The effect of diosmin-hesperidin combination treatment on the lipid profile and oxidative-antioxidative system in high-cholesterol diet-fed rats

Yüksek kolesterollü diyetle beslenen sıçanlarda diosmin-hesperidin kombinasyonu tedavisinin lipid profili ve oksidatif-antioksidatif sistem üzerindeki etkisi

Alptekin Yasım,[1] Davut Özbağ,[2] Metin Kılınç,[3] Harun Çıralık,[4] İsmail Toru[3]

Departments of [1]Cardiovascular Surgery, [2]Anatomy, [3]Biochemistry, [4]Pathology, Medicine Faculty of Kahramanmaraş Sütçü İmam University, Kahramanmaraş

Background: In this study, the effect of diosmin-hesperidin combination treatment on serum lipid profile and oxidative-antioxidative system in high-cholesterol diet-fed rats was investigated.

Methods: Thirty-six Sprague-Dawley rats, weighing between 220 and 280 g, were included in this study and were randomly assigned to three groups with 12 rats in each group. While rats in the control group (Group 1) were fed standard rat chow diet, those in group 2 (High cholesterol diet group) and group 3 (100 mg/kg/day diosmin-hesperidin group) were fed high-cholesterol diet for three months. After the study period, the levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured in serum. Malondialdehyde (MDA), glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase levels were measured in serum and heart tissue samples.

Results: The levels of total cholesterol, LDL-cholesterol and triglyceride were not significantly different between the groups. High-density lipoprotein cholesterol level was found to be significantly higher in group 3 than that in groups 1 and 2. Malondialdehyde levels were not significantly different between the groups. Glutathione peroxidase and SOD levels were found to be significantly higher in group 3 than those in groups 1 and 2. There were no significant differences between the groups with respect to catalase levels. Intracardiac measurements of MDA, GPX, SOD and catalase were not found significantly different between the groups.

Conclusion: Diosmin-hesperidin administration to high-cholesterol diet-fed rats significantly increased HDL-cholesterol levels but did not significantly affect other lipid parameters. Positive changes occurred in oxidative-antioxidative balance and administration of diosmin-hesperidin significantly increased the levels of GPX and SOD.

Key words: Diosmin-hesperidin; lipid profile; oxidative status; rat model.

Amaç: Bu çalışmada yüksek kolesterollü diyetle beslenen sıçanlarda diosmin-hesperidin kombinasyonu tedavisinin serum lipid profili ile oksidatif-antioksidatif sistem üzerindeki etkisi araştırıldı.

Çalışma planı: Çalışmaya ağırlıkları 220 ile 280 g arasında değişen 36 Sprague-Dawley cinsi sıçan alındı ve sıçanlar her bir grupta 12 sıçan olacak şekilde rasgele biçimde üç gruba ayrıldı. Kontrol grubundaki sıçanlar (Grup 1) standart sıçan yemi ile beslenirken, grup 2 (Yüksek kolesterollü diyet grubu) ve grup 3'teki (100 mg/kg/gün diosmin-hesperidin grubu) sıçanlar üç ay boyunca yüksek kolesterollü diyetle beslendi. Çalışma periyodu sonunda serumda total kolesterol, düşük yoğunluklu lipoprotein (LDL) kolesterol, yüksek yoğunluklu lipoprotein (HDL) kolesterol ve ve trigliserid düzeyleri ölçüldü. Serumda ve kalp dokusu örneklerinde malondialdehit (MDA), glutatyon peroksidaz (GPX), süperoksit dismutaz (SOD) ve katalaz düzeyleri ölçüldü.

