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Hibiscus sabdariffa Extract Inhibits the Development of Atherosclerosis in Cholesterol-Fed Rabbits

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Hibiscus sabdariffa L., a local soft drink material and medicinal herb, is usually used effectively in native medicines against hypertension, pyrexia, and liver disorders. Here, we report an extract, HSE (*H. sabdariffa* extract), which is designed to exhibit hypolipidemia and antiatherosclerotic 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% HSE 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 HSE than in the serum of rabbits fed HCD. Feeding HSE (0.5 and 1% in the diet) to rabbits significantly reduced severe atherosclerosis in the aorta. Histopathological examination showed that HSE reduced foam cell formation and inhibited smooth muscle cell migration and calcification in the blood vessel of rabbits. These results suggest that HSE inhibits serum lipids and shows an antiatherosclerotic activity.

KEYWORDS: ***Hibiscus sabdariffa* extract; hyperlipidemia; atherosclerosis; cholesterol-fed rabbit**

INTRODUCTION

There is a considerable amount of epidemiological evidence revealing an association between diets rich in fruits and vegetables and a decreased risk of cardiovascular disease (*1*−*4*). It is generally assumed that the active dietary constituents contributing to these protective effects are antioxidant nutrients such as α-tocopherol, β-carotene, polyphenolic acid, and anthocyanins (*5*−*8*). *Hibiscus sabdariffa* L. (Malvaceae) is a traditional Chinese rose tea and is used effectively in folk medicines against hypertension (*9*, *10*), inflammation (*11*), and mutagenicity (*12*).

Recently, it has gained an important position in the local soft drink market, although its biological and pharmacological effects are still poorly defined. The compositions contained in the flowers of *Hibiscus* species are polyphenolic acids, flavonoids, and anthocyanins. In our previous studies, *Hibiscus* anthocyanins and protocatechuic acid showed strong antioxidant (*13*, *14*) and antitumor effects (*15*, *16*). Many investigations highlight an additional role of polyphenolic acid, flavonoids, and anthocyanins that may act as antioxidants or via other mechanisms contributing to the cardioprotective actions (*17*−*19*). Recently, we found that HSEs (*H. sabdariffa* extracts) inhibit low-density lipoprotein (LDL) oxidation in vitro and decrease serum lipids in cholesterol- and high fructose-fed rats. Therefore, dietary HSE may reduce the incidence of heart disease, such as atherosclerosis, through their antioxidant activity. However, the antiatherogenic effect of HSE has never been assessed in vivo. In this study, we investigated the effect of HSE from *H. sabdariffa* on experimental atherosclerosis in rabbits.

MATERIALS AND METHODS

Preparation of HSE. HSE was prepared from *H. sabdariffa* (Malvaceae). Briefly, the *H. sabdariffa* (150 g) was macerated with hot water (95 °C, 6000 mL) for 2 h and the aqueous extract was evaporated under vacuum at −85 °C. The extracted solution was filtered and then lyophilized to obtain 75 g of HSE and stored at 4 °C before use. The concentration of total phenols was analyzed according to the Folin−Ciocalteau method (*20*). Briefly, HSE (0.1 mg) was dissolved in a test tube with 1 mL of distilled water; Folin−Ciocalteu reagent (2N, 0.5 mL) was added and mixed in thoroughly. After an interval of 3 min, 3 mL of 2% Na_2CO_3 solution was added, and the mixture was allowed to stand for 15 min with intermittent mixing. The absorbance of the mixture at 750 nm was measured on a Hitachi spectrophotometer (U-3210) with gallic acid as the standard. Total anthocyanins content in HSE was determined using the Fuleki and Francis method (*21*). Briefly, 10 mL of HSE (1 mg/mL) was diluted to 50 mL with the pH 1.0 and 4.5 buffer, respectively. The O.D. of the samples was measured at 535 nm, using distilled 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 the appropriate dilution and calculation factors. Total flavonoids content

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was determined by the Jia method (*22*) using rutin as a standard. Briefly, half of a milliliter of the HSE (1 mg/ml) was diluted with 1.25 mL of distilled water. Then, 75 μL of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150 μL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. A 0.5 mL amount of 1 M NaOH was added, and the total was made up to 2.5 mL with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm. The final extract (HSE) was composed of 2.5% anthocyanins, 1.7% polyphenolic acid, and 1.43% flavonoids, as measured by quantitative analysis.

