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ORIGINAL ARTICLE

The blood lipid regulation of *Monascus*-produced monascin and ankaflavin via the suppression of low-density lipoprotein cholesterol assembly and stimulation of apolipoprotein A1 expression in the liver

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KEYWORDS
ankaflavin;
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Abstract *Background/Purposes:* Monascin (MS) and ankaflavin (AK) produced by *Monascus purpureus* NTU 568 were proven to show excellent hypolipidemic effects in our previous studies; however, the mechanism is still unclear.
Methods: This study used MS, AK, and monacolin K as test substances and performed tests on rats fed high-fat and high-cholesterol diet for 8 weeks. The lipid levels and the related protein levels of the rats were assessed to understand the effects of MS, AK, and monacolin K on lipid metabolism.
Results: MS and AK lowered low-density lipoprotein cholesterol (LDL-C) and preserved high-density lipoprotein cholesterol contents. MS and AK inhibited acetyl-coenzyme A acetyltransferase, microsomal triglyceride transfer protein, and apolipoprotein (apo) B-100 expression, thereby preventing LDL assembly. In addition, enhanced LDL-receptor expression increased the transport of LDL-C to the liver for metabolism. MS and AK also significantly increase apo A1 expression, which facilitates high-density lipoprotein cholesterol formation.
Conclusion: Monascus-fermented MS and AK can perform blood lipid regulation via the suppression of LDL-C assembly and stimulation of apo A1 expression in liver.
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Introduction

Low-density lipoprotein cholesterol (LDL-C) within the vascular walls is an important risk factor for the development of atherosclerosis.[1] The occurrence of atherosclerosis is widely attributed to poor eating habits, cardiovascular risk factors, or genetic predisposition. Long-term consumption of a high cholesterol diet can easily result in hyperlipidemia, which induces atherosclerosis and cardiovascular disease.[2] In 1979, the *Monascus*-fermented metabolite monacolin K (MK) was found to be an effective hypolipidemic agent.[3] MK can effectively suppress the activity of the cholesterol synthetic enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase by preventing its activation and thus limiting the accumulation of cholesterol. However, because a long-term, high-dose MK may pose safety concerns (e.g., causing rhabdomyolysis),[4] the development of new, safe hypolipidemic compounds from *Monascus*-fermented products is crucial.

Studies have shown that long-term consumption of *Monascus*-fermented red mold rice (RMR) prevents cardiovascular disease by increasing apolipoprotein (apo) A1 and decreasing apo B100, which subsequently increases high-density lipoprotein cholesterol (HDL-C) and LDL-C levels.[5] Wei et al[6] (2006) also discovered that the primary active ingredient of *Monascus*-fermented RMR is not MK. However, numerous studies have shown that *Monascus*-fermented red mold dioscorea (RMD) exhibits comparatively superior hypolipidemic and antiatherosclerosis effects than *Monascus*-fermented RMR. *Monascus*-fermented RMD significantly reduces the total cholesterol (TC) and LDL-C levels in the blood while increasing the level of HDL-C. In addition, RMD exhibits a favorable total antioxidative status, catalase, and superoxide dismutase activity. Research results also show that RMD exerts favorable hypolipidemic and antiatherosclerosis effects.[7]

Related studies have confirmed that the blood lipid regulation of RMD results from monascin (MS) and ankaflavin (AK), which, similar to MK, significantly reduces TC, triglyceride (TG), and LDL-C levels in serum as well as lipid plaque accumulation in the heart aorta ($p < 0.05$).[8] Compared with MK, the effects of AK on reducing TC and TG in serum exhibited no significant differences ($p > 0.05$). However, the antifatty liver and antioxidative stress efficacy of AK exceeds that of MK. More importantly, MS significantly enhances HDL-C concentrations, whereas MK exerts the opposite effect.[9] Concerning safety, MK significantly increases creatine phosphokinase activity ($p < 0.05$), which is highly correlated with rhabdomyolysis. By contrast, this side effect is not observed for MS and AK.[9] Additionally, MS and AK do not pose any potential harm to the liver, kidneys, or muscle tissue.[9] Furthermore, the relatively higher MS and AK content in RMD fermented with deep ocean water rendered the RMD a superior agent for the prevention of cardiovascular disease.[10]

Although MS and AK were proven to perform excellent hypolipidemic effects and safety in our previous studies,[8,9] the blood lipid regulation of the two substances is still unknown. Therefore, this study is the first to investigate the blood lipid regulation of MS and AK. This is a novel and important topic for a natural *Monascus*-fermented product. To assess their effects on lipids and body fat, this study used the yellow pigments of RMR MS and AK, as well as cholesterol-lowering agent MK, as test substances and performed tests on Sprague-Dawley (SD) rats that were fed a high-fat and high-cholesterol (HFC) diet. Additionally, the lipid and body fat levels of the rats were assessed to understand the effects of MS, AK, and MK on lipids and fat-related metabolism. Subsequently, the protein levels of microsomal triglyceride transfer protein (MTP), acetyl-coenzyme A acetyltransferase (ACAT), low-density lipoprotein receptor (LDLR), apo A1, and apo B100 proteins in liver tissues were determined for investigating the lipid-regulating mechanisms of MS and AK.

Materials and methods

Chemicals

Liquid chromatography grade acetonitrile, chloroform, methanol, and dimethyl sulfoxide were purchased from Merck Co. (Darmstadat, Germany). Tryptone, yeast extract, peptone, malt extract, potato dextrose agar, and Bacto-agar were purchased from Difco Co. (Detroit, MI, USA). Monoclonal apo A1 antibody (sc-135837), MTP antibody, and polyclonal apo B (sc-25542) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal ACAT antibody (EP1375Y) and monoclonal LDLR antibody (ab60107) were purchased from Epitomics, Inc. (Burlingame, CA, USA).

