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# Monascin and Ankaflavin Act as Novel Hypolipidemic and High-Density Lipoprotein Cholesterol-Raising Agents in Red Mold Dioscorea

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*Monascus*-fermented red mold dioscorea (RMD) has been proven to possess greater hypolipidemic effect than red mold rice (RMR) even though they include equal levels of cholesterol-lowering agent monacolin K. However, higher concentrations of yellow pigments (monascin and ankaflavin) were found in RMD than in RMR. In this study, purified monascin and ankaflavin were administered to hyperlipidemic hamsters for 8 weeks, respectively, to test whether these two compounds were novel hypolipidemic ingredients. In the statistical results, monascin and ankaflavin showed significant effect on lowering cholesterol, triglyceride, and low-density lipoprotein cholesterol levels in serum, as well as aorta lipid plaque ($p < 0.05$). Importantly, monascin and ankaflavin, unlike monacolin K, were able to perform up-regulation rather than down-regulation on high-density lipoprotein cholesterol (HDL-C) levels in serum. This finding not only explained why RMD showed greater hypolipidemic and HDL-C-raising effect than RMR but also proved that monascin and ankaflavin would act as novel and potent hypolipidemic ingredients.

**KEYWORDS: Red mold dioscorea; monascin; ankaflavin; monacolin; cholesterol**

## INTRODUCTION

*Monascus* species have been used as traditional food fungi in eastern Asia for several centuries. *Monascus*-fermented rice, known as red mold rice (RMR), was gradually developed as a popular functional food for hypolipidemia. However, red mold dioscorea (RMD), a novel and valuable *Monascus*-fermented dioscorea, was first studied in our previous research (*1*). More yellow pigment content was found in RMD rather than in red mold rice (RMR). Furthermore, monascin and ankaflavin were found as the major pigments using LC-MS analysis (*1*). Monascin and ankaflavin are both yellow pigments consisting of an azaphilonoid structure. Monascin, known as an anti-inflammation agent, had been proven to protect the liver from chemical damage (*2*). In the previous study, monascin exhibited potent inhibitory activity on both PN- and UVB-induced mouse skin carcinogenesis tests (*3*). This study suggested that monascin may be valuable as a potential cancer chemopreventive agent in chemical and environmental carcinogenesis (*3*). Furthermore, ankaflavin was also proven to result in an arrest in the sub G1 phase in the cell cycle of Hep G2 cell viability via an apoptosis mechanism (*4*). These studies suggested that monascin and ankaflavin should be anticancer agents with antioxidiation and anti-inflammation ability. With regard to the safety of pigments, *Monascus* pigment has been used as a food colorant for centuries. In the previous study, a high dosage of *Monascus* pigment extract was orally fed to mice for 28 days to evaluate the safety (*5*, *6*). The results showed no damage in organs and no abnormalities in blood biochemistry analysis.

*Monascus*-fermented products such as RMR have been regarded as hypolipidemic functional foods because of the metablolite monacolin K, identified as lovastatin. However, lovastatin, a HMG CoA reductase inhibitor, was proven to lower cholesterol content in liver and serum (*7*), but more and more studies have indicated that lovastatin treatment at high dosage for long term would cause many side effects such as depletion of coenzyme Q10 levels (*8*) and rhabdomyolysis (*9*, *10*). These side effects also occurred in many research studies associated with monacolin K of red mold rice (*8*, *11*). In addition, lovastatin was able to lower the cholesterol level, but it also lowered high-density lipoprotein cholesterol (HDL-C) levels. Many studies found that RMR treatment always showed more potent effects on lowering cholesterol levels and keeping HDL-C levels high than pure monacolin K treatment, even though the two treatments used equal monacolin K content (*12*). These research studies suggested that unknown functional ingredients should exist in *Monascus*-fermented products and act as important factors in the hypolipidemic effect and in keeping HDL-C levels high (*12*, *13*).

Our previous study was the first to ferment *Monascus* species under dioscorea substrate and further produce more yellow pigment concentration including monascin and ankaflavin (*1*, *12*). The following study found that RMD showed greater hypolipidemic

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and antiatherosclerotic effects than RMR showed in the hyperlipidemic model hamster (*14*). However, other ingredients in RMD should express well hypolipidemic effects in addition to monacolin K. Therefore, the RMD should be different from RMR on the composition so as to perform greater hypolipidemic effect. The yellow pigments monascin and ankaflavin were found as the most different compounds on the content between RMD and RMR (*14*). Research into monascin and ankaflavin associated with hypolipidemic effect has not been conducted. On these bases, this study isolated monascin and ankaflavin from RMD and further tested the hypolipidemic effect via the hyperlipidemic model hamster. In addition, monacolin K, monascin, and ankaflavin existed in RMD. An important goal of this study was to investigate which functions in hypolipidemic effect were performed by these three metabolites, respectively.

## MATERIALS AND METHODS

**Chemicals.** Monascin and ankaflavin (99.9% purity) were provided from Sunway Biotechnology Co, (Taipei, Taiwan, ROC). Monacolin K (mevanolin), thiobarbituric acid (TBA), and malondialdehyde (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO). 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 Bactoagar were purchased from Difco Co. (Detroit, MI).

