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Anti-hyperlipidemic Effect of Insamsansa-eum in Mice

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Abstract – Hypolipidemic effect of Insamsansa-eum (ISE, Ren-Shen-Shan-Zah-Yin *in Chinese*) consisted of Red ginseng (RG; the steamed root of *Panax ginseng* C.A. Meyer) and Crataegii fructus (CF, the fruit of *Crataegus pinnatifida* BGE) is used frequently in China and Korea as a herbal medicine to treat arteriosclerosis, was investigated. Treatment of ISE significantly reduced blood triglyceride (TG) and total cholesterol (TC) levels in Triton WR-1339-induced hyperlipidemic mice and serum TG levels in corn oil-induced hypertriglyceridemic mice. ISE significantly lowered the high blood TG and TC levels as well as body and epididymal mass weights in hyperlipidemic mice induced by long-term feeding of a high-fat diet and increased blood HDL cholesterol level. ISE and its ingredients, RG and CF, inhibited pancreatic lipase and HMG-CoA reductase activities. Of its ingredients, RG reduced the blood TC level and HMG-CoA reductase activity more than CF. CF reduced blood TG level and pancreatic lipase activity more than RG. These finding suggest that the antihyperlipidemic effect of ISE may be due to synergistic inhibition of HMG-CoA reductase and pancreatic lipase by RG and CF, and that ISE may be effective hypolipidemic agents.
Keywords – Insamsansa-eum, red ginseng, *Crataegus pinnatifida*, hyperlipidemia, pancreatic lipase, HMG-CoA reductase

Introduction

Lipid metabolism normally maintains an elegant balance between synthesis and degradation. When this balance is lost, hypercholesterolemia and hyperlipidemia may develop, leading to arteriosclerosis, hypertension, obesity, diabetes, and functional depression of select organs (Goldstein *et al*., 1973). The rate-limiting enzyme of cholesterol biosynthesis from acetate is HMG-CoA reductase (Heller and Shrewsburg, 1976; White and Rudney, 1970). Pancreatic lipase is a key enzyme for lipid breakdown and fatty acid absorption (Birari and Bhutani, 2007). Inhibitors of HMG-CoA reductase and pancreatic lipase are anti-hypercholesterolemic agents (Birari and Bhutani, 2007; Endo *et al*., 1992; Davidson, 2003), such as orlistat and lovastatin, and reduce the absorption of dietary triglycerides and inhibit cholesterol biosynthesis, respectively. However, repeated use of these agents causes side effects (Davidson, 2003; Ballinger, 2000). Thus, herbal medicines have been receiving increased attention as alternative treatments for hyperlipidemia (Zhou *et al*., 2005).

Red ginseng (RG), which is the steamed root of *Panax ginseng* C.A. Meyer, showed hypolipidemic effects in cyclophosphomide- and Triton WR-1339-induced mice (Trinh *et al*., 2007; Inoue *et al*., 1999). Its main ingredients, the ginsenosides Rg3 and Rh2, reduced blood TG and TC levels in Triton WR-1339-induced hyperlipidemic mice (Trinh *et al*., 2007). Crataegii fructus (CF, the fruit of *Crataegus pinnatifida* BGE) also showed antihyperlipidemic effects in rats and humans (Chang *et al*., 2005; Ia Cour *et al*., 1995). Its main ingredient, ursolic acid, reduced blood TC levels in experimental arteriosclerosis in rats and rabbits (Liu, 1995). Of many herbal medicinal formulas, Insamsansa-eum (ISE, Ren-Shen-Shan-Zah-Yin *in Chinese*) is composed of RG and CF, and frequently used in China and Korea to treat arteriosclerosis, brain ischemia, and angina pectoris. Most herbal formulas may work via synergistic or antagonistic interactions of the herbal constituents. Nevertheless, its pharmacological effects have not been studied.

Therefore, we investigated the hypolipidemic effects of ISE on corn oil-, Trition WR-1339- or high-fat diet-induced experimental hyperlipidemic mice.

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Experimental

Materials – Triton WR-1339, tributyrin, and Pancreatic lipase were purchased from Sigma Chemical (St. Louis, MO, USA). Total cholesterol and triglyceride assay kits were purchased from Asan Pharmaceutical Co. Ltd. (Korea). Orlistat (Xenical) was kindly donated by Dr. B W Song of Kyung Hee Medical Center, Kyung Hee University (Seoul, Korea).

