Hypolipidemic effects of Alismatis rhizome on lipid profile in mice fed high-fat diet

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ABSTRACT

الأهداف: التحقق من تأثير خلاصة أليزماتيز ريزوم (Alismatis rhizome) على الفئران التي أُعطيت حمية عالية الدهون.

الطريقة: أُجريت هذه الدراسة في مختبر المجمع الصيني للوصفات الطبية التابع لجامعة هيوبي للعلوم الطبية، وزارة التعليم، ووهان، الصين، واستمرت خلال الفترة من ديسمبر 2009م إلى يونيو 2010م. شملت الدراسة فئران كيومينغ التي تبلغ من العمر 48 أسبوعاً والتي أُخضعت للتجربة على مدى 4 أسابيع وذلك بعد تقسيمها إلى المجموعات التالية: المجموعة 1 وهي مجموعة الشاهد التي كانت حميتها طبيعية ولم تُعط شيئاً، والمجموعة 2 وهي مجموعة الشاهد التي تناولت حمية دسمة ولم تُعط شيئاً، والمجموعة 3 وهي مجموعة الشاهد الإيجابية التي تناولت حمية دسمة مع عقار سيمفاستاتين، والمجموعة 4 وهي مجموعة الدراسة التي تناولت حمية دسمة وأُعطيت المقدار 2.62 غرام/كلغ من خلاصة أليزماتيز ريزوم. لقد تم تقييم دور هذه الخلاصة في تخفيض دهون الدم من خلال الاختبارات التالية: مستوى الدهون في مصل الدم، ودهون الكبد، والتفاعل التسلسلي المبلمر ذو النسخ العكسي، بالإضافة إلى قياس مستويات ناقل الأمينات في مصل الدم، والتغيرات التي حصلت في الأنسجة.

النتائج: أشارت نتائج الدراسة إلى أن تناول خلاصة أليزماتيز ريزوم قد أدى إلى تخفيض الكولسترول وثلاثي الجليسريد في مصل الدم والكبد، كما أنه أدى إلى زيادة مستويات الكولسترول عالي الكثافة لدى الفئران التي تناولت الحمية الدسمة. وقد أظهر تحليل الأنسجة بأن حالة الفجوات الشحمية لدى الفئران المُعالجة كانت مشابهة للفئران في مجموعة الشاهد التي كانت حميتها طبيعية. كما أدت هذه الخلاصة إلى تقليل مستويات ناقلة الألانين، وناقلة الأسبارتات، بالإضافة إلى إنقاص وزن الكبد النسبي.

خاتمة: أثبتت هذه الدراسة فعالية خلاصة أليزماتيز ريزوم في علاج ارتفاع مستوى الدهون في الجسم، حيث أنها تعمل على تقليل تمثيل الكبد للكولسترول وليس زيادة تقويض الكولسترول.

Objectives: To investigate the effect of Alismatis rhizome (AR) extract on lipid profile in mice fed high-fat diet.

Methods: The study was performed in Key Laboratory of Chinese Medicine Resource and Compound Prescription (Hubei University of Chinese Medicine), Ministry of Education, Wuhan, China, between December 2009 and June 2010. Forty male Kunming mice (8-week-old) were randomly divided into 4 groups and were treated for 4 weeks: Group 1: normal control, Group 2: high-fat control, Group 3: positive control and Group 4: AR 2.26 g/kg. The hypolipidemic effects of AR were evaluated by serum lipids, liver lipids, and reverse transcriptase polymerase chain reaction. Serum aminotransferases and histopathological changes were also measured.

Results: Alismatis rhizome treatment resulted in an obvious decrease in serum and liver cholesterol, triglyceride along with elevated serum high-density lipoprotein cholesterol in hyperlipidemic mice. The histopathological results showed that adipose vacuoles in AR treated mice liver were almost identical to those of normal control mice. Serum alanine transaminase, aspartate aminotransferase and the relative liver weight in AR treated mice were decreased significantly. Alismatis rhizome substantially decreased the mRNA expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmgcr), while the expressions of sterol regulatory element binding factor 2 (Srebf2) and cholesterol 7alpha-hydroxylase (Cyp7a1) were marginally affected.

Conclusion: These results confirmed the efficacy of AR in the treatment of hyperlipidemia. Alismatis rhizome may act by decreasing the liver synthesis of cholesterol, rather than by increasing the cholesterol catabolism.