**Preparation of Red Mold Dioscorea and Red Mold Rice.** *Monascus purpureus* NTU 568 fermented product has been proven to ppssess a potent hypolipidemic effect in our previous study (*12*, *14*). 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 red mold dioscorea using the method of solid-state culture. After fermentation, the crushed and dried product with the mold was used for the experiments (*15*).

**Animals and Diets.** Sixty-four 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 eight 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 by Boyd (*16*). 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 (*14*). 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 (*17*). 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 (*14*). H-RMD-1×, H-RMD-2×, and H-RMD-5× groups were fed the high-cholesterol diet and orally given a 1-fold dosage of RMD (96 mg/kg of BW/day), 2-fold dose of RMD (192 mg/kg of BW/day), and 5-fold dose of RMD (480 mg/kg of BW/ day), respectively. RMD included 2892 mg/kg monacolin K, 9822 mg/kg monascin, and 1428 mg/kg ankaflavin. The MK group, also a positive control group, was fed monacolin K at 1.56 mg/kg of BW/day equaling that of the 5-fold dose of RMD. The MS group was fed monascin at 5.30 mg/kg of BW/day equaling that of the 5-fold dose of RMD. The AF group was fed ankaflavin at 0.77 mg/kg of BW/day equaling that of the 5-fold dose of RMD.

Twenty-four hours before sacrifice, all food was removed. Animals were anesthetized and sacrificed by carbon dioxide inhalation, and whole blood, plasma, and serum samples were collected, prepared, and then stored at −80 °C. Liver tissue was lavaged and rinsed frequently with an 0.8% sodium chloride solution for eliminating 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 was immersed in 10% formalin stock and then examined for pathology using H&E staining. The other 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: the TC assay kit (CH 200, Randox Laboratories Ltd., Antrim, U.K.), the TG assay kit (TR-210, Randox Laboratories Ltd.), and the HDL-C assay kit (CH-203, Randox Laboratories Ltd.). Serum low-density lipoprotein cholesterol (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/ethanol = 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 (*19*, *20*). According to the procedure of the previous study, the TBARS levels of serum and liver were determined by the method of thiobarbituric acid (TBA) colorimetric analysis, and the optical density (OD) value was measured at 532 nm (*21*).

**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, 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 7.0 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 (*14*) as follows:

$$\text{aortic plaque (\%)} = \text{pixel of stained plaque area/pixel of whole aorta} \times 100\%$$

**Plasma Liver Index Analysis.** Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured in triplicate using commercial enzymatic kits. The kits were as follow: AST assay kit (Part MP2-113, Johnson and Johnson), ALT assay kit (Part MP2-36, Johnson and Johnson).

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

## RESULTS

**Concentrations of Monacolin K, Monascin, and Ankaflavin in RMD and RMR.** Higher monacolin K and monascin productions were found in RMD than in RMR (*1*). The following research further proved that RMD had greater hypolipidemic and antiatherosclerotic effect than RMR and might not only associate with higher monacolin K concentration (*14*). It was interesting to determine the difference between the metabolites of RMD and RMR, which might aid the finding of new hypolipidemic ingredients. Therefore, a further determination in this study proved that not only monacolin K and monascin but also ankaflavin was expressed in higher concentration in RMD than in RMR (**Table 1**). Therefore, monascin and ankaflavin were reasonably supposed as the possible unknown hypolipidemic ingredients. To evidence this fact, monascin and ankaflavin were respectively isolated and used to study the role and effect on the hyoplipidemic examination in the hyperlipidemic hamster model.

**Total Cholesterol and Triglyceride in Serum.** In this study, RMD and it metabolites monascin, ankaflavin, and monacolin K were administered, respectively, to hyperlipidemic hamsters for

8 weeks to evaluate and compare each effect and role in hypolipidemic function. The results of serum TC and TG levels are shown in **Table 2**. RMD and monacolin K, known as the cholesterol-lowering agent, significantly lowered the TC and TG levels as seen in our previous study. In addition, a significant lowering effect, as compared with the HC group, was also found in the group treated with monascin or ankaflavin. However, the feeding content of monascin (5.30 mg/kg/day), ankaflavin (0.77 mg/kg/day), or monacolin K (1.56 mg/kg/day) in the MS, AF, or MK group was equal to that in the RMD-5× group. Therefore, the results indicated that monascin (by 29.9 and 63.4%) and ankaflavin (by 28.6 and 58.2%) exhibited a more significant effect on lowering TC and TG levels than monacolin K (by 19.6 and 42.1%), as compared with the HC group ($p < 0.05$). This means that the TC- and TG-lowering effects performed by RMD should be contributed more by monascin and ankaflavin than monacolin K.