Animals and Diets. Thirty male New Zealand white rabbits (Animal Center of Chung Shan Medical University) weighing 2000−2200 g were randomly divided into five experimental groups. 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. Experimental rabbits were fed for 10 weeks on a diet containing 95.7% standard Purina Chow (Purina Mills, Inc.), 3% lard oil, and 1.3% cholesterol (HCD) to provoke an atherosclerotic process. At the same time, two of the groups were orally treated with HSE at doses of 0.5 and 1.0%. The selection of HSE dose was based on the suitable pharmacological dose for human in the daily diet. To observe the side effect of HSE, only one group was given 1% HSE in diet. When handled 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 exsanguinations after deep anesthesia with pentobarbital (30 mg/kg i.v.) via the marginal ear vein. Serum was stored at −80 °C until serum lipid analysis and measurement of serum values. The aorta arch and thoracic aortas were carefully removed to protect the endothelial lining and were collected and cleaned carefully off adhering soft tissue.

Evaluation of Atherosclerotic Lesions. Thoracic aortas were rapidly dissected and opened longitudinally and stained with Oil Red. Then, the photographs of the inner surface were taken and copied onto graph paper with magnification (×2) and athermanous plaques were delineated. Numbers of small squares surrounded by the line were counted on the graph paper, and the percentages of the areas of athermanous plaque were calculated. Aortic arches were rapidly dissected out and kept in −80 °C or kept in 10% neutral buffer formalin. For the pathological analysis, paraffin-embedded tissue sections of aortic arch were stained with hematoxylin and eosin. 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 intensively semiquantative scale (−, absence; +, mild; ++, moderate; +++, intense) for each damage.

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

Statistical Analysis. Results were reported as means ± SD, and statistical analysis was obtained using an unpaired *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Growth and Diet Intake. The acceptance of the diet supplemented with HSE was that no significant differences were observed between the groups in the daily consumption of the diet. No significant difference in body weights was observed during the experimental period (**Table 1**).

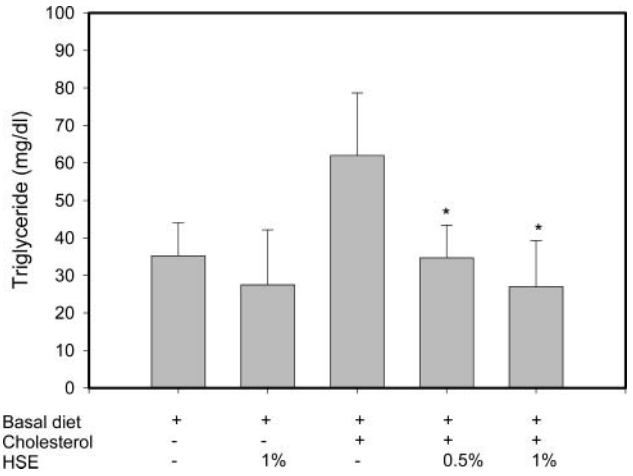

Figure 1. Effect of HSE on plasma triglyceride levels in 10 week period HCD-fed rabbit. The data are presented as means ± SD from six rabbits per group. The HCD contained 1.3% cholesterol and 3% lard oil. *$p < 0.05$ as compared with the group of HCD-fed rabbits.

Table 1. Effect of HSE in Rabbit Weight and Daily Diet Intake

group	weight of rabbit (g/rabbit)	food intake of daily diet (g/rabbit)
normal	2170 ± 125[a]	149 ± 13[a]
HSE 1%	2200 ± 230	150 ± 22
HCD[b]	2180 ± 325	158 ± 18
HCD + HSE 0.5%	2160 ± 235	152 ± 20
HCD + HSE 1%	2100 ± 340	146 ± 15

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

Effect of HSE on Serum Lipid Levels. The serum triglyceride level was increased in HCD-fed rabbits but significantly decreased by 46−59% in the 0.5% and 1% HSE groups ($p < 0.05$, **Figure 1**). Total cholesterol and LDL cholesterol (LDL-C) levels in the HSE groups were significantly lower than in the HCD-fed rabbits (**Figure 2**). No significant changes in high-density lipoprotein cholesterol (HDL-C) levels were observed in HCD- and HSE-fed groups, but the LDL-C/HDL-C ratio decreased in HCD- and 0.5% HSE-treated groups ($p < 0.05$, **Figure 2**).

