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Enhanced Hypolipidemic Effect and Safety of Red Mold Dioscorea Cultured in Deep Ocean Water

Chun-Lin Lee,[†] Yi-Hsin Kung,[†,§] Jyh-Jye Wang,[#] Tzu-Ying Lung,[†] and Tzu-Ming Pan*[,⊥]

[†]Department of Life Science, National Taitung University, Taitung, Taiwan, Republic of China

[§]R&D Division, Sunway Biotechnology Company Limited, Taipei, Taiwan, Republic of China

[#]Department of Nutrition and Health Science, Fooyin University, Kaohsiung, Taiwan, Republic of China

[⊥]Department of Biochemical Science and Technology, National Taiwan University, Taipei, Taiwan, Republic of China

ABSTRACT: Red mold dioscorea (RMD) produced by *Monascus* sp. was proven to be a hypolipidemic functional food. Deep ocean water (DOW), that is, water obtained from over 200 m deep in the ocean, was found to promote the growth of fungus via its mineral richness. On the basis of the advantages, this study used 650 m DOW as the culture water to culture *Monascus purpuresus* NTU 568 and produce the DOW-RMD. The goal of this study is to compare the difference between DOW-RMD and reverse osmosis water-cultured RMD (ROW-RMD) on the hypolipidemic effect. Hyperlipidemic hamsters were fed a high-cholesterol diet and administered various doses of DOW-RMD or ROW-RMD for 8 weeks. After sacrifice, biochemical analyses in serum, liver, and feces were carried out. The results showed that DOW-RMD had a greater effect on lowering cholesterol levels and lipid peroxidation in serum and lipid plaque in heart aorta than ROW-RMD. However, DOW was likely to modulate the *Monascus* metabolite biosynthesis pathway toward the formation of hypolipidemic yellow pigments (such as monascin and ankaflavin) rather than red pigments and the mycotoxin citrinin. In addition, the DOW with higher Mg^{2+} ion was proven to absorb into DOW-RMD; however, the accumulation of Mg^{2+} ions should contribute a greater hypolipidemic effect to DOW-RMD. Comprehensively, the DOW-induced metabolism modulation and the ions of DOW were a benefit to the development of safe DOW-RMD with low citrinin levels and high hypolipidemic, antiatherosclerosis, and anti-fatty liver effects.

KEYWORDS: red mold dioscorea, *Monascus*, hypolipidemic

INTRODUCTION

Monascus species has been used as the traditional food fungus in Eastern Asia for several centuries. *Monascus*-fermented products such as red mold rice (RMR) or red mold dioscorea (RMD) were gradually developed as the popular functional food for the prevention of hypolipidemia. RMD, a novel and beneficial *Monascus*-fermented dioscorea, was first studied in our previous research.[1] Because a stronger hypolipidemic effect was found in RMD rather than in RMR, RMD became a popular *Monascus* product.[2] With regard to the functional ingredients for hypolipidemic effect, monacolin K, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitor, is proven as the functional ingredient of the *Monascus*-fermented product for lowering cholesterol.[3] Furthermore, the two yellow pigments, monascin and ankaflavin, were proven to have a significant effect on lowering serum cholesterol, triglyceride, and low-density lipoprotein cholesterol (LDL-C) levels and raising high-density lipoprotein cholesterol (HDL-C) levels in the hyperlipidemic hamster model in our previous study.[4]

According to the above, the formation of secondary metabolites is very close to the health function of *Monascus*-fermented product. Culture conditions or the components added would significantly affect protein expression and levels of the secondary metabolites of *Monascus* species.[5−7] However, production of the functional hypolipidemic ingredients of *Monascus*-fermented product including monacolin K, monascin, and ankaflavin was increased by supplementation of the special components to stimulate the hypolipidemic effect.[8] Meanwhile, citrinin, known as the mycotoxin involving damage of the liver and kidney, was found in RMR.[9] Although the *Monascus*-fermented product has not been found to cause servere damage in the liver or kidney, the safety of *Monascus*-fermented products including higher citrinin concentrations was always of concerned. Therefore, lowering the citrinin concentration was a basal and important mission for the production of safe *Monascus*-fermented product. However, altering the composition of the medium was regarded as a useful method for mediating the citrinin concentration of *Monascus*-fermented product.

Deep ocean water (DOW) generally means ocean water from a depth of more than 200 m. The character of DOW includes high purity, cold temperature, abundant nutrients, and minerals.[10,11] Currently, DOW has been applied in the food, agriculture, cosmetic, and medical fields due to its high contents of minerals such as magnesium (Mg), calcium (Ca), potassium (K), and zinc (Zn).[12−16] The application of DOW in fermentation has been little studied. However, the minerals rich in DOW are likely to promote the growth rate or metabolite production of microorganisms via acting as cofactors of the key enzymes. For these reasons, the application of DOW in the stimulation of the biomass formation and functional metabolite production of microorganism is probably useful.[11,17]

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According to the above, the minerals and trace elements of DOW probably acted as the nutrients of microorganisms. Furthermore, DOW was proven to have a hypolipidemic effect in previous studies.[15,16] Therefore, *Monascus*-fermented DOW-RMD may have a greater hypolipidemic effect if DOW was chosen as the culture water. DOW may directly promote the hypolipidemic effect by itself or indirectly modulate the production of functional metabolites of DOW-RMD. DOW had never been used as a material of functional microorganisms to produce a functional product. Therefore, the goal of this study is to compare the hypolipidemic effects between DOW-RMD and ROW-RMD. Furthermore, the metabolites of DOW-RMD and ROW-RMD were measured and compared using high-performance liquid chromatography−photodiode array (HPLC-PDA) to investigate which metabolites mediated by DOW resulted in the alteration of the hypolipidemic effect of RMD. In addition, the citrinin concentration of DOW-RMD would be monitored due to safety concerns.

MATERIALS AND METHODS

Chemicals. Thiobarbituric acid (TBA) and malondialdehyde (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO). Liquid chromatography (LC) grade acetonitrile, chloroform, methanol, and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). Tryptone, yeast extract, peptone, malt extract, potato dextrose agar (PDA), and Bacto-agar were purchased from Difco Co. (Detroit, MI).

