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Study on the Effect of Grape Seed Extract on Hypercholesterolemia: Prevention and Treatment

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Abstract: The present research proved that grape seed extract could be used as hypocholesterolemic agent. Research: GSE was subjected to a series of experiments to document its AA, safety for use as a food supplement and/or phytochemical drug. We further compared the effects of GSE on the prevention and treatment of hypercholesterolemia. Our GSE was proven to have a potent AA and more efficient than ascorbic acid (Vitamin C), in addition to its safety for use as nutritional supplement where the LD₅₀ value was 6.3 g kg⁻¹. On other word, GSE is not toxic. The hypolipidemic effect of GSE was investigated at two levels, the preventive level and treatment level. It was found that GSE could prevent the hypercholesterolemia on which GSE lowered serum Total Cholesterol (TC) by 32%, Low-Density Lipoprotein (LDL) by 40% and elevated the High-Density Lipoprotein (HDL) by 23%. The current research, shows for the first time that GSE could be used for the treatment of hypercholesterolemia on which GSE reduced the serum TC by about 42% and LDL-C about 56%, while had elevation effect on the serum HDL-C by 56%. Therefore, both the LDL-C/HDL-C ratio and TC/HDL-C ratio were decreased by more than half, thus determining an improvement in the atherosclerotic risk index. In conclusion, GSE showed an obvious hypocholesterolemic effect that may have important pharmaceutical applications in the prevention and treatment of cardiovascular disease and atherosclerosis

Key words: GSE, LDL-C, HDL-C, TC, TG, atherosclerosis, antioxidant

INTRODUCTION

In 2001, chronic conditions, including Cardiovascular Diseases (CVD), diabetes, obesity, cancers and respiratory diseases, account for 59% of the 56.5 million deaths annually and 45.9% of the global burden of disease. It has been projected that, by 2020, chronic diseases will account for almost three-quarters of all deaths worldwide (WHO, 2002). An estimated 16.6 million or one-third of total global deaths-result from the various forms of CVD. By 2010, CVD will be the leading cause of death in developing countries. The major CVD include coronary (or ischaemic) heart disease (heart attack), cerebrovascular disease (stroke), hypertension (high blood pressure), heart failure and rheumatic heart disease, many of which are preventable by action on the major primary risk factors: Unhealthy diet, physical inactivity and smoking. Accumulating evidence suggests that people with high dietary intakes of fruits and vegetables are less likely to develop CVD than people who have low dietary intakes of these foods (Kris-Etherton *et al.*, 2002;

Temple and Gladwin, 2003). 2.7 million Deaths are attributable to low fruit and vegetable intake. Of the disease burden attributable to low fruit and vegetable intake, more than four fifths is from heart diseases and the balance from cancers (WHO and FAO, 2003 a,b). As a consequence, on 11 November 2003, the World Health Organization (WHO) and the UN Food and Agriculture Organization (FAO) announced a unified approach to promote greater consumption of fruits and vegetables. In 1995, the American Dietetic Association (ADA) formulated in a position statement that specific substances in foods (e.g., phytochemicals as naturally occurring components and functional food components) may have a beneficial role in health as part of a varied diet (Bloch and Thomson, 1995). They are produced in the secondary metabolism of many plants and play a role for instance in the defense against micro-organisms, as signaling compounds, etc. Therefore they are called secondary plant products (Watzl and Leitzmann, 1995), phytochemicals (Agarwal and Mukhtar, 1996; Newmark, 1996; Yang *et al.*, 1996), or chemo-preventers (Zumbé,

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1998). Such chemo-preventers are believed to have the potential to delay, prevent, or even reverse many conditions from cancer to dental caries. They have the potential for inclusion into manufactured foods as an added ingredient, or exist as an intrinsic ingredient of the food in question (Zumbé, 1998). Among phytochemicals, polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known (Bravo and Sauro-Calixto, 1998). Grape seed extract is a naturally occurring plant substance that contains a concentrated source of antioxidant nutrients known as Oligomeric Proanthocyanidins (OPCs). Grape seed proanthocyanidins comprise approximately 60 to 70% of the polyphenol content of grapes. (Bagchi *et al.*, 2000). OPCs are not limited to grape seeds but are found in approximately 80% of woody plants and 20% of leguminous plants. However, none has been found with higher quantities than grape-seeds (Santos-Buelga and Scalbert, 2000).