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 HSE groups (**Figure 3**). The lesions in most rabbits were relatively uniform in appearance and consisted of initimal foam cell, smooth muscle cells, and calcification. The most remarkable 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 HCD- and HSE-fed rabbits. Smooth muscle cells migration was observed in the atherosclerotic lesions in the HCD-fed rabbits but very few smooth muscle cell migration in the HCD- and HSE-fed rabbits. The media of all of the arteries showed mild calcification in HCD-fed group but not in HCD- and HSE-fed rabbits, and the results are summarized in **Table 2**.

Effect of HSE 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 (**Figure 4A**). The average percentage of the ratio of area of lipid deposits to intimal surface area (thoracic aorta) was 39.0

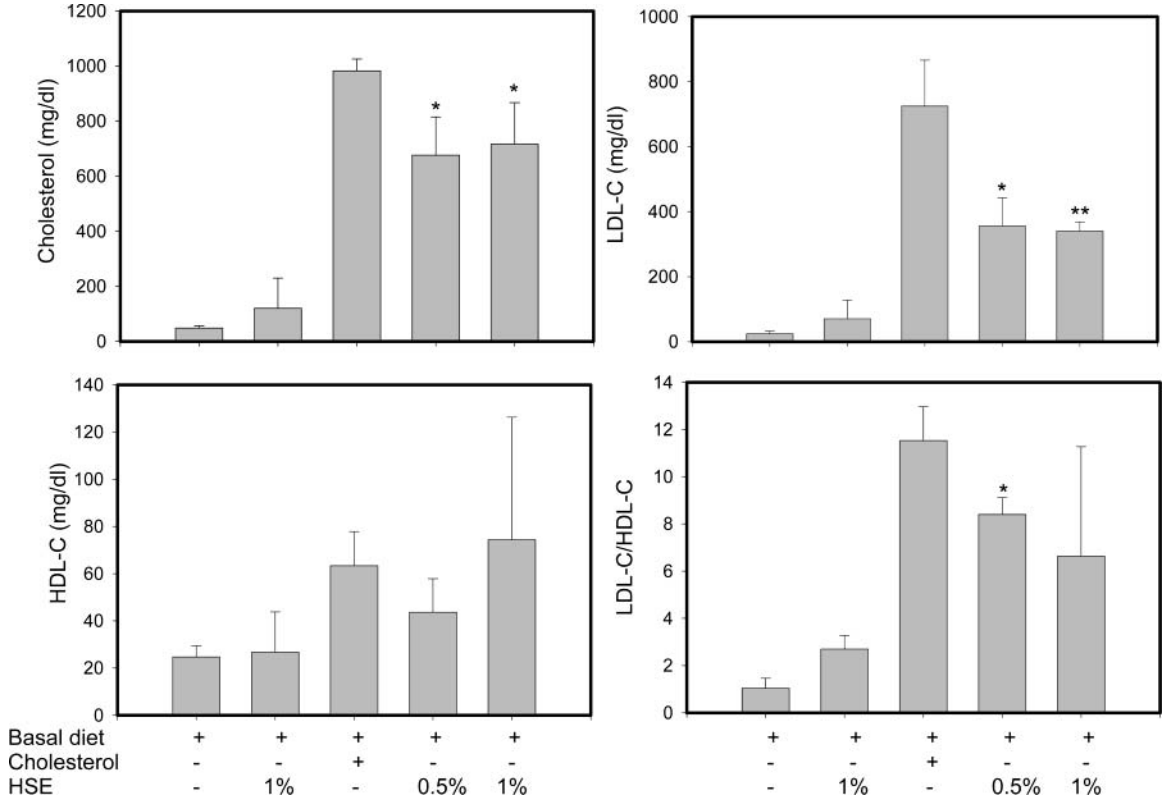

Figure 2. Effect of HSE on plasma cholesterol levels in 10 week period HCD-fed rabbit. The data are presented as means ± SD from six rabbits per group. The HCD contained 1.3% cholesterol and 3% lard oil. *$p < 0.05$; **$p < 0.01$ as compared with HCD-fed rabbits.