Source of DOW. The DOW purchased from the Taiwan Yes Deep Ocean Water Co. (Hualien, Taiwan) was pumped from a depth of 670 m in the Pacific Ocean near eastern Taiwan and processed through electrodeionization. The measurement of the concentration of the trace elements and minerals in DOW including Al, Cu, Zn, As, Ba, Cd, Cr, Pb, Hg, Se, Ag, Ca, Mg, K, Na, Sb, Tl, Be, fluoride, nitrate (as N), sulfate, chloramines, and chlorine was carried out by the commission agent Tze-Chiang Foundation of Science and Technology (Hsinchu, Taiwan).

Preparation of DOW-RMD and ROW-RMD. *Monascus purpureus* NTU 568 fermented product has been proven to have a potent hypolipidemic effect in our previous study.[2,18] The culture strain was maintained on PDA slant at 10 °C and transferred monthly. The dioscorea root (*Dioscorea batatas* Dence) purchased from a local supermarket in Taiwan was used to produce RMD using the method of solid-state culture. DOW was used as all of the water used in the production of DOW-RMD. Five hundred grams of dioscorea substrates was soaked in distilled water for 8 h. After that, excess water was removed with a sieve. The substrate was autoclaved for 20 min at 121 °C in a "koji-dish" (the koji-dish is made of wood with dimensions of 30 × 20 × 5 cm). After being cooled, the substrate was inoculated with a 5% (v/w) spore suspension (10^7 spores/mL) and 0.3% (v/w) ethanol. The inoculated substrate was cultured at 30 °C for 10 days. In addition, during the culturing stage, 100 mL of water waadded once every 12 h for a total of three times and the addition of water started on the fifth day of culture. After fermentation, the crushed and dried product with the mold was used for the experiments.[19]

Animals and Diets. Forty male Golden Syrian hamsters weighing 100−120 g were housed in individual plastics cages and subjected to a 12 h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C. The animals were given free access to regular rodent chow and water for 4 weeks to adapt to the new environment. Hamsters were weighed and randomly assigned to five groups of eight animals each before the commencement of the animal experiment.

Dose and Grouping. The dose of RMD powder was calculated in accordance with Boyd's formula of body surface area as recommended.[20] This study used 1 g of RMD as the reference dose of an adult with a weight of 65 kg and a height of 170 cm to calculate the hamster dose according to our previous study.[2] After the prebreeding stage for 4 weeks, all test samples were respectively suspended in 1 mL of water and orally administered to the hamsters using a stomach tube for 8 weeks. Food intake was recorded daily, and animals were weighed weekly.

Experimental diets were provided in accordance with AIN-76 diet formulation with modification. The control group was fed a normal diet via AIN-76 formulation, and the HC group was given a high-cholesterol diet including 0.2% cholesterol.[2] ROW-RMD, DOW-R1X, and DOW-R2X groups were fed the high-cholesterol diet and orally given a 1-fold dosage of ROW-RMD (107.83 mg/kg/day), a 1-fold dosage of DOW-RMD (107.83 mg/kg/day), or a 2-fold dosage of DOW-RMD (215.66 mg/kg/day), respectively.

Twenty-four hours before sacrifice, all food was removed. Animals were anesthetized and sacrificed by carbon dioxide inhalation. Whole blood was collected from the celiac vein and divided to heparin tubes for plasma preparation and nonheparin tubes for serum preparation. After centrifugation (5000*g*, 15 min), the plasma and serum samples were collected and then stored at −80 °C. Liver tissue was lavaged and rinsed frequently with a 0.8% sodium chloride solution to eliminate any blood. The biggest leaf of liver tissue was ground in ice-cold phosphate-buffered saline (PBS) and then centrifuged (8000*g*, 15 min). The supernatant was collected and stored at −80 °C for the assay of superoxide dismutase (SOD) activity and thiobarbituric acid reactive substances (TBARS). Part of the liver tissue were immersed in 10% formalin stock and then examined for pathology using H&E staining. The rest of the liver tissue was immersed in liquid nitrogen and then stored at −80 °C.

Serum, Liver, and Fecal Lipid Analysis. Serum total cholesterol (TC), triglyceride (TG), and HDL-C levels were measured in triplicate using commercial enzymatic kits. These kits were as follows: a TC assay kit (CH 200, Randox Laboratories Ltd., Antrim, U.K.), a TG assay kit (TR-210, Randox Laboratories Ltd.), and an HDL-C assay kit (CH-203, Randox Laboratories Ltd.). Serum LDL-C levels were gained via the following calculation:[18] LDL-C (mg/dL) = TC − TG/5 − HDL-C. Liver tissue and feces (0.5 g) were ground in 10 mL of ice-cold Folch solution (chloroform/methanol = 2:1; v/v) and incubated for 30 min at room temperature. The aqueous layer was aspirated and discarded, and the fixed volume of the organic layer was then evaporated to dryness. The dried lipid layer was dissolved with an equal volume of DMSO and then used to determine the TC and TG levels using commercial enzymatic kits.

Determination of TBARS Content. The TBARS assay is also regarded as the accepted determination for in vivo lipid peroxidation.[21,22] According to the procedure of the previous study, the TBARS levels of serum and liver were determined by the method of TBA colorimetric analysis, and the optical density (OD) value was measured at 532 nm.[23]

Stain of Aortic Plaque in Heart Aorta. The heart aorta was cut open longitudinally along the anterior side, and the lipid-rich lesions on the surface of the aorta were stained with 2% Sudan IV and then successively washed with a gradient concentration of methanol (100, 90, 80, 70, and 60%) and PBS. The whole surface area of the thoracic aorta was stained by Sudan IV and photographed using a digital camera. The aortic surface area and its stained plaque area (red) were selected and quantitated by the Posterize program of Photoshop CS2 software (Adobe Systems Inc., San Jose, CA). The selected pixel of the plaque area and whole aorta was used to calculate the percent area of the aortic plaque,[2] as follows:

$$\text{aortic plaque (\%)} = (\textit{pixelofstainedplaquearea}/\textit{pixelofwholeaorta}) \times 100\%$$

Plasma Liver Index Analysis. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured in triplicate using an automatic biochemical analyzer (Beckman-700, Fullerton, CA).