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Hyperlipemia is consistently associated with an increased risk for all forms of atherosclerotic disease and its clinical sequelae, including coronary heart disease, stroke, and sudden death.[1] Statins and fibrates are the most commonly used lipid-lowering medications in primary and secondary prevention of atherosclerotic disease. Although these classical hypolipidemic agents are generally well tolerated, considerable attention has been focused on their side-effects such as gastrointestinal symptoms, serum aminotransferase elevations, a reversible rise in creatinine, and myositis.[2,3] In addition, a number of patients did not respond well to the therapy.[4] Thus, novel approaches are in development to battle the world epidemics of hyperlipidemia. As medicinal plants have been proven to be promising resources for the discovery of new drugs, many researchers are interested in the hypolipidemic effects of natural substances, which are traditionally used as folk remedies. Alisma orientalis (Sam.) Juzep is a hardy aquatic plant in China, Japan, Mongolia, and Russia.[5] The dried rhizome of the plant, known as Alismatis rhizome (AR), is a popular traditional chinese medicine. Alismatis rhizome has been used for diuretics and some symptoms of hyperlipidemia and diabetes in China for centuries. Phytochemical studies have revealed that protostane-type triterpenes and sesquiterpenes are the principal constituents of AR.[6] Alismatis rhizome and its active ingredient candidates have also been reported to have a variety of biological effects, including antihypertensive, antimultidrug resistance, anti-hepatitis B, and anti-tumor activities.[6-8] Currently, a number of published reports have demonstrated that AR is efficacious in the treatment of hyperlipemia in the clinic.[9-11] However, scientific data on the hypolipidemic efficacy of AR are scare, and the mechanism of action is unknown. Therefore, the present study was carried out to investigate the effects of AR extract on lipid profile in mice fed high-fat diet.

Methods. Forty male Kunming mice (8-week-old; 18-22g) were purchased from Wuhan Institute of Biological Products, China (certificate No.: SCXK 2008-0003). Animal care and treatment were approved prior to the study by the Animal Care Committee of Hubei University of Chinese Medicine, Hubei, China. The study was carried out in Key Laboratory of Chinese Medicine Resource and Compound Prescription (Hubei University of Chinese Medicine), Ministry of Education, Wuhan, China, during the period December 2009 to June 2010.

Animals were randomly divided into 4 groups (10 mice per group). The first group received a standard diet, while the other groups (groups 2-4) were given a high-fat diet for 3 weeks. The high-fat diet consists of 1% cholesterol, 0.2% cholate, 10% lard, 10% egg yolk, 78.8% standard laboratory diet. At the end of week 3, the blood withdrawn from caudal vein and was centrifuged to get serum samples, and sera were investigated to determine lipid levels. In the following 4 weeks, all of the groups were maintained on a diet the same as before. In addition, Group 1 received distilled water as normal control (NC), Group 2 also received distilled water as high-fat control (HF), Group 3 received 6.7mg/kg of simvastatin as positive control, and Group 4 received 2.26g/kg/d of AR extract. The body weight and food consumption were recorded weekly. At the endpoint of the study, rats were anesthetized with phenobarbital and euthanized, and blood was collected without anticoagulant. The liver and spleen were removed and weighed. The relative weights of liver and spleen were calculated by the following equation: the relative weight of liver or spleen = absolute liver weight or spleen weight/body weight at sacrifice × 100.

The levels of serum cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined by commercial kits according to the manufacturer's protocol. The concentration of low density lipoprotein cholesterol (LDL-C) was calculated by the following equation: LDL-C=TC-(HDL-C+0.2TG). The lipids of liver tissue were determined according to the minor modifications methods.[12] Briefly, 200 mg of liver tissue was homogenized with 4 mL dichloromethane-methanol (2:1, v/v), and sodium chloride was added to homogenate and mixed. The mixture was centrifuged and aliquot of the organic phase was mixed with Triton X-100. After evaporation of the organic solvents, the TC and TG contents in the detergent phase were determined using the previously mentioned test kits. The results were expressed as mg/g wet tissue. A portion of the liver was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histopathological examination.

Detection of liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmgcr), cholesterol 7α-hydroxylase (Cyp7a1), sterol regulatory element binding factor 2 (Srebf2) mRNA expressions was performed by reverse transcriptase polymerase chain reaction (RT-PCR). Total ribonucleic acid (RNA) was extracted from liver using Simply P Total RNA Extraction kit (Bioflux, Japan) according to the manufacturer's protocol.

