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Full Length Research Paper

Lipid-Lowering mechanisms of grape seed extract (*Vitis vinifera* L) and its antihyperlidemic activity

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Grape Seed Extract (GSE) has been targeted to promote beneficial health effects, especially the prevention of obesity and hyperlipidemia. In present study, we investigated the mechanisms of GSE on inhibition of lipid digestion and absorption. GSE significantly inhibited pancreatic lipase and cholesterol esterase in dose-dependent manner with the IC_{50} values of 3.71 ± 0.03 and 27.27 ± 4.12 mg/mL, respectively. In addition, GSE also inhibited the formation of cholesterol micelles, and bound to bile acid. The oral administration of GSE significantly decreased serum triglyceride and cholesterol in rat fed high fat emulsion through inhibition of lipid digestion and absorption. GSE may be a feasible therapeutic strategy for prevention and treatment of patients with hyperlipidemia and obesity.

Key words: Grape seed extract, bile acid binding, cholesterol micellization, pancreatic cholesterol esterase, pancreatic lipase, mechanisms.

INTRODUCTION

Hyperlipidemia is a group of metabolic disorders characterized by hypertriglyceride and/or hypercholesterol in blood circulation. The long-term hyperlipidemia is an important contributor to develop the progression of micro- and macrovascular complications including microangiopathy, cardiovascular, cerebrovascular and metabolic syndrome diseases. The prevalence of hyperlipidemia has dramatically increased worldwide due to a modern lifestyle and an increase of consumption of a high-fat diet (Jacobson et al., 2007). Consumption of dietary plant foods and their ingredients could be a more effective strategy for management of hyperlipidemia. Currently, fruits and vegetables have been screened for lipid digestion and absorption inhibitory agents.

Functional foods and medicinal plants have been targeted to promote beneficial health effects, especially the prevention of pathophysiologic conditions such as dyslipidemia, diabetes, hypertension and cancer. Grape seed (*Vitis vinifera* Linn.) contains important vitamins, minerals and polyphenols including flavonoids, proanthocyanidins and procyanidins (Weber et al., 2007). It has recently become clear that Grape Seed Extract (GSE) has shown various pharmacological effects such as chemoprotective (Nandakumar et al., 2008) and oxidative stress as well as being anti-inflammatory (Terra et al., 2009), anti-bacterial (Mayer et al., 2008), anti-cancer (Kaur et al., 2006), and anti-diabetic activities (Pinent et al., 2004). Recent study indicates that GSE inhibits pancreatic α-amylase, and intestinal α-glucosidases related to delay postprandial hyperglycemia (Adisakwattana et al., 2010).

The study has recently shown that GSE significantly reduces plasma cholesterol in rabbits fed a high-

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cholesterol diet. This action reduces the risk of atherosclerosis and coronary heart disease (Yamakoshi et al., 1999). Many attempts to find the responsible mechanisms for anti-hyperlipidemic activities of GSE have been studied, particularly in the inhibition of lipase (Moreno et al., 2003), and cellular cholesterol uptake (Leifert et al., 2008). Although, a great deal of work has been carried out to investigate its mechanisms, we hypothesize that GSE may play other roles for controlling postprandial hyperlipidemia by inhibition of lipid digestion and absorption. The aim of this study was to determine the inhibitory effects of GSE related to lipid digestion and absorption such as cholesterol micellization, bile acid binding, pancreatic lipase and pancreatic cholesterol esterase. Furthermore, acute anti-hyperlipidemic effect of GSE was also performed in normal rats by feeding high-fat emulsion containing corn oil and cholesterol.

MATERIALS AND METHODS

Chemical

(+)-Catechin, (-)-epicatechin, gallic acid, *p*-nitrophenylpalmitate (*p*-NPP), *p*-nitrophenylbutylrate (*p*-NPB), oleic acid, phosphatidylcholine, glycodeoxycholic acid, taurodeoxycholic acid, taurocholic acid, porcine cholesterol esterase, porcine pancreatic lipase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Triglyceride and cholesterol test kits were purchased from HUMAN GmbH Co. (Wiesbaden, Germany). Total bile acid kit was purchased from Bio-Quant Co. (San Diego, CA, USA). All other chemical reagents used in this study were of analytical grade.