Determination of Monascin, Ankaflavin, and Citrinin Concentrations of RMD. DOW-RMD or ROW-RMD (1 g) was extracted respectively with 10 mL of ethanol at 60 °C for 30 min. The extracts (10%, w/v) were further filtered with 0.45 μm pore size filter and analyzed by HPLC (model L-2130, Hitachi Co., Tokyo, Japan). HPLC was performed according to the method described previously[24] in triplicate. Yellow pigments (monascin and ankaflavin) and red pigments were detected using a photodiode array detector (model L-2455 DAD, Hitachi Co.) set at 231 nm and full wavelength. For citrinin analysis, the fluorescence detector (model L-2485 FL detector, Hitachi Co.) was set with an excitation wavelength of 330 nm and an emission wavelength of 500 nm.

Determination of the Mg^{2+} and Ca^{2+} of DOW-RMD and ROW-RMD. The concentrations of Mg^{2+} and Ca^{2+} ions in RMD were measured in triplicate using the commercial kit of magnesium (MG 531, Randox Laboratories Ltd.) and calcium (CA 2390, Randox Laboratories Ltd.).

Statistics. Data are expressed as the mean ± SD. The statistical significance in the biochemical effects was determined by one-way analysis of variance (ANOVA), followed by ANOVA with Duncan's multiple test.

RESULTS

Final Body Weight and Daily Intake. In this study, DOW-RMD and ROW-RMD were administered respectively to hyperlipidemic hamsters for 8 weeks to investigate whether DOW promotes *M. purpureus* NTU 568 to produce RMD with a greater hypolipidemic effect. The final body weight and average daily intake of hamsters are shown in Table 1. The results indicate that the final body weight and daily intake of the hamsters showed no significant differences among the various groups during the period of the practical experiment. In addition, the externals and health of all experimental animals had a normal expression.

Table 1. Body Weight and Daily Feed Intake of Experimental Hamsters[a]

group	body weight (g)	daily feed intake (g/day)
NOR	106.4 ± 8.1 a	6.10 ± 0.65 a
HC	105.2 ± 10.0 a	5.96 ± 0.71 a
ROW-R1X	102.3 ± 7.7 a	5.82 ± 0.59 a
DOW-R1X	103.2 ± 6.2 a	6.02 ± 0.61 a
DOW-R2X	103.7 ± 8.5 a	5.98 ± 0.60 a

[a] Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group). Mean values within each column with different letters are significantly different ($p < 0.05$).

Total Cholesterol and Triglyceride in Serum. The results of serum TC and TG levels are shown in Table 2. ROW-RMD significantly lowered the TC and TG levels as seen in our previous study involving the hypolipidemic test of RMD.[2] In addition, a significant lowering effect, as compared with the HC group, was also found in the group treated with ROW-RMD or DOW-RMD. However, the dosage of DOW-R1X group (107.83 mg/kg/day) was equal to that of the ROW-R1X group. Therefore, the results indicated that DOW-RMD exhibited a more significant effect (by 24.11 and 50.53%, $p < 0.05$) in lowering TC and TG levels than ROW-RMD (by 13.98 and 42.69%, $p < 0.05$), as compared with the HC group. This means that the TC- and TG-lowering effects performed by DOW-RMD should be promoted by the use of DOW during the fermentation process.

HDL-C, LDL-C, and the LDL-C/HDL-C Ratio in Serum. *Monascus*-fermented RMD and RMR were proven to enhance HDL-C levels and decrease LDL-C levels in our previous studies.[2] In Table 2, the treatment with a 1-fold dosage of ROW-RMD had a significant effect on decreasing LDL-C levels (by 23.37%, $p < 0.05$), but not on raising HDL-C levels (by 7.53%, $p > 0.05$), as compared with the HC group. However, significant effects on both lowering LDL-C levels and raising HDL-C levels were seen (by 29.73 and 19.79%, $p < 0.05$) in the treatment with 1-fold dosage of DOW-RMD (DOW-R1X), respectively, as compared with the HC group. The 2-fold dosage of DOW-RMD (DOW-R2X) also resulted in significantly increased HDL-C levels (by 18.35%, $p < 0.05$), as well as significantly decreased LDL-C levels (by 35.21%, $p < 0.05$).

The ratio of LDL-C to HDL-C is another criterion for the development of cardiovascular diseases. If the ratio is low, then the TC level would include a higher protective factor HDL-C level and a lower risk factor LDL-C level. The results in Table 2 indicate that feeding a high-cholesterol diet for 8 weeks would lead to an increase in the ratio of LDL-C to HDL-C, as compared to that of the NOR group ($p < 0.05$). Therefore, the results obtained by a statistical analysis showed that the ratio of LDL-C to HDL-C was less by 40.80% in the DOW-R1X group than in the HC group ($p < 0.05$). The 1-fold dosage of ROW-RMD (ROW-R1X) showed less of an effect on lowering the ratio of LDL-C to HDL-C by 35.06% ($p < 0.05$), as compared with the HC group.

Total Cholesterol and Triglyceride in Liver and Feces. ROW-RMD was proven to decrease liver TC and TG concentrations as seen in our previous study.[2] As expected, hamsters

Table 2. Effect of ROW-RMD and DOW-RMD on Levels of TC, TG, HDL-C, and LDL-C and the LDL-C/HDL-C Ratio in Serum of Hyperlipidemic Hamsters Fed a High-Cholesterol Diet[a]

group	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	LDL-C/HDL-C ratio
NOR	113.8 ± 6.9 a	126.3 ± 23.7 b	61.7 ± 5.5 a	20.0 ± 2.5 a	0.310 ± 0.035 a
HC	223.1 ± 15.0 d	197.7 ± 45.3 c	97.0 ± 7.7 b	67.6 ± 10.8 d	0.696 ± 0.159 d
ROW-R1X	191.9 ± 6.2 c	113.3 ± 14.3 ab	104.3 ± 9.4 b	51.8 ± 4.4 c	0.452 ± 0.050 c
DOW-R1X	169.3 ± 33.2 b	97.8 ± 13.1 a	116.2 ± 7.3 c	47.5 ± 3.3 bc	0.412 ± 0.023 bc
DOW-R2X	173.5 ± 16.8 bc	102.9 ± 16.3 ab	114.8 ± 7.4 c	43.8 ± 3.9 b	0.381 ± 0.036 b

[a] Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group). Mean values within each column with different letters are significantly different ($p < 0.05$).