MS and AK purification

MS and AK (99.9% purity) were purified from the RMR fermented by *Monascus purpureus* NTU 568 according to the method of our previous study.[11]

Animal experiments

Animal experiment protocol is according to our previous study.[12] Forty male SD rats at 6—8 weeks of age were purchased from the BioLasco Co. (Taipei, Taiwan). The animals were housed individually and allowed free access to a standard laboratory chow (Ralston Purina, St Louis, MO, USA) and water. Three weeks later, the rats were randomly assigned to one of the following diets for 8 weeks: standard chow (control group, NOR; 4.5% fat, 3.34 kcal/g), HFC diet consisting of 26.7% butter powder (Gene Asia Biotech Co., Ltd., Nang-Tou, Taiwan) in standard chow (HFC group; 30% fat, 0.2% cholesterol, 4.85 kcal/g), HFC + MK (MK group), HFC + MS (MS group), and HFC + AK (AK group). The recommended dosage of RMR for lowering cholesterol is suggested as 2 g/d for humans in our previous study.[12] RMR included 9.82 mg/g MS, 1.425 mg/g AK, and 2.89 mg/g MK. The dosage of MS, AK, and MK were based on the concentration of RMR. The adult dose of MS (19.64 mg) in the MS group, the adult dose of AK (2.85 mg) in the AK group, and the adult dose of MK (5.78 mg) in the MK group were equal to the MS, AK, and MK contents of 2-g RMR, respectively. The doses of the test substances used in this study were

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calculated according to Boyd's formula for body surface area for adult humans (weight: 65 kg; height: 170 cm).[13] The rats of the MS, AK, and MK groups were orally administrated 0.55 mg/d MS, 0.08 mg/d AK, and 0.16 mg/d MK for 8 weeks, respectively.

Feces were collected from rats on three consecutive days and oven dried (65°C) to a constant weight for the determination of fat content. At the end of the study, the rats were deprived of food for 16 hours before being scarified by CO_2 asphyxiation. Blood samples were collected from the posterior vena cava and centrifuged at 700*g* for 10 minutes; the serum was stored at −20°C until analyzed. The livers were excised and stored at −20°C for the measurement of lipids. The experiment was reviewed and approved by the Animal Care and Research Ethics Committee of the National Taitung University.

Serum, liver, and fecal lipid analysis

Serum TC, 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 LDL-C levels were gained via the following calculation: 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; volume/volume) and incubated for 30 minutes at room temperature.[9] The aqueous layer was aspirated and discarded, and the fixed concentration of the organic layer was then evaporated to dryness. The dried lipid layer was dissolved with an equal concentration of dimethyl sulfoxide and then used to determine the TC and TG levels using commercial enzymatic kits (Randox Laboratories Ltd.).

Fecal bile acid analysis

Feces powder (0.5 g) were ground in 5 mL of 100% ethanol and extracted for 18 hours at room temperature. After centrifugation (1800*g*, 15 minutes), the supernatant was then evaporated to dryness in a vacuum. The above extraction process was carried out three times. Petroleum ether (10 mL) was added for extracting the organic layer. After centrifugation (1800*g*, 15 minutes), the supernatant was removed. The deposition was dissolved by methanol (10 mL), and then used to carry out the determination of bile acid using a commercial kit (BXC00581, Fortress Diagnostics Limited, Antrim, UK).

Immunoblotting

Protein concentration was determined using the bicinchoninic acid method. A total of 40 μg of total protein was applied as a Western blot representative of three independent experiments according to the previous studies.[14] The samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinyldiene fluoride membranes. After blocking in a gelatin-NET solution (gelatin 0.5%, NaCl 0.15 M, EDTA·2Na 5 mM. Tween 20 0.05%, Tris base 50 mM, pH 8.0), blots were incubated with monoclonal MTP antibody (1:200), monoclonal ACAT antibody (1:25000), monoclonal LDLR antibody (1:1000), and monoclonal apo A-1 antibody (1:1000) at room temperature for 1 hour. Then bands were incubated with specific horse radish peroxidase-conjugated secondary antibodies (1:100,000) at room temperature for 1 hour and visualized by enhanced chemiluminescence substrate with UVP AutoChemi Image system (UVP Inc., Upland, CA, USA). Protein loading was evaluated by anti-actin antibody (1:3,000). Band intensities were quantified using UVP LabWork 4.5 software (UVP Inc.).[15]

Immunohistochemical stain

The liver tissue was fixed in 10% formalin at pH 7.4. Livers were blocked and serial 35-μm thick frozen sections cut on a sledge microtome were collected sequentially and without interruption into wells. Sections from regions containing liver were processed for polyclonal apo B-100 immunohistochemical stain. The tissue section was dried at 60°C for 1 hour, then deparaffinized and hydrated, following a dip of xylene, gradient concentration of ethanol, and phosphate buffered saline (PBS). These sections were treated for 30 minutes in 3% hydrogen peroxide in absolute methanol, followed by several washes with cold PBS. After blocking in 10% normal goat serum in PBS, sections were placed in primary monoclonal apo B-100 antibody (1:50) diluted in antibody diluent at 4°C overnight. Labeling was detected by using the nonbiotin polyhydrogen peroxidase immunohistochemistry detection kit (BioGenex, San Ramon, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride. Counterstaining of the section was then carried out with hematoxylin for 3 minutes and stopped with a PBS wash. Sections were then mounted, air dried, and cover slipped. The apo B-100 expression in liver were monitored by intermittent microscope examination.[16]

Statistical analysis

Data are expressed as means ± standard deviation. Analysis of variance with Duncan's test and Pearson's product-moment correlation coefficient test were determined using SPSS version 10.0 software (SPSS Institute, Inc., Chicago, IL, USA). Differences with $p < 0.05$ were considered statistically significant.