Preparation of grape seed extract

Grape seeds obtained from Siam Winery (Samutsakhon, Thailand) were extracted according to literature procedure (Saito et al., 1998). The aqueous solution was freeze dried and GSE was kept at -20°C. The amount of total flavanols was measured according to the vanillin method using (+)-catechin as a reference (Broadhurst, 1978). The content of monomeric flavanols was obtained as the sum of each monomeric flavanol's amount such as (+)-catechin and epicatechin by a High-Performance Liquid Chromatography (HPLC) method using an Intertsil® ODS-3 C18 column (Li et al., 2008). The content of procyanidins was calculated as the difference between total flavanols and monomeric flavanols (Saito et al., 1998). Total flavanols, monomeric flavanols and procyanidins of GSE were 50.81 ± 0.63, 1.02 ± 0.01 and 49.79 ± 0.63 g/100 g of GSE, respectively.

Pancreatic lipase inhibition

The pancreatic lipase activity was performed according to previous method (Slanc et al., 2004). Briefly, various concentrations of GSE were incubated with 0.30 mg/mL of porcine pancreatic lipase, 3.33 mM substrate (*p*-NPP) in 0.061 M Tris-HCl buffer, pH 8.5 at 37°C for 25 min. The absorbance of released *p*-nitrophenol was measured at 405 nm by using spectrophotometer. Orlistat was used as positive control in this study.

Pancreatic cholesterol esterase inhibition

The pancreatic cholesterol esterase inhibition was performed spectrophotometrically at 25°C (Pietsch et al., 2005). GSE was incubated with mixtures containing 5.16 mM taurocholic acid, 0.2 mM *p*-NPB in 100 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0. The reaction was initiated by adding porcine pancreatic cholesterol esterase (1 mg/mL). After incubation for 5 min at 25°C, the mixtures were measured the absorbance at 405 nm. Simvastatin was used as positive control for this study.

Cholesterol micellization of grape seed extract

Artificial micelles were prepared according to previous method (Kirana et al., 2005) with minor modifications. In brief, the mixtures (2 mM cholesterol, 1 mM oleic acid and 2.4 mM phosphatidylcholine) were dissolved in methanol and dried under nitrogen before adding 15 mM phosphate-buffered saline (PBS) containing 6.6 mM taurocholate salt, at pH 7.4. The suspension was sonicated twice for 30 min using a sonicator. The micelle solution was incubated overnight at 37°C. GSE and equivalent PBS as control were added to the mixed micelle solution and incubated for a further 2 h at 37°C. The solution was then centrifuged at 16,000 rpm for 20 min. The supernatant was collected for the determination of cholesterol by using total cholesterol test kits. Gallic acid was used as positive control.

Bile acid binding of grape seed extract

The bile acid binding assay was slightly modified according to previous method (Yoshie-Stak et al., 2004). Briefly, GSE (1 mg/mL) was incubated with bile acid (2 mM) containing in 0.1 M PBS, pH 7 at 37°C for 90 min. The mixtures were filtered through 0.2 □m filter and frozen at -20°C until analysis was carried out. The bile acid concentration was analyzed spectrophotometrically at 540 nm by using bile-acid analysis kit. Cholestyramine was used as a positive control in this study.

Animals

Male Wistar rats (180 - 200 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. Animal facilities and protocol were approved by the Laboratory Animal Care and Use Committee at Faculty of Veterinary Science, Chulalongkorn University, Thailand. Wistar rats were housed in individual stainless steel cages in a room maintained at 25 ± 1°C on a 12:12 h light-dark cycle. They were fed standard laboratory chow with water *ad libitum* and fasted overnight before the experiments.

