JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 243−248 **243**

Amelioration of Obesity and Glucose Intolerance in High-Fat-Fed C57BL/6 Mice by Anthocyanins and Ursolic Acid in Cornelian Cherry (*Cornus mas*)

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Much attention has been focused on food that may be beneficial in preventing diet-induced body fat accumulation and possibly reduce the risk of diabetes and heart disease. Cornelian cherries (*Cornus mas*) are used in the preparation of beverages in Europe and also to treat diabetes-related disorders in Asia. In this study, the most abundant bioactive compounds in *C. mas* fruits, the anthocyanins and ursolic acid, were purified, and their ability to ameliorate obesity and insulin resistance in C57BL/6 mice fed a high-fat diet was evaluated. Mice were initially fed a high-fat diet for 4 weeks and then switched to a high-fat diet containing anthocyanins (1 g/kg of high-fat diet) and ursolic acid (500 mg/kg of high-fat diet) for an additional 8 weeks. The high-fat diet induced glucose intolerance, and this was prevented by anthocyanins and ursolic acid. The anthocyanin-treated mice showed a 24% decrease in weight gain. These mice also showed decreased lipid accumulation in the liver, including a significant decrease in liver triacylglycerol concentration. Anthocyanin and ursolic acid treated mice exhibited extremely elevated insulin levels. Both treatments, however, showed preserved islet architecture and insulin staining. Overall, these data suggest that anthocyanins and ursolic acid purified from *C. mas* fruits have biological activities that improve certain metabolic parameters associated with diets high in saturated fats and obesity.

KEYWORDS: Cornelian cherry; *Cornus mas*; anthocyanins; ursolic acid; glucose tolerance; insulin resistance; islet morphology; liver triglycerides

INTRODUCTION

Type-2 diabetes mellitus is an increasingly common disorder; ∼150−300 million people suffer worldwide from this debilitating disease (*1*). Insulin is secreted from pancreatic β-cells in response to nutrients and hormones to maintain normal physiological glucose concentrations (*2*). Insulin resistance, commonly associated with obesity, leads to the failure of liver, muscle, and adipose tissue to respond to physiological doses of insulin (*3*). Type-2 diabetes ultimately results from a relative reduction in insulin secretion necessary to overcome insulin resistance at these peripheral tissues. Both insulin deficiency and resistance are associated with health problems such as hyperlipidemia, atherosclerosis, and hypertension and are often linked to impaired carbohydrate and lipid metabolism (*4*). The complex interplay among genetic, environmental, and social factors causes obesity and diabetes (*5*). Some of the complications resulting from social and environmental factors, however, may be delayed or prevented by exercise and proper diet (*6*). Epidemiological studies show that diets rich in fruits and vegetables reduce the incidence of cancer, cardiovascular disease, diabetes, cataracts, and inflammatory disease (*7*−*13*). There has been an increased interest in natural hypoglycemic compounds derived from generally regarded as safe (GRAS) plants, fruits, and vegetables because they are considered to be less toxic and have fewer side effects (*14*). These bioactive compounds present in food can alter gene expression and cellular events, resulting in the modification of proteins and their functions (*15*). Although several studies have suggested that the phytochemicals present in fruits and vegetables are beneficial in ameliorating adverse health risks, their anecdotal protective effects are not well understood.

Anthocyanins are one of the major classes of dietary polyphenols present and widely consumed in fruits and vegetables. Although several beneficial effects of these compounds have been reported, very few anthocyanins are reported for their effect on obesity-related disorders. For example, pelargonidin 3-*O*-rhamnoside exhibited an antidiabetic effect in diabetic rats

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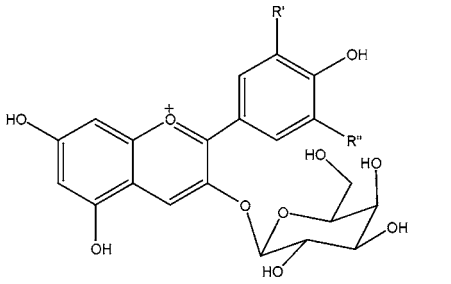
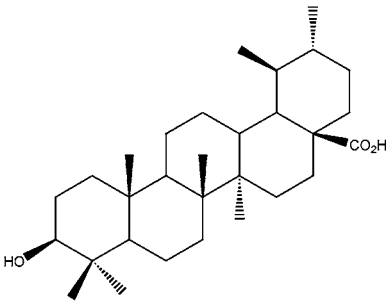
10.1021/jf0520342 CCC: $33.50
Published on Web 12/08/2005