Results

Body weight and food intake

With regards to the body weight, the animal experiment commenced under the condition that the initial average body weight (0^{th} week) did not differ significantly ($p > 0.05$; Table 1). However, regarding the final body weight (8^{th} week), the body weight of the HFC group was significantly higher than rats fed a normal diet (NOR) group ($p < 0.05$). The body weight of the MS and AK groups were significantly lower than that of the HFC group ($p < 0.05$) and MK group ($p < 0.05$). The final body weight was not significantly decreased by MK administration as compared with the HFC

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Table 1 Effect of monacolin K (MK), monascin (MS), and ankaflavin (AK) on body weight and food intake in male Sprague-Dawley rats fed with high-fat and high-cholesterol diet. Two groups of the Sprague-Dawley rats were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic rats were administrated with MK (0.16 mg/d 400 g body weight; MK group), MS (0.55 mg/d 400 g body weight; MS group), and AK (0.08 mg/d 400 g body weight; AK group), respectively.

Groups	Initial body weight 0_{th} wk (g)	Final body weight 8^{th} wk (g)	Food intake,(g/d)
NOR	334 ± 23	447 ± 22*,***	30.7 ± 0.8*,**
HFC	330 ± 21	481 ± 24	23.1 ± 1.5
MK	337 ± 18	489 ± 20	25.3 ± 2.2
MS	336 ± 24	424 ± 19**,****	21.3 ± 3.0
AK	328 ± 18	408 ± 25**,****	21.7 ± 2.7

Data are presented as means ± standard deviation ($n = 8$).
* $p < 0.05$ versus the high-fat and high-cholesterol diet group.
** $p < 0.01$ versus the high-fat and high-cholesterol diet group.
*** $p < 0.05$ versus the monacolin K group.
**** $p < 0.01$ versus the monacolin K group.

group ($p > 0.05$). The result was similar to our previous study in that MS and AK were proven to perform antiobesity effects in obese rats fed a high fat diet.[15]

In Table 1, the food intake of the HFC group was significantly lower than the NOR group ($p < 0.05$). This may be because the unit calorie level of the high fat diet is higher (HFC: 4.17 kcal/g and NOR: 3.34 kcal/g). However, the food intake of the MS and AK groups was slightly lower than that of the HFC group and the MK group without significant difference ($p > 0.05$), even though they were fed the same high fat diet. The reason may be that the MS and AK groups had a lower body weight so their food intake was lower.

Serum TC, TG, HDL-C, and LDL-C levels

Although our previous studies have shown that MS and AK can perform significant hypolipidemic effects in a hamster model,[9] the blood lipid regulation of MS and AK in a rat model has never been investigated. Therefore, in this study, the cholesterol-lowering effect of MS and AK should be confirmed again before the investigation of blood lipid regulation. The changes in serum lipid levels following an 8-week animal experiment are shown in Table 2. Compared with NOR, although a significant increase in TG levels of the HFC group was not observed, the levels of TC ($p < 0.01$) and LDL-C ($p < 0.05$) and the LDL-C/HDL-C ratio ($p < 0.01$) in the HFC group all exhibited a significant increase, indicating that hyperlipidemia was successfully induced in the rats. The cholesterol-lowering effects observed in each group following the consumption of the test substances are summarized below: MK, MS, and AK significantly reduced the cholesterol level by 13.1% ($p < 0.05$), 16.0% ($p < 0.05$), and 21.9% ($p < 0.05$), respectively. Concerning serum TG, AK reduced TG levels by 23.1% and with a significant difference ($p < 0.05$).

Regarding the LDL-C reduction, compared with the HFC group, the MK, MS, and AK groups showed a LDL-C reduction ($p < 0.05$). Among the three groups, the MS and AK groups exhibited a higher rate of LDL-C reduction compared with that of the MK group, although this difference was not significant ($p > 0.05$). HDL is negatively correlated with the risk of cardiovascular diseases. Because the TC level of the HFC group increased significantly ($p < 0.05$), the HDL-C level of the HFC group significantly exceeded that of the NOR group ($p < 0.05$). The decline in TC levels for the MK group

Table 2 Effect of monacolin K (MK), monascin (MS), and ankaflavin (AK) on the serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) levels, and LDL-C/HDL-C ratio of male Sprague-Dawley rats fed with high-fat and high-cholesterol diet. Two groups of the Sprague-Dawley rat were fed a normal diet (NOR group) or a high-fat and high-cholesterol diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic rats were administrated with MK (0.16 mg/d 400 g body weight; MK group), MS (0.55 mg/d 400 g body weight; MS group), and AK (0.08 mg/d 400 g body weight; AK group), respectively.

Groups	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	LDL-C/HDL-C ratio
NOR	63.1 ± 7.8**,***	70.3 ± 22.8	14.6 ± 3.5*,***	40.9 ± 9.4*	0.38 ± 0.16**,****
HFC	98.4 ± 16.2***	83.9 ± 7.1	38.3 ± 9.2	48.6 ± 2.7***	0.78 ± 0.16
MK	85.5 ± 4.9*	74.6 ± 6.0	31.1 ± 3.2	39.6 ± 3.5*	0.79 ± 0.10
MS	82.6 ± 13.3*	74.4 ± 6.8	21.8 ± 5.7*	47.7 ± 5.4***	0.47 ± 0.16*,***
AK	76.9 ± 6.1*	64.5 ± 14.4*	20.0 ± 8.0*	45.2 ± 2.8	0.44 ± 0.18*,***

Data are presented as means ± standard deviation ($n = 8$).
* $p < 0.05$ versus high-fat and high-cholesterol diet group.
** $p < 0.01$ versus high-fat and high-cholesterol diet group.
*** $p < 0.05$ versus the monacolin K group.
**** $p < 0.01$ versus the monacolin K group.

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significantly reduced their HDL-C level by 18.5% ($p < 0.05$). Conversely, a slight decrease was observed for the MS and AK groups, and without significant difference compared with the HFC group ($p > 0.05$). The ability of MS and AK to reduce HDL-C levels in serum was not affected by declines in TC levels. Regarding the LDL-C/HDL-C ratio, compared with the HFC group, a significant reduction was exhibited in the MS and AK groups ($p < 0.05$) but not the MK group ($p > 0.05$). These results showed that test substances MS and AK exert superior lipid-regulating effects than MK does.