A high-fat diet containing 25% beef tallow [American Institute of Nutrition (AiN)-76 fat-diet #180337] was purchased from Dyets, Inc. (Bethlehem, PA, USA).

RG and CF were purchased from Kyung Dong Market (Seoul, Korea) and identified by Dr. Nam-Je Kim, East-West Medical Research Institute, Kyung Hee Medical Center, Kyung Hee University.

Extraction of RG, CF and ISE – RG (500 g), CF (500 g), or ISE (500 g) consists of RG (125 g) and CF (375 g) were extracted twice with 5 L of boiling water, filtered, evaporated in a rotary vacuum evaporator, and freeze-dried. The yields were 23.9, 20.2, and 22.3%, respectively. The main component of each extract was analyzed using a Younglin HPLC system (Younglin instrument, Korea). The sample extracts were subjected to Develosil C30-UG-5 (4.6 × 250 mm, 5 μm, Nomura Chem. Co. Ltd., Japan). The mobile phases were water (solvent A) and acetonitrile (solvent B). Gradient elutions were performed as follows: solvent B increasing from 0% to 100% in 20 min and then held at 100% for 5 min (Fig. 1). The injected volume was 20 μL, the flow rate was 1 mL/min, and the UV absorbance was monitored at 203 nm. Ursolic acid and Rg3 were 0.18 and 0.19% of the ISE extract, respectively, and those of ursolic acid in CF extract and Rg3 in RG extract were 0.68 and 0.27%, respectively.

Assay of PL activity – The enzyme activity assay was performed according to a previously reported method (Bae *et al*., 2002). The reaction mixture (3.06 mL) contained 135 mM triolein emulsified in gum acacia, 2 mM sodium thioglycolate, and PL (0.6 U using triacetin as a substrate), adjusted to pH 8.8 with 0.1 M NaOH, incubated at 25 °C, and adjusted to pH 8.8 with 10 mM NaOH. The inhibitory activity of the sample was calculated from the titrated volume.

Assay of HMG-CoA reductase activity – HMG-CoA reductase was partially purified according to the method of Edwards *et al.* (1980). Male Sprague-Dawley rats (250 - 300 g body weight) were housed one per cage in a room where the lights were off from 7:00 AM to 7:00 PM. Food and water were available *ad libitum*. For 3 days prior to killing, the rats were fed powdered rat chow containing 5% cholestyramine. Animals were killed at 1:00 PM, the peak of the HMG-CoA reductase cycle. The livers were homogenized at 4 °C in 25 mL buffer A (0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate, and 0.03 M potassium EDTA; pH 7.2) with a motor-driven, tight-fitting, glass-Teflon Potter-Elvejhem homogenizer, and microsomes were prepared. Three-milliliter aliquots of the microsomal suspension were frozen in glass tubes at a rate of 6 - 8 °C per min unless otherwise stated, and stored at –20 °C for up to 2 months. For optimal solubilization of the reductase, the frozen microsomes were allowed to thaw to 37 °C before addition of an equal volume of 50% glycerol in buffer B (buffer A plus 10 mM DTT) preheated to 37 °C. The suspension was rehomogenized with 10 downward passes of a hand-driven, all-glass Potter-Elvehjem homogenizer and then incubated at 37 °C for 60 min. The suspension was diluted three fold with buffer B heated to 37 °C for a final glycerol concentration of 8.3%, rehomogenized with 10 downward passes of the glass homogenizer pestle, and

