293 cells. Cells were treated with citrinin (50 or 100 μM), DFC (50 or 100 μg/mL), or both for 24 h. Data were expressed as the mean ± SD (n = 3). (*) Significantly different (p < 0.01) versus the treatment of citrinin (50 μM). (#) Significantly different (p < 0.01) versus the treatment of citrinin (100 μM).

Decrease of Citrinin-Induced Cell Deaths by DFC on HEK-293 Cells. To study alternations of the cell cycle, we treated HEK-293 cells with citrinin (100 μM), DFC (100 μg/mL), or both for 24 h. After the harvesting of cells, we used

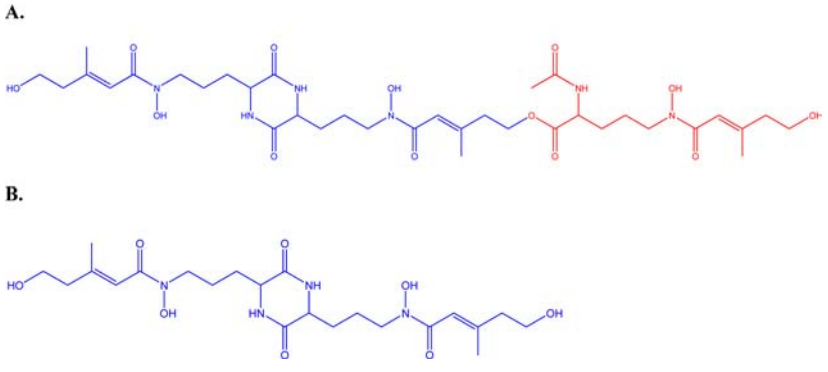

Figure 2. Structures of (A) deferricoprogen and (B) dimerumic acid.

dx.doi.org/10.1021/jf301889q | J. Agric. Food Chem. 2012, 60, 7880−7885

flow cytometry (propidium iodide staining) to analyze the ratio of the sub-G_1 area (Figure 5). Citrinin significantly induced about 5-fold greater cell death than the control group, while DFC showed no differences. However, treatment of HEK-293 cells with DFC revealed a 60% reduction of citrinin-induced cell deaths. The results suggested that DFC could prevent cell deaths induced by citrinin and maintain the survival of cells. In the next step, we followed a standard apoptotic approach to investigate the caspase activations by citrinin and DFC treatment.

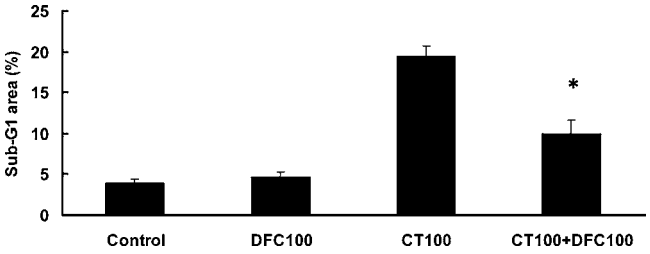

Figure 5. Flow cytometric analysis of the sub-G_1 area of HEK-293 cells. Cells were treated with citrinin (100 μM) or DFC (100 μg/mL) or co-treated with both of them for 24 h. Data were expressed as the mean ± SD (n = 3). (*) Significantly different (p < 0.05) versus the treatment of citrinin (100 μM).

Decrease of Citrinin-Induced Caspase Activations by DFC in HEK-293 Cells. HEK-293 cells were treated with citrinin (100 μM), DFC (100 μg/mL), or both for 24 h. After the treatments, we analyzed the enzyme activities of caspase-3 and caspase-9 using a colorimetric assay kit (Figure 6). Citrinin significantly induced about 3-fold greater caspase-3 activation than the control group, while DFC showed no differences. However, treatment with DFC decreased the citrinin-induced caspase-3 activation to basal levels. Treatment of HEK-293 cells with DFC also exhibited a significant decrease in citrinin-induced caspase-9 activation. Thus, DFC demonstrated an anti-apoptotic potential in preventing citrinin-induced apoptotic events, such as caspase-3 and caspase-9 activations.