	R′	R″
Cyanidin galactoside	OH	H
Pelargonidin galactoside	H	H
Delphinidin galactoside	OH	OH



Ursolic acid

Figure 1. Structures of anthocyanins and ursolic acid.

(*16*), and several anthocyanin-containing extracts showed α-glucosidase inhibitory activity (*17*). In another study, purple corn color (PCC), rich in cyanidin 3-*O*-glucoside, was reported to ameliorate the insulin resistance, hyperglycemia, hyperinsulinemia, and hyperlipidemia in high-fat-fed mice (*18*). Recent studies have also shown that the aglycone cyanidin and its glucoside up-regulated the adipocyte-specific gene expression (*19*) and genes involved in lipid metabolism (*20*).

The fruits of *C. mas* or Cornelian cherry are similar to popular tart cherries (*Prunus cerasus*). Although *Cornus* fruits are used to treat diabetes in China (*21*), the active compounds present in it are not fully characterized. Our studies have revealed that *C. mas* fruits contained primarily the anthocyanins pelargonidin, cyanidin, and delphinidin galactosides (*22*). We have shown that several anthocyanins and related anthocyanidins stimulate insulin release from a rodent pancreatic *β*-cell line (*23*). In the present study, we report the impact of a mixture of pure anthocyanins, cyanidin 3-*O*-galactoside, pelargonidin 3-*O*-galactoside, and delphinidin 3-*O*-galactoside, and ursolic acid present in *C. mas* fruits on weight loss, insulin resistance, glucose tolerance, islet function, islet morphology, liver triglycerides, and cholesterol levels in high-fat-fed C57BL/6 mice.

MATERIALS AND METHODS

Purification of Anthocyanins and Ursolic Acid from *C. mas* Fruits. Cyanidin 3-*O*-galactoside, pelargonidin 3-*O*-galactoside, and delphinidin 3-*O*-galactoside (**Figure 1**) were isolated as a pure mixture (2.0 g/kg of fresh fruits) of anthocyanins from *C. mas* fruits, as published earlier from our laboratory (*22*). The *C. mas* fruits were collected at the Michigan State University campus during September 2003. Briefly, the seeds (1 kg) were separated from the fruit (8 kg), the resulting pulp was blended with water (pH 3) and filtered, and the residue was lyophilized. The filtrate was passed through XAD-16 Amberlite resin and the resin washed repeatedly with water to remove sugars and organic acids. The adsorbed anthocyanins were then eluted with acidic methanol (MeOH) (3 M HCl, pH 3). The anthocyanins mixture obtained was purified by medium-pressure liquid chromatography (MPLC) column (C_{18} silica) using MeOH/H_2O (pH 3) under step gradient conditions. The fractions were eluted with solvent system MeOH/H_2O (65:35, v/v) and evaporated to dryness under vacuum. The purity of anthocyanins was confirmed by HPLC (Waters Corp.) using a Capcell C_{18} analytical column and detected at 520 and 280 nm (PDA, Waters Corp.) (**Figure 2**). The lyophilized residue was successively extracted with *n*-hexane (3 × 1 L), ethyl acetate (EtOAc) (3 × 1 L), and MeOH (3 × 1 L). The EtOAc (3.5 g) extract was purified over column chromatography (silica gel) using *n*-hexane and EtOAc under gradient conditions. The hexane/EtOAc (7:3) eluates were evaporated to dryness under vacuum, and crystallization of the resulting residue from MeOH yielded ursolic acid (2.2 g). Ursolic acid was characterized by ^{1}H and ^{13}C NMR spectral experiments (*24*).