Liver TC and TG levels

Cholesterol in the body is derived from food and the products of the cholesterol biosynthesis pathway in the liver. Cholesterol converts into bile acids and is eliminated from the body via fecal excretion. Changes in the liver lipid levels are shown in Table 3. Compared with the NOR group, the results in Table 3 show a significant increase in TC and TG levels in the liver of the HFC group ($p < 0.05$). However, significantly decreased liver TC levels were found in the MK ($p < 0.01$), MS ($p < 0.05$), and AK ($p < 0.05$) groups.

Concerning the liver TG level, compared with the HFC group, the MK ($p < 0.001$) and AK ($p < 0.05$) groups demonstrated the ability to significantly reduce TG levels. The extent of this decrease for the MK group was significantly greater than for the AK group ($p < 0.05$). However, no significant difference was observed between the MS and HFC groups. The cause of the decline in liver TC and TG may be attributed to MK, MS, and AK inhibiting cholesterol biosynthesis in the liver.

Fecal TC, TG, and bile acid levels

Table 3 shows the fecal TC and TG results. The amount of fecal TC and TG may be attributable to the transportation of lipids consumed from food to the liver via enterohepatic circulation. Furthermore, because the HFC group was fed a high-fat and high-cholesterol diet, their levels of TC and TG increased, which increased TC and TG levels in the excreted feces. Regarding the test substances, the TC level in the excreted feces increased slightly for the MK and AK groups compared with that for the HFC group ($p > 0.05$). In contrast, the TC levels in the excreted feces of the MS group significantly decreased by 26.6% ($p < 0.05$ vs. the HFC group). Regarding fecal TG, the AK group exhibited a lower excretion concentration ($p < 0.05$), and an insignificant reduction ($p > 0.05$) was observed for the MK and MS groups. Regarding fecal bile acid, compared with the HFC group, the excretion concentration of the MS and AK groups significantly increased by 41.6% ($p < 0.05$) and 49.2% ($p < 0.05$), respectively. Meanwhile, the significant difference was not observed in the MK groups ($p > 0.05$).

LDLR protein level in the liver

Cardiovascular diseases and LDL-C are positively correlated, and negatively with the LDLR, the receptor that recognizes LDL. Therefore, an increase of LDLR in the cell can improve LDL-C metabolism, reducing the LDL-C levels in blood.[9]

Figure 1B shows the effects that MK, MS, and AK have on LDLR protein levels in the liver, where the LDLR protein level of the HFC group was lower than that of the NOR group ($p < 0.05$). This may be because the increase in liver cholesterol levels for the HFC group increased LDL synthesis, generating a negative feedback effect on LDLRs in the liver. Compared with the HFC group, the MK, MS, and AK groups all exhibited an increase in LDLR protein level. Clinically, MK is a statin-type cholesterol-lowering drug that not only inhibits cholesterol biosynthesis, but also increases LDLR feedback.[3,17] The 39.4% increase in LDLR protein level observed in this experiment is consistent with that reported in related literature.[3,17] Furthermore, MS and AK increased LDLR protein level ($p < 0.01$ vs. the HFC group), exceeding that achieved by MK ($p < 0.05$). The substantial increase in LDLR protein level of the MS and AK groups may be attributed to decreased cholesterol biosynthesis in the liver, which reduces LDL synthesis, causing a feedback that simulates an increase in the synthesis of LDLRs in liver cells.

Table 3 Effect of monacolin K (MK), monascin (MS), and ankaflavin (AK) on the levels of liver total cholesterol (TC) and triglyceride (TG) and fecal TC, TG, and bile acid of male Sragu-Dawley rats fed with high-fat and high-cholesterol diet (HFC). Two groups of the Sragu-Dawley rats were fed a normal diet (NOR group) or a HFC diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic rats were administrated with MK (0.16 mg/d 400 g body weight; MK group), MS (0.55 mg/d 400 g body weight; MS group), and AK (0.08 mg/d 400 g body weight; AK group), respectively.

Groups	Liver		Feces		
	TC (mg/g)	TG (mg/g)	TC (mg/g)	TG (mg/g)	Bile acid (mg/g)
NOR	20.0 ± 3.4**,***	74.7 ± 9.9**,***	50.8 ± 1.9**,****	67.8 ± 6.2	177.8 ± 44.1*,***
HFC	49.1 ± 8.3***	145.9 ± 18.9****	96.8 ± 6.9	71.3 ± 7.4	342.6 ± 74.9
MK	12.2 ± 1.5**	55.1 ± 4.7**	104.0 ± 7.2	60.8 ± 5.3	388.9 ± 100.1
MS	34.9 ± 14.9*,***	141.1 ± 31.8****	71.1 ± 18.8*,***	61.8 ± 18.1	485.2 ± 105.6*,***
AK	32.5 ± 13.3*,***	116.4 ± 22.4*,****	87.4 ± 7.9***	46.1 ± 7.5*	511.1 ± 120.4*,***

Data are presented as mean ± standard deviation ($n = 8$).
* $p < 0.05$ versus high-fat and high-cholesterol diet group.
** $p < 0.01$ versus high-fat and high-cholesterol diet group.
*** $p < 0.05$ versus the monacolin K group.
**** $p < 0.01$ versus the monacolin K group.