**HDL-C and LDL-C in Serum.** Both RMD and RMR were proven to enhance HDL-C levels and decrease LDL-C levels in our previous studies. The same tendency is found in **Table 2**: treatment with RMD at at least 1-fold dosage was able to enhance HDL-C levels and decrease LDL-C levels, significantly. A 5-fold dosage of RMD resulted in increased HDL-C levels (by 20.7%), as well as decreased LDL-C levels (by 31.3%). These effects should be contributed by the metabolites in RMD. Both monascin and ankaflavin had significant effects on increasing HDL-C levels by 14.1 and 17.3% and decreasing LDL-C levels by 33.9 and 42.3% as compared to HC groups. Monacolin K at a concentration equal to that in the 5-fold dosage of RMD resulted in a significant lowering effect in LDL-C levels (by 34.1%) and ineffectiveness in raising HDL-C levels. The results evidenced that the LDL-C-lowering effect should be contributed by monascin, ankaflavin, and monacolin K, but the HDL-C-raising effect of RMD should be contributed by monascin and ankaflavin rather than monacolin K.

**Total Cholesterol and Triglyceride in Liver and Feces.** In **Table 3**, RMD dosages above 0.5-fold were proven to decrease liver TC and TG concentrations as seen in our previous study. However, monascin, ankaflavin, and monacolin K showed significant declines in liver TC and TG levels, as compared to the HC group ($p < 0.05$). Monascin and ankaflavin had better effects on lowering liver TC levels than monacolin K, but without significant difference. However, monacolin K would perform greater effect on lowering liver TG levels and with significant difference ($p < 0.05$). The results clarified that monascin and ankaflavin could be used as functional ingredients like monacolin K in RMD to lower liver TC and TG levels.

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, monascin, ankaflavin, and monacolin K also had similar tendencies to decrease fecal TC and TG excretion. This probably implied that monascin and ankaflavin should decrease serum cholesterol

**Table 1.** Concentrations of Monacolin K, Monascin, and Ankaflavin in RMD and RMR Fermented by *Monascus purpureus* NTU 568

| | monacolin K (mg/kg) | monascin (mg/kg) | ankaflavin (mg/kg) |
|---|---|---|---|
| RMR | 990 | 3897 | 797 |
| RMD | 2892 | 9822 | 1428 |

**Table 3.** Effect of RMD and Its Metabolites, Monascin, Ankaflavin, and Monacolin K, on the Levels of TC and TG in the Liver and Feces of Hyperlipidemic Hamsters Fed a High-Cholesterol Diet[a]

| | liver | | feces | |
|---|---|---|---|---|
| group | TC level (mg/g) | TG level (mg/g) | TC level (mg/g) | TG level (mg/g) |
| NOR | 0.85 ± 0.12 a | 0.71 ± 0.17 a | 1.92 ± 0.13 c | 5.78 ± 1.43 ab |
| HC | 2.34 ± 0.13 c | 1.61 ± 0.32 c | 1.62 ± 0.25 c | 9.17 ± 1.71 c |
| MK | 1.32 ± 0.19 b | 0.75 ± 0.13 a | 1.30 ± 0.25 b | 6.45 ± 1.22 b |
| MS | 1.25 ± 0.25 ab | 1.16 ± 0.28 b | 1.20 ± 0.25 b | 3.69 ± 0.61 a |
| AF | 1.23 ± 0.20 ab | 1.09 ± 0.21 b | 0.72 ± 0.23 a | 7.15 ± 1.95 abc |
| RMD-0.5× | 1.33 ± 0.20 ab | 1.10 ± 0.14 b | 1.65 ± 0.45 bc | 9.25 ± 2.52 c |
| RMD-1× | 1.14 ± 0.10 ab | 1.09 ± 0.21 b | 1.52 ± 0.23 c | 6.55 ± 2.32 ab |
| RMD-2× | 1.10 ± 0.11 ab | 0.79 ± 0.14 a | 1.38 ± 0.07 b | 6.43 ± 1.61 b |
| RMD-5× | 1.06 ± 0.07 ab | 0.97 ± 0.23 ab | 1.26 ± 0.21 b | 4.50 ± 1.12 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 monacolin K (1.56 mg/kg/day) (MK group), monascin (5.30 mg/kg/day) (MS group), ankaflavin (0.77 mg/kg/day) (AF group), a 0.5-fold dose of RMD (53.92 mg/kg/day including 0.16 mg of monacolin K, 0.53 mg of monascin, and 0.08 mg of ankaflavin) (RMD-0.5× group), a 1-fold dose of RMD (107.83 mg/kg/day including 0.31 mg of monacolin K, 1.06 mg of monascin, and 0.15 mg of ankaflavin) (RMD-1× group), a 2-fold dose of RMD (215.66 mg/kg/day including 0.62 mg of monacolin K, 2.12 mg of monascin, and 0.31 mg of ankaflavin) (RMD-2× group), or a 5-fold dose of RMD (539.15 mg/kg/day including 1.56 mg of monacolin K, 5.30 mg of monascin, and 0.77 mg of ankaflavin) (RMD-5× group). Data are presented as means ± SD ($n = 8$). Mean values within each column with different letters are significantly different ($p < 0.05$).