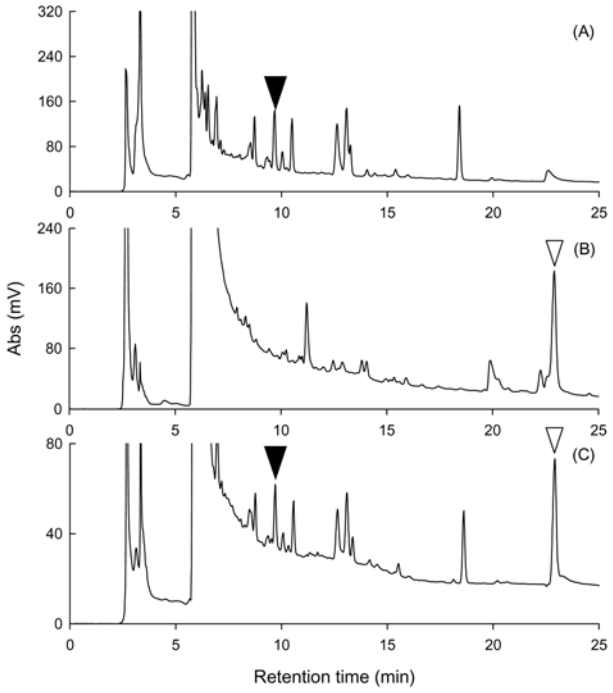

Fig. 1. Content analysis of the main constituents, Ginsenoside Rg3 and ursolic acid, of red ginseng The chromatogram of Insamsansa-eum (C) and its ingredients: red ginseng (A) and crataegii fructus (B). The black and white arrows indicate Rg3 and ursolic acid, respectively. The constituent analyses of ISE and its ingredients were conducted on a Younglin HPLC system: column, Develosil C30-UG-5 (4.6 × 250 mm, 5 μm); mobile phase, water (solvent A) and acetonitrile (solvent B)-the gradient elution from 0% to 100% solvent B in solvent A for 20 min and 100% solvent B for 5 min; inject volume, 20 μL; flow rate, 1 mL/min; detection wavelength, 203 nm.

centrifuged at 100,000 × g for 60 min at 25 °C. The supernatant containing solubilized HMG-CoA reductase was used for the enzyme inhibitory activity assay as the crude enzyme.

The inhibitory activity assay of HMG-CoA reductase was performed according to the method of Edwards *et al.* (1979). Its activity was determined at 37 °C in a total volume of 0.5 mL using a Beckman spectrophotometer (Shimazu). The cell path length was 1.0 cm. The oxidation rate of NADPH was initially determined in the absence of HMG-CoA, and this blank value was subtracted from the rate obtained with both substrates. The activity assay reaction mixture contained 0.2 M KCl, 0.16 M potassium phosphate, 0.004 M EDTA, and 0.001 M DTT, 0.2 mM NADPH, and 0.1 M RS-HMG-CoA; pH 6.8.

Animals – Male ICR mice (20 - 25 g) were purchased from Orient Charles River Co. (Korea) and fed a commercial diet (Orient Charles River Co., Korea). These animals were kept for at least 7 days prior to the experiments. All animals were housed in wire cages at 20 - 22 °C, 50 ± 10% humidity, fed standard laboratory chow (Samyang, Seoul, Korea), and allowed water *ad libitum*. All procedures relating to animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85 - 23 revised 1985).

To evaluate hypolipidemic effects, three kinds of hyperlipidemic animal models were established. Six mice were used per group. First, a hyperlipidemic mouse model based on corn oil was established according to the method of Duhault *et al.* (1976). Corn oil (1 g/kg) was orally administered 2 h after each sample was administered orally. Two hours after the administration of corn oil, blood samples of the mice were drawn by cardiac puncture under ether anesthesia. Second, a hyperlipidemic mouse model based on Trition WR-1339 was established according to the method of Kusama *et al.* (1988). Triton WR-1339 was injected at the end of the regular 16 h fasting period as a 10% solution in saline at a dose of 200 mg/kg body weight into the tail veins of mice under light ether anesthesia. These mice were anesthetized with ether 18 h after Triton WR-1339 injection, and 1-1.5 mL of blood was drawn by cardiac puncture. Sera were obtained by centrifugation (1500 × g, 10 min). Tested samples, lovastatin or orlistat, were administered orally once a day for 3 days before Triton WR-1339 injection. The final administration of the samples was performed 1 h before Triton WR-1339 injection. Third, a hyperlipidemic mouse model based on high-fat diets was established. Mice were classified into 6 groups. The high-fat control group was fed a high-fat diet for 5 weeks. The normal group received a solid, normal diet. ISE, RG, and CF were orally administered for 5 weeks at 50 mg/kg/day, and xenical at 10 mg/kg/day. After a 16 h fasting period following the final administration of samples, blood samples were drawn by cardiac puncture under ether anesthesia.