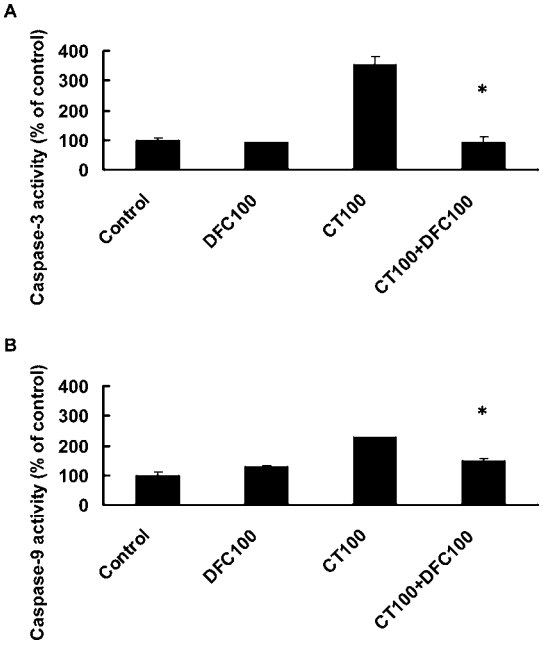

Figure 6. Protective effects of DCF against citrinin-induced caspase activations on HEK-293 cells. Cells were treated with citrinin (100 μM) or DFC (100 μg/mL) or co-treated with both of them for 24 h. (*) Significantly different (p < 0.05) versus the treatment of citrinin (100 μM).

DISCUSSION

RMR was recognized to contain various bioactive components, which were associated with chemoprevention. In prior studies, our group demonstrated that yellow pigments in *Monascus* species possessed antitumor properties.[26,27] In this study, we used bioactivity-directed isolation to obtain antioxidative components from the fermented products of *M. purpureus* NTU 568 and further investigated its protective effects. We have successfully identified an antioxidative compound called DFC, which directly removed DPPH free radicals at low dosages (IC_{50} = 15.37 μg/mL). In a previous study, an antioxidant dimerumic acid was identified in *Monascus anka*, and it was effective in reducing carbon tetrachloride-induced liver injury in a mice model.[28] Recently, a type of *Monascus* pigment, monascin, was shown to diminish oxidative damage in a *Caenorhabditis elegans* model.[29] All evidence suggest that RMR is a natural food with excellent protective effects against oxidative or other chemical stresses.

In this study, we selected citrinin as a chemical challenge to stimulate the human kidney cell line HEK-293. Using the MTT assay and flow cytometric analysis, we found that citrinin inhibited the cell viability of HEK-293 cells, resulting in their accumulation in the sub-G_1 area. Moreover, we also observed that citrinin induced several apoptotic hallmarks, such as caspase-3 and caspase-9 activations. These results were consistent with the findings of several prior studies reporting that citrinin increased ROS levels and caspase-3 activity in HEK-293 cells[30] and a human osteoblast cell line.[31] The results of all of these studies have confirmed that citrinin-induced apoptosis was potentially triggered by ROS-related damage.

In this study, we also co-incubated the isolated DFC with citrinin in HEK-293 cells. During the co-treatment, DFC effectively reduced citrinin-induced loss of cell viability. After analyzing the upstream events, we found that caspase-3 and caspase-9 activations induced by citrinin were further down-regulated by DFC. Similarly, resveratrol was reported to inhibit citrinin-induced apoptosis in human hepatoma G_2 cells.[21] This evidence has suggested that the protective effects of DFC or other antioxidants primarily resulted from their antioxidative properties. It is possible that these antioxidants inhibited ROS levels and subsequently reduced several mitochondria-mediated apoptotic responses,[32] such as caspase-9 and caspase-3 activations.[33]