Bulgular: Total kolesterol, LDL-kolesterol ve trigliserid düzeyleri açısından gruplar arasında anlamlı farklılık yoktu. Yüksek yoğunluklu lipoprotein kolesterol düzeyi ise grup 3'te, grup 1 ve 2'dekinden anlamlı ölçüde yüksekti. Malondialdehit düzeyleri açısından gruplar arasında anlamlı farklılık yoktu. Glutatyon peroksidaz ve SOD düzeylerinin grup 3'te grup 1 ve 2'ye göre anlamlı ölçüde yüksek olduğu saptandı. Katalaz düzeyleri açısından gruplar arasında hiçbir anlamlı farklılık yoktu. İntrakardiyak MDA, GPX, SOD ve katalaz ölçümlerinin gruplar arasında anlamlı ölçüde farklı olmadığı görüldü.

Sonuç: Yüksek kolesterollü diyetle beslenen sıçanlara diosmin-hesperidin kombinasyonu verilmesi HDL-kolesterol düzeylerini anlamlı ölçüde artırdı, fakat diğer lipid parametrelerini anlamlı düzeyde etkilemedi. Oksidatif-antioksidatif dengede olumlu değişiklikler meydana geldi ve diosmin-hesperidin uygulaması GPX ve SOD düzeylerinde anlamlı ölçüde artış sağlandı.

Anahtar sözcükler: Diosmin-hesperidin; lipid profili; oksidatif durum; sıçan modeli.

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Correspondence: Alptekin Yasım, M.D. Kahramanmaraş Sütçü İmam Üniversitesi Tıp Fakültesi Kalp ve Damar Cerrahisi Anabilim Dalı, 46100 Kahramanmaraş, Turkey. Tel: +90 344 - 221 23 37 e-mail: alpyasim@ksu.edu.tr

Hyperlipidemia and hypercholesterolemia are important risk factors for the development of coronary heart disease. Nowadays prevention of coronary heart disease has become more important than treatment of the disease. For this reason, the prevention of risk factors including hypercholesterolemia is quite important.

Flavonoids are a group of natural compounds found in plants commonly consumed by human beings. These phenolic compounds, especially found in fruits and vegetables, have many biological and pharmacological activities like enzyme inhibition, free radical scavenging, anti-inflammatory and anti-estrogenic activity and tumor promotion inhibition.[1-6] It has been reported in epidemiological studies that long-term dietary intake of flavonoids reduce the incidence of coronary heart disease and mortality due to this disease.[7-9] Some biological activities of flavonoids, like anti-thrombotic, anti-inflammatory, anti-oxidative and vasorelaxant effects may contribute to the prevention of coronary heart disease.[2] Furthermore, flavonoids are known to have a high content of vitamins and minerals. In spite of this, many investigators have related their cardioprotective effects mainly to their antioxidative and vasodilatator properties.[3,10-12] On the other hand, it has been suggested that flavonoids have an antiatherosclerotic activity through the inhibition of distinct stages in the pathogenesis of atheromatous plaque formation.[3]

Purified micronized flavonoid fraction contains 90% diosmin and 10% hesperidin. It is a potent venotropic drug that has been used in the treatment of chronic venous diseases for a long time. It increases venous tone, improves lymph drainage, reduces mast cells, suppresses leukocyte activation, inhibits prostaglandin secretion, reduces inflammatory response, inhibits free oxygen radical synthesis and acts as a scavenger of these free radicals; so it improves capillary permeability and capillary resistance which are increased by free oxygen radicals.[13,14] Furthermore, it has been noted in various experimental studies that this potent agent prevents distant organ injury in ischemia reperfusion condition, reduces oxidative damage due to hyperglycemia and inhibits intraperitoneal adhesion formation.[14,15]

In this study, we aimed to investigate the effects of diosmin-hesperidin combination treatment on the lipid profile and oxidative-antioxidative system in rats fed high cholesterol diet for three months.