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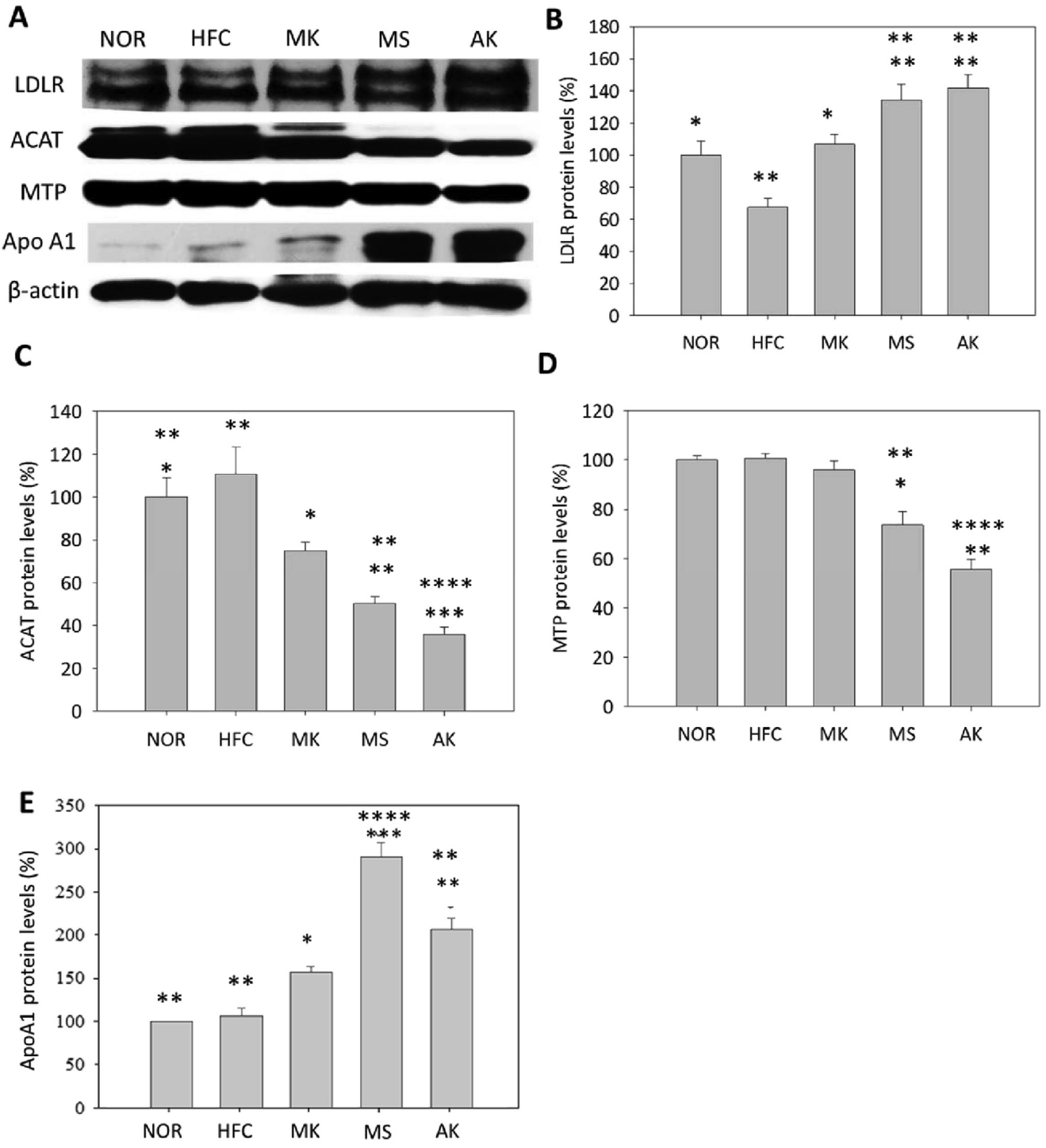

Figure 1. Effect of monacolin K (MK), monascin (MS), and ankaflavin (AK) on low density lipoprotein receptor (LDLR), acetyl-coenzyme A acetyltransferase (ACAT), microsomal triglyceride transfer protein (MTP), and apolipoprotein (apo) A1 protein level in liver of male Sprague-Dawley rats fed with high-fat and high-cholesterol diet (HFC). Two groups of the Sprague-Dawley rats were fed a normal diet (NOR group) or a HFC diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic rats were administrated with MK (0.16 mg/d 400 g body weight; MK group), MS (0.55 mg/d 400 g body weight; MS group), or AK (0.08 mg/d 400 g body weight; AK group), respectively. * $p < 0.05$ versus the high-fat and high-cholesterol diet group. ** $p < 0.01$ versus the high-fat and high-cholesterol diet group. *** $p < 0.05$ versus the monacolin K group. **** $p < 0.01$ versus the monacolin K group.

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ACAT protein level in the liver

The primary function of ACAT is to catalyze long-chain fatty acids and cholesterol into cholesteryl ester, which is then transferred using MTP to newly synthesized apo B to form very-LDL (VLDL).[18] Therefore, ACAT is a key enzyme in LDL-C synthesis and positively correlates with cardiovascular disease.[19] Figure 1C shows the effect that MK, MS, and AK consumption has on ACAT protein level in liver tissues. Compared with the NOR group, the ACAT protein level of the HFC group increased slightly ($p > 0.05$). Considering the effects of consummation of the test substances on the reduction of ACAT protein levels, the reduction yielded by AK was the highest ($p < 0.001$), followed by that yielded by MS ($p < 0.01$) and MK ($p < 0.05$).

MTP expression in the liver

MTP can transport TG, cholesteryl ester, and phospholipids in cells to newly synthesized apo B.[20] This process is critical for the formation and secretion of lipoproteins containing apo B, such as VLDL, LDL, and chylomicrons (CM).[21] Because apo B is the apolipoprotein of LDL-C, MTP is positively correlated with cardiovascular disease.

Figure 1D shows the effects that MK, MS, and AK consumption has on MTP expression in liver tissues. No significant difference in MTP level was observed between the HFC and NOR groups ($p > 0.05$). This indicates that HFC diets do not exert a considerable effect on the transfer of lipids by MTP. MK was default to decrease the high fat diet-raised MTP level ($p > 0.05$ vs. the HFC group). However, compared with the HFC and MK groups, the MS ($p < 0.05$) and AK ($p < 0.01$) groups showed a significant reduction in MTP expression. This may be because the earlier metabolism phase limited cholesterol synthesis, lowering the overall level of cholesterol in the body, which decreased the conversion of cholesterol into cholesteryl ester by ACAT, thereby reducing the expression of MTP for transferring cholesteryl ester.

