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Mulberry extract inhibits the development of atherosclerosis in cholesterol-fed rabbits

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Abstract

Mulberry (*Morus alba L.*) fruit is used effectively in Chinese medicines against fever, to protect the liver and to lower blood pressure. Here, we report a water extract, MWE (*Mulberry* water extract), which is designed to exhibit anti-hyperlipidemia and anti-atherosclerotic effects in rabbits with experimental atherosclerosis. New Zealand white rabbits were fed with a normal diet, high cholesterol (1.3%), lard oil (3%) diet (HCD) with or without 0.5 or 1% MWE for 10 weeks. The levels of triglyceride, cholesterol and low-density lipoprotein cholesterol (LDL-C) were lower in the serum of rabbits fed HCD plus MWE than in the serum of rabbits fed HCD. Feeding MWE (0.5 or 1% in the diet) to rabbits significantly reduced severe atherosclerosis in the aorta by 42–63%. Histopathological examination showed that MWE reduced aortic atherosclerotic lesion in the blood vessel of rabbits. From previous data and present results, we suspect that MWE not only inhibits LDL-oxidation but also has a direct effect on the anti-hyperlipidemia in animals.

Keywords: *Mulberry* water extract; Anti-hyperlipidemia; Atherosclerosis; Cholesterol-fed rabbit

1. Introduction

Hyperlipidemia, resulting from lipid metabolic changes, is a major cause of cardiovascular disturbance (Chobanian, 1991), such as atherosclerosis and coronary heart disease. Although a high level of serum cholesterol has been clearly identified as a risk factor for atherosclerosis and coronary heart disease (Kannel, Castelli, & Gordon, 1979), the role of a high level of triglycerides has only recently been established as an independent risk factor for such diseases (Austin, 1989; Cambien et al., 1986). Hypercholesterolemia, or more specifically an elevated plasma low-density lipoprotein cholesterol (LDL-C), is an important risk factor for the development and progression of atherosclerosis, (Kannel, Castelli, Gordon, & McNamara, 1971; Keys, 1970). On the other hand, it has been reported that the oxidative modified LDL might be important in the progression of atherosclerosis, due to the observations that oxidized LDL is cytotoxic, chemotactic and chemostatic. Monocyte macrophages in an environment of oxidized LDL would avidly remove LDL from the interstitium and generate macrophage foam cells, a major cell type present within the fatty streaks and fibrous plaque (Napoli et al., 1997; Napoli et al., 1999; Steinberg, 1997). Therefore, it has been proposed that inhibition of the generation of the

Abbreviations: MWE, *Mulberry* water extract; HCD, high cholesterol diet; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

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oxidative LDL-generated foam cells, and reductions in the level of triglyceride, cholesterol and LDL, by naturally occurring compounds, would result in retardation of atherosclerostic lesion development.

Mulberry fruit is a traditional Chinese edible fruit that is used effectively in folk medicines to treat fever, protect liver from damage, strengthen the joints, facilitate discharge of urine and lower blood pressure. Recently, it has gained an important position in the local soft drink market, although its biological and pharmacological effects are still poorly defined. Important constituents of *mulberry* fruits are the anthocyanins (Gerasopoulos & Stavorulakis, 1997). In our recent studies, *Mulberry* extract showed a strong anti-oxidative ability against LDL-oxidation, and an inhibition on oxidized LDL-induced macrophage cell death and foam cell formation (unpublished data). Therefore, dietary consumption of *Mulberry* extract may reduce the incidence of heart diseases, such as atherosclerosis, through their antioxidant activity. However, the anti-atherogenic effect of *Mulberry* extract has never been assessed in vivo. In this study, we investigated the effect of *Mulberry* water extract (MWE) on experimental atherosclerosis in rabbits.

