

Effects of a Purified Saponin Mixture from Alfalfa on Plasma Lipid Metabolism in Hyperlipidemic Mice

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Alfalfa (*Medicago sativa* L.) is a genus distributed widely throughout the world and considered as the richest land source of nature products. In this study, we obtained a purified saponin mixture (PSM) through a simple, repeatable and controllable separation method from alfalfa. The compounds of the saponin mixture were identified as Azukisaponin V and Soyasaponin I, which was determined as 79.26 and 20.74% respectively by HPLC-evaporative light scattering detection (ELSD) method. The effects of PSM on plasma lipid metabolism were studied in hyperlipidemic mice induced by Triton WR-1339. Results showed that PSM could significantly inhibit the increase of total cholesterol (TC), triacylglycerol (TG) and low density lipoprotein cholesterol (LDL-C) levels, and the decrease of plasma high density lipoprotein cholesterol (HDL-C) level in plasma of hyperlipidemic mice induced by Triton WR-1339. Further study demonstrated that PSM not only enhanced the activities of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) in liver, but also increased their mRNA levels in hyperlipidemic mice. The results above indicate that PSM from alfalfa exerts effects on plasma lipid metabolism through elevating the activity of liver lipases in hyperlipidemic mice. The obtained data would be a good contribution to the development and utilization of alfalfa in the world.

Key words — alfalfa, saponin, Triton WR-1339, lipid metabolism

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a genus distributed widely throughout the world and considered as the richest land source of nature products.¹⁾ It is traditionally used to treat hyperlipidemia in Chinese folk²⁾ and modern pharmacological studies indicated it has hypolipidemic activities.^{3,4)} Many studies proved that saponins are the main active compounds in alfalfa.^{5,6)} However, the relatively low content of saponins in alfalfa extracts leads to the decrease of hypolipidemic activities, and results in the unavailable medicinal and clinical usages. In this study, we obtained a purified saponin

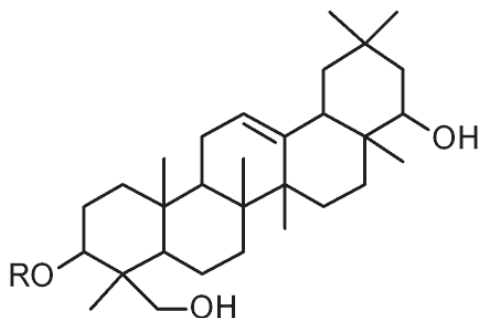
mixture (PSM) through a simple, repeatable and controllable separation method. The compounds of the saponin mixture were identified as Azukisaponin V and Soyasaponin I (AS). The contents of each saponins was determined by HPLC method. Moreover, the hypolipidemic activities of PSM on plasma lipid metabolism were investigated in hyperlipidemic mice induced by Triton WR-1339.

MATERIALS AND METHODS

Plant Material and Preparation of Saponin Mixture — Alfalfa was obtained from leaves and stems of *Medicago sativa* L. growing in a natural field in the Hebei province, northern China, in July 2009. The plant was verified by the herbarium of South China Botanical Garden, Chinese Academy of Sciences (Guangzhou, China). The air-dried plant material was powdered and extracted exhaus-

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Soyasaponin I R= GlcA-Gal-Rha
Azukisaponin V R= GlcA-Glc-Rha

Fig. 1. Chemical Structures of AS in Isolated Saponin Mixture from Alfalfa

PSM was obtained from alfalfa after extraction by ethanol and purification by D101 macroporous adsorption resin and ODS column chromatography. The chemical structures of PSM were identified as AS by spectrophotometer, mass spectrometer and NMR.