Following the nephrotoxicity concerns of citrinin, several studies have been performed by our group, including the selection of *Monascus* strains with low citrinin production levels,[34] adjustment of fermentation procedures to reduce the production of citrinin,[35−37] or establishment of the quantification methods of citrinin.[38] A prior toxicity study has suggested that citrinin concentrations within 200 ppm in *Monascus*-fermented products will not negatively affect the functions of the liver and kidneys.[39] However, the citrinin level in *Monascus*-fermented products remains strictly limited as 2 ppm in European countries. In this study, we showed that 100 μg/mL DFC was sufficient to diminish the nephrotoxicity induced by 100 μM citrinin on HEK-293 cells. In comparison to the general content of citrinin (2−20 ppm) in *Monascus*-fermented

products, the DFC content (175 ppm) in RMR from *M. purpureus* NTU 568 was about 10-fold greater. These results strongly suggest that RMR fermented from *M. purpureus* NTU 568 is considered very safe because of the available amount of DFC.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +886-2-2820-1999, ext. 7061 (Y.-H.K.); +886-2-3366-4519, ext. 10 (T.-M.P.). Fax: +886-2-2823-6150 (Y.-H.K.); +886-2-3366-3838 (T.-M.P.). E-mail: kuoyh@nricm.edu.tw (Y.-H.K.); tmpan@ntu.edu.tw (T.-M.P.).

Funding

The research grant was supported by the National Science Council (NSC) and the National Research Institute of Chinese Medicine (NRICM), Taiwan, Republic of China.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Lee, C. L.; Tsai, T. Y.; Wang, J. J.; Pan, T. M. In vivo hypolipidemic effects and safety of low dosage *Monascus* powder in a hamster model of hyperlipidemia. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 533−540.

(2) Wang, J. J.; Shieh, M. J.; Kuo, S. L.; Lee, C. L.; Pan, T. M. Effect of red mold rice on antifatigue and exercise-related changes in lipid peroxidation in endurance exercise. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 247−253.

(3) Lee, C. L.; Kuo, T. F.; Wu, C. L.; Wang, J. J.; Pan, T. M. Red mold rice promotes neuroprotective sAPPalpha secretion instead of Alzheimer's risk factors and amyloid beta expression in hyperlipidemic Aβ40-infused rats. *J. Agric. Food Chem.* **2010**, *58*, 2230−2238.

(4) Lee, C. L.; Kuo, T. F.; Wang, J. J.; Pan, T. M. Red mold rice ameliorates impairment of memory and learning ability in intracerebroventricular amyloid β-infused rat by repressing amyloid β accumulation. *J. Neurosci. Res.* **2007**, *85*, 3171−3182.

(5) Chen, W. P.; Ho, B. Y.; Lee, C. L.; Lee, C. H.; Pan, T. M. Red mold rice prevents the development of obesity, dyslipidemia and hyperinsulinemia induced by high-fat diet. *Int. J. Obes.* **2008**, *32*, 1694−1704.

(6) Tsai, R. L.; Ho, B. Y.; Pan, T. M. Red mold rice mitigates oral carcinogenesis in 7,12-dimethyl-1,2-benz[*a*]anthracene-induced oral carcinogenesis in hamster. *Evidence-Based Complementary Altern. Med.* **2011**, DOI: 10.1093/ecam/nep215.

(7) Hsu, W. H.; Lee, B. H.; Pan, T. M. Red mold dioscorea-induced G_2/M arrest and apoptosis in human oral cancer cells. *J. Sci. Food. Agri.* **2010**, *90*, 2709−2715.

(8) Ho, B. Y.; Wu, Y. M.; Hsu, Y. W.; Hsu, L. C.; Kuo, Y. H.; Chang, K. J.; Pan, T. M. Effects of *Monascus*-fermented rice extract on malignant cell-associated neovascularization and intravasation determined using the chicken embryo chorioallantoic membrane model. *Integr. Cancer Ther.* **2010**, *2*, 204−212.

(9) Li, Y. G.; Zhang, F.; Wang, Z. T.; Hu, Z. B. Identification and chemical profiling of monacolins in red yeast rice using high-performance liquid chromatography with photodiode array detector and mass spectrometry. *J. Pharm. Biomed. Anal.* **2004**, *35*, 1101−1112.