Determination of serum total cholesterol (TC), triglyceride (TG), and HDL cholesterol – Total cholesterol was measured by the enzymatic method of Allain *et al.* (1974). Serum triglycerides were measured by the method designed by Kim *et al.* (2008). HDL cholesterol was measured by the enzymatic method of Kim *et al.* (2008).

Statistical analysis – All data are expressed as the mean ± standard deviation, and statistical significance was analyzed by one-way ANOVA followed by the Student-Newman-Keuls test.

Results

***In vivo* antihyperlipidemic activity of ISE** – We measured the hypolipidemic effects of ISE on Triton WR-1339-induced hyperlipidemic mice (Fig. 2). Serum TG and TC levels increased after treatment with Triton WR-1339. ISE treatment significantly reduced the Triton WR-1339-induced increases.

We also measured the inhibitory effects on corn-oil-induced hyperlipidemic mice (Fig. 3). Corn oil feeding significantly induced serum TG level, but not cholesterol level. ISE treatment reduced serum TG level, but not back

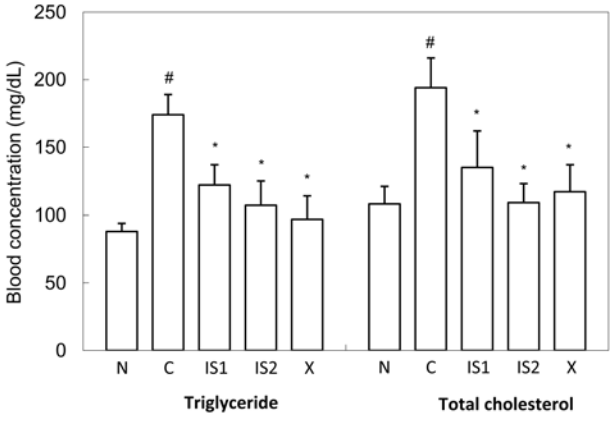

Fig. 2. Effect of Insamsansa-eum (IS) in Trition WR-1339-induced hyperlipidemic mice. The normal group (N) received a vehicle alone. The control group (C) was treated with Triton WR-1339 alone. The samples were orally administered with Triton WR-1339: IS1, 50 mg/kg IS; IS2, 100 mg/kg IS; X, 10 mg/kg Xenical. Values indicate mean ± S.D. (n = 6).

#Significantly different, compared with normal group ($p < 0.05$).

*Significantly different, compared with control group ($p < 0.05$).

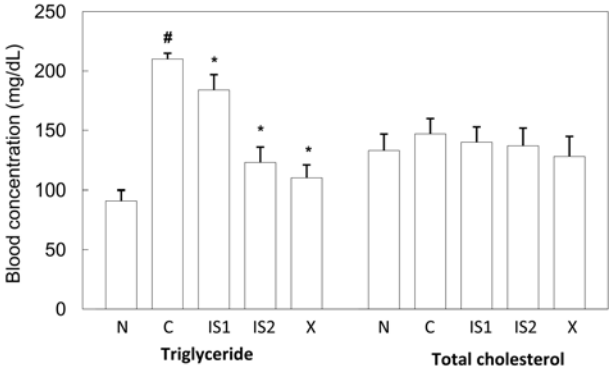

Fig. 3. Hypolipidemic effect of Insamsansa-eum (IS) in corn oil-induced hyperlipidemic mice. The normal group (N) received a vehicle alone. The control group (C) was treated with corn oil alone. The samples were orally administered with corn oil: IS1, 50 mg/kg IS; IS2, 100 mg/kg IS; X, 10 mg/kg Xenical. Values indicate mean ± S.D. (n = 6).

#Significantly different, compared with normal group ($p < 0.05$).

*Significantly different, compared with control group ($p < 0.05$).

Table 1. Inhibitory Effect of Red Ginseng (RG), Crataegii Fructus (CF), and ISE for Pancreatic Lipase and HMG CoA Reductase Activities

Samples	IC_{50}	
	Pancreatic lipase (mg/mL)	HMG CoA reductase (μg/mL)
RG	1.8	2
CF	1.6	5
ISE	1.7	4
Xenical	0.8 (1.6)	0.2 (0.4)

Values in parenthesis are those indicated in mmole for pancreatic lipase and mmole for HMG CoA reductase.

to control levels.