Acute effect of grape seed extract on serum triglyceride and cholesterol concentration in normal rat by high-fat emulsion loading test

The effect of GSE on the reduction of triglyceride and cholesterol was done by the oral fat-loading method with slight modification (Ueshima et al., 2004). Briefly, rats were fasted for 12 h and divided into 6 groups containing 6 animals. They were orally administered with 5 mL/kg body weight of olive oil emulsion (5 mL of emulsion containing 3.33 ml of olive oil, 44.3 mg of cholic acid, 0.48 g of cholesterol and 1.67 mL of distilled water) with or without various doses of GSE (100, 250 and 500 mg/kg). Blood samples were collected before and after 2, 4, 6, 8 and 10 h after loading of high-fat emulsion. Serum was separated for the measurement of triglyceride and total cholesterol by assay kits. The area under the curve ($AUC_{0-10\,h}$) of serum triglyceride and cholesterol levels was calculated using a modification of the trapezoidal rule.

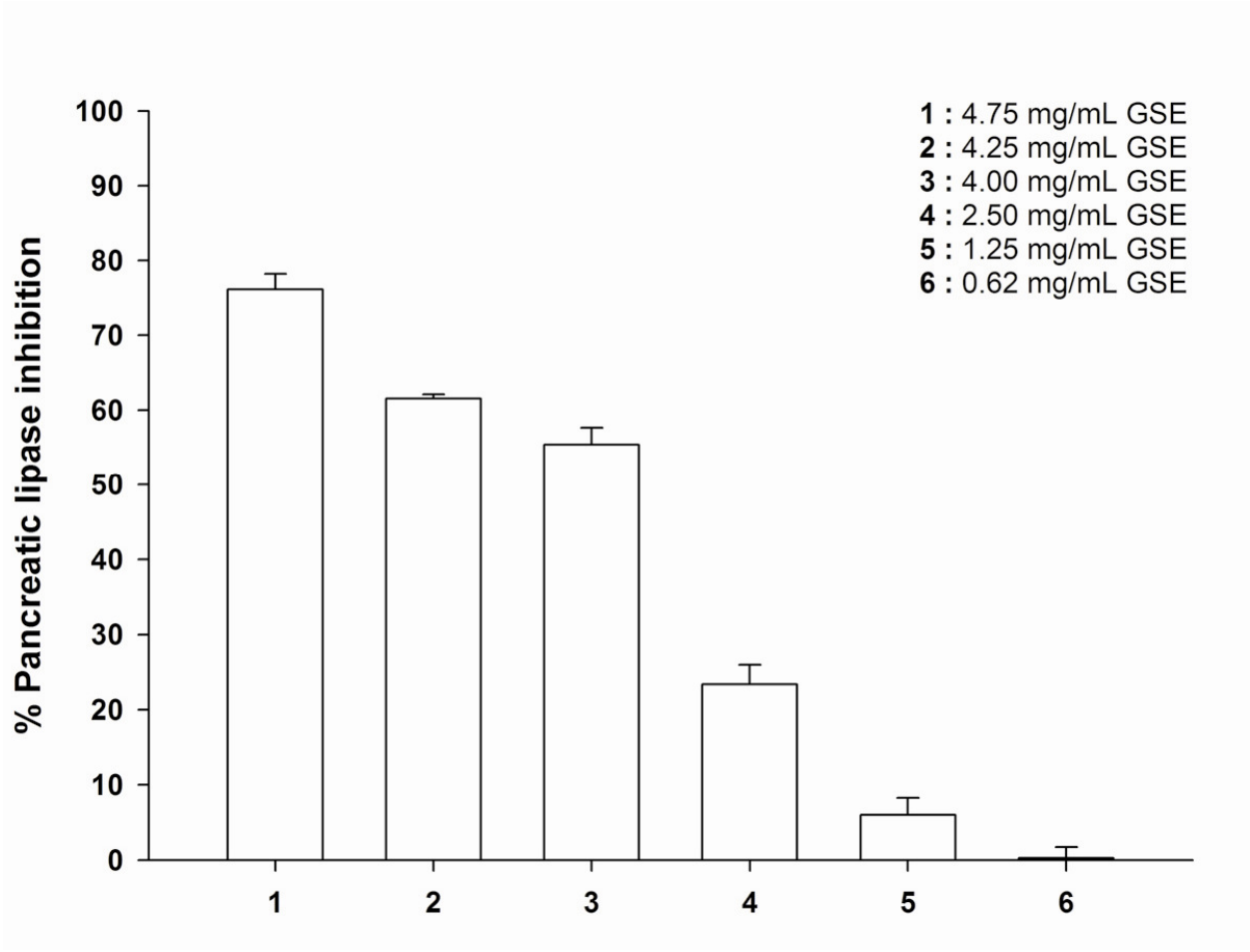

Figure 1. The effect of GSE on pancreatic lipase by using *p*-NPP as a substrate. Results were expressed as mean ± S.E.M., n= 4.