2. Materials and methods

2.1. Preparation of MWE

MWE was prepared from fruit of *Morus spp.* (*Mulberry*). Briefly, the fruit (100 g) was macerated with water (200 ml) for 24 h, and the aqueous extract was filtered and centrifuged (3000 rpm, 15 min), then lyophilized (−80 °C, 12 h) to obtain 1.5 g of MWE, and stored 4 °C before use. Total anthocyanins were determined using the Fuleki and Francis method (Fuleki & Francis, 1969). Briefly, 10 ml of MWE (1 mg/ml) was diluted to 50 ml with pH 1.0 or 4.5 buffer. The O.D. of the sample was measured at 535 nm, using distillted water as blank. The O.D. difference was obtained by subtracting the total O.D. at pH 4.5 from the total O.D. at pH 1.0. Both values were calculated from the O.D. readings using appropriate dilution and calculation factors.

2.2. Animals and diets

Thirty male New Zealand white rabbits (Animal Center of Chung Shan Medical University), weighing 2000–2200 g were used. They were individually housed in metal cages in an air-conditioned room (22 ± 2 °C, 55 ± 5%, humidity), under a 12 h light/12 h dark cycle with free access to food and water. Water was allowed ad libitum and 150 g/day of food was provided. All experimental rabbits were randomly divided into five groups, each group containing 6 animals. Group I rabbits were fed with standard chow. Group II, rabbits were fed with standard chow with 1% MWE in the daily diet. Group III, rabbits were fed with HCD diet (containing 95.7% standard Purina chow (Purina Mills, Inc.), 3% lard oil and 1.3% cholesterol) for 10 weeks, to provoke an atherosclerotic process. At the same time, two of the groups were treated with HCD plus MEW with different doses of 0.5% and 1.0% in the daily diet, as Groups IV and V. The selection of MWE dose was based on a suitable pharmacological dose for humans in a daily diet. During the 10 week feeding period, all animals used were handled according to the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMC) for the care and use of laboratory animals.

At the end of 10 weeks, the rabbits were sacrificed by exsanguination after deep anesthesia with pentobarbital (30 mg/kg i.v.) via the marginal ear vein. Serum was stored at −80 °C prior to serum lipid analysis and measurement of serum values. The aortic arch and thoracic aortas were carefully removed to protect the endothelial lining, and were collected and freed of adhering soft tissue.

2.3. Evaluation of atherosclerotic lesions

Thoracic aortas were rapidly dissected, opened longitudinally and stained with Oil Red. Then the photographs of the inner surface were taken and copied onto graph paper with magnification (2x) and atheromatous plaques were delineated. Numbers of small squares surrounded by the line were counted on the graph paper, and the percentages of the areas of atheromatous plaques were calculated. Aortic arches were rapidly dissected out and kept in −80 °C or kept in 10% neutral-buffer formalin (NBF). For the pathological analysis, paraffin-embedded tissue sections of aortic arch were stained with hematoxylin and eosin (H & E). Experienced pathologists evaluated the presence of fatty streak and medical calcifications and smooth muscle cell in examined preparations. Lesions were scored on a four point intensity semiquantative scale (−, absence; +, mild; ++, moderate; +++, intense) for each damage.

2.4. Serum lipid measurements and toxicity assessment

Serum and lipoprotein levels of cholesterol and triglyceride were measured by enzymatic colorimetric methods using commercial kits (Boeheringer Mannheim, Germany). Several hepatic enzymes, such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), were used as biochemical markers for hepatic damage. The levels of creatinine, blood urea nitrogen (BUN) and uric acid (UA) were used for evaluating renal damage in MWE-treated rabbits.

The activities of enzymes and biochemical values were determined by enzymatic colorimetric methods, using a standard Sigma commercial kit (St. Louis, MO, USA).

2.5. Statistical analysis

Results were collated as means ± SD and statistical analyses were obtained using the unpaired *t test*. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Concentrations of anthocyanins in MWE

MWE were extracted with water as described in materials and methods. To establish the quality of MWE extracted from dried fruit of *Mulberry*, the concentrations of anthocyanins were determined. Studies showed that the yield of MWE was 1.5–2%. MWE contains about 2.5%, of anthocyanins.

3.2. Growth and diet intake

Acceptance measurements of the diet supplemented with MWE showed that no significant differences existed between the groups in the daily consumption of the diet. No significant difference in body weights was observed during the experimental period (Table 1).