Effect of long-term feeding of ISE and its ingredients RG and CF on high-fat diet-induced hyperlipidemia in mice – We next measured the hypolipidemic effects of long-term feeding of ISE and its ingredients RG and CF on high-fat diet-induced hyperlipidemic mice (Fig. 4). TG and TC levels in serum were increased by treatment with a high-fat diet for 5 weeks. ISE and its ingredients RG and CF significantly decreased these levels. The high-fat diet decreased HDL-cholesterol, with ISE restoring HDL levels more potently than its ingredients alone. ISE also was most potent at reducing the body and epididymal mass weights induced by the high-fat diet.

***In vitro* inhibitory effect of ISE and its ingredients RG and CF on pancreatic lipase and HMG-CoA reductase activities** – To understand the hypolipidemic mechanism of ISE and its ingredients RG and CF, we measured their inhibitory effects on pancreatic lipase and

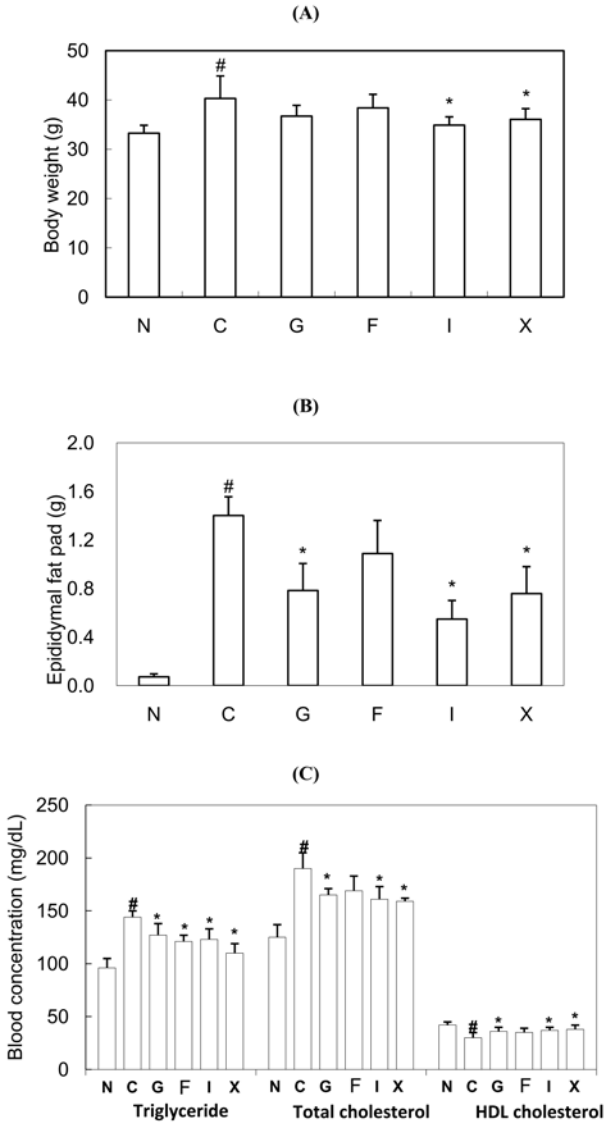

Fig. 4. Effect of red ginseng (RG), Crataegii fructus (CF), and Insamsansa-eum (IS) on body (A) and epididymal fat (B) weights and serum triglyceride, Total cholesterol and high density lipoprotein (HDL) cholesterol levels (C) in hyperlipidemic mice induced by high-fat diets. Each group contained 6 mice with body weights of 24.3 ± 0.93 g (mean ± standard deviation) per a mice. The normal group (N) received a normal, solid diet. The control group (C) was fed the high-fat diet for 5 weeks, respectively. These agents were orally administered once a day with the high-fat diet for 5 weeks: G, 50 mg/kg RG; C, 50 mg/kg CF; I, 50 mg/kg IS; X, 10 mg/kg Xenical. Body weight was measured before the final administration of the samples. Epididymal fat pads were taken under ether anesthesia and their weights were measured.

Values indicate mean ± S.D. (n = 6).