Statistical analysis

Data were expressed as means ± S.E.M. Statistical analysis was performed by one-way ANOVA. The AUCs for the treatment period were calculated using transforms and regressions. The Least Significant Difference (LSD) test was used for mean comparisons and $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of GSE on pancreatic lipase and cholesterol esterase

As shown in Figure 1, GSE exhibited the pancreatic lipase inhibitory activity with IC_{50} value of 3.71 ± 0.03 mg/mL when used *p*-NPP as a substrate. However, it was less potent activity than orlistat (IC_{50} = 44.52 ± 3.17 mg/mL) which was used as a pancreatic lipase inhibitor. However, the results in Figure 2 show the inhibitory effect of GSE on cholesterol esterase. GSE significantly inhibited cholesterol esterase in dose-dependent manner with the IC_{50} value of 27.27 ± 4.12 µg/mL. In addition, the IC_{50} value of simvastatin was 0.08 ± 0.01 µg/mL.

Effect of GSE on cholesterol micellization and bile acid binding

As shown in Figure 3, GSE (10, 20 and 40 mg/mL) markedly inhibited the solubility of cholesterol in artificially prepared micelles by 3.18 ± 1.45, 6.84 ± 1.16 and 11.87 ± 1.90%, respectively, whereas gallic acid (0.2 mg/mL) inhibited the formation of cholesterol micelles about 27.26 ± 2.17%. The bile acid binding by GSE is shown in Figure 4. The results showed that glycodeoxycholic acid and taurodeoxycholic acid were bound by GSE to a degree of 70 and 25%, respectively, nearly the same as the binding degree of cholestyramine. In the meantime, taurodeoxycholic acid was slightly bound by GSE which had lower binding capacity than cholestyramine at the