3.3. Effect of MWE on serum lipid levels

The serum triglyceride level was increased in Group III but significantly decreased by 46% and 56% in Groups IV and V ($p < 0.05$ and $p < 0.01$, receptively, (Fig. 1). Total cholesterol and LDL-C levels in Groups IV and V were significantly lower ($p < 0.05$ in Group IV and $p < 0.01$ in Group V) than in Group III (Fig. 2). No significant changes in HDL-C levels were observed in Group III compared with Groups IV and V,

Table 1
Effect of MWE on rabbit weight and daily diet intake

Group	Weight of rabbit (kg/rabbit)	Food intake of daily diet (g/rabbit)
Group I	2.25 ± 0.32[a]	149 ± 23[a]
Group II	2.12 ± 0.24	154 ± 28
Group III[b]	2.29 ± 0.34	155 ± 18
Group IV	2.26 ± 0.31	153 ± 25
Group V	2.23 ± 0.28	156 ± 13

[a] Means ± SD, $n = 6$.
[b] HCD containing 1.3% cholesterol and 3% lard oil in Purina Lab chow.

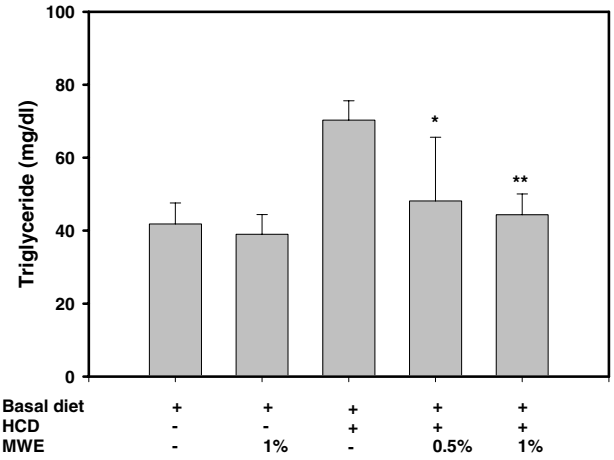

Fig. 1. Effect of MWE on plasma triglyceride levels in 10 week-period HCD-fed rabbits. The data are presented as means ± SD from 6 rabbits per group. *$p < 0.05$, compared with Group III.

but the LDL-C/HDL-C ratio significantly decreased in Group V ($p < 0.01$, Fig. 2).

3.4. Extent of atherosclerosis

The extent of atherosclerosis in the aorta was evaluated as the area of fatty region using the detection of the formation of foam cells in the atherosclerotic lesions. The number of foam cells in atherosclerotic lesions in the thoracic aorta decreased in Groups IV and V (Fig. 3). The lesions in most rabbits were relatively uniform in appearance and consisted of initial foam cell, smooth muscle cells and calcification. The most marked changes were observed in the aortic arch. The percentage of oxidized LDL-positive macrophage-derived foam cells in atherosclerotic lesions in the aortic arch decreased in Groups VI and V. Smooth muscle cell migration was observed in the atherosclerotic lesions in Group III, but very few smooth muscle cell migrations in Groups IV and V. The results are summarized in Table 2.

3.5. Effect of MWE on atherosclerosis development

An evaluation of atherosclerotic lesion on the inner surface of the thoracic aorta revealed the control rabbit aorta to be covered with opaque material (Fig. 4a). The average percentage of the ratio of area of lipid deposits to initial surface area (thoracic aorta) was 56.1 ± 12.6% in Group III, 41.9 ± 11.7% in Group IV and 25.3 ± 16.3% in Group V. Groups I and II only had no lipid lesion developed during this study. There were differences in the extents of lesions among Groups III, IV and V rabbits that are summarized in Fig. 4b. These lesions were approximately 56% of thoracic aorta in Group III and, for those fed with MWE (Groups IV