Table 3. Effect of ROW-RMD and DOW-RMD on the Levels of TC and TG in Liver and Feces of Hyperlipidemic Hamsters Fed a High-Cholesterol Diet[a]

	liver		feces	
group	TC levels (mg/g)	TG levels (mg/g)	TG levels (mg/g)	TG levels (mg/g)
NOR	22.01 ± 3.34 ab	61.87 ± 12.23 a	30.76 ± 2.03 ab	25.31 ± 2.02 a
HC	49.52 ± 11.91 d	83.77 ± 12.32 b	60.38 ± 6.38 d	88.61 ± 5.83 d
ROW-R1X	33.09 ± 4.14 c	65.40 ± 11.67 a	50.19 ± 4.68 c	72.50 ± 2.41 c
DOW-R1X	26.15 ± 7.52 b	60.50 ± 10.41 a	28.80 ± 2.42 a	67.70 ± 5.38 c
DOW-R2X	15.18 ± 5.51 a	61.27 ± 11.70 a	35.88 ± 4.88 b	55.21 ± 7.44 b

[a] Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group). Mean values within each column with different letters are significantly different ($p < 0.05$).

treated with a high-cholesterol diet for 8 weeks showed a remarkable increase in liver TC and TG levels as compared to the NOR group ($p < 0.05$) (Table 3). A 1-fold dose of DOW-RMD (DOW-R1X) had a greater effect on lowering liver TC levels (by 47.19%, $p < 0.05$) than did ROW-R1X (by 33.18%, $p < 0.05$), respectively. However, both DOW-RMD and ROW-RMD showed significant effects on lowering high-cholesterol diet-raised liver TG levels ($p < 0.05$) as compared to the HC group, but there was no significant effect between them ($p > 0.05$).

The decline of cholesterol levels might result from the blocking of the cholesterol biosynthesis pathway or the promotion of fecal cholesterol excretion. Therefore, fecal TC was regarded as another marker for investigating the metabolism of cholesterol. As shown in Table 3, tendencies toward the decrease in fecal TC and TG excretion were found in RMD treatment groups. However, DOW-RMD and ROW-RMD had similar tendencies to decrease fecal TC and TG excretion. This probably implied that DOW-RMD and ROW-RMD should decrease serum cholesterol levels through the inhibition of liver cholesterol biosynthesis rather than the stimulation of fecal cholesterol excretion. However, TC and TG excretions in the DOW-RMD treatment were lower than in the ROW-RMD treatment, which may result from the DOW-R1X group having lower serum and liver TC and TG levels than the ROW-R1X group.

Lipid Peroxidation in Serum. The serum LDL would be transformed into oxidative LDL because of lipid peroxidation and would result in atherosclerosis development. Figure 1 indicates that feeding a high-cholesterol diet stimulated the increase of MDA levels in the HC group. However, the treatment with DOW-RMD or ROW-RMD was helpful for repressing the occurrence of lipid peroxidation. Importantly, the 1-fold dosage of DOW-RMD (DOW-R1X) performed a stronger suppression in high-cholesterol-diet-induced MDA content than did ROW-RMD, which suggested that DOW should act as stimulant for raising the potential of RMD on the prevention of lipid peroxidation.

Lipid Plaque in Heart Aorta. The deposition content of lipid plaque was associated with the occurrence of atherosclerosis development. The photograph and the statistical deposition contents of lipid plaque of various groups are shown in Figure 2. The HC group had the greatest content of plaque deposition in the aorta among all groups. The deposition was decreased by treatment with DOW-RMD or ROW-RMD. A significant lowering effect by 37.32% was shown in the ROW-R1X group, as compared to the HC group ($p < 0.05$). However, the 1-fold dosage of DOW-RMD contributed more potent levels by 64.34%

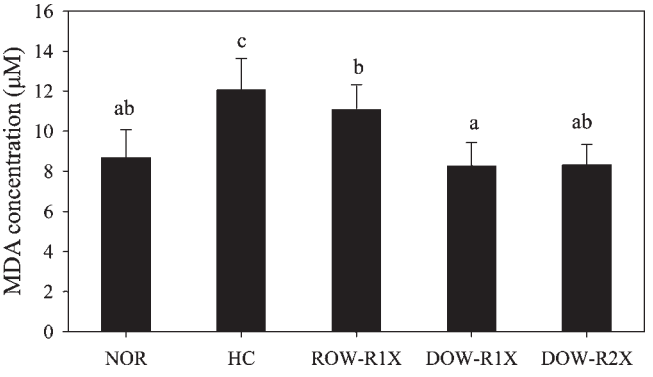

Figure 1. Effect of ROW-RMD and DOW-RMD on lipid peroxidation in serum of hyperlipidemic hamsters fed a high-cholesterol diet. Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group). Mean values with different letters are significantly different ($p < 0.05$).

($p < 0.05$, as compared with the HC group) to lower the deposition of lipid plaque in the surface of heart aorta. Therefore, the trend that DOW-RMD performed stronger inhibition of atherosclerosis-associated lipid plaque deposition than ROW-RMD should be caused by the use of DOW during the fermentation process.

Liver Function Test and Pathological Examination. Serum ALT and AST activities regarded as markers in the liver function test were measured in this study to evaluate the protection of DOW-RMD against high-cholesterol-diet-induced liver damage. As shown in Figure 3, feeding a high-cholesterol diet resulted in an increase in serum ALT and AST activities of the HC group, and with significant difference ($p < 0.05$, as compared with the NOR group). However, treatment with DOW-RMD would not intensify the cholesterol-increased ALT and AST activity even though a 2-fold dosage was used. Contrarily, the damage was reversed by the treatment with DOW-RMD, which was more potent than that by the treatments with ROW-RMD. The results implied that DOW was able to strengthen the protection of RMD against cholesterol-induced liver damage.