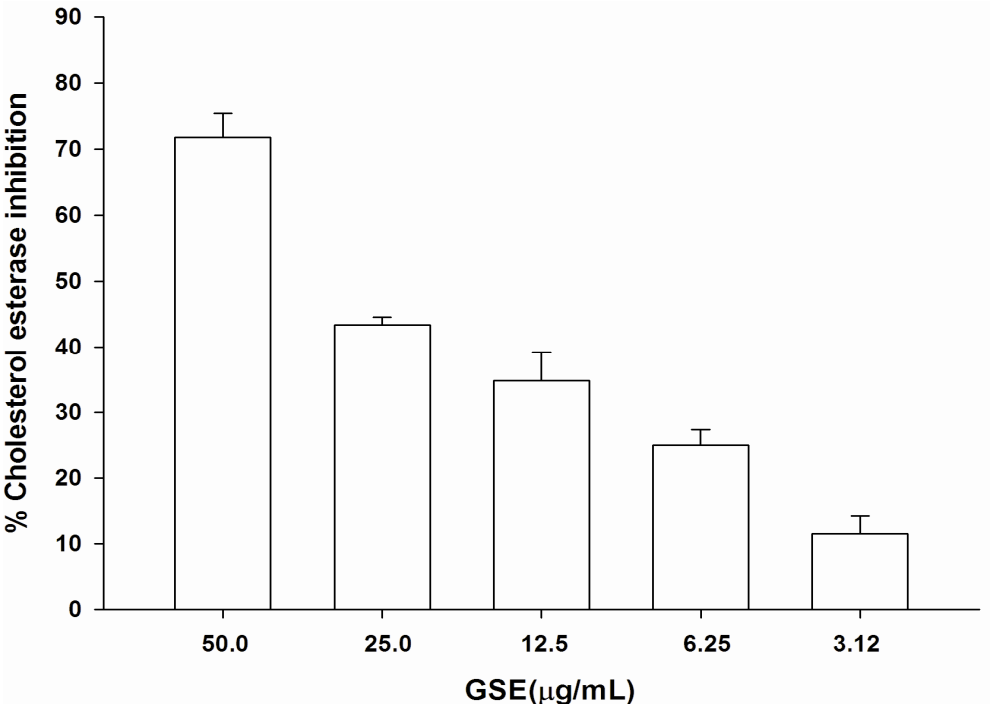

Figure 2. The effect of GSE on pancreatic cholesterol esterase. Results were expressed as mean ± S.E.M., n= 4.

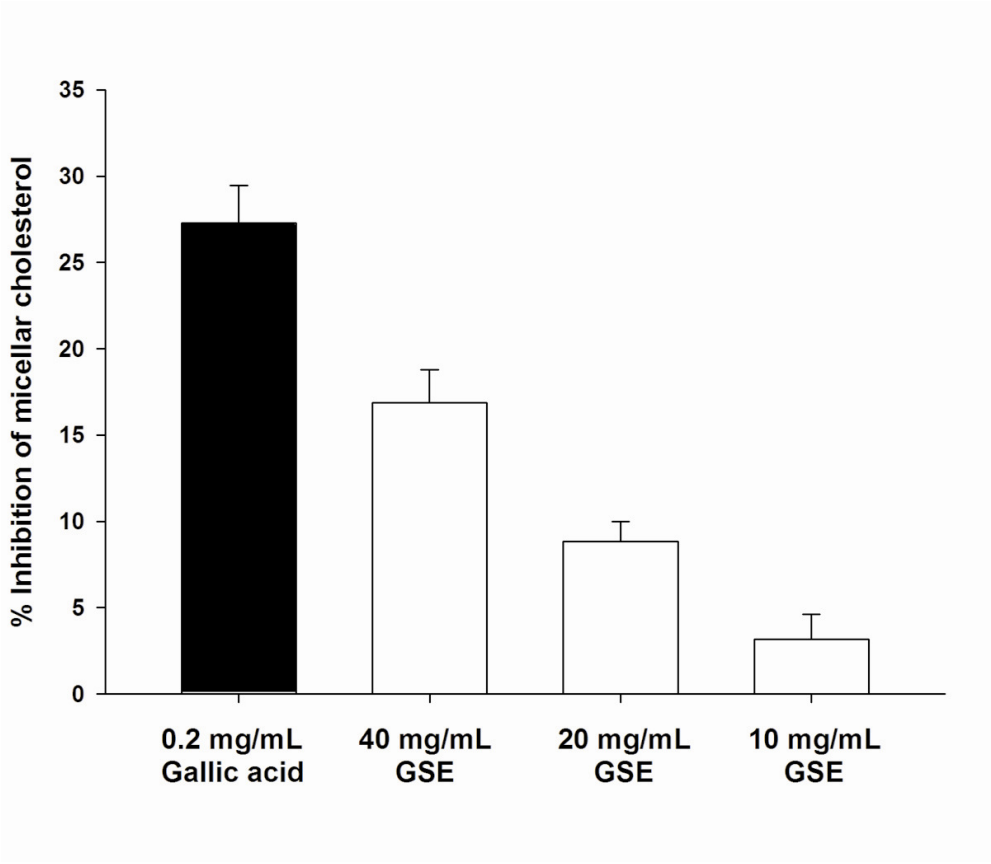

Figure 3. The effect of GSE on cholesterol micellization. Results were expressed as mean ± S.E.M., n= 4.