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Single-strand cDNA was synthesized from 2μg RNA by reverse transcription (RevertAidTM First strand cDNA Synthesis Kit, Fermentas, EU) using an oligo dT primer. Specific PCR primers were either identical to those described previously[13] or were designed using Primer 5. All PCR primer sequences are shown in **Table 1**. Polymerase chain reaction (PCR) using the Hmgcr primer was performed with an initial cycle of 4 min at 94°C followed by 29 cycles of 30 seconds at 94°C, 1 minute at 57°C, 1 minute at 72°C, and a final extension for 5 min at 72°C. Polymerase chain reaction using the Cyp7a1, Srebf2, and β-actin primers was performed similarly, with the exception of the annealing temperature (Cyp7a1, 50°C; Srebf2, 56°C; β-actin, 56°C) and the number of cycles (Cyp7a1, 35cycles; Srebf2, 30cycles; β-actin, 30cycles). β-actin was used as an internal standard to monitor loading variations. The PCR products were separated on 1.2% agarose gel and visualized with the Gel Doc-ITTM imaging system (Ultra-Violet Products Ltd., CA, USA) in the presence of GelRedTM (Biotium, USA).

Data are presented as means±SD. Statistical analysis was carried out by one-way analysis of variance (SPSS Version 16.0 [SPSS, Inc., Chicago, USA]). A *p*-value of less than 0.05 was considered statistically significant.

Results. There were no significant differences in the food intake as well as the body weight gain among the groups (data not shown). As shown in **Figure 1**, a high-fat diet resulted in a significant increase in both the relative liver weight and spleen weight of mice. In comparison with the HF control, the relative liver weight in AR treated mice was reduced by 11.1% (p=0.002), and the relative spleen weight, 15.4% (p=0.02). Simvastatin also significantly decreased the relative liver weight and spleen weight. Animals fed high-fat diet exhibited a remarkable elevation of serum ALT and AST activities in comparison with the normal group (**Figure 2**). Alanine transaminase activity in AR treated mice was lowered to 86.3% of the HF control (p=0.01), and AST activity, 80.5% (p=0.0009). However, ALT activity in simvastatin treated mice was increased to 1.1-fold of the HF control (p=0.02), and AST activity, 1.3-fold (p=0.0008).

Table 2 shows the general effects of AR on serum and hepatic lipid levels. Three weeks of high-fat diet induced hypercholesterolemia and hypertriglyceridemia

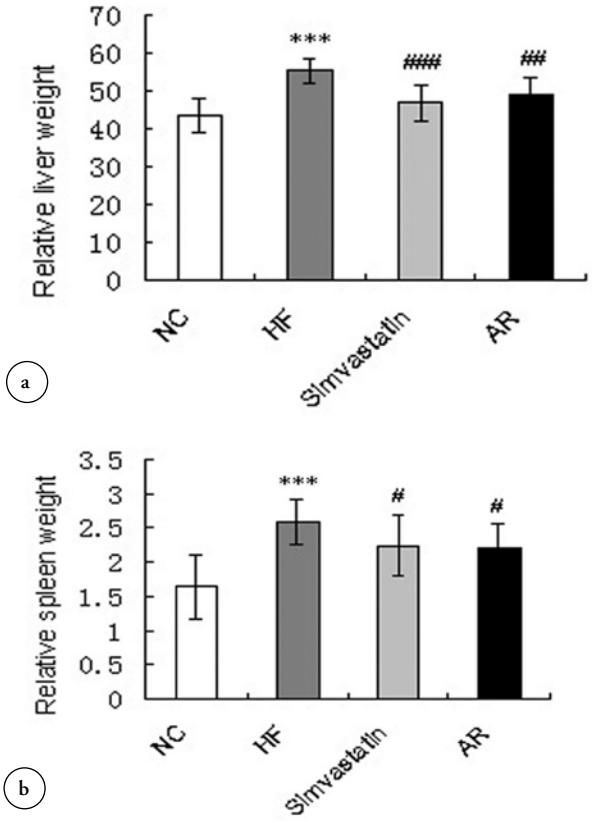

Figure 1 - Effects of Alismatis rhizome (AR) on: **a)** Relative liver weight and **b)** Relative spleen weight. Data are expressed as means ± SD (n=10). $^{***}p$<0.001 compared with normal control (NC). $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 compared with high-fat control (HF).