According to the pathological examination of the liver section, the HC group fed a high-cholesterol diet was found to show

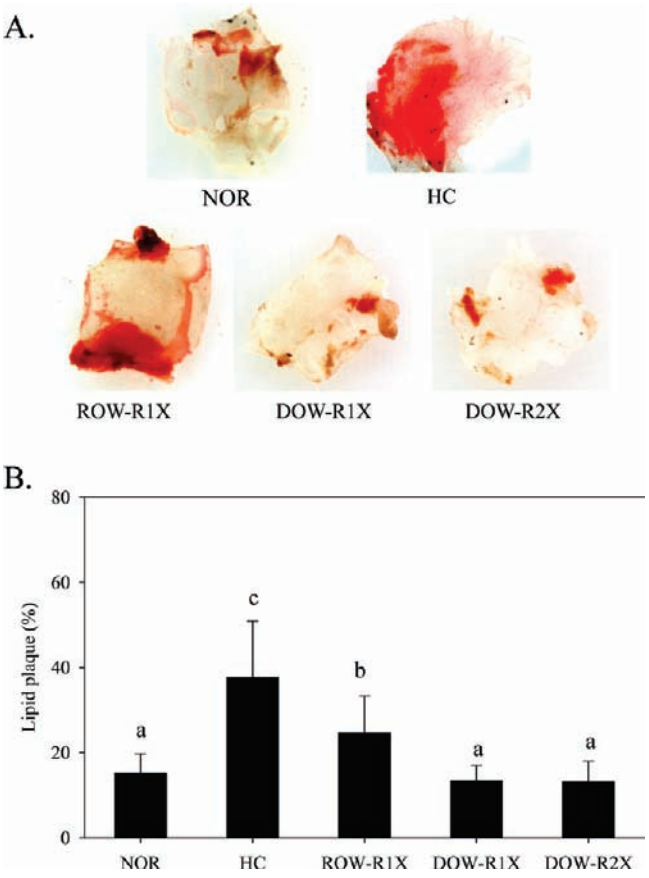

Figure 2. Effect of ROW-RMD and DOW-RMD on the atherosclerotic plaque in the heart aorta of hyperlipidemic hamsters: (A) atherosclerotic plaque presented as the red dye in the graph; (B) proportion of the area of the atherosclerotic plaque in the aorta. Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group). Mean values with different letters are significantly different ($p < 0.05$).

serious fatty liver (Figure 4). However, feeding DOW-RMD had more potent prevention for fatty liver development than feeding ROW-RMD. Feeding a 2-fold dosage of DOW-RMD (DOW-R2X) showed a dose-dependent effect on the prevention of fatty liver.

Comparison of Metabolites between DOW-RMD and ROW-RMD. To determine why DOW-RMD had a greater hypolipidemic effect than ROW-RMD, the metabolites in DOW-RMD and ROW-RMD were measured and compared using HPLC-PDA, as shown in Figure 5. Yellow pigments had two maximum absorption wavelengths at 230 and 400 nm, and the red pigments had three maximum absorption wavelengths at 300, 400, and 520 nm. According to the full wavelength and standards comparison, the two peaks (retention times at 14.8 and 29.7 min) were identified as the two yellow pigments, monascin and ankaflavin, which were proven to be the hypolipidemic agents in our previous study.[4] The two components were found to express higher area amount in DOW-RMD than in ROW-RMD. However, the peaks of red pigment (retention times within 0−5 min) had higher peak areas in ROW-RMD than in DOW-RMD. In addition, the monacolin

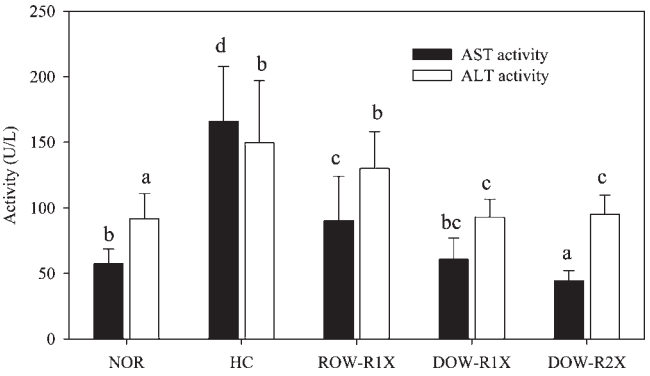

Figure 3. Effect of various samples on the serum aspartate aminotransferase (AST) and alanine transferase (ALT) activity of experimental hamsters. Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group). Mean values with different letters are significantly different ($p < 0.05$).

K peak (with a single maximum absorption wavelength at 237 nm) was not found in the photodiode array chromatogram of ROW-RMD or DOW-RMD because *Monascus* was not cultured under a monacolin K-producing condition. This consideration was based on the concern for the safety and side effects of monacolin K involved in liver injury,[26] rhabdomyolysis,[27,28] Q10 deficit,[29,30] etc. Therefore, the hypolipidemic effect of ROW-RMD or DOW-RMD was not contributed from monacolin K in this study. However, the effect of DOW on the monacolin K production is one of the important areas associated with DOW-modulated change in *Monascus* metabolite biosynthesis, which should be studied in the future. In addition to monacolin K, modulation of the *Monascus*-fermented secondary metabolites by DOW is shown in Table 4. DOW-RMD fermentation using 100% DOW had greater monascin and ankaflavin production by 72.07 and 25.12% than ROW-RMD ($p < 0.05$). With regard to the variation of mycotoxin citrinin formation, increasing the concentration of DOW was useful in lowering citrinin formation during the fermentation of RMD. DOW-RMD had lower citrinin levels than ROW-RMD by 31.14% ($p < 0.05$). The results suggested that DOW mediated the secondary metabolites of *Monascus* fermentation toward the formation of hypolipidemic pigments (monascin and ankaflavin), as well as the inhibition of citrinin biosynthesis.