Apo A1 protein level in the liver

HDL transports cholesterol from surrounding tissues to the liver for metabolization, thereby cleaning the vascular walls. The higher the serum HDL-C level, the better the body's ability to transport serum cholesterol to the liver for metabolism, which reduces the likelihood of developing atherosclerosis.[22] Apo A1 is an apolipoprotein of HDL. Increased level of apo A1 increases HDL synthesis, which subsequently reduces the risk of developing cardiovascular disease. Thus, apo A1 and cardiovascular disease are negatively correlated.

Figure 1E shows the effects that MK, MS, and AK consumption have on apo A1 protein levels in liver tissue. No significant differences in apo A1 protein level were observed between the HFC group and the NOR group ($p > 0.05$). Compared with the HFC group, the increase in apo A1 protein level of the MS group was the most significant ($p < 0.001$), followed by those of the AK ($p < 0.01$) and MK ($p < 0.05$). By increasing the expression of apo A1 protein, HDL synthesis also increased. To understand the mechanism of how increases in MS and AK increase the expression of apo A1 protein, further studies must be performed.

Apo B protein level in the liver

Numerous studies have confirmed that LDL is responsible for atherosclerosis development, and apo B-100 is indispensable in the formation of VLDL and LDL.[23,24] Therefore, reducing apo B-100 decreases the formation of VLDL and LDL, thereby minimizing the development of cardiovascular disease.[25] Thus, apo B and cardiovascular disease can be considered positively correlated.

Figure 2 shows the effects that MK, MS, and AK consumption have on apo B-100 protein level in the liver. Compared with the NOR group, apo B-100 protein level in the HFC group rose significantly ($p < 0.001$), which indicates that LDL synthesis in the HFC group had increased. However, compared with the HFC group, MK, MS, and AK groups all achieved significant reductions in apo B-100 protein level ($p < 0.001$). Literature shows that lovastatin can lower apo B-100 protein levels,[26] which is consistent with the results obtained for the MK group in this study. Furthermore, the experiment conducted in this study was the first time that MS and AK were observed to effectively reduce the expression of apo B-100 protein in the liver. The effects of both MS and AK significantly exceeded those of MK ($p < 0.05$).

Discussion

MS and AK are nature *Monascus*-fermented yellow pigments without liver damage that are abundantly produced in RMD.[8] Relevant studies have shown that MS and AK can significantly reduce LDL-C and increase HDL-C.[9] Furthermore, unlike MK, MS and AK do not increase creatine phosphokinase activity, which elevates the risk of rhabdomyolysis.[9] Therefore, MS and AK are safe and effective lipid-lowering agents. In Table 2, the blood biochemical assay results show that both MS and AK can significantly lower TC and TG concentrations, which is consistent with that reported by previous studies.[8,9] The MS and AK groups showed significant reductions in LDL-C levels ($p < 0.05$), respectively. However, the blood lipid regulation mechanism of MS and AK remain unknown. Therefore, this study examined the influence of MS and AK on lipoprotein formation and metabolism, to understand the path through which MS and AK regulate blood lipids.

Concerning the absorption and regulation of cholesterol in the body, 30% of cholesterol from food is absorbed by the small intestine. Bile acid is also absorbed by the small intestine before being transferred to the liver through enterohepatic circulation and stored in the gallbladder as reserve. Only a small portion of bile acid is excreted in feces. The bile acid that assists food fat absorption is also derived from cholesterol.[27] The largest proportion of endogenous cholesterol in the human body is secreted by the liver, accounting for 50% of the total concentration, followed by the small intestine, which accounts for approximately 15% of all cholesterol. The cholesterol absorbed by the small intestine is converted into cholesteryl ester and then CM before entering the enterohepatic

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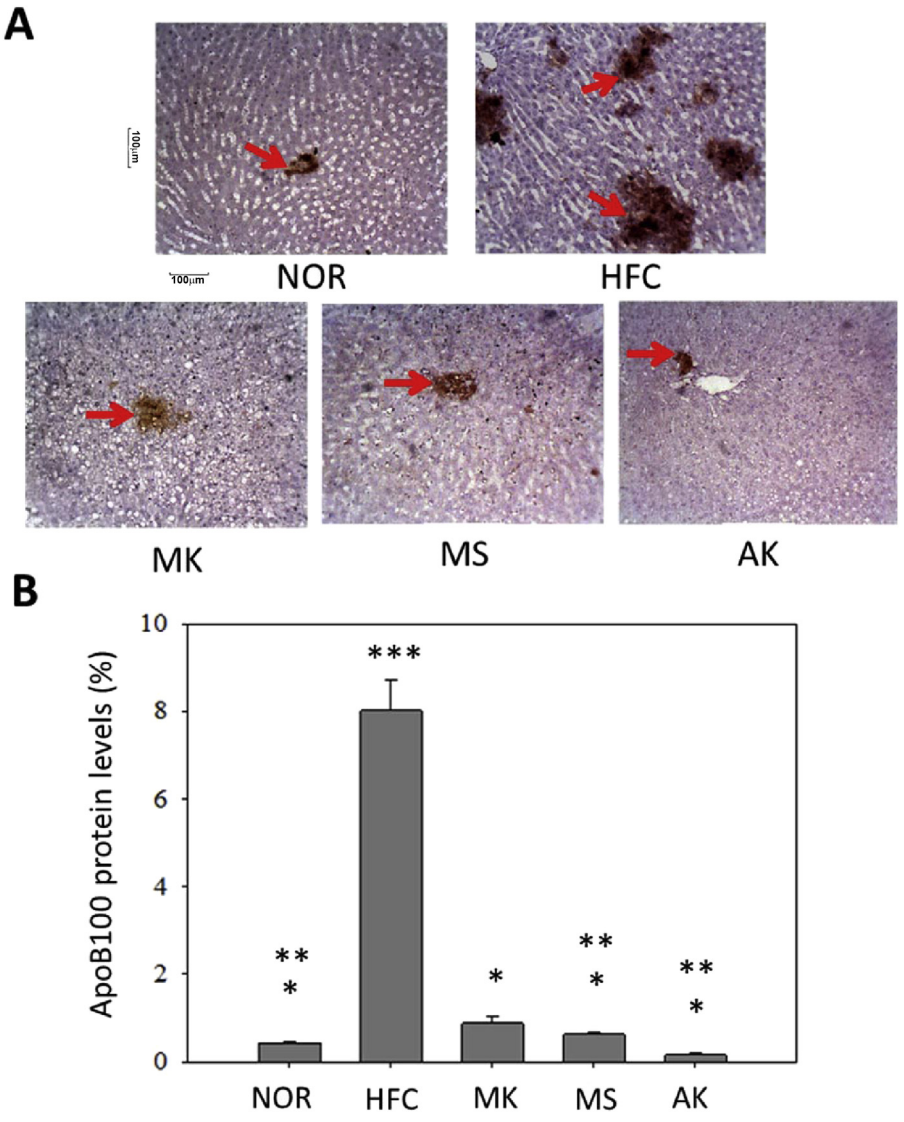