In the present study, we have investigated the effect of GSE on the prevention and treatment of hypercholesterolemia on experimental Wistar strain male *Albino* rats.

MATERIALS AND METHODS

Chemicals: Linoleic acid, ammonium thiocyanate, ferrous chloride and ascorbic acid were obtained from Sigma-Aldrich, Germany. Lipid profile kit was purchased from Bio-Diagnostic, Cairo-Egypt.

Total phenolics determination: The total phenol in the extract was determined by 4-ammonium antipyrine method (Ettinger *et al.*, 1951). The absorbance of the sample solution was measured spectrophotometrically at 500 nm. The polyphenol content was determined using the standard curve previously prepared.

Determination of antioxidant activity by the ferric thiocyanate method: The FTC method was adopted from Osawa and Namiki (1981). Samples (20 mg mL^{-1}) dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%v/v) in 99.5%(w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screw-cap containers at 40°C in the dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture. The absorbance at 500 nm of the resulting red solution was measured every 24 h until maximum absorbance value of the control was attained.

The percent inhibition of linoleic acid per oxidation was calculated as: (%) inhibition = $100 - [(\text{absorbance increase of the sample/absorbance increase of the control}) \times 100]$. All tests were run in duplicate and analysis of all samples were run in triplicate and averaged.

Test animals: Part I (Toxicity test): Group of 36 Wistar strain male Albino rats were received at 28 days of age from Tudor Bilharz Institute (Cairo, Egypt). The animals were quarantined and acclimatized for 7 days prior to the initiation of the treatment. During this acclimatization period, the animals were fed *ad libitum* and each animal was examined to confirm suitability for study. Criteria for suitability included acceptable physical examination and body weight. When placed on study, the animals weighed 100-120 g.

Part II (Hypolipidemic effect study): Group of 48 Wistar strain male *Albino* rats were received at the same age and weight in the previous part. Animals were examined for suitability for the study as we mentioned before.

Rats considered suitable for study were divided into groups by a computerized random sort program. Each rat was identified with an ear tag bearing its assigned animal number. The assigned animal number plus the study number comprised the unique number for each animal. In addition, each cage was provided with a cage card which was coded for dose level identification and contained study number and animal number information.

Housing and environment: Pairs of rats were housed in elevated stainless-steel, wire-mesh cages during the experimental period. Diets were dispensed in cage cups which were refilled every day. Water, was likewise available without restriction via an automated watering system. There were no known contaminants in the food or water which could interfere with the results of the study. The animal room was maintained on a 12-h light/dark cycle controlled via an automatic timer. Temperature and humidity were monitored for maintenance within specified ranges of 18-26°C and 30-70 relative humidity.

Grape seed extract (GSE): GSE was prepared by extraction with aqueous ethanol from dried ground grape seeds at up to 70°C for at least 120 min (Youssef and El-Adawi, 2006). This extract contained 598.8 mg of polyphenol per gram. The stock solution of GSE was then prepared by dissolving 35 g in 250 mL sterile distilled water.

Experimental design

Oral toxicity study: According to a dietary concentration of approximately 1789 mg kg^{-1} body weight/day GSPE in

male rats was considered to be a No-Observed-Adverse Effect Level (NOAEL). The logarithmic progression of interval around 1.04 was calculated for that concentration to choose four doses above. They were 1796.4, 2773, 3820, 5394.2 and 7575 mg poly phenol, which equivalent 3.0, 4.63, 6.38, 9 and 12.65 mg GSE/g body wt.

A total of 36 rats were divided into six groups of six rats each, after twenty hours fasting period. A single oral administration of GSE was given by oral gavages. As for the control group, oral administration of water was given and the observations were continued for 14 days.

The hypolipidemic effect study: A total of 48 rats were divided into three groups, one group (24 rats) were fed a High Cholesterol Diet (HCD). Another group (12 rats) received the same HCD supplemented with 0.3% GSE w/w (one-fifth of the LD₅₀) to test the preventive effect of GSE on hypercholesterolemia. The third group (12 rats) was given the basic diet and served as controls. The lipid profiles were assayed for all groups till we got a marked hypercholesterolemia in HCD group, followed by subdivision of this group (HCD) into two groups in order to test whether the GSE could treat the hypercholesterolemia or not. In order to test this hypothesis, one group received the basic diet and the other received the basic diet enriched with 0.3% GSE (w/w).