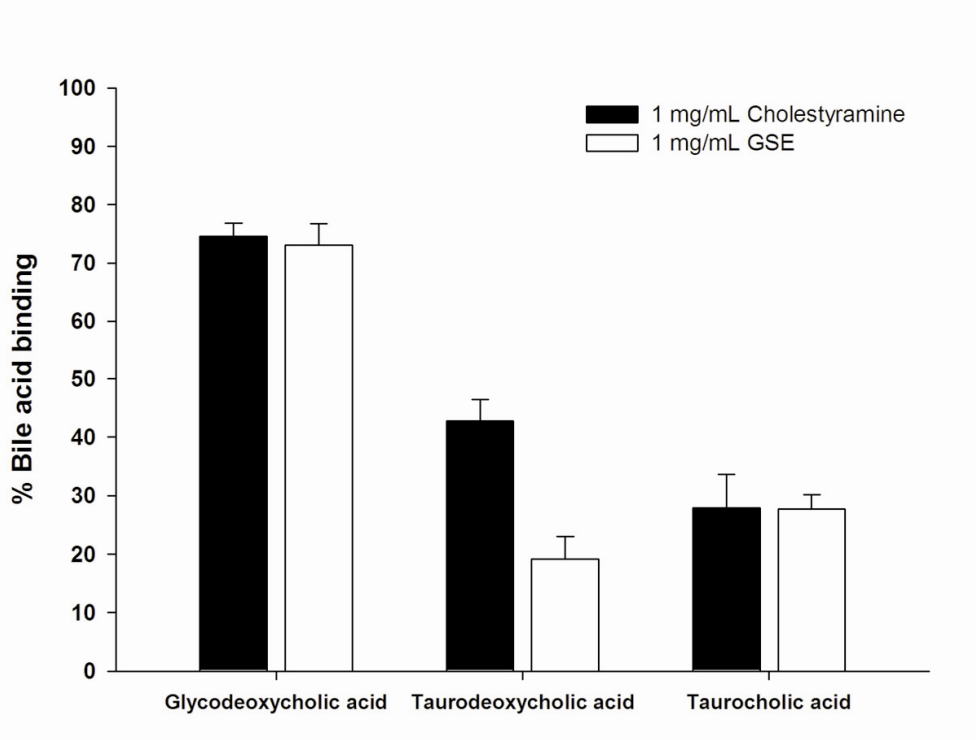

Figure 4. The effect of GSE on bile acid binding. Results were expressed as mean ± S.E.M., n= 4.

same concentration.

Effect of GSE on serum triglyceride and cholesterol concentration in normal rats fed with high-fat emulsion

The serum triglyceride and cholesterol concentrations of normal rats by feeding with high-fat emulsion are shown in Figure 5. The normal rats treated with 250 and 500 mg/kg GSE ($P < 0.05$) resulted in the significant lowering of serum triglyceride concentrations at 2 until 6 h after administration (Figure 5A). The Area Under Curves (AUC) of groups treated with GSE were significantly lower than those of control group by 19 and 27% (Figure 5C), respectively (AUC for control group = 1,921.9 ± 33.3 mg $dL^{-1}.h^{-1}$; AUC for group treated with GSE 250 mg/kg = 1.526.0 ± 46.5 mg $dL^{-1}.h^{-1}$, 500 mg/kg = 1.384.5 ± 12.7 mg $dL^{-1}.h^{-1}$). The serum cholesterol concentrations of normal rats are shown in Figure 5B. The serum cholesterol concentrations were raised to maximum levels 6 h after loading fat emulsion. The results showed that GSE produced the significant suppression in serum cholesterol concentration after 4 h of loading fat emulsion. As shown in Figure 5D, the AUC of normal rats treated with GSE (250 and 500 mg/kg) were 804.9 ± 4.6 and 788.4 ± 6.0 mg $dL^{-1}.h^{-1}$, respectively which lower than those of control group by 8 and 11% (AUC for normal control group = 867.4 ± 17.8 mg $dL^{-1}.h^{-1}$). There were no significant differences between the AUC of serum triglyceride and cholesterol between the groups treated with GSE (100 mg/kg) and control group.