± 6.8% in HCD-fed rabbits, 19.6 ± 2.1% in HSE fed 0.5% in diet, and 12.6 ± 2.0% in HSE fed 1% with rabbits. The normal and HSE 1% groups only had no lipid lesion develop during this study. There were differences in the extent of lesions among the HCD- and HSE-fed rabbits that are summarized in **Figure 4B**. These lesions are approximately by 39% of thoracic aorta in HCD-fed rabbits, and for rabbits fed with HSE (0.5 or 1%) in the daily diet, the lesion decreased approximately 20−25% in rabbit thoracic aorta (**Figure 4B**).

Effect of HSE on Liver and Renal Function. The results in **Table 1** represent the effect of administration of HSE. No significant difference in weight was observed in the high cholesterol diet with or without HSE. After 10 weeks of continuous administration of HSE, no alteration in serum creation of the HSE was shown and HSE also remained relatively constant in liver and renal function markers (AST, ALT, ALP, creatinine, BUN, and UA). These results reveal that HSE shows no toxicity in the liver and renal function.

DISCUSSION

HSE compositions contained polyphenolic acids (1.7%), flavonoids (1.43%), and anthocyanins (2.5%). Previously, we reported that HSE and its components (anthocyanins and protocatechuic acid) display remarkable antioxidative activity (*13*, *14*) and can inhibit LDL oxidation and TBARs formation in vitro (*23*, *24*). We also investigated the suppressive effect of HSE on serum lipids and lipoproteins in rats fed a high cholesterol diet or a high fructose diet (*24*). However, their pharmacological properties have not been fully elucidated. In this study, we showed that HSE inhibits progression of atherosclerosis in cholesterol-fed rabbits.

Yamakoshi et al. (*25*) reported that probucol (1%) decreased serum cholesterol by 14% and LDL-C by 17% in cholesterol-fed rabbits for 8 weeks but had no effect on HDL-C and triglyceride. In this study, HSE (1%) inhibits cholesterol by 28% and LDL-C by 55% and also inhibits triglyceride by 53%. In our study, the triglyceride level was reduced to a nearly normal range when the animals were treated with 0.5 and 1% HSE. The effect on cholesterol and LDL-C levels was similar for both doses. It is possible that the treatment of 0.5% HSE had reached the maximal pharmacological effect. Therefore, there was no significant difference between these two doses.

Hence, the hypolipidemic potential of HSE was higher as compared with that of polyphenol in HCD-fed rabbits. The antiatherosclerotic activity of probucol (1%) in thoracic aorta was 52% (*25*). The antiatherosclerotic activity of HSE was almost the same as that of probucol in HCD-fed rabbits.

Anthocyanins, a major component of HSE, are a color pigment obtained from *H. sabdariffa* and used in Chinese herb and food coloring. Besides anthocyanins, the main active components of HSE are polyphenolic acids and flavonoids with antioxidant activity (*5*−*8*). Our group recently published that the HSE increases in vitro inhibition of human LDL to oxidation (*24*). In this study, we first describe a positive and direct effect of HSE in the atherosclerotic process. Feeding HSE decreased in serum lipids, in the number of oxidized LDL-positive foam cells and in the migration of smooth muscle cells. These results suggested that the HSE might trap reactive oxygen species in aqueous series such as plasma and interstitial fluid of the arterial wall, thereby inhibiting oxidation of LDL.