tively with 65% ethanol. After filtration and evaporation, the solvent was condensed under reduced pressure to obtain crude extract. The crude extract was subjected to chromatographic separation on D101 macroporous adsorption resin (Hualing Resin Co. Ltd., Shanghai, China) and eluted with water, 35%, and 85% EtOH-H₂O to give three fractions. The major saponins were obtained in 85% EtOH-H₂O fraction. This fraction was subjected to octadecylsilane (ODS) HPLC column chromatography and eluted with 50 and 70% methanol. The 70% methanol elution was collected and solvent was removed by rotary evaporation under low temperature (45°C) to yield PSM. The recovery rate of PSM was 0.99%. The chemical structures of the saponin constituents were identified as AS (shown in Fig. 1) by spectrophotometer (Waters Corp., Milford, MA, U.S.A.), mass spectrometer (Bruker Daltonics, Billerica, MA, U.S.A.), and NMR (Bruker Daltonics, Billerica, MA, U.S.A.). Standard compounds of Soyasaponin I and Azukisaponin V were purchased from Tauto Biotech (Shanghai, China).

Quantification of AS by HPLC-ELSD— The quantification of AS was analyzed by HPLC-ELSD (Waters Alliance 2695-2996+SEDEX 75 ELSD), under the following conditions: column, Ultimate™ XB-C₁₈ (250 mm × 4.6 mm, 5 μm); mobile phase, 40% acetonitrile and 60% water with 1% acetic acid; flow rate, 0.8 ml/min; column temperature, 30°C. The ELSD conditions were 100°C and 3.2 l/min flow rate. Quantification of each saponin was calculated by standard calibration curves. Data were obtained as average values in triplicate experi-

ments.

Animals and Treatment— Five-week-old male Kunming mice (18.0–20.0 g)^{7,8)} were purchased from Center of Laboratory Animal Science Research (Guangzhou, China). All mice were housed in plastic cages (29.5 cm × 17.5 cm × 13.0 cm) under controlled temperature (23 ± 1°C), humidity (60 ± 5%), with a 12-hr day/night cycle (lights on at 06:00) and fed with a normal diet and water *ad libitum*. The animals were allowed to acclimatize to the environment for 1 week before the experiment. Procedures for animal experiments were conducted in accordance with the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health (NIH). Sixty mice were randomly divided into 6 groups with 10 animals each: normal control group, Triton WR-1339 model group, Fenofibrate group (FEN, 100 mg/kg), low, middle and high doses of PSM groups (100, 200, 400 mg/kg). FEN was product of Laboratories Fournier S.A. (Daix, France). Mice in the PSM groups were orally administered with PSM for consecutively 7 d. On the 7th day, all animals except normal control group were fasted for 12 hr prior and then treated with Triton WR-1339 (300 mg/kg), which was injected into a tail vein of mice 1 hr after final administration. Triton WR-1339 was obtained from Sigma (St. Louis, MO, U.S.A.). Blood was drawn from heart and livers were quickly removed under anesthesia 20 hr after Triton WR-1339 treatment.⁸⁾

Measurement of Plasma Lipids Levels— Blood samples kept in a tube containing 2% sodium heparin were centrifuged at 5000 rpm for 5 min to obtain plasma and stored at -20°C until the assay. As previously described,^{9,10)} the levels of total cholesterol (TC), triacylglycerol (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in plasma were enzymatically determined with commercial assay kits (Nanjing Jiancheng Bioengineering Co. Ltd., Nanjing, China).

Determination of Liver Lipases Activities— Liver samples were prepared according to the report of He *et al.*¹¹⁾ Briefly, a 100-mg sample of tissue was removed immediately from each liver and kept in phosphate buffered saline (PBS) solution under an ice-cold condition. Then the tissues were minced with scissors and homogenized with a homogenizer at the maximum setting for 30 s under an ice-cold condition in 1 ml of solution [containing 0.25 M sucrose, 1 mM ethylenediaminete-

traacetic acid (EDTA), 10 mM Trizma hydrochloric acid (Tris-HCl), and 12 mM deoxycholate, pH 7.4]. The homogenate was collected by centrifuging at 12000 rpm 4°C for 20 min and diluted with 4 volumes of the homogenization solution, then decanted and immediately stored at -20°C until assay. As previously described,^{10, 11} the activities of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) in liver were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Co. Ltd.) and expressed as units per milligram protein.