MATERIALS AND METHODS

Rat model

The Ethics Committee of our Medical Faculty approved this study. Thirty-six Sprague-Dawley rats, weighing between 220 and 280 g, were obtained from the Animal Research Center in our University. The animals were individually housed in stainless steel cages in a room with controlled temperature (22-24 ºC) and lighting (alternating a 12-hour period of light and dark). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Six-month-old Sprague-Dawley rats were randomly divided into three groups (n=12 in each group). The animals in the control group (Group 1) were fed a palletized commercial chow diet for three months. The animals of group 2 (High cholesterol diet group) and group 3 (Diosmin-hesperidin group) were given an atherogenic diet for three months. The diet compositions are shown in table 1. In addition, the rats of group 3 were administered 100 mg/kg diosmin-hesperidin (Daflon tablet, Servier Ilac, Istanbul, Turkey) by means of an orogastric tube. A placebo was also given to groups 1 and 2, through oral gavage, during the same experimental period. The study finished at the end of three months. The animals were given food and distilled water ad libitum during the entire experimental period.

At the end of the experimental period, the animals were anesthetized with ketamine (10 mg/kg) and xylazine (3 mg/kg). Blood samples were taken from the heart for the determination of plasma lipid profiles and oxidative status. Blood samples were centrifuged at 4000 rpm for 10 minutes. In addition, the hearts were removed and rinsed with physiological saline for the determination of oxidative status. All samples were stored at –70 ºC until the day of analysis.

Determination of plasma lipids

Plasma cholesterol levels were determined using a commercial kit (Dade Behring Co., Germany). Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride tests were measured using a Behring RXL autoanalyser (Dade Behring Co., Germany).

Table 1. Normal and high-cholesterol diet composition

Content	Normal diet (%)	High-cholesterol diet (%)
Wheat	23	14
Soybean flour	28	18
Corn	18	12
Sunflower flour	11	10
Barley	10	6
Soybean oil	2	2
Marble powder	1	1
Meat and bone flour	4	4
Sugar beet powder	2	2
Vitamin-mineral	1	1
Cotton oil	0	30

Very-low-density lipoprotein (VLDL) was calculated using the ratio of triglyceride/5 and low-density lipoprotein (LDL) cholesterol was calculated with Friedewald formula. All parameters were calculated according to their standard curves.

Determination of oxidative status in plasma

Malondialdehyde assay: Malondialdehyde levels were measured by a spectrophotometer. The reaction mixture contained 0.1 ml sample, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The pH of the mixture was adjusted to 3.5 and the volume was finally made up to 4.0 ml with distilled water, and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the absorbance of the organic layer was measured at 532 nm wavelength.

Glutathione peroxidase assay: Blood samples were diluted to 1/20 with distilled water. Ten milliliter diluted blood samples were taken and added to reaction mixture (100 ml tris- EDTA, 20 ml glutathione, 100 ml glutathione reductase, 100 ml NADPH and 660 ml distilled water). Samples were incubated at 37 °C in water bath for 10 minutes. Then 10 ml t-buthyl-hydroperoxide was added, and the measure was performed in the spectrophotometer at 340 nm wavelengths.

Superoxide dismutase activity assay: Superoxide dismutase was determined as described by Fridovich[16] This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitro phenol-s-phenyl tetrazolium chloride) to form a red formazan dye. Superoxide dismutase is measured at 505 nm via spectrophotometric by the degree of inhibition of this reaction. Assay medium contains 0.01 molar phosphate buffer and CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer. Xanthine 0,05 mm, 0.025 mm INT [2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium cloride] and xanthine oxidase (80 U/L) were used as substrates. All samples were calculated according to a standard curve and SOD activity was expressed as U/mg creatinine.

Catalase activity assay: Catalase activities were determined by measuring the decrease in hydrogen peroxide concentration at 230 nm. Assay medium consisted of 1M Tris-HCl; 5 mm Na2EDTA buffer solution (pH 8.0), 1M phosphate buffer solution (pH 7.0), and 10 mm H_2O_2. Catalase activity was expressed as U/mg creatinine.

Determination of oxidative status in heart tissue

Tissue samples were homogenized with 10 volumes of ice-cold 0.25 M sucrose and centrifuged at 14,000 g to measure the levels of MDA, activities of SOD, GPX and catalase.