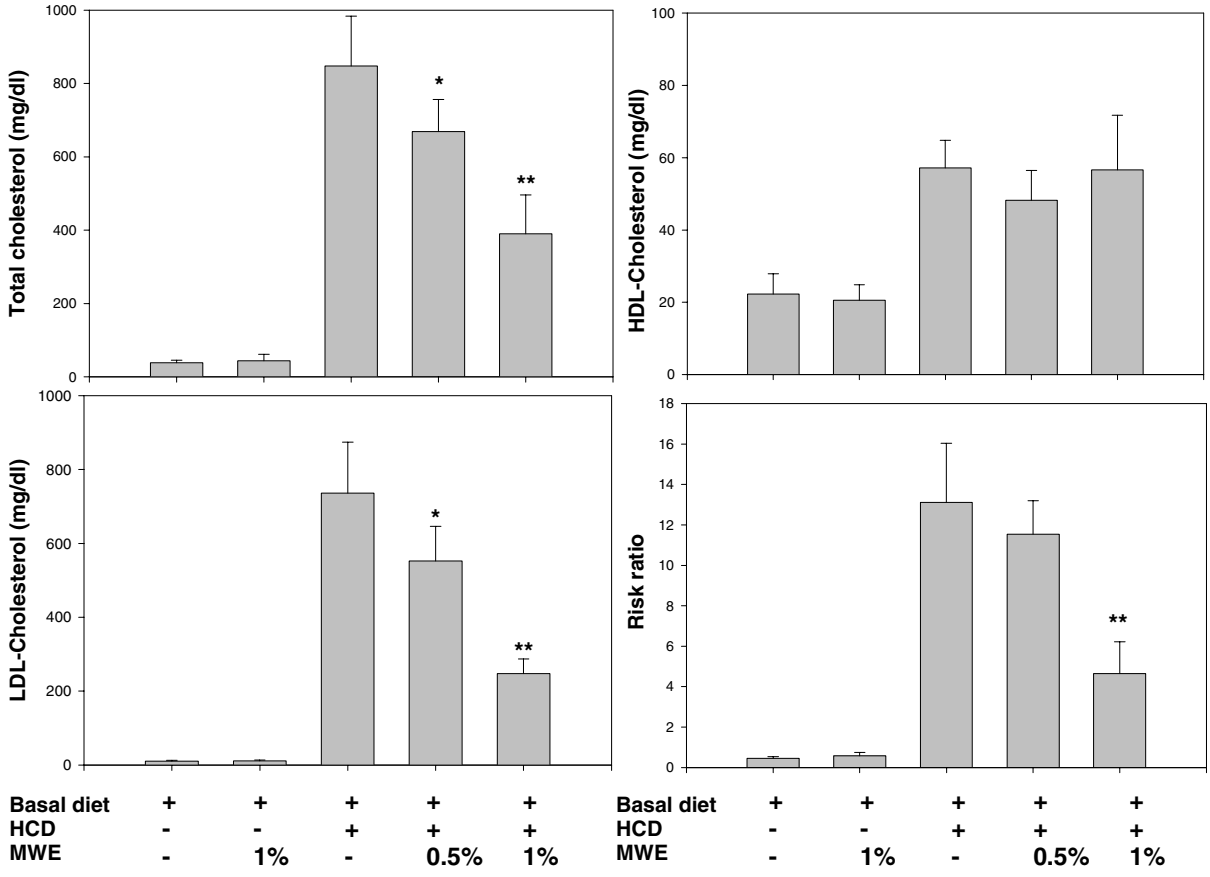

Fig. 2. Effect of MWE on plasma cholesterol levels in 10 week-period HCD-fed rabbits. The data are presented as means ± SD from 6 rabbits per group. *$p < 0.05$, **$p < 0.01$ compared with Group III.

and V) in the daily diet, the lesion was decreased by approximately 15–25% in rabbit thoracic aorta (Fig. 4b).

3.6. Effect of MWE on liver and renal function

The results in Table 3 show the effect of administration of MWE. No significant difference in weight was observed for the high cholesterol diet, with or without MWE. Ten weeks of continuous administration of MWE showed no alteration in serum creatinine MWE also retained relatively constant liver and renal function markers (AST, ALT, ALP, creatinine, BUN and uric acid). These results reveal that MWE shows no toxicity in liver on kidney (Table 3).