Comparison of the Ratio of Ca^{2+}/Mg^{2+} between DOW-RMD and ROW-RMD. During the fermentation process of DOW-RMD, DOW was used instead of ROW and supplemented to the substrate. DOW including the ions and trace elements should be absorbed into the RMD. However, DOW was proven to prevent the development of cardiovascular disease because of the low Ca^{2+}/Mg^{2+} ratio.[16] To understand whether DOW contributed this advantage to DOW-RMD, the Ca^{2+} and Mg^{2+} ions of DOW-RMD and ROW-RMD were measured. The DOW used in this study included 20.65 mg/L Mg^{2+} ions and 5.02 mg/L Ca^{2+} ions (Table 5). However, the DOW-RMD had greater Mg^{2+} concentration (2.53 ± 0.27 mg/g) than the ROW-RMD (1.90 ± 0.02 mg/g) (Table 6). In addition, the accumulated Ca^{2+} concentration in DOW-RMD (1.58 ± 0.11 mg/g) showed no significant difference as compared to ROW-RMD (1.53 ± 0.24 mg/g)

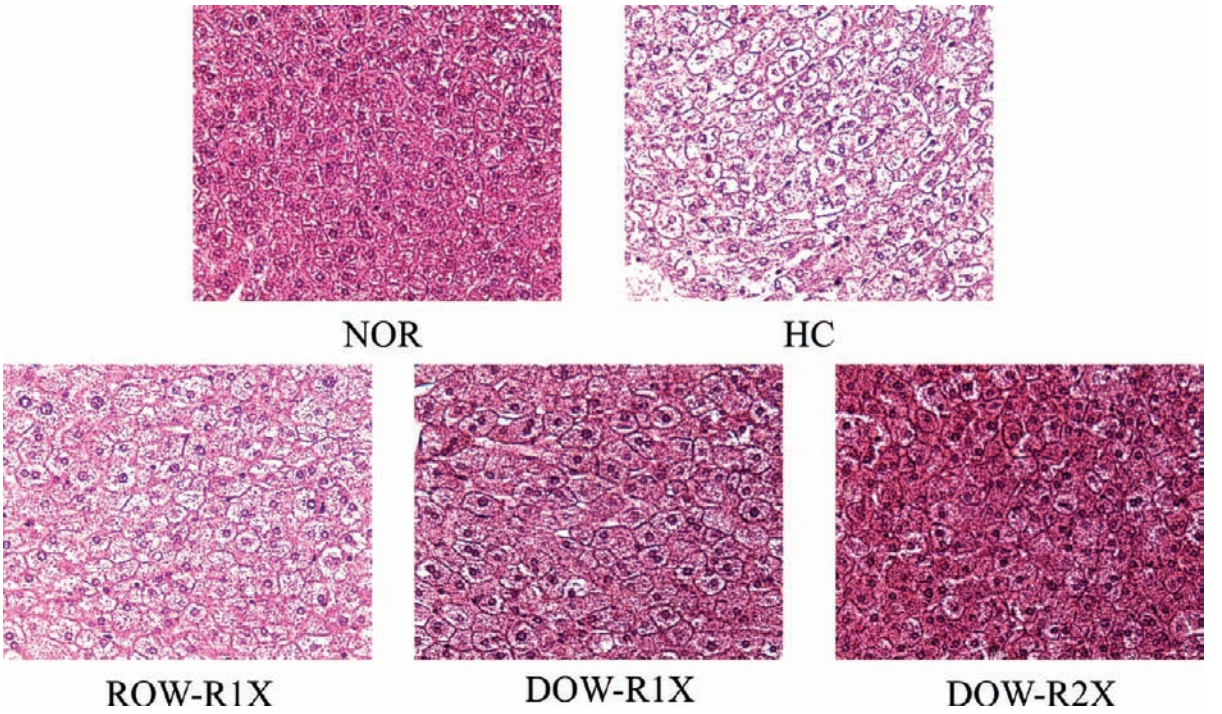

Figure 4. Pathological examination of liver of experimental hamsters in the ×400 power field. The liver sections were stained using H&E and observed in the light microscope. Two groups of hamsters were fed a normal diet (NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered a 1-fold dose of ROW-RMD (107.83 mg/kg/day) (ROW-R1X group), a 1-fold dose of DOW-RMD (107.83 mg/kg/day) (DOW-R1X group), or a 2-fold dose of DOW-RMD (215.66 mg/kg/day) (DOW-R2X group).

($p > 0.05$). However, the ratios of Ca^{2+}/Mg^{2+} in DOW-RMD and ROW-RMD were obtained at 0.63 ± 0.11 and 0.81 ± 0.14, respectively. The lower Ca^{2+}/Mg^{2+} ratio in DOW-RMD should be contributed from the supplementation of DOW during the fermentation process, which may advance the hypolipidemic effect of RMD.

DISCUSSION

DOW, a rare water source, was developed as a biotechnology material and functional food in many previous studies.[12,15,16,31−33] This study is the first to use DOW as the culture water of functional microorganisms to strengthen the healthy function. DOW-RMD has greater hypolipidemic effect than ROW-RMD. The advantage should be contributed from the minerals and trace elements of DOW. The DOW is proven to prevent cardiovascular disease via cholesterol-lowering and triglyceride-lowering effects in the previous study,[33] which was probably the result of the lower ratios of Ca^{2+}/Mg^{2+} in DOW. During the production process of the DOW-RMD, the ions and trace elements of DOW would be absorbed and further accumulated in the RMD via the daily supplementation of DOW. The DOW used in this study included 20.65 mg/L Mg^{2+} ions and 5.02 mg/L Ca^{2+} ions (Table 5). Furthermore, higher concentrations of Mg^{2+} and Ca^{2+} were measured in DOW-RMD than in ROW-RMD, suggesting that the ions in DOW would be absorbed and accumulated in the DOW-RMD. Therefore, the health function of DOW should contribute to the DOW-RMD. The accumulation of high concentrations of ions and trace elements may be one reason DOW-RMD showed greater hypolipidemic effect than ROW-RMD.