Glutathione peroxidase activity in tissue homogenate supernatants was measured according to the Beutler method.[17] The method was based on an nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-coupled reaction, whereby oxidized glutathione (GSSG) produced by GSH-Px (GPX) and hydroxyperoxide were reduced by exogenous glutathione reductase and NADPH. Enzyme activity was measured at 340 nm and expressed in units, each representing the oxidation of 1 μmol NADPH per minute per ml supernatant.

Malondialdehyde method was based on measurement of the absorbance of thiobarbituric acid-MDA modified according to Ohkawa et al.[18] In our modification, the reaction mixture was heated at 95 °C for 60 min instead of 45 min.

Superoxide dismutase activity was determined as described by Fridovich[16] employing xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol-S-phenyltetrazolium chloride) to form a red formazan dye. Superoxide dismutase activity is then measured by the degree of inhibition of this reaction. Protein was measured according to Lowry et al.[19] The catalase activity was spectrophotometrically measured by the disappearance of H_2O_2 at 230 nm. Results were expressed in nanomoles per milligram protein (nmol/mg protein).

Statistical analysis

All data presented as mean ± standard error of the mean (SEM). Statistical comparisons between groups were performed using analysis of variance (ANOVA) on the log-transformed data with differences between groups assessed with Tukey significant difference test. Statistical significance was considered as a p value of <0.05.

RESULTS

Animals gained approximately 20-25 percent weight at the end of the experimental period but there were no statistically significant differences between the groups in respect of weight gain.

Levels of total cholesterol, LDL cholesterol and triglycerides were found to be higher in group 2 than in group 1. These levels were also found to be higher in group 3 than in group 1, but lower than in group 2. However there was no statistically significant difference between the groups. High-density lipoprotein cholesterol levels were found to be lower in group 2 than group 1. However, there was no statistically significant difference between groups 1 and 2. In group 3, HDL cholesterol levels were found to be higher than groups 1 and 2.

Table 2. Total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglyceride levels in serum

	Group 1	Group 2	Group 3	*p*
	Mean±SD	Mean±SD	Mean±SD	
Total cholesterol (mg/dl)	55.8±9.1	62.5±11.1	58.5±13.0	NS
LDL cholesterol (mg/dl)	19.7±7.3	24.8±8.1	21.5±8.4	NS
Triglyceride (mg/dl)	62.4±22.8	80.2±30.1	67.6 ±19.1	NS
HDL cholesterol (mg/dl)	24.5±5.6	23.7±5.4	29.3±4.7*	p<0.05

*: Statistically significant versus groups 1 and 2; SD: Standard deviation; NS: Not significant; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

Although the difference regarding HDL cholesterol levels between group 3 and group 1 was not statistically significant (p>0.05), the difference between groups 3 and 2 was statistically significant (p<0.05). The results are shown in table 2.

Malondialdehyde levels (showing oxidative damage) were higher in groups 2 and 3 than in group 1 but the difference was not statistically significant. Although there was no significant difference between groups 1 and 2 with respect to GPX and SOD levels (showing antioxidative status), these levels were found to be significantly higher in group 3 than in groups 1 and 2 (p<0.05). There was no significant difference between the groups regarding catalase levels. The results are shown in table 3.

The results of intracardiac measurements showed that MDA, GPX, SOD and catalase levels were not significantly different between the groups. The results are shown in table 4.

DISCUSSION

Flavonoids, commonly found in plants consumed by human beings, are phenolic compounds, which have antithrombotic, anti-inflammatory, and vasorelaxant effects.[1,2,10] They have also been reported to be the scavengers of superoxide, hydroxyl and peroxyl radicals and to be the inhibitor of several enzymes that play essential roles in superoxide radical production.[1,2,10,11,20,21] Flavonoids can also reduce prooxidative activity by binding metal ions.[11,20] Thus, they can help to support the antioxidative defense of the body against free radicals. This may support the explanation of low coronary heart disease incidence in patients taking a rich diet of flavonoids. The Zutphen Elderly Study has shown that mortality risk due to coronary heart disease and relative risk for myocardial infarction were approximately 50% lower in patients taking a rich diet of flavonoids than in patients taking a low diet.[7] Again, it has been shown that dietary intake of flavonoid rich cocoa and cocoa-based products improve endothelial function in both compromised and healthy individuals.[8] These effects are related to the preventive effect of flavonoids on atherosclerosis as a result of their antioxidative properties. An imbalance between antioxidative defense mechanism and production of reactive oxygen radicals may contribute to atherosclerosis development by causing oxidative stress.