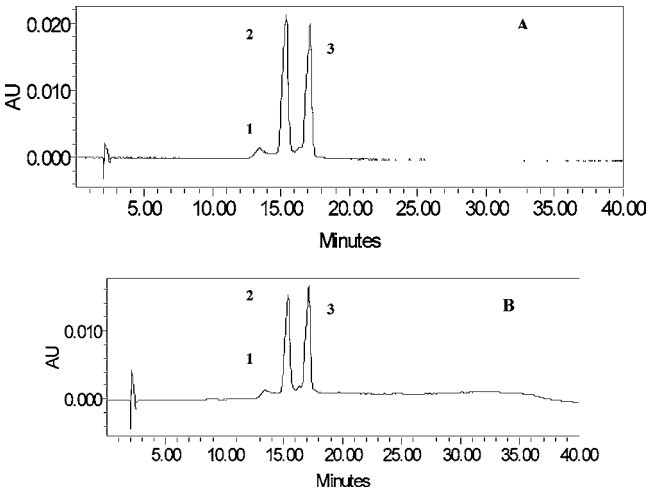


Figure 2. HPLC profiles of purified *C. mas* anthocyanins at (**A**) 520 nm and (**B**) 280 nm. Peaks: 1, delphidin 3-*O*-galactoside; 2, cyanidin 3-*O*-galactoside; 3, pelargonidin 3-*O*-galactoside.

Table 1. Composition of Diets.

ingredient	low fat (g %)	high fat (g %)
casein, lactic	200	200
L-cystein	3	3
cornstarch	315	0
maltodextrin	35	125
sucrose	350	68.8
cellulose	50	50
soybean oil	25	25
lard	20	245

Animals and Diet. Four-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). The diets, D12450B (10% kcal, low fat) and D12492 (60% kcal, high fat), were purchased from Research Diets (New Brunswick, NJ) (**Table 1**).

The mice were individually housed under controlled temperature (70 °F) and 12 h light−dark cycles. The mice had free access to water and standard rodent diet (Teklad 8640) for 5 days. The mice were then randomly divided into four groups ($n = 8$) for the study. Group 1 mice were fed with normal diet; group 2 mice were fed with the high-fat diet; group 3 mice were fed with the high-fat diet plus anthocyanins; and group 4 mice were fed with the high-fat diet plus ursolic acid. The control animals received normal (10% kcal fat) and high-fat (60% kcal fat) diets, respectively, throughout the study. The food was prepared for each treatment separately by mixing 1 g of pure anthocyanin mixture and 500 mg of ursolic acid per kilogram of high-fat diet, respectively. Food was changed at intervals of 3 days to avoid oxidation of the fat or compounds. Daily food intake and weekly body weight for each mouse were determined throughout the study. The experiments were carried out according to the ethical guidelines of University Laboratory Animal Resources (ULAR) at Michigan State University, East Lansing, MI.

Glucose Tolerance Test (GTT) and Fasting Blood Glucose Levels. Glucose tolerance tests were performed on ad libitum fed mice ($n =$

5) after 6 weeks of treatment with the compounds. Blood glucose levels were measured with a Free Style Flash (TheraSense, Inc., Alameda, CA) handheld glucometer using Free Style test strips (TheraSense, Inc.). For GTT, a sterile solution containing 2 g of glucose/kg of body weight was injected intraperitoneally (ip). Blood glucose levels were sampled from tail vein blood at 5, 10, 15, 30, 60, and 90 min, respectively. Fasting blood glucose levels were measured after 7 weeks of treatment with compounds. Mice were deprived of food for 6 h, and the blood glucose levels were determined in blood obtained from the tail vein.

Oil Red O Staining of Mouse Liver Sections. Frozen mouse liver was cut at 8 μm thickness on a Leica Cryostat (Bannockburn, IL). The cut sections were put on RNase Away-treated glass slides and fixed in 10% formalin for 20 min. Sections were then stained with 0.2% Oil Red O solution for 30 min. The sections were rinsed with 60% 2-propanol and water and then counterstained with hemotoxylin (Vector Laboratories, Burlingame, CA). Images were obtained using a Nikon Epi-fluorescent microscope equipped with a SPOT-RT digital camera and analyzed with Spot Advanced software (Diagnostic Instruments, Detroit, MI).