DISCUSSION

It is well known that a successful strategy for treatment of dyslipidemia is primary prevention of postprandial hyperlipidemia by aggressive delaying dietary fat digestion and absorption (Ros, 2000). Our findings showed that GSE markedly inhibited pancreatic lipase with *p*-NPP which was used as a substrate. Previously, it has shown that oligomeric procyanidins containing in apple suppress triglyceride absorption by inhibiting pancreatic lipase activity in mice and human (Sugiyama et al., 2007). It was found that degree of polymerization of oligomeric procyanidins was an important factor to increase potency on pancreatic lipase inhibition. Thus, it is possible that oligomeric procyanidins in GSE cause a marked inhibition of pancreatic lipase in this study. Pancreatic cholesterol esterase plays a pivotal role in hydrolyzing dietary cholesterol esters (Brodt-Eppley et al., 1995). Generally, hydrolysis of cholesterol ester in the lumen of the small intestine is catalyzed by pancreatic cholesterol esterase which liberates free cholesterol. Moreover, it enhances the incorporation of cholesterol

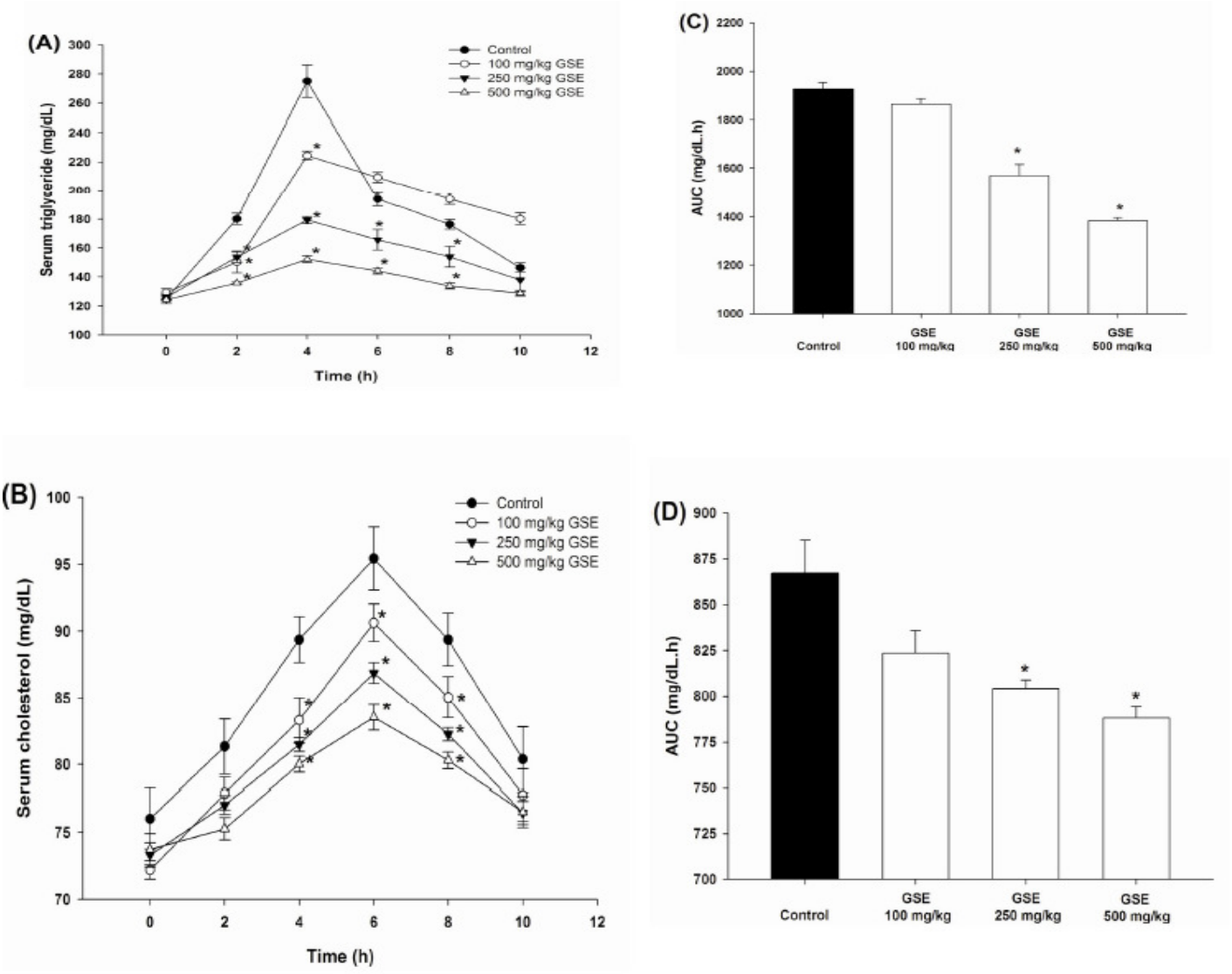

Figure 5. The effects of GSE on serum triglyceride (A) and cholesterol (B) in normal rats by feeding with high-fat emulsion. Total areas under triglyceride (C) and cholesterol (D) response curves were calculated for each group of rats. Results were expressed as means ± S.E.M., n = 6. *P < 0.05 compared with control.

into mixed micelles (Myers-Payne et al., 1995). This is the first study to show significant evidence that GSE inhibits pancreatic cholesterol esterase. This inhibitory action may enhance to control the bioavailability of dietary cholesterol derived from cholesterol esters and the limitation of absorption of free cholesterol into blood circulation.

Generally, the principal steps in the absorption of dietary cholesterol are emulsification, hydrolysis of the ester bond by a pancreatic esterase, micellar solubilization, and absorption in the proximal jejunum (Hui et al., 2005). It has recently reported that reduction of cholesterol absorption by inhibiting cholesterol micellization in the intestinal lumen is a new target site of intervention for treatment of hyperlipidemia and obesity (Kirana et al., 2005). Previous studies have shown the inhibitory effect of GSE on cholesterol uptake by inhibition of specific cholesterol uptake/transporters such as the Niemann-Pick C1-like 1 cholesterol transporter (Leifert et al., 2008). As the results mentioned above, it clearly indicates that GSE presents cholesterol-lowering effect by inhibition of cholesterol micelles.