4. Discussion

In our previous studies, MWE and its components (anthocyanins) displayed a marked anti-oxidative activity and inhibited LDL-oxidation and foam cell formation *in vitro* (unpublished data). However, their pharmacological properties have not been fully elucidated. In this follow up study, we now show that MWE inhibited the progression of atherosclerosis in cholesterol-fed rabbits.

Yamakoshi, Kataoka, Koga, & Ariga (1999) reported that probucol (1%) decreased serum cholesterol by 14% and LDL-C by 17%, but had no effect on HDL-C or triglyceride in rabbits fed with cholesterol for eight weeks. In this study, we observed that 1% MWE (Group V) was able to reduce cholesterol by 53% and LDL-C by 66%. Triglyceride also declined, by 56%, to a level near the normal range when the animals were treated with MWE (Group V). The effect of MWE on total cholesterol and LDL-C levels was dose-dependent. Hence, the anti-hyperlipidemic potential of MWE was better than that of probucol in the cholesterol-fed rabbits. The anti-atherosclerotic activity in the thoracic aorta of MWE was also stronger than probucol (1%) which was shown to be 52% (Brown & Goldstein, 1983).

MWE contains 2.5% anthocyanins and 4.6% total phenol. The anti-oxidative activity of MWE, along with the observed positive and direct effects in decreasing serum lipids and in inhibiting smooth muscle cell

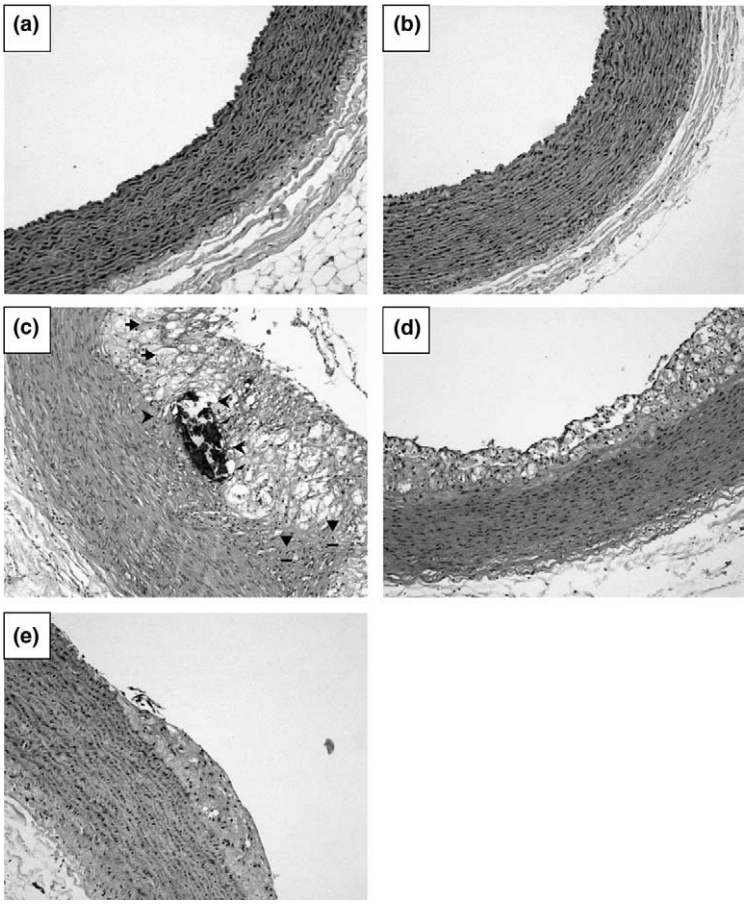

Fig. 3. Histological analysis of a representative atherosclerotic lesion from HCD-fed rabbits treated for 10 weeks with MWE (0.5%) and MWE (1%). Group I, rabbits fed with basal diet (a); Group II, rabbits fed basal diet with MWE (1%, wt/wt) (b); Group III, rabbits fed with HCD (c). The arrow (➔) is shown for foam cell and (▼) for smooth muscle cell migration. Calcification core is shown as arrow (➤) in (c); HCD-fed rabbits and those treated with MWE (0.5%) (Group IV, d) and MWE (1%) (Group V, e) in daily diet.