To test this proposition, we investigated the effect of diosmin-hesperidin (a flavonoid used in the treatment of chronic venous diseases) combination treatment on both serum lipid levels and oxidative-antioxidative system in high cholesterol diet-fed rats. Although the results were not statistically significant, serum levels of total cholesterol, LDL cholesterol and triglycerides were found to be higher in high cholesterol diet-fed rats than those in normal diet-fed rats. The resistance of rats to atherosclerosis may explain this insignificant change in the lipid profile even in the presence of high cholesterol diet. In most experimental studies, bile acids were added to diet in an attempt to elevate the lipid levels. Bile acids would increase the absorption of cholesterol from the

Table 3. Malondialdehyde, glutathione peroxidase, superoxide dismutase and catalase levels in plasma

	Group 1	Group 2	Group 3	*p*
	Mean±SD	Mean±SD	Mean±SD	
MDA (nmol/mg)	0.9±0.2	1.3±0.4	1.2±0.3	NS
GPX (U/Hb)	18.1±6.9	19.2±4.5	26.0±6.7*	p<0.05
SOD (U/ml)	94.5±17.0	93.6±18.0	111.7±14.5*	p<0.05
Catalase (U/ml)	90.4±26.5	88.1±18.7	90.5±15.6	NS

*: Statistically significant versus groups 1 and 2; SD: Standard deviation; MDA: Malondialdehyde; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; NS: Not significant

Table 4. Malondialdehyde, superoxide dismutase, glutathione peroxidase and catalase levels in heart tissues

	Group 1	Group 2	Group 3	*p*
	Mean±SD	Mean±SD	Mean±SD	
MDA (nmol/mgprt)	12.99±3.91	11.92±3.91	11.82±1.55	NS
GPX (U/mgprt)	0.0006±0.0002	0.0008±0.0004	0.0009±0.0004	NS
SOD (U/mgprt)	7.08±1.59	7.86±1.31	7.29±1.68	NS
Catalase (U/mgprt)	0.13±0.03	0.12±0.02	0.14±0.03	NS

SD: Standard deviation; MDA: Malondialdehyde; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; NS: Not significant.

intestines. Since we did not aim to render the intestine to absorb large amounts of cholesterol in a short period, bile acids were not added to the diet. Instead, we preferred to constitute a condition where the intestine absorbed the cholesterol in a slow manner. It is noteworthy to say that bile acids could affect the oxidative stress parameters, which could confound the interpretation of our results.

By treating high cholesterol diet-fed rats with diosmin-hesperidin combination, the levels of total cholesterol, LDL cholesterol and triglyceride decreased in an insignificant manner, but interestingly, the levels of HDL cholesterol increased significantly. The increase in HDL cholesterol levels after diosmin-hesperidin combination treatment may be explained by the increase in the levels of human serum paroxonase 1 (PON1). Paroxonase 1 enzyme, which protects HDL cholesterol against oxidation, is inactivated under oxidative stress. If oxidative stress is inhibited, PON1 levels may increase and this may protect HDL cholesterol against oxidation. Aviram[22] has reported that PON1 levels increased in rats fed with red wine flavonoids. Paroxonase 1 also reduces the oxidation of LDL cholesterol by hydrolyzing lipid peroxides.[5,22] Similar with this study, other experimental and clinical studies have reported that HDL cholesterol levels were increased by the increased intake of flavonoids.[8,20]

The key event in atherosclerosis is the oxidative modification of LDL cholesterol. Oxidized LDL is more atherogenic than native LDL.[5,21] Oxidative and modified LDL is a potent ligand for the receptors on macrophages and thus it causes the formation of foam cells derived from macrophages.[3-6,21] Antioxidative compounds provide resistance to this process and reduce atherogenicity by the inhibition of lipoprotein oxidation.