Another key reason involving higher hypolipidemic effect of DOW-RMD strengthened by DOW use should result from the alteration and mediation of secondary metabolite biosynthesis in *Monascus* spp. In this study, the metabolites of DOW-RMD were significantly different from those of ROW-RMD according to the comparison of the chromatograms of HPLC-PDA. When DOW was used as the culture water of *M. purpureus* NTU 568, an increased tendency was found for the formation of yellow pigments and a decreased tendency was found for the formation of red pigments (Figure 5). The results suggested that the DOW mediated the biosynthesis of *Monascus*-fermented secondary metabolites toward the formation of yellow pigments rather than the formation of red pigments. This study found that both monascin and ankaflavin production of DOW-RMD were increased by the supplementation of increasing DOW concentration during the fermentation process. DOW was suggested to stimulate the growth of microorganisms because of the rich ions and trace elements.[34] In this study, the components of DOW included various minerals such as Mg^{2+}, Ca^{2+}, K^{+}, Fe^{2+}, Na^{+}, Zn^{2+}, Cu^{2+}, and other trace elements (Table 5). However, high Mg^{2+} and Ca^{2+} concentrations were found in the DOW. Park et al. indicated that the biomass, polysaccharide, and the secondary metabolite production of fungus were stimulated with supplementation of some ions and trace elements to the culture medium, especially Mg^{2+}, Ca^{2+}, and PO_4^{3-}.[35] These ions may act as cofactors of enzymes to regulate the biosynthesis and metabolism of *Monascus* toward the formation of functional yellow pigment rather than red pigment.

Therefore, another reason involving the strengthened hypolipidemic effect of DOW-RMD should be contributed by the increase of monascin and ankaflavin production. The two yellow

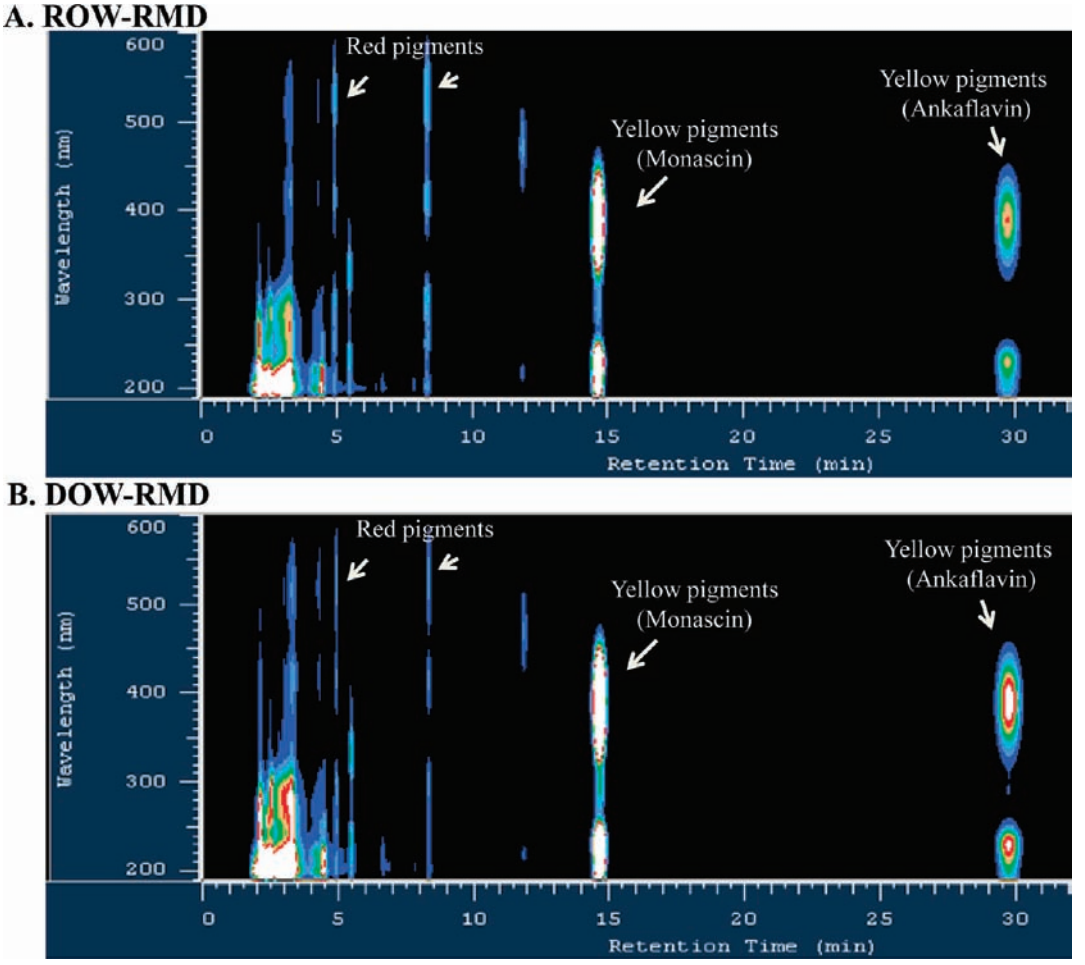

Figure 5. Comparison of the metabolite formation levels between ROW-RMD and DOW-RMD using the HPLC-PDA.

Table 4. Effect of DOW Concentration on the Production of Monascin, Ankaflavin, and Citrinin of *Monascus*-Fermented RMD[a]

water source	monascin (mg/kg)	ankaflavin (mg/kg)	citrinin (ppb)
100% ROW	3520 ± 632 a	1075 ± 234 a	2903 ± 143 b
20% DOW	3954 ± 126 a	1153 ± 132 a	2254 ± 132 a
60% DOW	4435 ± 247 b	1285 ± 121 b	2082 ± 149 a
100% DOW	6057 ± 292 c	1345 ± 124 b	1999 ± 264 a

[a] Mean values within each column with different letters are significantly different ($p < 0.05$).