Lipid profile analysis: For assessment of the lipid profile, blood samples were drawn from marginal ear veins at 2-week intervals and centrifuged at 1000xg 10 min at 4°C to obtain serum.

Total Cholesterol (TC) was assayed according to the method of Richmond (1973) and Allain *et al.* (1974). In brief, after enzymatic hydrolysis and oxidation of cholesterol, the resultant hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form a quinonimine, which was measured spectrophotometrically at 500 nm.

Total Triglycerides (TG) were determined following the method of Fossati P. and Prencipe, (1982). This method resides simply on the enzymatic hydrolysis of triglycerides to glycerol, which reacts with ATP to form hydrogen peroxide; in turn the resultant hydrogen peroxide reacts with 4-amino antipyrine in the presence of p-chlorophenol to form a quinonimine which is measured colorimetrically at 505 nm.

High-Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL) cholesterol fractions were determined according to Burstein *et al.* (1970) and Lopez-Virella *et al.* (1977). In which Phosphotungstic acid and magnesium ions selectively precipitating all lipoproteins except the

HDL fraction-cholesterol present in the supernatant can be determined by the same method used for total cholesterol. LDL was computed mathematically according to Friedwald's equation (Friedwald, 1972): LDL = TC-[HDL+(TG/ 5)]

Clinical observations: Animals were observed in their cages daily for mortality and signs of severe toxic effect. Examinations included observations of general conditions of skin, fur, eyes, nose, oral cavity, abdomen and external genitalia, occurrence of secretions and autonomic activity (e.g., pupil size, respiratory pattern). Changes in gait and handling as well as presence of clonic or tonic movements, stereotype (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards) were recorded if observed.

RESULTS

Total antioxidant activity: Figure 1 shows the inhibition of linoleic peroxidation by GSE in comparison with ascorbic acid as a standard. GSE has higher antioxidant activity than ascorbic acid.

Acute oral LD₅₀ toxicity: At the dose of 3.0 and 4.63 mg GSE/g body wt, no mortality or clinical signs of toxicity during the 14 days post dose were recorded. With the Exception of reduced food consumption, no adverse effects were observed at 6.38, 9.0 and 12.65 mg GSE/g body wt. On the sixth day we started to record mortality

Table 1A: The total numbers of animals died in each group during the entire experiment (14 days)

Days	Doses (mg GSE/g body wt.)				
	3.0	4.63	6.38	9.0	12.65
Day 1-5	-	-	-	-	-
Day 6	-	-	-	-	2
Day 7	-	-	-	-	-
Day 8	-	-	-	-	2
Day 9	-	-	-	1	-
Day 10	-	-	-	-	-
Day 11	-	-	-	1	-
Day 12	-	-	-	-	-
Day 13	-	-	1	-	-
Day 14	-	-	-	-	-
Total no. of animals died in each group	-	-	1	2	4

Table 1B: The mortality percentage for each dose and the corresponding probit units

Dose mg polyphenol/kg	Log dose	Total no. of animals	Animals died	Mortality (%)	Probit units
1796.4	3.25	6	0	0.00	0.00
2773.0	3.44	6	0	0.00	0.00
3820.0	3.58	6	1	16.67	4.03
5394.2	3.73	6	2	33.34	4.57
7575.0	3.88	6	4	66.67	5.43

as shown on Table 1A. In this study the highest dose 12.65 mg GSE/g body wt represents the worse case scenario in which the total survival rats were two out of six rats. The lower dose 9.0 mg GSE/g body wt recorded four survivals out of six. Since LD₅₀ is the dose of a chemical which kills 50% of a sample population, in our study LD₅₀ will be the dose between 9.0 and 12.65 mg GSE/g body wt. By converting the response probabilities to probit units using a probit transformation table and converting all doses into log dose units Table 1B, we could draw a graphic estimation of LD₅₀ by probit analysis Fig. 2.