It is clear that oxidized LDL has a significant role in the initial endothelial damage that leads to atherogenesis. Because endothelial dysfunction is important in the pathogenesis of cardiovascular diseases and it is an early precursor of atherosclerosis,[12] protection of endothelial function is an important defense mechanism against atherosclerosis. For this reason, improvement in endothelial function may reduce the risk of atherosclerotic events. It has been suggested that antioxidants may inhibit arterial disease development by preserving endothelial function.[23] Being exposed to increased levels of reactive oxygen metabolites compromises hemostatic balance and this results in endothelial dysfunction.[24] Machha and Mustafa[12] have reported that aortas of hypertensive rats treated with flavonoids showed more relaxation in response to acetylcholine, flavonoids caused a decrease in blood pressure and these investigators have reported that the chronic treatment of hypertensive animals with flavonoids preserved vascular endothelial function.

In the present study, the serum levels of GPX and SOD significantly increased by the addition of diosmin-hesperidin combination to the diet, indicating that this drug has antioxidative properties. Many authors support this opinion. Previous studies have shown that flavonoids having antioxidative properties inhibit lipid peroxidation on vascular endothelial cell membranes by reducing the oxidative stress on macrophages and the effects of free radicals on the lipid layer.[3-6,21] It has been shown in various studies that dietary intake of flavonoids may reverse endothelial dysfunction and has high antioxidative effects.[3-5,8,12,21] In addition, various experimental studies showed a micronised purified flavonoid fraction, diosmin-hesperidin combination, also reduces oxidative stress.[15,20,24]

We found a slight increase in the serum MDA levels (showing oxidative stress) in response to a high cholesterol diet given to rats and a minimal decrease due to the addition of diosmin-hesperidin to the high cholesterol diet. But these increases or decreases were found to be statistically insignificant. Serum levels of GPX, SOD and catalase (showing antioxidative defence system) were not found to be different as compared with high cholesterol diet-fed rats and normal diet-fed rats. But serum levels of GPX and SOD significantly increased by adding diosmin-hesperidin combination to the diet. This may be related to the antioxidative effect of diosmin-hesperidin combination. Finding no difference

between groups in respect of MDA levels may be attributed to the stress that may have been occurred during orogastric gavage.

Sato et al.[25] reported that although cholesterol levels did not significantly change, a vascular dysfunction occurred and aorta relaxation was impaired in high fat-fed rats. They stated that this effect may be improved with the use of antioxidants and high fat diet may impair endothelial functions despite normal cholesterol levels. The results of our study were concordant with this; because we did not find significant important changes in LDL cholesterol levels. However, the antioxidant levels were increased by diosmin-hesperidin treatment and this probably preserved endothelial functions in early period by inhibiting LDL cholesterol oxidation.

We are strongly of the opinion that determining no difference between the three groups in intracardiac measurements may be related to the relatively short time period (i.e., three months) in performing our study which possibly was insufficient for the formation of a change in the heart. Furthermore, the very small values of intracardiac parameters measured may prevent making a reliable judgment.

As a conclusion, treating the high cholesterol-fed rats with diosmin-hesperidin combination may reduce the development of atherosclerosis by increasing HDL cholesterol levels and antioxidative defenses despite the lack of significant differences in serum lipid levels. However, our results should be supported by further clinical studies.