Analysis of Liver LPL and HTGL mRNAs Expression—The mRNA levels of liver LPL and HTGL were determined by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) method using Maxima™ Synergy Brands, Inc. (SYBR) Green/Fluorescein quantitative PCR (qPCR) Master Mix (Fermentas, Hanover, MD, U.S.A.) via IQ™5 real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). The PCR primers for mice LPL mRNA were F: 5'-CTAACTGCCACTTCAACC-3' and R: 5'-CAGACTTCCTGCTACGC-3', and the product size was 320 bp. The PCR primers for mice HTGL mRNA were F: 5'-TGGACGGGAAGAACAAG-3' and R: 5'-GGAGTCAATGAAGAGGTGC-3', and the product size was 324 bp. The primers for the mice housekeeping gene 18S mRNA were F: 5'-GGGAGAGCGGGTAAGAGA-3' and R: 5'-ACAGGACTAGGCGGAACA-3', and the product size was 241 bp. The final products were analyzed by Ct method.

Statistical Analysis—Data were presented as mean ± S.D. The differences between experimental groups were evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's test using SPSS 13.0 software. Differences between the group means were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

In this study, PSM was obtained from alfalfa after extraction by ethanol and purification by D101 macroporous adsorption resin and ODS column chromatography. The compounds of the saponin mixture were identified as AS (shown in Fig. 1), the proportions of which were determined as 79.26 and 20.74% by HPLC-ELSD, respectively.

We further studied the effects of PSM on plasma lipid metabolism in hyperlipidemic mice induced by Triton WR-1339. As shown in Table 1, when compared with normal group, Triton WR-1339 treatment caused a marked increase in levels of plasma TC, TG, and LDL-C ($p < 0.01$) and a decrease in HDL-C level ($p < 0.01$), indicating hyperlipidemia was induced by Triton WR-1339. However, plasma TC level was significantly reduced by oral administration of PSM (200, 400 mg/kg) in mice treated with Triton WR-1339. Besides, all doses of PSM remarkably decreased the levels of plasma TG and LDL-C. In contrast, PSM (200, 400 mg/kg) significantly enhanced plasma HDL-C level ($p < 0.05$). The positive drug FEN (100 mg/kg) also resisted all the changes of plasma lipids caused by Triton WR-1339. Triton WR-1339 has been known to increase plasma levels of TC and TG by up-regulating 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity and decreasing lipases activity.^{12, 13} Therefore, Triton WR-1339 is widely used to study lipid metabolism and induce acute hyperlipidemia in animals to screen potential hypolipidemic drugs, in particular natural or chemical drugs.^{9, 14} It is well known that high levels of plasma TC, TG and LDL-C are primary risk factors for hyperlipidemia and related vascular diseases, but high plasma HDL-C level confers a protective effect against their developments.¹⁵ Accordingly, our results demonstrated the effect of PSM on plasma

Table 1. Effect of PSM on Plasma Lipid Level and Liver Lipase Activity

Groups	Plasma lipid level (g/l)				Liver lipase activity (U/mg protein)	
	TC	TG	LDL-C	HDL-C	LPL	HTGL
Normal	113.03 ± 9.90	85.3 ± 14.69	32.19 ± 7.53	117.89 ± 17.17	1.18 ± 0.30	1.22 ± 0.16
Model	272.53 ± 41.03 ^{##}	570.85 ± 85.12 ^{##}	86.06 ± 14.21 ^{##}	89.78 ± 21.11 ^{##}	0.85 ± 0.23 [#]	1.05 ± 0.17 ^{##}
FEN 100	138.79 ± 10.64 ^{**}	58.02 ± 5.87 ^{**}	47.34 ± 9.42 ^{**}	123.70 ± 25.70 ^{**}	1.54 ± 0.22 ^{**}	1.10 ± 0.11
PSM 100	252.73 ± 79.87	405.1 ± 103.69 ^{**}	57.67 ± 9.09 [*]	95.37 ± 11.55	1.39 ± 0.17 ^{**}	1.16 ± 0.24
PSM 200	220.71 ± 43.90 [*]	342.12 ± 77.09 ^{**}	62.38 ± 17.39 [*]	122.20 ± 33.05 [*]	1.44 ± 0.18 ^{**}	1.17 ± 0.15
PSM 400	174.21 ± 70.85 ^{**}	224.79 ± 79.95 ^{**}	45.13 ± 8.52 ^{**}	105.86 ± 18.49 [*]	1.74 ± 0.13 ^{**}	1.23 ± 0.12 [*]