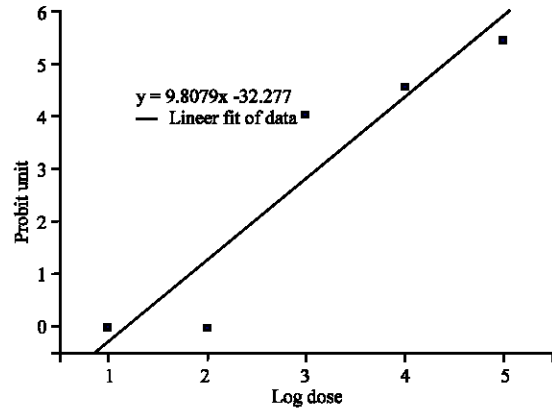


Fig. 2: The LD₅₀ of GSE

Effect of GSE on the development of hypercholesterolemia (preventive effect [PE]): Feeding rats with an HCD for 8 weeks resulted in hypercholesterolemia, as evidenced by high serum total cholesterol, which was more than double (216.14 mg dL⁻¹) that of the control animals (86.21 mg dL⁻¹). The total serum triglycerides (TG) reduced by about 41% (49.08 mg dL⁻¹) following the HCD (Fig. 3).

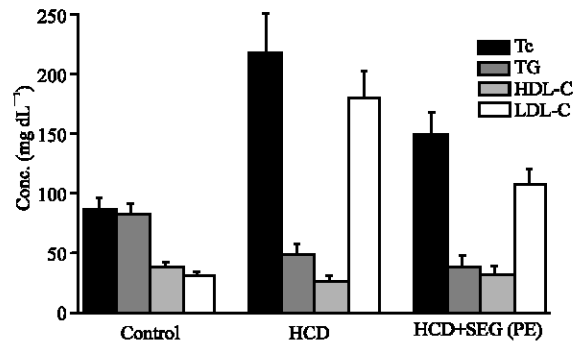


Fig. 3: Representative comparison of lipid profile in control group, HCD group and HCD supplemented with GSE group [PE]. The bar graph shows that the TC and LDL-C was significantly increased in HCD group compared to control and a significant decrease in case of PE group $p < 0.05$. Values are mean \pm SD, N = 24 for HCD group and 12 for control and PE group

Serum HDL-C was reduced by about 30% (27.09 mg dL⁻¹) following the HCD, compared to control animals (38.25 mg dL⁻¹). Serum LDL-C, however, was markedly increased, more than 5.6 fold (179.22 mg dL⁻¹) compared to normal serum values (31.5 mg dL⁻¹). There was an 8-fold increase in the LDL-C/HDL-C risk ratio compared to control animals. Likewise, the TC/HDL-C was significantly elevated by about 3.54 fold in the group fed the HCD compared to control animals.

Supplementation of the HCD with 0.3% GSE (w/w) reduced the serum TC by about 32% (148.79 mg dL⁻¹) compared to rats fed HCD alone. However, there was still an increase in TC by about 72% compared to control animals. GSE-supplemented HCD noticeably reduced the

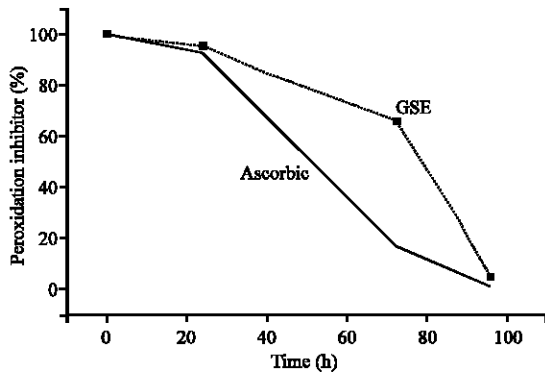


Fig. 1: Inhibition of linoleic peroxidation by Ascorbic acid as a standard and GSE, as measured by the FTC method. Absorbance values represent means of triplicates of different samples analyzed

serum TG by about 18% (39.82 mg dL⁻¹) compared to animals fed GSE-free HCD (49.08 mg dL⁻¹). There was a marked elevation in serum HDL cholesterol, amounting to 23% (33.5 mg dL⁻¹) compared to animals fed the GSE-free HCD (27.09 mg dL⁻¹).

GSE-supplemented HCD noticeably reduced the serum LDL-C by 40% (107.33 mg dL⁻¹) compared to animals fed a GSE-free HCD, but the serum LDL-C was still more than 3.4-fold higher than control values.

The LDL-C/HDL-C risk ratio was lowered by 52% in GSE-enriched HCD group compared to animals fed HCD alone. The ratio however, was still more than 3.9 times greater than normal values. Similarly, there was a decrease in the TC/HDL-C ratio, amounting to 45% compared to GSE-free HCD.