#Significantly different, compared with normal group ($p < 0.05$).

*Significantly different, compared with control group ($p < 0.05$).

HMG-CoA reductase (Table 1). RG, CF, and ISE all inhibited pancreatic lipase and HMG-CoA reductase

activities with similar potencies. Of them, CF more potently inhibited pancreatic lipase activity than RG and RG more potently inhibited HMG-CoA reductase activity than CF.

Discussion

Hyperlipidemia, or hypertriglyceridemia and hypercholesterolemia, can cause arteriosclerosis, hypertension, obesity, diabetes, and functional organ depression (Goldin *et al*., 1973). Pancreatic lipase is a key enzyme in lipid breakdown and is required to absorb fatty acids (Thomson *et al*., 1993). It is one of the exocrine enzymes in the pancreatic juice and catalyzes the hydrolysis of emulsified glycerol esters and long-chain fatty acids. Short-chain fatty acids can be directly absorbed into the blood, but long-chain fatty acids and monoglycerides combine with bile salts to form water soluble micelles (Carey *et al*., 1983). Micelles are absorbed into the mucosal cells of the intestine, and the fatty acids and monoglycerides are resynthesized into triglycerides. Dietary TG is usually stored in the adipose tissue. HMG-CoA reductase catalyzes a key step in the mevalonate pathway, which is involved in the synthesis of sterols, isoprenoids, and other lipids (Espenshade and Hughes, 2007). In humans, HMG-CoA reductase is the rate-limiting step in cholesterol synthesis and is the major target of modern cholesterol-lowering drugs (Meigs *et al*., 1996).

Many researchers have been studying the hypolipidemic effects of herbal medicines to develop them as alternative treatments for hyperlipidemia because the repeated used of orlistat and lovastatin results in side effects (Davidson, 2003; Ballinger, 2000; Thompson Coon and Ernst *et al*., 2003). However, the antihyperlipidemic effects of ISE have not been thoroughly studied, although those of RG and CF, have the hypolipidemic effect (Trinh *et al*., 2007; Inoue *et al*., 1999). Here, their combined formula, ISE, had antihyperlipidemic effects on corn oil-, trition WR1339- and high-fat diet-induced hyperlipidemic mice. In hyperlipidemic mice induced by long-term feeding of high fat, ISE and its ingredients RG and CF reduced total blood cholesterol and TG levels. Compared to those of ISE ingredients, ISE synergistically and/or additively inhibited the increase in body and epididymal mass weights in hyperlipidemic mice induced by long-term feeding of high fat. RG more potently reduced total blood cholesterol levels than CF, whereas CF more potently reduced total triglyceride levels than RG.

RG, CF, and ISE all inhibited pancreatic lipase and HMG CoA reductase activities. CF and its constituent ursolic acid inhibited pancreatic lipase activity the most potently, followed by ISE and RG. RG inhibited HMG-CoA reductase activity the most potently. CF more potently inhibited pancreatic lipase activity than RG. These results suggest that the antihyperlipidemic effect of ISE may be due to synergistic inhibition of HMG-CoA reductase and pancreatic lipase by RG and CF. Finally, we propose that ISE may be effective as hypolipidemic agents by the synergistic interaction of its ingredients RG and CF.

References

Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C., Enzymatic determination of total serum cholesterol. *Clin. Chem*. **20**, 470-475 (1974).

Bae, E.A., Han, M.J., Choo, M.K., Park, S.Y., and Kim, D.H., Metabolism of 20(S)- and 20(R)-ginsenoside Rg3 by human intestinal bacteria and its relation to in vitro biological activities. *Biol. Pharm. Bull*. **25**, 58-63 (2002).

Ballinger, A., Orlistat in the treatment of obesity. *Expert. Opin. Pharmacother*. **1**, 841-847 (2000).

Birari, R.B. and Bhutani, K.K., Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug Discovery Today* **12**, 879-889 (2007).

Carey, M.C., Small, D.M., and Bliss, C.M., Lipid digestion and absorption. *Annu. Rev. Physiol*. **45**, 651-677 (1983).

Chang, W.T., Dao, J., and Shao, Z.H., Hawthorn: potential roles in cardiovascular disease. *Am. J. Chin. Med*. **33**, 1-10 (2005).