The results represent the mean ± S.D. of values in each group ($n = 10$ animals). The significance of differences from the normal control at [#] $p < 0.05$, ^{##} $p < 0.01$ and model control at ^{*} $p < 0.05$, ^{**} $p < 0.01$.

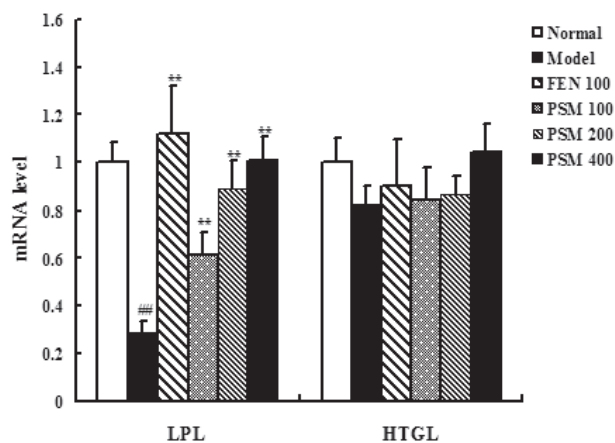


Fig. 2. Effects of PSM on mRNA Expression of Liver LPL and HTGL

The results represent the mean \pm S.D. of values in each group ($n = 10$ animals). The significance of differences from the normal control at ^{###} $p < 0.01$ and model control at ^{**} $p < 0.01$.

lipid metabolism in hyperlipidemic mice.

When compared with normal mice, Triton WR-1339 treatment significantly lowered the activity of liver LPL ($p < 0.05$) and HTGL ($p < 0.01$) in mice (shown in Table 1). However, FEN (100 mg/kg) and all doses of PSM obviously increased LPL activity ($p < 0.01$) in mice treated with Triton WR-1339. Moreover, high dose of PSM (400 mg/kg) also increased HTGL activity ($p < 0.01$). Previous studies demonstrated that LPL plays a central role in lipoprotein metabolism by hydrolyzing TG.¹⁶ Moreover, HTGL, which is another form of lipase expressed in the liver and adrenal glands, could prevent the accumulation of cholesterol and serve as a ligand that mediates the interaction of lipoproteins to cell surface receptors and proteoglycans.¹⁷ Thus our results suggested that PSM treatment may modulate plasma lipid metabolism under hyperlipidemic condition *via* up-regulating liver lipases activity. Whether the enhancement of liver lipases activity was linked to their up-regulation of gene expression was continuously examined. Interestingly, our results (shown in Fig. 2) found that Triton WR-1339 significantly decreased the mRNA levels of LPL ($p < 0.01$) and HTGL ($p < 0.05$) when compared to normal control, while all doses of PSM remarkably increased LPL mRNA level ($p < 0.01$) and high dose of PSM (400 mg/kg) also increased HTGL mRNA level ($p < 0.05$). The results above suggested that enhanced expression of liver lipases seems to mediate its up-regulated activity.

In conclusion, we obtained a purified saponin mixture through a simple, repeatable and control-

lable separation method from alfalfa. The contents and structures of the saponin mixture were identified as 79.26% Azukisaponin V and 20.74% Soyasaponin I. The recovery rate of PSM was as high as 0.99%, and it also showed significant activities on abnormal plasma lipid metabolism in hyperlipidemic mice *via* up-regulating the activity of liver lipases. The obtained data would be a good contribution to the development and utilization of alfalfa in the world. However, Triton WR-1339-induced hyperlipidemia in animals don't resemble the formation of human hyperlipidemia and could only used to screen potential candidate health foods or drugs. The anti-hyperlipidemia effects of PSM should be examined in further studies with more hyperlipidemia models.

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