Table 2
Effect of MWE on pathological analysis of rabbit aortic arch lesions

Group	Fatty streak	calcification	SMC migration
Group I	–[a]	–	–
Group II	–	–	–
Group III[b]	+++	++	++
Group IV	++	–	+
Group V	+	–	–

[a] Lesions were scored on a four point intensity semiquantitative scale : –, absence; +, mild; ++, moderate; +++, intense.

[b] HCD containing 1.3% cholesterol and 3% lard oil in Purina Lab chow.

migration, suggested that MWE might trap reactive oxygen species in aqueous environments such as plasma and interstitial fluid of the arterial wall and, thereby, prevent the oxidation of LDL. The ability of *mulberry* anthocyanin extract to inhibit LDL-oxidation was 10-fold higher than that of MWE (containing 2.5% anthocyanins). These results indicate that the anti-atherosclerotic activity of the MWE was due to *mulberry* anthocyanins.

According to the oxidative hypothesis of atherosclerosis (Badimon, Fuster, Chesebrom, & Badimon, 1993; Brown & Goldstein, 1983; Glass & Witztam, 2001; Ross, 1993; Witzum & Steinberg, 1991), LDL entrapped in the subendoethelial space of lesion-prone arterial sites is slowly oxidized through the action of resident vascular cells. Oxidation of LDL in the arterial wall is thought to be a very important step in atherogenesis. The present pathological examination showed that the number of foam cells decreased after the administration of MWE. These results suggested that MWE inhibited the oxidation of LDL in the arterial wall, thereby exerting an anti-atherosclerotic effect. The transition from a relatively simple fatty streak to a more complex lesion is characterized by the immigration of smooth muscle cells from the medial layer of the artery wall past the internal elastic lamina and into the intima, or subendoethelial space (Glass & Witztam, 2001). Immunohistological and pathological examination demonstrated that MWE suppressed smooth muscle cell migration in the HCD-fed rabbits.

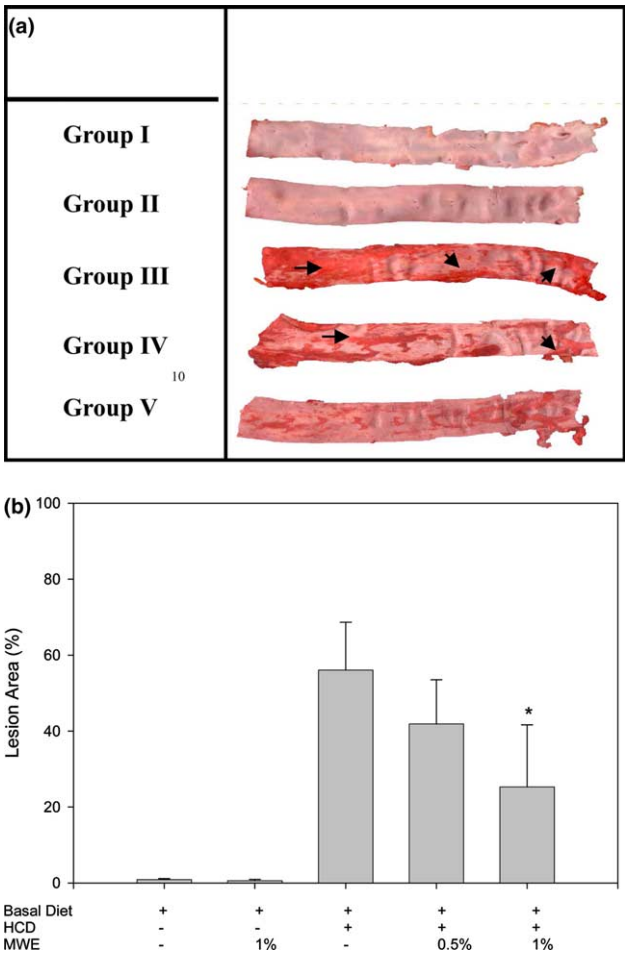

Fig. 4. The extent of the development of atherosclerotic lesions: (a) and densitometric analysis of the relative area of atherosclerotic lesion in the thoracic aorta; (b) after feeding with the experimental diet. Results are shown as means ± SD. *$p < 0.01$, compared with Group III.