Table 1 - Primer Sequences of 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmgcr), cholesterol 7α-hydroxylase (Cyp7a1), sterol regulatory element binding factor 2 (Srebf2) and β-actin.

Gene name	GenBank Accession No.	Primer sequence (5'–3')
Hmgcr	NM_008255.2	Forward: GTTCTTTCCGTGCTGTGTTCTGGA
		Reverse: CTGATATCTTTAGTGCAGAGTGTGGCAC
Cyp7a1	NM_007824.2	Forward: AGTTACTCTTCCCGTTTC
		Reverse: ATCACCTCCAGCCTCTAC
Srebf2	NM_033218.1	Forward: AAATCCACGGTCCAAGCC
		Reverse: GTGCGTCTATCAAGTCCAGAAT
ß-actin	NM_007393.3	Forward: CACTGTGCCCATCTACGA
		Reverse: CAGGATTCCATACCCAAG

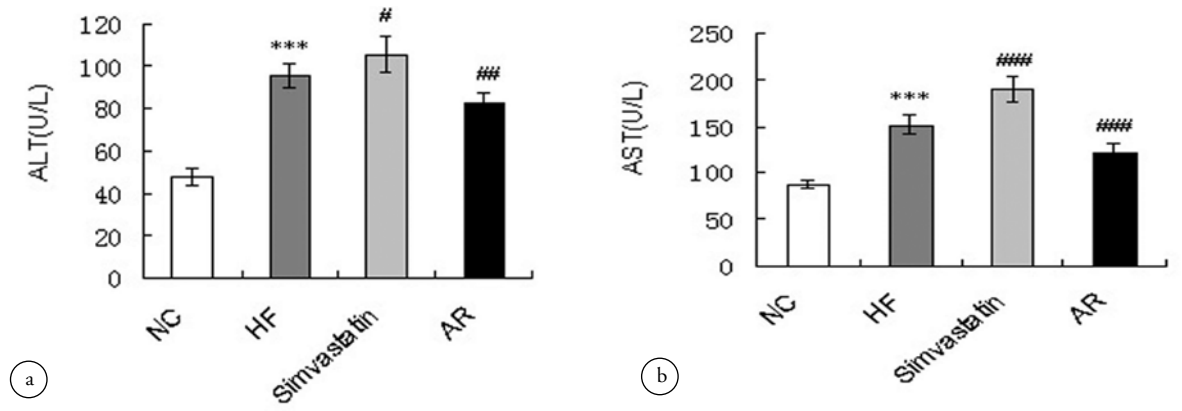

Figure 2 - Effects of Alismatis rhizome (AR) on **a**) serum alanine aminotransferase (ALT) and **b**) serum aspartate aminotransferase (AST). Data are expressed as means ± SD (n=10). ***p<0.001 compared with normal control (NC). $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 compared with high-fat control (HF).

Table 2 - Effects of Alismatis rhizome (AR) on serum total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), liver TC and TG.

Lipid profile	NC	HF	Simvastatin	AR
Serum TC (mmol/L)				
0 week	3.77 ± 0.56	7.68 ± 1.73†	8.07 ± 1.74	8.18 ± 1.99
2 weeks	3.51 ± 0.77	7.27 ± 1.69†	6.83 ± 1.15	6.39 ± 1.34
4 weeks	3.83 ± 0.66	6.52 ± 1.32†	4.42 ± 0.59§	5.15 ± 1.03‡
Serum TG (mmol/L)				
0 week	0.57 ± 0.13	1.16 ± 0.17†	1.15 ± 0.21	1.14 ± 0.24
2 weeks	0.59 ± 0.14	1.20 ± 0.24†	0.94 ± 0.16‡	0.95 ± 0.08‡
4 weeks	0.63 ± 0.11	1.22 ± 0.16†	0.68 ± 0.09**	0.76 ± 0.08**
Serum LDL-C (mmol/L)				
4 weeks	0.36 ± 0.67	3.96 ± 1.79†	1.78 ± 0.97§	2.12 ± 1.41§
Serum HDL-C (mmol/L)				
4 weeks	3.00 ± 0.57	2.19 ± 0.37†	3.14 ± 0.43**	3.29 ± 0.73**
Liver TC (mg/g)				
4 weeks	3.05 ± 0.40	45.56 ± 8.80†	32.21 ± 9.33‡	34.42 ± 4.67‡
Liver TG (mg/g)				
4 weeks	13.17 ± 2.19	17.30 ± 4.26*	11.53 ± 1.64§	14.20 ± 2.51‡