(10) Tsuji, K.; Ichikawa, T.; Tanabe, N.; Obata, H.; Abe, S.; Tarui, S.; Nakagawa, Y. Extraction of hypotensive substance from wheat benikoji. *J. Jpn. Soc. Food Sci. Technol.* **1992**, *39*, 913−918.

(11) Wu, C. L.; Lee, C. L.; Pan, T. M. Red mold dioscorea has a greater antihypertensive effect than traditional red mold rice in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2009**, *57*, 5035−5041.

(12) Aniya, Y.; Yokomakura, T.; Yonamine, M.; Shimada, K.; Nagamine, T.; Shimabukuro, M.; Gibo, H. Screening of antioxidant action of various molds and protection of *Monascus anka* against experimentally induced liver injuries of rats. *Gen. Pharmacol.* **1999**, *32*, 225−231.

(13) Hsu, Y. W.; Hsu, L. C.; Chang, C. L.; Liang, Y. H.; Kuo, Y. H.; Pan, T. M. New anti-inflammatory and anti-proliferative constituents from fermented red mold rice *Monascus purpureus* NTU 568. *Molecules* **2010**, *15*, 7815−7824.

(14) Hsu, L. C.; Hsu, Y. W.; Liang, Y. H.; Kuo, Y. H.; Pan, T. M. Anti-tumor and anti-inflammatory properties of ankaflavin and monaphilone A from *Monascus purpureus* NTU 568. *J. Agric. Food Chem.* **2011**, *59*, 1124−1130.

(15) Ciegler, A.; Vesonder, R. F.; Jackson, L. K. Production and biological activity of patulin and citrinin from *Penicillium expansum*. *Appl. Environ. Microbiol.* **1977**, *33*, 1004−1006.

(16) Bragulat, M. R.; Martinez, E.; Castella, G.; Cabanes, F. J.; Ochratoxin, A and citrinin producing species of the genus *Penicillium* form feedstuffs. *Int. J. Food Microbiol.* **2008**, *126*, 43−48.

(17) Gupta, M.; Sasmal, D.; Bandyopadhyay, S.; Bagchi, G.; Chatterjee, T.; Dey, S. Hematological changes produced in mice by ochratoxin A and citrinin. *Toxicology* **1983**, *26*, 55−62.

(18) Kanisawa, M. Synergistic effect of citrinin on hepatorenal carcinogenesis of ochratoxin A in mice. *Dev. Food Sci.* **1984**, *7*, 245−254.

(19) Riveiro, S. M. R.; Chagas, G. M.; Campello, A. P.; Kluppel, L. W. Mechanism of citrinin-induced dysfunction of mitochondria. V. Effect on the homeostasis of the reactive oxygen species. *Cell Biochem. Funct.* **1997**, *15*, 203−209.

(20) Iwahashi, H.; Kitagawa, E.; Suzuki, Y.; Ueda, Y.; Ishizawa, Y.; Nobumasa, H.; Kuboki, Y.; Iwahashi, Y. Evaluation of toxicity of the mycotoxin citrinin using yeast ORF DNA microarray and oligo DNA microarray. *BMC Genomics* **2007**, *8*, 95−99.

(21) Chen, C. C.; Chan, W. H. Inhibition of citrinin-induced apoptotic biochemical signaling in human hepatoma G_2 cells by resveratrol. *Int. J. Mol. Sci.* **2009**, *10*, 3338−3357.

(22) Sokmen, M.; Angelove, M.; Krumova, E.; Pashova, S.; Ivancheva, S.; Sokmen, A. *In vitro* antioxidant activity of polyphenol extracts with antiviral properties from *Geranium sanguineum* L. *Life Sci.* **2005**, *76*, 2981−2993.

(23) Edmondson, J. M.; Armstrong, L. S.; Martinez, A. O. A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *Methods Cell Sci.* **1988**, *11*, 15−17.