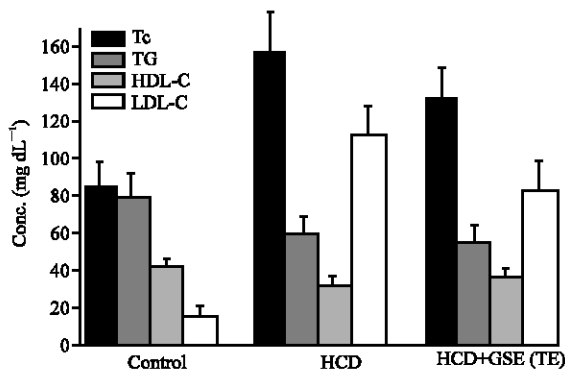


Fig. 4: Representative Comparison of lipid profile in control group, HCD group and HCD supplemented with GSE group [TE]. The bar graph shows that the TC and LDL-C was significantly increased in HCD group compared to control and a significant decrease in case of PE group $p < 0.05$. Values are mean \pm SD, N = 12 for all groups

Effect of GSE after the development of hypercholesterolemia (treatment effect [TE]): After eight weeks, GSE-supplemented basic diet could reduce the serum TC on HCD group by 42% (132.4 mg dL^{-1}), while the basic diet could only reduce it to 33% (157.3 mg dL^{-1}). HDL-C was elevated to 56% (37.31 mg dL^{-1}) on HCD group fed GSE-supplemented basic diet (group-A), where an elevation to 23% (31.91 mg dL^{-1}) was recorded on HCD group fed GSE-free basic diet (group-B) (Fig. 4). However, on group-A, GSE-supplemented basic diet noticeably reduced the serum LDL-C by 56% (83.92 mg dL^{-1}) compared to the previous record, but the serum LDL-C was still more than 3.2 fold higher than control values. On the other hand, the serum LDL-C on group-B, reduced by about 43% ($113.25 \text{ mg dL}^{-1}$) compared to the previous record. The basic diet noticeably elevated the TG to 41% (60.65 mg dL^{-1}) on group-B and 18% (55.64 mg dL^{-1}) on group-A.

The LDL-C/HDL-C risk ratio was lowered by 66% in group-A and 46% in group-B compared to previous values. Similarly, there was a decrease in the TC/HDL-C ratio, amounting to 55 and 44% for group-A and group-B, respectively.

DISCUSSION

Low-Density Lipoprotein (LDL) peroxidation has been reported to contribute to atherosclerosis development (Steinbrecher, 1987). Therefore, delay or prevention of LDL peroxidation is an important function

of antioxidants. The percentage of inhibition in linoleic acid peroxidation from 20 mg GSE was 100, 95.5, 66.16 and 4.4%, respectively along 96 h incubation. These values were slightly higher than obtained from 20 mg ascorbic acid, indicating that GSE possess strong antioxidant activity and persists for longer time than ascorbic. The LD_{50} (median lethal dose) value of the GSE found to be greater than 4 g kg^{-1} in the male wistar-albino rats in the oral acute toxicity study. In the current research the LD_{50} value of GSE was 6.3 g kg^{-1} . Therefore, GSE was not toxic in this study. The possible explanation for the apparent toxicity of high-GSE dose may lie in the inhibition of post-digestive metabolism, or a systemic effect. In an elegant study using 14 C-labeled proanthocyanidins, Abia and Fry (2001) have shown that 18 hrs after gavages 90-94% of the label was in the gut contents and/or feces. More than half of this originated in condensed tannins with higher Degree of Polymerization (DP) becoming insoluble, mainly in the form of protein-tannin complexes. In agreement with that reports, Vallet *et al.* (1994) found that tannins reduced dry matter and nitrogen digestibility,

Butler and Rogler (1992) also suggested possible systemic effects to include direct inhibition of a key metabolic pathway and/or the diversion of metabolism into detoxification of polyphenols or their degradation products.

Overall, these results indicated a lack of toxicity up to 4.3 g kg^{-1} and supported use of GSE in various food supplements.