Data are expressed as means±SD (n=10). $^{*}p$<0.05, $^{\dagger}p$<0.001 compared with normal control (NC). $^{\ddagger}p$<0.05, $^{\S}p$<0.01, $^{**}p$<0.001 compared with high-fat control (HF)

in the experimental mice indicated by the increased serum TC and TG. In comparison with the HF control, the serum TC was decreased by 21.0% (p=0.04), TG 37.7% (p=0.00001), and LDL-C 46.5% (p=0.007), whereas HDL-C was increased by 50.2% (p=0.0005) after 4 weeks of consecutive treatment with AR. Similarly, simvastatin decreased the serum TC, TG, and LDL-C, but increased the serum HDL-C. To determine an ectopic accumulation in liver, the hepatic TC and TG levels were compared. The hepatic TC and TG of the HF group were dramatically increased. Compared to the HF control, AR treatment decreased the hepatic TC by 24.5% (p=0.03) and TG by 17.9% (p=0.04). The hepatic TC of simvastatin treated mice was decreased by 29.3% (p=0.04) and TG by 33.4% (p=0.004).

Figure 3 displays a typical RT-PCR result showing the mRNA expression of 3 key regulators of cholesterol level: (A) Hmgcr, (B) Cyp7a1, and (C) Srebf2. As indicated in **Figure 3a**, mRNA expression of Hmgcr in the HF group was significantly enhanced comparable to normal control (p=0.002), while AR decreased the mRNA expression of Hmgcr by 64.0% (p=0.005). The mRNA expression of Cyp7a1 and Srebf2 were marginally affected by the feeding of high-fat diet. Alismatis rhizome treatment had no significant effects on Cyp7a1 and Srebf2 mRNA expression (**Figures 3b & 3c**).