Davidson, M.H., Ezetimibe: a novel option for lowering cholesterol. *Expert. Rev. Cardiovasc. Ther*. **1**, 11-21 (2003).

Duhault, J., Boulanger, M., Beregi, L., Sicot, N., and Bouvier, F., 780 SE: a new type of hypolipemic agent. *Atherosclerosis* **23**, 63-72 (1976).

Edwards, P.A., Lemongello, D., and Fogelman, A.M., Improved methods for the solubilization and assay of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res*. **20**, 40-46 (1979).

Edwards, P.A., Lemongello, D., and Kane, J., Properties of purified rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and regulation of enzyme activity. *J. Biol. Chem*. **255**, 3715-3725 (1980).

Endo, A., The discovery and development of HMG-CoA reductase inhibitors. *J. Lipid Res*. **33**, 1569-1582 (1992).

Espenshade, P.J. and Hughes, A.L., Regulation of sterol synthesis in eukaryotes. *Annu. Rev. Genet*. **41**, 401-427 (2007).

Goldstein, J.L., Schrott, H., Hazzard, E., Bierman, E., and Motuski, A., Hyperlipidemia in coronary heart disease. I. Lipid levels in 500 survivors of myocardial infarction. *J. Clin. Invest*. **52**, 1544-1568 (1973).

Heber, D., Herbs and atherosclerosis. *Curr. Atheroscler. Rep*. **3**, 93-96 (2001).

Heller, R.A. and Shrewsburg, M.A., 3-Hydroxy-3-methylglutaryl coenzyme A reductase from rat liver. Its purification, properties, and immunochemical studies. *J. Biol. Chem*. **251**, 3815-3822 (1976).

Ia Cour, B., Molgaard, P., and Yi, Z., Traditional Chinese medicine in treatment of hyperlipidaemia. *J. Ethnopharmacol*. **46**, 125-129 (1995).

Inoue, M., Wu, C.Z., Dou, D.Q., Chen, Y.J., and Ogihara, Y., Lipoprotein lipase activation by red ginseng saponins in hyperlipidemia model animals. *Phytomedicine* **6**, 257-265 (1999).

Kim, H.Y., Jeong da M., Jung, H.J., Jung, Y.J., Yokozawa, T., and Choi, J.S., Hypolipidemic effects of Sophora flavescens and its constituents

in poloxamer 407-induced hyperlipidemic and cholesterol-fed rats. *Biol. Pharm. Bull.* **31**, 73-78 (2008).

Liu, J., Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.* **49**, 57-68 (1995).

Meigs, T.E., Roseman, D.S., and Simoni, R.D., Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo. *J. Biol. Chem.* **271**, 7916-7922(1996).

Thomson, A.B., Schoeller, C., Keelan, M., Smith, L., and Clandinin, M.T., Lipid absorption: passing through the unstirred layers, brush-border membrane, and beyond. *Can. J. Physiol. Pharmacol.* **71**, 531-555 (1993).

Thompson Coon J.S. and Ernst, E., Herbs for serum cholesterol reduction: a systematic view. *J. Fam. Pract.* **52**, 468-478 (2003).

Trinh, H.T., Han, S.J., Kim, S.W., Lee, Y.C., and Kim, D.H., Bifidus fermentation increases hypolipidemic and hypoglycemic effects of red ginseng. *J. Microbiol. Biotechnol.* **17**, 1127-1133 (2007).

White, L.W. and Rudney, H., Regulation of 3-hydroxy-3-methylglutarate and mevalonate biosynthesis by rat liver homogenates. Effects of fasting, cholesterol feeding, and triton administration. *Biochemistry* **9**, 2725-2731 (1970).

Zhou, L., Zuo, Z., and Chow, MS., Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J. Clin. Pharmacol.* **45**, 1345-1359 (2005).

Kusama, H., Nishiyama, M., and Ikeda, S., Pharmacological investigation of bezafibrate, a hypolipidemic agent (1). Effects of bezafibrate on normal and experimental hyperlipidemia in rats. *Folia Pharmacol. Jpn.* **92**, 175-180 (1988).

Sardesai, V.M. and Mannig, J.A., The determination of triglycerides in plasma and tissues. *Clin. Chem.* **14**, 156-158 (1968).

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