Table 5. Concentrations of Minerals and Trace Elements of Deep Ocean Water

element/mineral	concn	element/mineral	concn
Na	7.71 mg/L	Zn	0.019 mg/L
Mg	20.65 mg/L	Al	2.27 μg/L
Ca	5.02 mg/L	Pb	<0.53 mg/L
K	0.22 mg/L	Cd	<0.072 mg/L
B	0.37 mg/L	Cu	<0.018 mg/L
Fe	0.0062 mg/L	As	<1.54 mg/L
Ba	<2.42 mg/L	Cr	<0.17 μg/L
fluoride	0.11 mg/L	Hg	<0.017 μg/L
chloramines	<0.021 mg/L	Tl	<0.45 μg/L
SO_3	51.86 mg/L	Sb	<1.11 μg/L
NO_3 (as N)	<0.1 mg/L	Be	<0.068 μg/L
PO_4 (as P)	0.0023 mg/L		

pigments were found as the novel hypolipidemic agents in our previous study.[4] Monascin and ankaflavin purified from RMD were orally administered to hyperlipidemic hamsters for 8 weeks, respectively. The results of serum biochemical analysis indicated that monascin and ankaflavin had significant effects on lowering serum TC, TG, and LDL-C levels. In addition, monascin and ankaflavin were proven to raise HDL-C levels, and with significant difference as compared with the HC group in this study. This fact clarified that the particular function for raising HDL-C levels was performed by monascin and ankaflavin rather than monacolin K.[4] Comparison of the hypolipidemic effect of DOW-RMD in this study with that of monascin and ankaflavin reported in a previous study[4] revealed that they all showed similar hypolipidemic trends on the suppression of TC, TG, and LDL-C levels as well as the stimulation in HDL-C levels in the same hyperlipidemic hamster animal models and treatment environment. Therefore, monascin and ankaflavin production of DOW-RMD were increased because of the DOW supplementation

Table 6. Comparison of the Mg^{2+} and Ca^{2+} Concentrations between ROW-RMD and DOW-RMD[a]

	Mg^{2+} concn (mg/g)	Ca^{2+} concn (mg/g)	Ca^{2+}/Mg^{2+} ratio
ROW-RMD	1.90 ± 0.02 a	1.53 ± 0.24 a	0.81 ± 0.14 b
DOW-RMD	2.53 ± 0.27 b	1.58 ± 0.11 a	0.63 ± 0.11 a

[a] Mean values within each column with different letters are significantly different ($p < 0.05$).

during the fermentation, which should be the key reasons why DOW-RMD had greater hypolipidemic effect than ROW-RMD.

Furthermore, DOW-RMD showed greater antioxidative ability in the prevention of serum lipid peroxidation than ROW-RMD. The serum lipid peroxidation caused from the formation of oxidative LDL is involved in the development of atherosclerosis. However, monascin and ankaflavin are proven antioxidative and anti-inflammatory agents.[4] The previous study also proved that the serum lipid peroxidation of hyperlipidemic hamsters was repressed by the administration of monascin or ankaflavin.[4] Monascin and ankaflavin were both yellow pigments having an azaphilonoid structure. Monascin, known as an anti-inflammation agent, had been proven to protect the liver from chemical damage.[36] These studies suggested that monascin and ankaflavin should be the anticancer agents with antioxidation and anti-inflammation abilities. Therefore, the effect of DOW-RMD on the prevention of cholesterol-diet-induced lipid peroxidation and the occurrence of atherosclerosis should be contributed by the DOW-raised production of monascin or ankaflavin.

In addition to the promotion in hypolipidemic effect, lower levels of the mycotoxin citrinin were found in DOW-RMD than in ROW-RMD. Therefore, DOW-RMD with lower citrinin levels should have greater safety than ROW-RMD. Citrinin, a known mycotoxin formed by *Monascus*, *Aspergillus*, and *Penicillium*, causes damage in the liver and kidney.[9] The citrinin levels of *Monascus*-fermented RMR or RMD are always of concern.[2S]Furthermore, many researchers used various methods including the alteration of culture conditions,[19,37,38] mutations of the strains,[39] and physical or chemical detoxinification[40] to lower or remove the citrinin of *Monascus*-fermented product. In this study, citrinin levels in the DOW-RMD were found to be significantly lower than in the ROW-RMD ($p < 0.05$). This significant effect on lowering citrinin levels should be an important finding in *Monascus* studies. This effect should be contributed from the use of DOW during the fermentation. However, the specific ingredients in DOW may modulate or repress the citrinin biosynthesis of *Monascus*. DOW including high levels of Mg^{2+} and Ca^{2+} was proven to promote the growth of fungi or yeasts. However, DOW probably contributed a suitable culture environment to *Monascus*, leading stimulated growth of biomass rather than the production of the mycotoxin citrinin. Determining the functional ingredients and ions of DOW involved in the citrinin-lowering effect should be an important area of investigation in the future.

In this study, DOW-RMD has been proven to show a greater hypolipidemic effect than ROW-RMD, but the hypolipidemic effect of DOW-RMD was default to be stronger as using 2-fold dose. This problem also occurred in our previous study associated with the hypolipidemic effect of RMD.[2] The unobvious effect is possible in that DOW-RMD had shown the greatest activity in the hypocholesterolemic and hypotriglyceridemic effects. Furthermore, the compensation regulation of cholesterol biosynthesis may affect the RMD.[2] The problem involved in the obvious dose−response in hypolipidemic effect of RMD should be resolved in future study.

In conclusion, DOW-RMD fermentation using DOW as the culture water has greater effect in lowering serum TC, TG, and LDL-C levels and raising HDL-C levels than ROW-RMD. Furthermore, greater antiatherosclerosis and anti-fatty liver effects were found in the DOW-RMD treatment groups than in the ROW-RMD treatment group. The advantages of DOW-RMD with greater hypolipidemic effect should be contributed by the DOW-stimulated monascin and ankaflavin biosynthesis of *Monascus* sp. The ions of DOW probably modulated the *Monascus*-fermented metabolite biosynthesis toward the formation of functional yellow pigments rather than red pigments and the mycotoxin citrinin. In addition, DOW with lower ion ratio of Ca^{2+}/Mg^{2+} was absorbed by DOW-RMD, so the accumulation of the DOW ions should contribute to the advantage of the prevention of cardiovascular disease to DOW-RMD. Comprehensively, the DOW-induced metabolism modulation and the ions of DOW benefitted the development of safe DOW-RMD with low citrinin levels and high hypolipidemic, antiatherosclerosis, and anti-fatty liver effects. This study provided a model which suggested that DOW may be a novel biomaterial with specific ions or trace elements for the promotion of the hypolipidemic effect or other health functions of microorganism-fermented product via the mediation of metabolite biosynthesis and its functional effect.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Department of Biochemical Science and Technology, National Taiwan University, 1, Sec. 4, Roosevelt Road, Taipei, Taiwan 106. Phone: +886-2-33664519. Fax: +886-2-3366-3838. E-mail: tmpan@ntu.edu.tw.

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