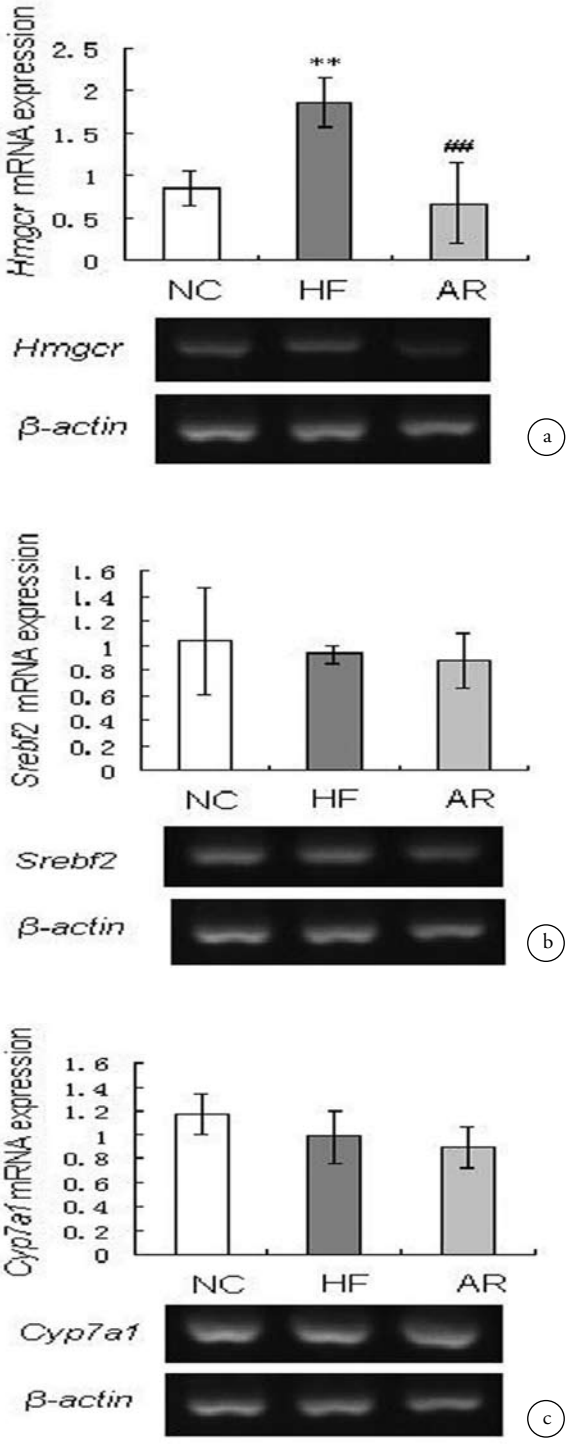

Figure 3 - Effects of Alismatis rhizome (AR) on the mRNA expression of key regulators of cholesterol level: **a)** 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmgcr) mRNA expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). **b)** Cholesterol 7α-hydroxylase (Cyp7a1) mRNA expression was analyzed by RT-PCR. **c)** Sterol regulatory element binding factor 2 (Srebf2) mRNA expression was analyzed by RT-PCR. β-actin was used as an internal standard. Each column represents the mean ± SD of four animals. $^{**}p<0.01$ compared with normal control (NC). $^{\#\#}p<0.01$ compared with high-fat control (HF).

Figure 4 shows the photomicrographs of histopathological examination of 4 groups: normal control, high-fat control, simvastatin, and Alismatis rhizome. The hepatic lobules were well-arranged without liquid droplets in the liver tissues of the mice in NC group (**Figure 4a**). The widespread distribution of fat vacuoles in the cytoplasm of liver cells was observed in HF mice (**Figure 4b**). The number of these fatty droplets was reduced noticeably in the livers of hyperlipidemic mice treated with AR and simvastatin (**Figures 4c & 4d**). Livers in AR and simvastatin groups possessed several similarities to the normal liver structures in NC group.

Discussion. Our results clearly demonstrate that consumption of high-fat diet for 3 weeks led to hypercholesterolemia as indicated by significant increase in serum TC, TG. Alismatis rhizome treatment in hyperlipidemic mice caused an obvious decrease in serum TC, TG, and LDL-C in spite of continued access to the high-fat diet for 4 weeks. Atherosclerosis often associates with accumulation of lipoproteins along blood vessels, leading to cardiovascular disease morbidity and mortality. Several recent meta-analyses of numerous lipid-lowering outcome trials confirm the direct relationship between LDL-C lowering and cardiovascular risk reduction.[14] Therefore, prime consideration in the therapy for atherosclerosis is to attenuate the elevated lipid levels. We found that AR consumption attenuated the elevated lipid levels in hyperlipidemic mice. Moreover, AR consumption raised the low level of HDL-C, which is a continuous inverse cardiovascular risk factor.[15] High-density lipoproteins protect against atherosclerosis by reversing cholesterol transport, and excess cellular cholesterol is returned to the liver for excretion in the bile. It is of importance since quite a number of antihyperlipidemic drugs are causing significant reduction in both TC and HDL-C levels.[16] Our data demonstrated that AR consumption decreased the liver lipids as evidenced by histopathological examination as well as the obvious reduction of hepatic TC and TG. Whole body cholesterol homeostasis is controlled by supply and removal pathways, including dietary cholesterol uptake, hepatic cholesterol biosynthesis, cholesterol catabolism.[17] The food intake did not differ among the groups, which eliminated the influence of exogenous cholesterol uptake. The relative activity of the supply and removal pathway can be assessed by quantifying relevant cellular biomarkers. We, therefore, tested the mRNA expression of several key regulators of cholesterol level. We found that AR substantially decreased the expression of Hmgcr, while the expression of Srebf2 and Cyp7a1 were marginally affected. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR)