On the other hand, a cholesterol lowering property of HSE has been described. We have found that HSE treatment is useful in achieving and maintaining low plasma levels of total cholesterol and triglyceride in high cholesterol- or high fructose-fed rats, respectively (*24*). According to these studies, rabbits fed HSE had significantly lower cholesterol and triglyceride concentrations in plasma than the control group. We suggested that HSE has a hypolipidemia effect on rabbits fed HCD to

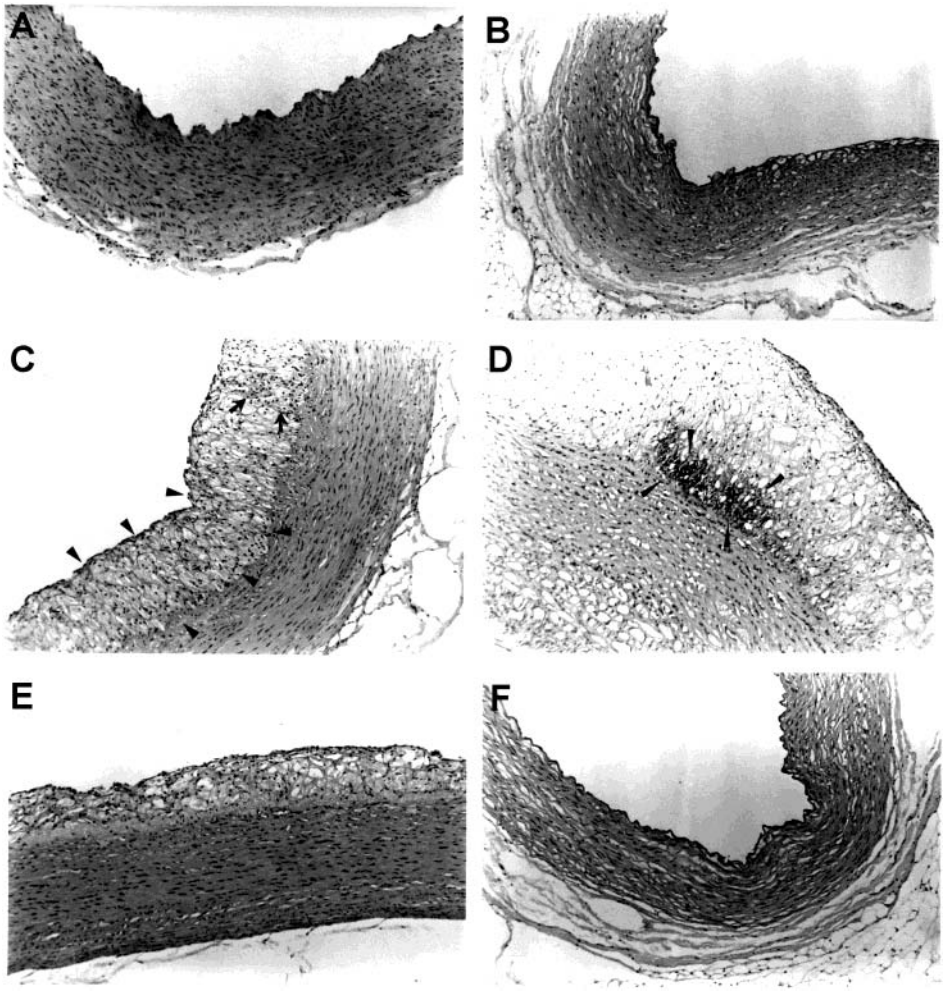

Figure 3. Histological analysis of a representative atherosclerotic lesion from HCD-fed rabbits treated for 10 weeks with HSE 0.5% and HSE 1%. Basal diet (A); rabbits fed basal diet with HSE 1% (wt/wt) (B); rabbits fed with HCD (C and D). The arrow was shown for foam cell (▲) and smooth muscle cell migration (→) in (C). The calcification core was shown as an arrow (▲) in (D); HCD-fed rabbits were treated with HSE 0.5% (E) and 1% (F) on daily diet.

Table 2. Effect of HSE on Pathologic Analysis of Rabbit Aortic Arch Lesions

group	fatty streak	calcification	SMC migration
normal	–[a]	–	–
HCD[b]	+++	++	++
HSE 1%	–	–	–
HCD + HSE 0.5%	+	–	+
HCD + HSE 1%	+	–	–

[a] Lesions were scored on a four point intensively semiquantative scale: –, absence; +, mild; ++, moderate; +++, intense. [b] HCD containing 1.3% cholesterol and 3% lard oil in Purina Lab chow.

provoke experimental atherosclerosis. At present, this hypolipidemic effect is still unclear and will be a goal for future research.