Figure 2. Effect of monacolin K (MK), monascin (MS), and ankaflavin (AK) on apolipoprotein (apo) B100 protein level in the liver of male Sprague-Dawley rats fed with a high-fat and high-cholesterol (HFC) diet. Two groups of the Sprague-Dawley rats were fed a normal diet (NOR group) or a HFC diet (HFC group) without the administration of test materials, respectively. The other hyperlipidemic rats were administrated with MK (0.16 mg/d 400 g body weight; MK group), MS (0.55 mg/d 400 g body weight; MS group), and AK (0.08 mg/d 400 g body weight; AK group), respectively. * $p < 0.001$ versus high-fat and high-cholesterol diet group. ** $p < 0.05$ versus the monacolin K group. *** $p < 0.001$ versus the monacolin K group.

circulation. Although the cholesterol-lowering mechanism remains unclear, the results of this study show that MS and AK can significantly enhance bile acid concentrations, which facilitate the excretion of cholesterol in feces. The increase in bile acid may be attributable to a high HDL-C content, collecting and transporting cholesterol from surrounding tissues to the liver where it is metabolized to form bile acid.[28] In addition, the increase in LDLR also prompts the transportation of LDL-C to the liver for metabolism.[29] However, MK only slightly increased the amount of bile acid (Table 3), which may be attributable to the significant decline in level of HDL-C, causing fewer amount of cholesterol to be transported to the liver for metabolism. Previous studies have shown that MS and AK can significantly reduce the expression of Niemann-Pick C1-like 1 (NPC1L1) protein.[15] NPC1L1 is a crucial enzyme that is absorbed by cholesterol in the small intestine. By inhibiting NPC1L1 expression, MS and AK can reduce the absorption of cholesterol. Therefore, the bile acid, which is excreted to the small intestine due to MS and AK induction, is less frequently absorbed by the small intestine because of the inhibited NPC1L1 expression, thereby reducing cholesterol concentrations.

The results of this study and previous studies confirm that MS and AK can reduce TC and TG concentrations in the serum and liver, which significantly decreases LDL-C content while retaining HDL-C. MS and AK were proven to increase HDL-C content in several studies but maintained in this study.[8–10] However, the animal model of this study is a high-fat diet-induced hyperlipidemic rat which is different to the hamster animal model in our previous studies. Cholesteryl ester transfer protein, which promotes the transfer

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of cholesteryl esters from HDLs to apoB-containing lipoproteins, including VLDLs, VLDL remnants, intermediate density lipoproteins, and LDLs, is expressed in the hamster model but not in the rat model. This discrepancy may lead to a different HDL regulation in hamster and rat models. This study further explored the effects that MS and AK have on the expression of proteins that regulate LDL-C and HDL-C concentrations to understand how MS and AK reduce LDL-C concentrations while maintaining the HDL-C concentration.

Cholesterol synthesized in the body may induce ACAT expression, which transforms cholesterol into cholesteryl ester. Subsequently, the cholesteryl ester is transported by MTP and combined with apo B-100 to form LDL. The LDL then carries cholesterol, forming LDL-C and leading to the accumulation of cholesterol on vascular walls, which results in atherosclerosis development.[23,24] LDL-C and cardiovascular disease are positively correlated. If the LDLR protein level increases, LDL metabolism is improved, reducing the level of LDL-C in the blood.[17] HDL, including the apo A1, collects and transports cholesterol from surrounding tissues to the liver for metabolism. Thus, HDL possesses vascular wall-cleaning efficacy, and the HDL-C level and apo A1 content are both the important factors for reducing the risk of cardiovascular disease.[30,31]

Based on the results in Table 3, the decline in liver TC levels can be inferred to result from the inhibition of endogenous cholesterol biosynthesis. MS can inhibit the merging of cholesterol and LDL in the liver, forming LDL-C. Figure 1 shows that both MS and AK can significantly suppress ACAT protein level. At this stage, MS and AK significantly suppress the conversion of free cholesterol into cholesteryl ester. MS and AK also reduce MTP expression, thereby limiting the transport of cholesteryl ester by MTP. Thus, cholesteryl ester cannot bond with apo B to form CM before entering enterohepatic circulation. Figure 2 shows that the regulating effects of MS and AK also significantly reduce apo B-100 content. Consequently, the CM required for LDL-C and downstream VLDL-C formation cannot be produced because ACAT, MTP, and apo B-100 expressions are inhibited by MS and AK. The serial effects of MS and AK prevent the conversion of free cholesterol into LDL-C. Additionally, regarding serum LDL-C removal, MK and AK increase the expression of LDLR protein in the liver by 67.1% and 74.5%, respectively. This effect enhances LDL metabolism, subsequently reducing LDL-C levels in the blood. Based on these results, MS and AK reduce LDL-C concentrations in the serum by inhibiting LDL-C formation and enhancing LDL-C metabolism.