Hence, MWE also attenuated the development of atherosclerosis in the HCD-fed rabbits.

It has been confirmed that many antioxidants inhibit the development of atherosclerotic lesions in rabbits fed with HCD. It is generally assumed that the ability of some antioxidants to prevent atherosclerosis is exerted by protecting LDL from oxidation and is associated with an anti-hypercholesterolemic effect. Because of their expected safety for long-term administration to healthy people, diet-derived compounds are of high interest as potential cardioprotective agents. Dietary components with cardioprotective activity are usually composed of complex mixtures, such as curcuminoids, tea polyphenol extract, and soy isoflavone mixtures (Clarkson, 2002; Crawford, Kirk, Rosenfeld, LeBoeuf, & Chait, 1998; Kondo, Suzuki, Ikeda, & Umemura, 2002; Laura, 1998; Naito et al., 2002; Scalbert & Williamson, 2000; Weisburger & Chung, 2002). MWE was able to prevent hypertriglyceridemia and hypercholesterolemia in the HCD-fed rabbits; this may indicate a specific inhibitory effect of MWE on fatty acid synthesis, especially triglycerides. Such an effect has been demonstrated by anka and green tea polyphenol (Chan et al., 1999; Wang, Lin-Shiau, Chen, & Lin, 2000; Yeh, Chen, Chiang, Lin-Shiau, & Lin, 2003). It is not clear by which mechanism MWE lowers serum cholesterol in HCD-fed rabbits. An increase in its excretion in the bile as bile acids or a decrease in its biosynthesis might be involved. Our recent work revealed that MWE could lower liver triglyceride and cholesterol, and increase hepatic lipase activity in hamsters fed with high fat diet for 12 weeks (unpublished data). However, the mechanism of the anti-hyperlipidemic role of MWE in HCD-fed rabbits is yet to be determined.

In this study, MWE was shown to decrease cholesterol and triglyceride and attenuate the development of atherosclerosis in HCD-fed rabbits. Additionally, MWE had no toxic effects on the liver or renal functions during the experimental period. Taken together, these data suggest a promising application of MWE in cardioprotection.

In conclusion, MWE was shown to lower the serum cholesterol and triglyceride and repress progression of atherosclerosis in HCD-fed rabbits. This outcome might be attributed to the preventative effect of anthocyanins against LDL-oxidation in the arterial wall and supports the use of MWE for lowering the incidence of atherosclerosis and coronary heart disease.

Table 3
Effect of MWE on rabbit liver and renal function after being fed with high cholesterol diet for 10 weeks

Group	Liver function			Renal function	
	ALT (mg/ml)	AST (mg/dl)	ALP (mg/dl)	CRE (mg/dl)	BUN (mg/dl)
Group I	16.5 ± 4.46[a]	35.2 ± 12.5	116 ± 29.12	1.50 ± 0.11	18.7 ± 1.90
Group II	18.5 ± 2.88	38.7 ± 6.77	126 ± 44.55	1.65 ± 0.24	20.7 ± 2.42
Group III[b]	19.0 ± 5.55	35.3 ± 8.80	118 ± 36.80	1.48 ± 0.15	19.1 ± 3.32
Group IV	16.8 ± 4.12	36.7 ± 13.02	114 ± 45.75	1.90 ± 0.28	19.9 ± 4.49
Group V	17.0 ± 2.76	36.0 ± 6.00	105 ± 12.63	1.92 ± 0.18	20.4 ± 4.32

ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; CRE, creatinine; BUN, blood urea nitrogen.
[a] Mean ± SD, $n = 6$.
[b] HCD containing 1.3% cholesterol and 3% lard oil in Purina Lab chow.

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