**Table 2.** Effect of RMD and Its Metabolites, Monascin, Ankaflavin, and Monacolin K, on the 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 | 111.8 ± 10.7 a | 168.8 ± 35.6 c | 66.6 ± 5.0 a | 19.9 ± 2.6 a | 0.28 ± 0.04 a |
| HC | 236.5 ± 18.9 e | 226.3 ± 76.5 d | 98.1 ± 8.9 b | 68.3 ± 9.8 d | 0.57 ± 0.09 e |
| MK | 190.3 ± 25.1 cd | 131.1 ± 36.7 b | 96.9 ± 8.8 b | 45.0 ± 7.0 bc | 0.49 ± 0.05 d |
| MS | 165.8 ± 10.6 b | 82.8 ± 9.0 a | 114.2 ± 9.4 c | 45.1 ± 3.4 bc | 0.42 ± 0.02 bc |
| AF | 168.9 ± 11.5 b | 94.5 ± 18.0 ab | 118.6 ± 8.1 cd | 39.4 ± 5.8 b | 0.36 ± 0.05 b |
| RMD-0.5× | 192.8 ± 14.8 d | 124.9 ± 18.8 b | 101.2 ± 4.7 b | 46.9 ± 4.5 c | 0.46 ± 0.04 c |
| RMD-1× | 177.6 ± 12.6 bcd | 114.0 ± 18.2 ab | 111.4 ± 8.1 c | 45.6 ± 5.5 bc | 0.45 ± 0.05 cd |
| RMD-2× | 176.5 ± 9.6 bc | 109.9 ± 21.1 ab | 112.7 ± 7.0 c | 40.5 ± 5.9 bc | 0.42 ± 0.04 cd |
| RMD-5× | 162.4 ± 8.3 b | 111.0 ± 19.4 ab | 123.6 ± 11.5 d | 41.9 ± 3.8 bc | 0.37 ± 0.04 c |

[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 monacolin K (1.56 mg/kg/day) (MK group), monascin (5.30 mg/kg/day) (MS group), ankaflavin (0.77 mg/kg/day) (AF group), a 0.5-fold dose of RMD (53.92 mg/kg/day including 0.16 mg of monacolin K, 0.53 mg of monascin, and 0.08 mg of ankaflavin) (RMD-0.5× group), a 1-fold dose of RMD (107.83 mg/kg/day including 0.31 mg of monacolin K, 1.06 mg of monascin, and 0.15 mg of ankaflavin) (RMD-1× group), a 2-fold dose of RMD (215.66 mg/kg/day including 0.624 mg of monacolin K, 2.118 mg of monascin, and 0.308 mg of ankaflavin) (RMD-2× group), or a 5-fold dose of RMD (539.15 mg/kg/day including 1.56 mg of monacolin K, 5.30 mg of monascin, and 0.77 mg of ankaflavin) (RMD-5× group). Data are presented as means ± SD ($n = 8$). Mean values within each column with different letters are significantly different ($p < 0.05$).

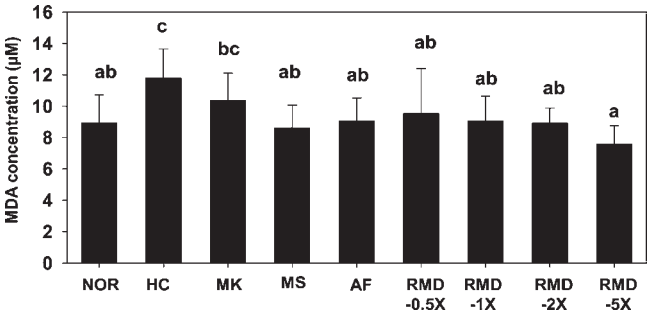


**Figure 1.** Effect of RMD and its metabolites, monascin, ankaflavin, and monacolin K, on lipid peroxidation in serum of hyperlipidemic hamsters fed a high-cholesterol diet. Two groups of hamsters were fed a normal diet (the NOR group) or a high-cholesterol diet (HC group) without the administration of test materials, respectively. The other hyperlipidemic hamsters were administered monacolin K (1.56 mg/kg/day) (MK group), monascin (5.30 mg/kg/day) (MS group), ankaflavin (0.77 mg/kg/day) (AF group), a 0.5-fold dose of RMD (53.92 mg/kg/day including 0.156 mg of monacolin K, 0.53 mg of monascin, and 0.08 mg of ankaflavin) (RMD-0.5× group), a 1-fold dose of RMD (107.83 mg/kg/day including 0.31 mg of monacolin K, 1.06 mg of monascin, and 0.15 mg of ankaflavin) (RMD-1× group), a 2-fold dose of RMD (215.66 mg/kg/day including 0.62 mg of monacolin K, 2.12 mg of monascin, and 0.31 mg of ankaflavin) (RMD-2× group), or a 5-fold dose of RMD (539.15 mg/kg/day including 1.56 mg of monacolin K, 5.30 mg of monascin, and 0.77 mg of ankaflavin) (RMD-5× group). Data are presented as means ± SD ($n$ = 8). Bars with different letters are significantly different ($p < 0.05$).

levels through the inhibition of liver cholesterol biosynthesis rather than the stimulation of fecal cholesterol excretion.