According to the oxidative hypothesis of atherosclerosis (*26*–*30*), 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. In our immunohistochemical examination, the number of foam cells originating from macrophages, which had taken in oxidized LDL, administered HSE. These results suggested that HSE inhibits oxidation of LDL in the arterial wall, thereby exerting an antiatherosclerotic effect. The transition from the relatively simple fatty streak to the 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 (*30*). In immunohistological and pathological examinations, HSE inhibits smooth muscle cells migration in HCD-fed rabbits. Hence, HSE also attenuates the development of atherosclerosis in HCD-fed rabbits.

It has been demonstrated that many antioxidants inhibit the development of atherosclerotic lesions in rabbits fed with HCD. It is generally assumed that some antioxidants can prevent atherosclerosis by protecting LDL from oxidation and are also associated with an antihypercholesterolemic effect. In this study, we also investigated whether HSE could inhibit LDL oxidation and decrease cholesterol and triglyceride and attenuate the development of atherosclerosis in HCD-fed rabbits. HSE showed no toxic effect in rabbit liver and renal function during the experimental period. 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 typically started as complex mixtures, such as mixture of curcuminoids, tea polyphenol extract, and soy isoflavone mixtures (*7*, *17*, *31*–*35*). HSE contains polyphenolic compounds, anthocyanins, and

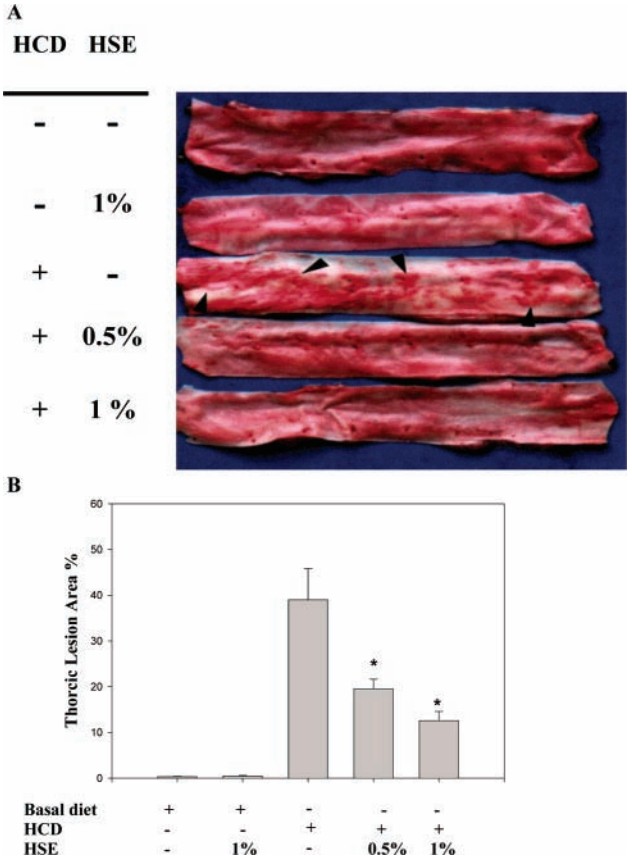

Figure 4. Extent of the development of atherosclerosis lesions (A) and densitometric analysis of the relative area of atherostic lesion in the thoractic aorta (B) after feeding with the experimental diet. Results are shown as the means ± SD. *P < 0.01 as compared with HCD-fed group.

flavonoids, and it may by used to demonstrate the potential efficacy of *H. sabdariffa*.

In conclusion, HSE inhibited serum cholesterol, triglyceride, LDL oxidation, and progression of atherosclerosis in HCD-fed rabbits. Our results suggest that the antiatherosclerotic activity of HSE, which contains anthocyanins, polyphenolic extracts, and flavonoids, was related to prevention of LDL oxidation in the arterial wall and that HSE might be beneficial in lowering the incidence of atherosclerosis and coronary heart disease.

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