We have demonstrated that feeding Wistar rats a HCD increased the plasma TC. Similar results have been reported by Monte and Jimenez (1993) and Sakuma *et al.* (1997). In our work, the serum level of TG following the HCD was reduced compared to control animals. This is inconsistent with the finding by Sakuma *et al.* (1997) who did not find any change in serum TG in either normal or diabetic rats fed a high-fat diet. Yumiko and Yasuhide (2002) however, documented that feeding rats a diet containing high concentration of cholesterol for 28 days significantly decreased plasma TG.

Serum HDL-C was reduced by 29% and serum LDL-C was elevated more than 4.6 fold following the HCD, compared to animals fed a normal diet. In addition, the LDL-C/HDL-C ratio was about 8-fold higher. Also, the TC/HDL-C ratio was increased by about 3.5 fold compared to normal values. A GSE-supplemented diet exhibited a notable hypolipidemic effect, as evidenced by its modulating effects on the serum lipid profile of rats. GSE lowered serum TC by about 32 and 42% on both PE group and TE group and serum TG by about 18% on both, but had elevation effect on the serum HDL-C by 23% on PE group and 56% on TE group, while reducing serum LDL-C

by about 40% on PE group and 42% on TE. Therefore, the LDL-C/HDL-C ratio was decreased by more than half on PE and about 66% on TE. The ratio TC/HDL-C was also decreased by about 45 and 55% on PE and TE groups, respectively, thus determining an improvement in the atherosclerotic risk index (Buchwald *et al.*, 2001). Similarly, Del Bas *et al.* (2005) has reported that oral administration of GSE to rats could significantly lower both ratios. Diet-induced hyperlipemia is the most relevant stimulus for the induction of atherosclerotic lesions in humans. Diet-induced hypercholesterolemia is almost always useful for the assessment of agents that interfere with absorption, degradation and excretion of cholesterol, with minimal effects on cholesterol biosynthesis. A priori, cholesterol levels in the body result from two sources: Absorption from the Gastrointestinal Tract (GIT) and endogenous *de novo* synthesis. Taken together, one could argue that the hypolipidemic effect of GSE observed in the current study could possibly be ascribed to an effect on the absorption of cholesterol in the gut, especially GSE was administered orally. Although this conclusion is possible, but it is contrary to our results of TE group where GSE could lower the lipid profile after the induction of hypercholesterolemia. Certainly, we can not exclude local action in the gastrointestinal tract in addition to the well-known anti-oxidant effects of GSE in many biological systems (Mittal *et al.*, 2003; Bagchi *et al.*, 2002; Rays *et al.*, 2001; Bagchi *et al.*, 2000). Another plausible explanation for the hypolipidemic effect of though speculative at present, is that it may have increased the rate of cholesterol catabolism by increasing the activity of hepatic cholesterol 7- α -hydroxylase enzyme. This enzyme is the rate-limiting enzyme of bile acid biosynthesis, thus suggesting that GSE could stimulate the conversion of cholesterol to bile acids, an important pathway of elimination of cholesterol from the body (Del Bas *et al.*, 2005). Tebib *et al.* (1994) reported that plasma total cholesterol and LDL-cholesterol levels were reduced in rats fed a diet containing a high dosage of 2% proanthocyanidins plus cholesterol. In our study, these results suggested that the water-soluble antioxidant, proanthocyanidins might trap reactive oxygen species in aqueous series such as plasma thereby inhibiting oxidation of LDL. Meunier *et al.* (1987) reported that proanthocyanidins inhibit angiotensin-converting enzyme *in vitro*. Moreover, Hernandez *et al.* (1998) showed that delapril, an inhibitor of angiotensin-converting enzyme, reduced the extent of atherosclerotic lesions in cholesterol-fed rabbits. Therefore, antiatherosclerotic effect of proanthocyanidins might be partly related to inhibition of angiotensin-converting enzyme. In conclusion, proanthocyanidin-rich extract inhibited progression of atherosclerosis. Our results suggested

that the antiatherosclerotic activity of proanthocyanidins, which are the major polyphenols in grape seeds, was related to prevention of LDL oxidation in the plasma. Proanthocyanidin-rich foods such as health foods containing grape seed extract may be beneficial in prevention, treatment and lowering the incidence of atherosclerosis and coronary heart disease.

The protection and therapeutic effect afforded by GSE is evident in the light of the decomposition of lipid peroxides and inhibition of lipid peroxidation in the plasma, providing a promising therapeutic phytochemical candidate for the hypercholesterolemia.

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