**Lipid Peroxidation.** The serum LDL would be transformed into oxidative LDL because of lipid peroxidation, which resulted 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 of RMD was helpful for repressing the occurrence of lipid peroxidation. In addition, the stimulated MDA levels were lowered by the treatment with monascin or ankaflavin.

**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 most content of plaque deposition in the aorta among all groups. The deposition was decreased with the increasing dosage of RMD use. A significant lowering effect by 84.3% is shown in the RMD-5X group, as compared to the HC group ($p < 0.05$). Monascin, ankaflavin, and monacolin K at a concentration equal to that in the 5-fold dosage of RMD, respectively, contributed 40.1% ($p < 0.05$), 59.6% ($p < 0.05$), and 16.5% ($p > 0.05$) to lower the deposition of lipid plaque in the surface of heart aorta, as compared with the HC group. However, this also implied that inhibition of atherosclerosis-associated lipid plaque deposition caused by the treatment with RMD was contributed more by monascin and ankaflavin than by monacolin K.

**Liver Function Test and Pathological Examination.** Serum ALT and AST activities are regarded as markers in liver function test and were measured in this study to evaluate the safety of monascin and ankaflavin. As shown in **Table 4**, 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 RMD would not intensify the cholesterol-increased ALT and AST activities even when using a 5-fold dosage. Contrarily,

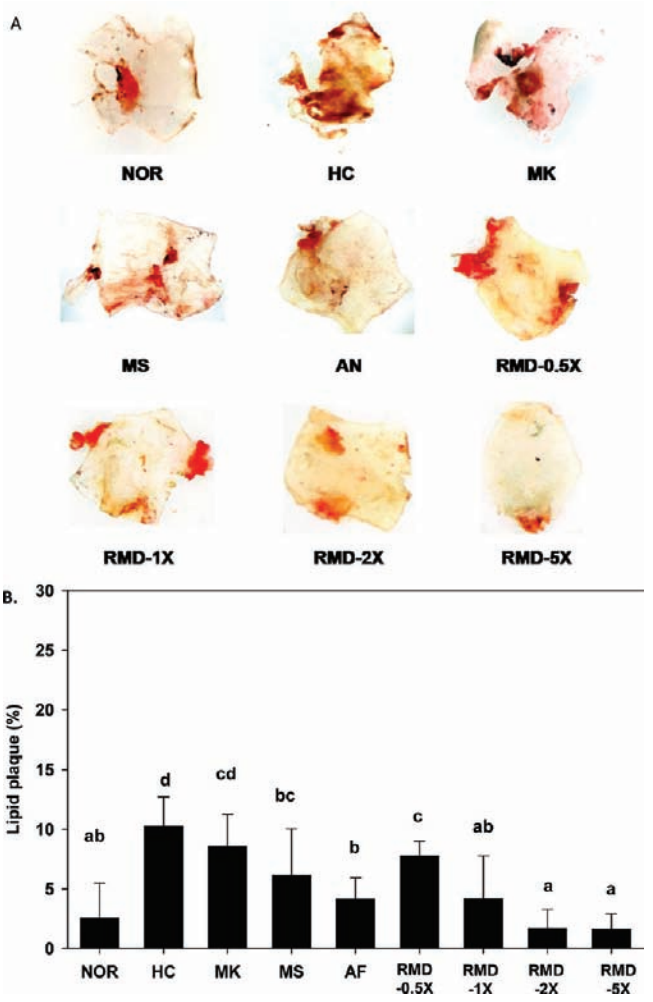


**Figure 2.** Effect of RMD and its metabolites, monascin, ankaflavin, and monacolin K, on the atherosclerotic plaque in the heart aorta of hyperlipidemic hamsters: (**A**) atherosclerotic plaque presented as the red dye in the graph; (**B**) proportion of the area of the atherosclerotic plaque in the aorta. 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 dye) were selected and quantitated by the Posterize program of Photoshop 7.0 software. The selected pixel of the plaque area and the whole aorta was used to calculate the percent area of the aortic plaque. 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 monacolin K (1.56 mg/kg/day) (MK group), monascin (5.30 mg/kg/day) (MS group), ankaflavin (0.77 mg/kg/day) (AF group), a 0.5-fold dose of RMD (53.92 mg/kg/day including 0.16 mg of monacolin K, 0.53 mg of monascin, and 0.08 mg of ankaflavin) (RMD-0.5× group), a 1-fold dose of RMD (107.83 mg/kg/day including 0.31 mg of monacolin K, 1.06 mg of monascin, and 0.15 mg of ankaflavin) (RMD-1× group), a 2-fold dose of RMD (215.66 mg/kg/day including 0.62 mg of monacolin K, 2.12 mg of monascin, and 0.31 mg of ankaflavin) (RMD-2× group), or a 5-fold dose of RMD (539.15 mg/kg/day including 1.56 mg of monacolin K, 5.30 mg of monascin, and 0.77 mg of ankaflavin) (RMD-5× group). Data are presented as means ± SD ($n$ = 8). Bars with different letters are significantly different ($p < 0.05$).

the damage was reversed by the treatment with RMD, and that also occurred in the treatments with monascin, ankaflavin, and monacolin K. The results implied that RMD and its metabolites, monascin, ankaflavin, and monacolin K, were able to protect against cholesterol-induced liver damage.