(24) Morgan, D. M. Tetrazolium (MTT) assay for cellular viability and activity. *Methods Mol. Biol.* **1998**, *79*, 179−183.

(25) Frederick, C. B.; Bentley, M. D.; Shive, W. Structure of triornicin, a new siderophore. *Biochemistry* **1981**, *20*, 2436−2438.

(26) Hsu, Y. W.; Hsu, L. C.; Liang, Y. H.; Kuo, Y. H.; Pan, T. M. Monaphilones A−C, three new antiproliferative azaphilone derivatives from *Monascus purpureus* NTU 568. *J. Agric. Food Chem.* **2010**, *58*, 8211−8216.

(27) Hsu, L. C.; Hsu, Y. W.; Liang, Y. H.; Liaw, C. C.; Kuo, Y. H.; Pan, T. M. Induction of apoptosis in human breast adenocarcinoma cells MCF-7 by monapurpyridine A, a new azaphilone derivative from *Monascus purpureus* NTU 568. *Molecules* **2012**, *17*, 664−673.

(28) Aniya, Y.; Ohtani, I. I.; Higa, T.; Miyagi, C.; Gibo, H.; Shimabukuro, M.; Nakanish, H.; Taira, J. Dimerumic acid as an antioxidant of the mold, *Monascus anka*. *Free Radical Biol. Med.* **2000**, *28*, 999−1004.

(29) Shi, Y. C.; Liao, V. H. C.; Pan, T. M. Monascin from red mold dioscorea as a novel antidiabetic and antioxidative stress agent in rats and *Caenorhabditis elegans*. *Free Radical Biol. Med.* **2012**, *52*, 109−117.

(30) Chang, C. H.; Yu, F. Y.; Wang, L. T.; Lin, Y. S.; Liu, B. H. Activation of ERK and JNK signaling pathways by mycotoxin citrinin in human cells. *Toxicol. Appl. Pharmacol.* **2009**, *237*, 281−287.

(31) Huang, Y. T.; Lai, C. Y.; Lou, S. L.; Yeh, J. M.; Chan, W. H. Activation of JNK and PAK2 is essential for citrinin-induced apoptosis in human osteoblast cell line. *Environ. Toxicol.* **2008**, *10*, 343−355.

(32) Hengartner, M. O. The biochemistry of apoptosis. *Nature* **2000**, *407*, 770−776.

(33) Budihardjo, I.; Oliver, H.; Lutter, M.; Luo, X.; Wang, X. Biochemical pathways of caspase activation during apoptosis. *Ann. Rev. Cell Dev. Biol.* **1999**, *15*, 269−290.
(34) Wang, J. J.; Lee, C. L.; Pan, T. M. Modified mutation method for screening low citrinin-producing strains of *Monascus purpureus* on rice culture. *J. Agric. Food Chem.* **2004**, *52*, 6977−6982.
(35) Wang, J. J.; Pan, T. M. Improvement of monacolin K, γ-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 669−676.
(36) Lee, C. L.; Hung, H. K.; Wang, J. J.; Pan, T. M. Improving the ratio of monacolin K to citrinin production of *Monascus purpureus* NTU 568 under dioscorea medium through the mediation of pH value and ethanol addition. *J. Agric. Food Chem.* **2007**, *55*, 6493−6502.
(37) Lee, C. L.; Chen, W. P.; Wang, J. J.; Pan, T. M. A simple and rapid approach for removing citrinin and remaining monacolin K in red mold rice. *J. Agric. Food Chem.* **2007**, *55*, 11101−11108.
(38) Lee, C. L.; Wang, J. J.; Pan, T. M. Synchronous analysis method for detection of citrinin and the lactone and acid forms of monacolin K in red mold rice. *J AOAC Int.* **2006**, *89*, 669−677.
(39) Lee, C. H.; Lee, C. L.; Pan., T. M. A 90-D toxicity study of *Monascus*-fermented products including high citrinin level. *J. Food Sci.* **2010**, *75*, 91−97.