Bile acids are synthesized in the liver from cholesterol. After conjugation with glycine or taurine, they are secreted into the duodenum. Bile acids are actively reabsorbed by the terminal ileum and undergo an enterohepatic circulation (Hofmann et al., 2008). Binding bile acids by forming insoluble complexes in the intestine and increasing their fecal excretion have been hypothesized as a possible mechanism of lowering plasma

cholesterol level. This phenomenon consequently releases a feedback inhibitory mechanism by inhibiting bile acid synthesis. As a result, greater amount of cholesterol is converted to bile acids to maintain a steady level in blood circulation (Insull, 2006). Importantly, high secondary bile acid has been associated with increased risk of developing colorectal cancer (Peterlik et al., 2008). As a result, GSE exhibits the strongest binding capacity against glycodeoxycholic acid, whereas it slightly bound to taurocholic acid and taurodeoxycholic acid, indicating that GSE may increase fecal bile acid excretion, resulting in the reduction of plasma cholesterol level. Moreover, GSE may reduce the risk factor in developing colorectal cancer.

Our findings showed that acute administration of GSE markedly suppressed the elevation of serum triglyceride and cholesterol in normal rats. These results confirm the *in vitro* activity of GSE, indicating that acute anti-hyperlipidemic activities of GSE may act through inhibition of lipid digestion and absorption. Interestingly, it has been reported that long-term supplementation of GSE reduces plasma lipid profiles and prevents a high-fat diet-induced obesity in hamster and related metabolic pathways by improving adipokine secretion and oxidative stress (Décordé et al., 2008). The supplemented with proanthocyanidin-rich extract from grape seed inhibits progression of atherosclerosis in cholesterol-fed rabbits. This mechanism of action is related to prevention of Low-density lipoprotein (LDL) oxidation in the arterial wall diet (Yamakoshi et al., 1998). We suggest that long-term supplementation of GSE may reduce plasma lipid profiles by mediating through inhibition of pancreatic lipase, cholesterol esterase, cholesterol micellization, and bile acid binding. The overall of the study, we have demonstrated the mechanisms of GSE on inhibition of pancreatic lipase, cholesterol micellization, pancreatic cholesterol esterase, and bile acid binding. From this point of view, an intake of GSE may be a feasible therapeutic strategy for prevention and treatment of patients with hyperlipidemia and obesity.

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