**Table 4.** Effect of RMD and Its Metabolites, Monascin, Ankaflavin, and Monacolin K, on the Activities of ALT and AST in Serum of Hyperlipidemic Hamsters Fed a High-Cholesterol Diet[a]

| group | ALT activity (U/L) | AST activity (U/L) |
|---|---|---|
| NOR | 87.6 ± 29.4 b | 115.5 ± 23.2 bc |
| HC | 111.4 ± 71.4 c | 149.5 ± 47.4 d |
| MK | 73.6 ± 26.6 ab | 113.5 ± 33.5 bc |
| MS | 76.1 ± 21.4 ab | 128.5 ± 30.6 cd |
| AF | 63.5 ± 17.3 ab | 84.6 ± 16.5 a |
| RMD-0.5× | 63.9 ± 13.8 ab | 87.8 ± 14.9 ab |
| RMD-1× | 57.1 ± 17.4 ab | 89.0 ± 25.1 ab |
| RMD-2× | 55.1 ± 16.5 abc | 79.0 ± 4.6 a |
| RMD-5× | 50.6 ± 23.4 a | 74.1 ± 17.6 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 monacolin K (1.56 mg/kg/day) (MK group), monascin (5.30 mg/kg/day) (MS group), ankaflavin (0.77 mg/kg/day) (AF group), a 0.5-fold dose of RMD (53.92 mg/kg/day including 0.16 mg of monacolin K, 0.53 mg of monascin, and 0.08 mg of ankaflavin) (RMD-0.5× group), a 1-fold dose of RMD (107.83 mg/kg/day including 0.31 mg of monacolin K, 1.06 mg of monascin, and 0.15 mg of ankaflavin) (RMD-1× group), a 2-fold dose of RMD (215.66 mg/kg/day including 0.62 mg of monacolin K, 2.12 mg of monascin, and 0.31 mg of ankaflavin) (RMD-2× group), or a 5-fold dose of RMD (539.15 mg/kg/day including 1.56 mg of monacolin K, 5.30 mg of monascin, and 0.77 mg of ankaflavin) (RMD-5× group). Data are presented as means ± SD (n = 8). Mean values within each column with different letters are significantly different ($p < 0.05$).

Although the mixed *Monascus*-fermented pigments were tested and proven as safe in a previous study (*6*), monascin or ankaflavin was never evaluated for their safety in the liver and kidney using in vivo animal tests. Sections of liver and kidney pathological examinations of various groups are shown in **Figures 3** and **4**. The results certainly indicate that administering a 5-fold dosage of monacolin K, monascin, or ankaflavin to hamsters would not induce significant damage in liver and kidney tissue.

## DISCUSSION

This study investigated the effects and roles of RMD metabolites, monascin and ankaflavin, on the treatment of hyperlipidemia. Monascin and ankaflavin, known as the yellow pigments, were proven to be the functional ingredients against inflammatory response and cancer (*1*−*4*). However, monascin and ankaflavin had never been found as the hypolipidemic agents before. When RMD was found to perform more potently on hypolipidemic effect than RMR in our previous study (*14*), the differences between their components of metabolites were extensively investigated. A lot of yellow pigments that RMD expressed on the exterior had implied that yellow pigments were possibly acting as the hypolipidemic agents in RMD. Our previous study was the first to find that more monascin content would be produced using RMD fermentation rather than RMR fermentation (*1*). Furthermore, another yellow pigment, ankaflavin, was also found with rich content in the RMD in this study. Therefore, monascin and ankaflavin should be regarded as the possible functional ingredients for explaining why RMD had greater effect on hypolipidemic effect than RMR.

RMD fermented by *M. purpureus* NTU 568 included various concentrations of monascin, ankaflavin, and monacolin K at 2892, 9822, and 1428 mg/kg, respectively. The three compounds at individual concentrations should contribute individual effects to prevent hypolipidemic and atherosclerotic effects. Therefore, this study was to investigate and compare the roles and effects among monascin, ankaflavin, and monacolin K on hypolipidemic function according to individual concentrations in RMD. The importance and effect shown by monascin, ankaflavin, or monacolin K at individual concentration in RMD could be found in this study, but comparison among the three compounds at