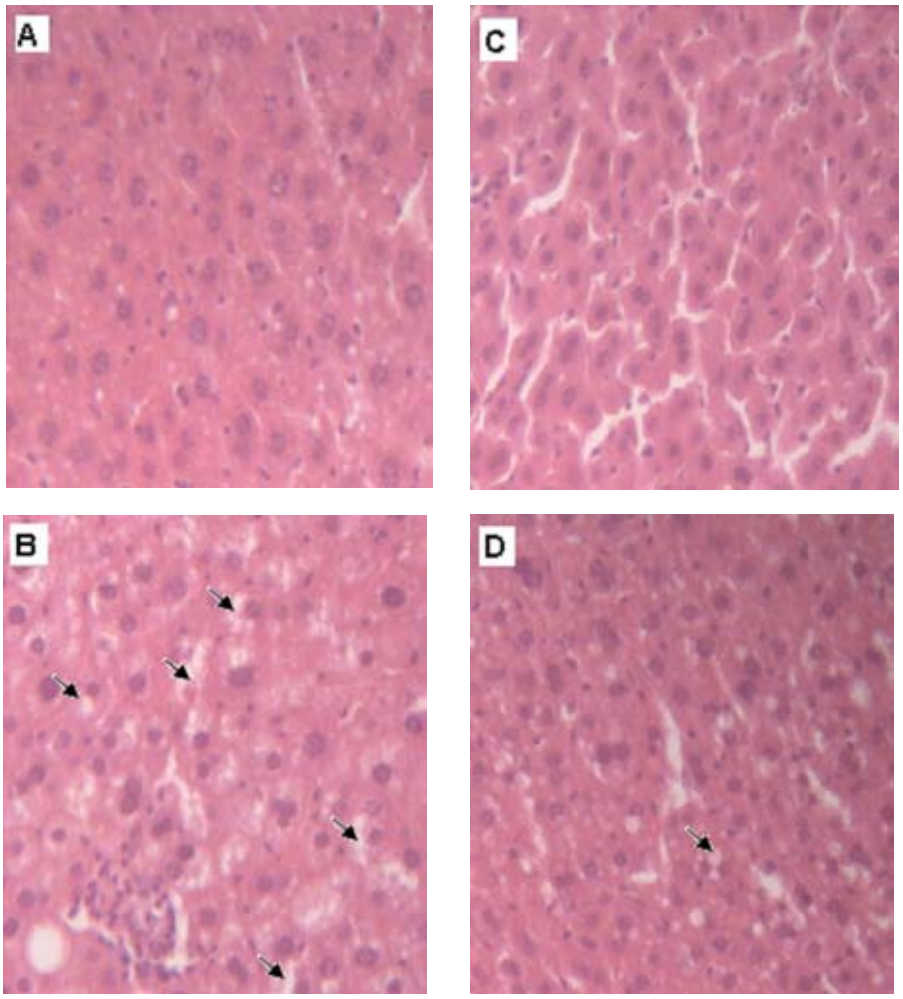

Figure 4 - Effects of Alismatis rhizome (AR) on the histological morphology of mice livers by hematoxylin and eosin staining (400×): **a)** Normal control , **b)** High-fat control, **c)** Simvastatin and **d)** Alismatis rhizome. Arrow indicates the adipose vacuoles in the liver.

is a major pharmacologic strategy to lower plasma cholesterol levels because the enzyme mediates a rate-limiting step in the hepatic cholesterol biosynthesis.[18] Sterol regulatory element binding factor 2 (SREBF2) is a membrane-bound transcriptional regulator of cholesterol synthesis modulating the expression of Hmgcr.[19,20] The inhibition of Hmgcr mRNA expression in AR treated mice indicated that the hypolipidemic effects of AR may result from decreasing endogenous synthesis of cholesterol in the liver. But, the mRNA expression of Hmgcr was not regulated by Srebf2. Cholesterol oxidation by cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting step in bile acid synthesis and therefore cholesterol catabolism.[21] The change of Cyp7a1 expression can provide information about the hepatic cholesterol catabolism rate.[22] In the present study, AR had no effect on Cyp7a1 mRNA expression, suggesting that the hypolipidemic effects of AR may not result from increasing the rate of cholesterol catabolism. Activities of ALT and AST were markedly elevated in the liver of mice fed high-fat diet, showing that liver damages were induced in the condition of hyperlipemia. The result in this study suggests that simvastatin may further worsen the liver damages induced by hyperlipemia as ALT and AST were increased dramatically comparable to HF control. The result is consistent with published reports that statins might cause hepatic adverse effects known as elevated liver aminotransferase levels clinically.[23,24] In this study, serum ALT and AST activities as well as the relative liver weight in AR treated mice were decreased significantly, indicating that AR had hepatoprotective effects in hyperlipidemic mice.

The study had certain limitations. We did not test the hypolipidemic effects of multiple dosages of AR. Further study should be carried out to investigate the dose dependence of this extract.

In conclusion, the morphological and biochemical results confirmed the efficacy of AR in the treatment

of hyperlipidemia. Moreover, AR showed a positive protective effect on the liver of hyperlipidemic mice. It seems that the hypolipidemic effect of AR is caused partly by inhibiting hepatic cholesterol biosynthesis. Further work is clearly needed to elucidate the mechanism of AR for development of new hypolipemic drugs.

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