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REFERENCES

1. Kim SY, Kim HJ, Lee MK, Jeon SM, Do GM, Kwon EY, et al. Naringin time-dependently lowers hepatic cholesterol biosynthesis and plasma cholesterol in rats fed high-fat and high-cholesterol diet. J Med Food 2006;9:582-6.
2. Woodman OL, Meeker WF, Boujaoude M. Vasorelaxant and antioxidant activity of flavonols and flavones: structure-activity relationships. J Cardiovasc Pharmacol 2005;46:302-9.
3. Wilcox LJ, Borradaile NM, Huff MW. Antiatherogenic properties of naringenin, a citrus flavonoid. Cardiovascular Drug Reviews 1999;17:160-78.
4. Miyake Y, Suzuki E, Ohya S, Fukumoto S, Hiramitsu M, Sakaida K, et al. Lipid-lowering effect of eriocitrin, the main flavonoid in lemon fruit, in rats on a high-fat and high-cholesterol diet. Journal of Food Science 2006;71:S633-S7.
5. Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. Ann N Y Acad Sci 2002; 957:146-61.
6. Safari MR, Sheikh N. Effects of flavonoids on the susceptibility of low-density lipoprotein to oxidative modification. Prostaglandins Leukot Essent Fatty Acids 2003;69:73-7.
7. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 1993;34:1007-11.
8. Wang-Polagruto JF, Villablanca AC, Polagruto JA, Lee L, Holt RR, Schrader HR, et al. Chronic consumption of flavanol-rich cocoa improves endothelial function and decreases vascular cell adhesion molecule in hypercholesterolemic postmenopausal women. J Cardiovasc Pharmacol 2006; 47:S177-86.
9. Marniemi J, Alanen E, Impivaara O, Seppanen R, Hakala P, Rajala T, et al. Dietary and serum vitamins and minerals as predictors of myocardial infarction and stroke in elderly subjects. Nutr Metab Cardiovasc Dis 2005;15:188-97.
10. Woodman OL, Chan ECh. Vascular and anti-oxidant actions of flavonols and flavones. Clin Exp Pharmacol Physiol 2004; 31:786-90.
11. Halliwell B, Rafter J, Jenner A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am J Clin Nutr 2005; 81:268S-276S.
12. Machha A, Mustafa MR. Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta. J Cardiovasc Pharmacol 2005;46:36-40.
13. Ting AC, Cheng SW, Wu LL, Cheung GC. Clinical and hemodynamic outcomes in patients with chronic venous insufficiency after oral micronized flavonoid therapy. Vasc Surg 2001;35:443-7.
14. Yilmaz HG, Tacyildiz IH, Keles C, Gedik E, Kilinc N. Micronized purified flavonoid fraction may prevent formation of intraperitoneal adhesions in rats. Fertil Steril 2005;84 Suppl 2:1083-8.
15. Unlü A, Sucu N, Tamer L, Coskun B, Yücebilgiç G, Ercan B, et al. Effects of Daflon on oxidative stress induced by hindlimb ischemia/reperfusion. Pharmacol Res 2003;48:11-5.
16. Fridovich I. Superoxide dismutases. Adv Enzymol Relat Areas Mol Biol 1974;41:35-97.
17. Beutler E. Red cell metabolism: a manual of biochemical methods. 2nd ed. New York: Grune & Stratton; 1978. p. 261-5.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
20. Jeon SM, Kim HK, Kim HJ, Do GM, Jeong TS, Park YB, et al. Hypocholesterolemic and antioxidative effects of naringenin and its two metabolites in high-cholesterol fed rats. Transl Res 2007;149:15-21.
21. Engler MB, Engler MM. The vasculoprotective effects of flavonoid-rich cocoa and chocolate. Nutrition Research 2004;24:695-706.
22. Aviram M. Flavonoids-rich nutrients with potent antioxidant activity prevent atherosclerosis development: the licorice example. Int Congr Ser 2004;1252:320-7.

23. Davis N, Katz S, Wylie-Rosett J. The effect of diet on endothelial function. Cardiol Rev 2007;15:62-6.
24. Lonchampt M, Guardiola B, Sicot N, Bertrand M, Perdrix L, Duhault J. Protective effect of a purified flavonoid fraction against reactive oxygen radicals. In vivo and in vitro study. Arzneimittelforschung 1989;39:882-5.
25. Sato J, O'Brien T, Katusic ZS, Fu A, Nygren J, Singh R, et al. Dietary antioxidants preserve endothelium dependent vasorelaxation in overfed rats. Atherosclerosis 2002; 16:327-33.