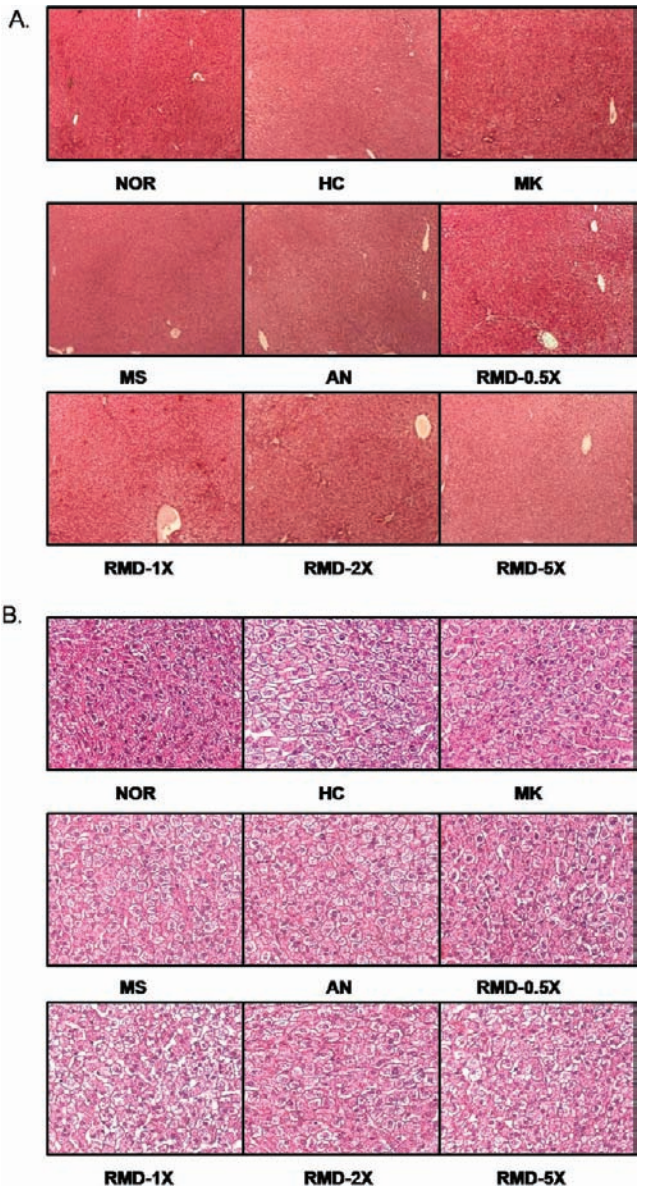


**Figure 3.** Pathological examination of liver of experimental hamsters in the 100× power field (**A**) and 400× power field (**B**). The liver section was stained using H&E and observed in the light microscope.

equal concentration was not the goal. Integrating the results of this study, monascin should result in the greatest effect on lowering serum TC and TG levels among the three compounds. However, ankaflavin had the lowest concentration among the three compounds in RMD, but it expressed the greatest effect on lowering LDL-C levels, AST and ALT activities, and lipid plaque levels in aorta, as well as increasing HDL-C levels. Monacolin K, known to be a cholesterol-lowering agent, not only contributed weaker effect to prevent hyperlipidemia than monascin and ankaflavin but also showed ineffective results in raising HDL-C levels. Therefore, monascin and ankaflavin acted as key factors for the prevention of hyperlipidemia and atherosclerosis. Furthermore, ankaflavin at 1428 mg/kg was lower than monascin at 9822 mg/kg in RMD (**Table 1**), but ankaflavin had greater effect on lowering LDL-C levels, AST and ALT activities, and lipid plaque levels in aorta, as well as increasing HDL-C levels, and showed similar effect without significance on lowering TC and TG levels, as compared with monascin.

However, the function of monascin and ankaflavin involved in the regulation mechanism of the lipid metabolism was of concern