HDL transports cholesterol from surrounding tissues to the liver for metabolism, thereby exhibiting vascular wall-cleaning efficacy.[31] Therefore, low HDL-C levels may increase the development of cardiovascular disease. A decrease in apo B-100 expression and increase in apo A1 expression guides free cholesterol to the biosynthesis pathway to form HDL-C. Figure 1E shows that MS and AK can significantly increase apo A1 protein level in the liver. The results of previous studies show that reducing the expression of ACAT, MTP, and apo B-100 inhibits the formation of LDL-C.[23,24] Therefore, free cholesterol may shift to undergo the biosynthesis pathway of HDL-C, which explains the result that HDL-C concentration in the presence of MS and AK did not decline because of a decrease in TC.

The results of this study show that MK, MS, and AK exhibit varying lipid-regulation effects. In addition, although all three substances can significantly reduce TC and TG, MK differs from MS and AK in that it possesses weaker LDL-C reduction effects and stronger HDL-C reduction results. However, regarding protein levels, MK

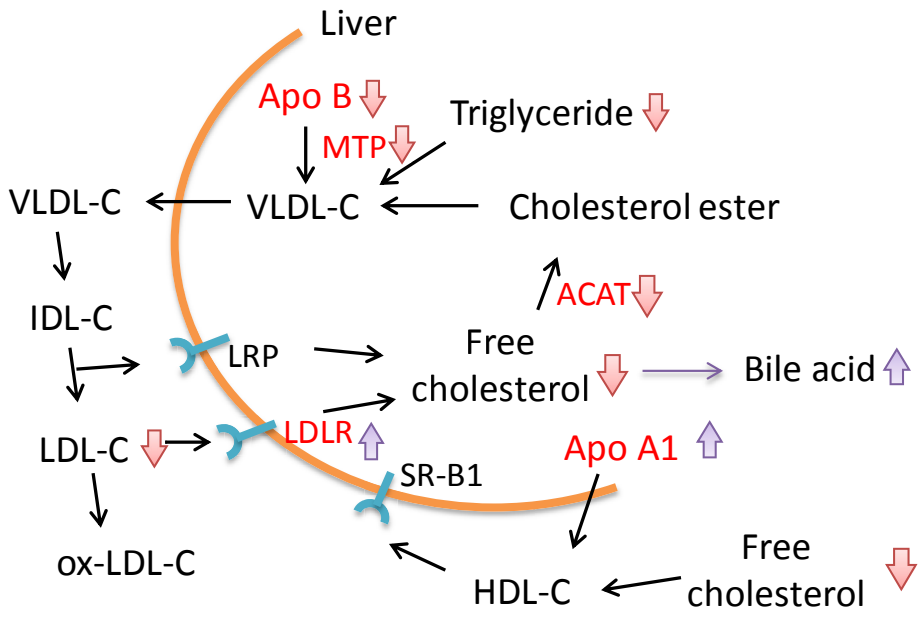

Figure 3. The regulation of low density lipoprotein (LDL) and high density lipoprotein (HDL) assembly by monascin and ankaflavin in the liver. apo B = apolipoprotein B; ACAT = acetyl-coenzyme A acetyltransferase; IDL-C = intermediate density lipoprotein-cholesterol; LDLR = low-density lipoprotein receptor; LRP = low density lipoprotein receptor-related protein; MTP = microsomal triglyceride transfer protein; ox-LDL-C = oxidized-low-density lipoprotein-cholesterol; SR-B1 = scavenger receptor class B1; VLDL-C = very low density lipoprotein-cholesterol.

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exhibits weaker ACAT, MTP, and LDLR inhibition effects, indicating that MK is inferior to MS and AK for inhibiting LDL-C formation and inducing LDL-C transport to the liver for metabolism. This indicates that MS and MK exert superior LDL-C reduction effects. Concerning HDL-C regulation, MK can significantly reduce HDL-C concentrations, possibly because MK cannot significantly increase apoA1 expression (Figure 1E). Consequently, declines in the TC concentration significantly reduce HDL-C concentrations. Based on the preceding discourse, this study infers that the lipid-regulating methods of MS and AK differ from that of MK. MK reduces the conversion of free cholesterol into LDL-C and HDL-C by limiting cholesterol synthesis. Therefore, despite lowering LDL-C concentrations, MK cannot prevent a decline in HDL-C, which consequently causes atherosclerosis. Conversely, by inhibiting LDL formation, accelerating the metabolic process of recycled LDL-C, and increasing apo A1 expression, MS and AK not only reduce TC and LDL-C content, but also preserve HDL-C concentrations. This study attempted to understand their regulation on LDL-C synthesis. In future studies, details on blood lipid regulation should continue to be carried out with *in-vitro* and *in-vivo* studies. MS and AK may be novel natural hypolipidemic compounds for functional food and medicine developments in the future.

Conclusion

This study confirms that MS and AK possess significant cholesterol-lowering effects and the ability to preserve HDL-C content. HDL-C induces the transport of cholesterol to the liver where cholesterol is metabolized into bile acid, which is subsequently excreted, lowering the overall cholesterol level. The lipoprotein regulations of MS and AK are concluded in Figure 3. MS and AK can inhibit ACAT, MTP, and apo B-100 expression, thereby preventing LDL formation. In addition, enhanced LDLR expression increases the transport of LDL-C to the liver for metabolism. Regarding HDL-C regulation, MS and AK significantly increase apo A1 expression, which facilitates HDL-C formation. The effects exerted by MS and AK on various lipoprotein regulatory factors significantly exceeded that of MK, particularly, in increasing apo A1 expression. This is also the reason that, unlike MK, MS and AK do not induce a significant decline in HDL-C. This study is the first to investigate the blood lipid regulation of MS and AK. This finding of blood lipid regulation will be of benefit to the development of the two novel hypolipidemic compounds produced by natural *M. purpureus* on functional food and medicine developments in the future.

Conflicts of interest

The authors have nothing to disclose.

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