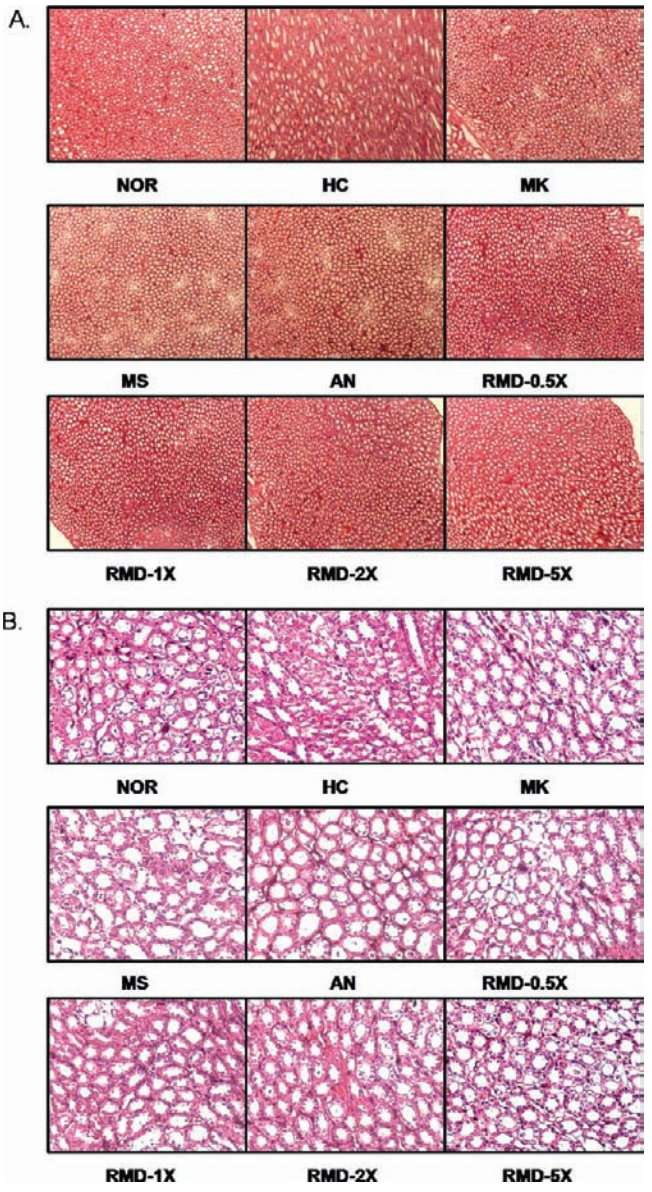


**Figure 4.** Pathological examination of kidney of experimental hamsters in the 100× power field (**A**) and 400× power field (**B**). The kidney section was stained using H&E and observed in the light microscope.

in this study. In general, the increased in vivo cholesterol levels were attributed to food intake and cholesterol biosynthesis. On the contrary, the decreased in vivo cholesterol levels mostly resulted from the stimulation of cholesterol excretion and the inhibition of cholesterol biosynthesis pathway. The results indicated that the fecal cholesterol excretion would be decreased by monascin and ankaflavin treatments, which had similar tendencies to that of monacolin K treatment. However, monacolin K was a typical competitive inhibitor to reduce HMG-CoA reductase activity in the cholesterol biosynthesis pathway (*7*). The decrease of TC levels in serum, liver, and feces implied that cholesterol levels were reduced through the inhibition of cholesterol biosynthesis rather than the increase of cholesterol excretion. According to the result, we could found that the lipid metabolism mediated by monascin should be the same as that of ankaflavin, in which the cholesterol levels were decreased in serum, liver, and feces. Therefore, monascin and ankaflavin might be supposed to lower cholesterol through the inhibition of cholesterol biosynthesis.

Although monascin or ankaflavin was supposed to have a similar hypolipidemic mechanism via the inhibition of cholesterol biosynthesis to monacolin K, different lipid metabolism was also be found. In particular, a HDL-C-raising effect could be expressed by monascin or ankaflavin rather than monacolin K. HDL-C was regarded as the good type of cholesterol toward the prevention of atherosclerosis development, because it transports mostly to the liver or steroidogenic organs such as the adrenals, ovaries, and testes. Several steps in the metabolism of HDL can contribute to the transport of cholesterol from lipid-laden macrophages of atherosclerotic arteries, termed foam cells, to the liver for secretion into the bile (*22*). This pathway has been termed reverse cholesterol transport and is considered to be the classical protective function of HDL toward atherosclerosis. However, HDL carries many lipid and protein species, several of which have very low concentrations but are biologically very active. For example, HDL and their protein and lipid constituents help to inhibit oxidation, inflammation, activation of the endothelium, coagulation, and platelet aggregation (*23*). Some medicine or functional ingredients were claimed to lower TC levels, but fewer were found to maintain HDL-C levels, let alone raise them. Monacolin K, known as the statin compound, led to lower serum TC, TG, and LDL-C levels but lacked a significant increase in HDL-C levels in this study, which is consistent with the previous studies (*24*, *25*). However, RMR or RMD was found to raise HDL-C levels in many previous studies, but the functional ingredient was never found. Although monacolin K had been evidenced as the hypolipidemic agent in *Monascus*-fermented products, it was not the functional ingredient for raising HDL-C levels. In this study, monascin and ankaflavin purified from RMD were orally administered to hyperlipidemic hamsters for 8 weeks, respectively. The results of serum biochemistry analysis indicated that monascin and ankaflavin had significant effect 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 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. However, it will be important to further investigate the mechanism in future study.

In addition to the hypolipidemic effect, liver protection might be found in monascin and ankaflavin treatment (**Table 4**). In previous studies, the AST and ALT activities of RMR-treated hamster were determined to address the misgiving involved in citrinin-induced hepatoxicity (*12*). However, there was no damage to be found whether in liver functional test or liver pathological examination in RMR- or RMD-treated hamsters, even though citrinin existed in that (*12*). The possible reasons were attributed to the fact that citrinin levels, in general, in *Monascus*-fermented products were not high enough to induce liver damage, or perhaps some functional ingredients protected against citrinin-induced liver damage. The former was suggested as the most likely reason in the previous study (*12*), but the latter was confirmed in this study because liver damage could not be observed in RMD. Liver damage induced by citrinin was not found, but that induced by the feeding of a high-cholesterol diet did occur in this study. According to the comparison between the NOR and HC groups, the AST and ALT activities would be increased because of feeding a high-cholesterol diet. However, this study indicated that RMD and its metabolites, monascin, ankaflavin, and monacolin K, would result in decreases instead of increases in the AST and ALT activities, which should result from the protection provided by monascin, ankaflavin, and monacolin K against liver damage induced by cholesterol-related risk factors such as oxidative LDL and lipid peroxidation.

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