Measurement of Liver Triacylglyceride (TAG) Levels. Flash-frozen liver was homogenized in phosphate-buffered saline (PBS) and centrifuged at 2000 rpm for 10 min at 4 °C. The pellet was then extracted overnight at 4 °C in chloroform/methanol (2:1 v/v), ddH_2O was added, and the mixture was centrifuged. The supernatant was transferred to a separate tube, and the chloroform layer was separated by centrifugation. The chloroform solution was then washed twice with H_2O and dried. The resulting residue was dissolved in polidocanol (Sigma) and diluted to 10% polidocanol with ddH_2O and incubated at 37 °C for 15 min. Liver TAGs were then quantified using a Wako TG kit (Richmond, VA) according to the manufacturer's instructions.

Plasma Insulin Levels. Mice were anesthetized with isofluorene after which blood was obtained by cardiac puncture. Insulin levels in heparinized plasma were measured by rat insulin RIA kit according to the manufacturer's recommended procedure (LINCO Research Inc., St. Charles, MO). Insulin levels were also measured by ELISA (ALPCO, Windham, NH).

Immunostaining of Insulin in Mouse Pancreatic Sections. Mouse pancreas was rapidly frozen in optimal temperature medium and cut at 8 μm thickness on a Leica Cryostat. Sections were mounted on RNase Away-treated glass slides and fixed in cold acetone (−20 °C) for 15 min, followed by washing with PBS and blocking with 10% normal goat serum. Sections were then incubated with guinea pig anti-human insulin antibody (1:200, LINCO) for 2 h at room temperature or overnight at 4 °C. Sections were washed with PBS and incubated for 2 h with FITC-conjugated goat anti-guinea pig secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Slides were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min. Fluorescent images were obtained using a Nikon Epi-fluorescent microscope equipped with a SPOT-RT digital camera and analyzed with Spot Advanced software (Diagnostic Instruments).

Determination of Plasma Cholesterol. The total plasma cholesterol was analyzed by the Clinical Pathology Laboratory at the Diagnostic Center for Population and Animal Health, College of Veterinary Medicine, Michigan State University, according to the established standard analytical protocol for total cholesterol.

Data Analysis. Data are represented as mean ± SEM. Statistical analyses were performed by Student *t* test using ProStat (Poly Software Int., Pearl River, NY). Probability values lower than 0.05 were accepted as significant.

RESULTS

C57BL/6 mice were fed control ($n = 8$) or high-fat diet ($n = 24$) for 4 weeks prior to treatment with anthocyanin or ursolic acid. Food intake by mice fed the normal diet was ∼4.5 g per day for the first 4 weeks, after which it remained steady at 3.5 g per day (**Figure 3**). Food intake by mice fed the high-fat, high-fat plus anthocyanins, and high-fat plus ursolic acid diets was ∼2.3 g per day throughout the experiment. It is important to note that control mice on normal diet consumed more food

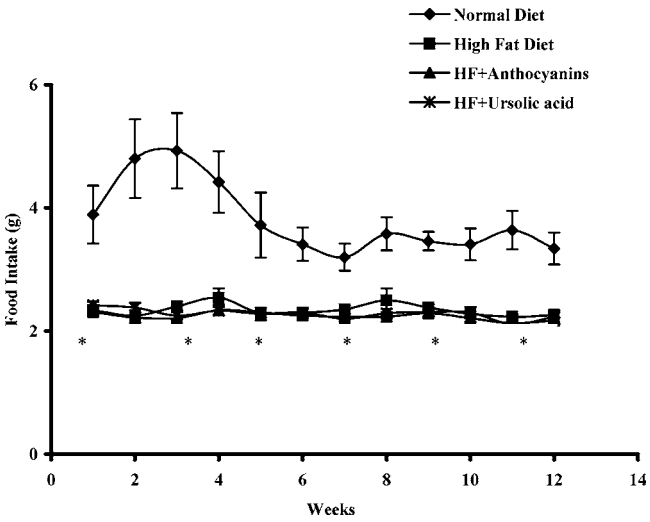


Figure 3. Food intake (grams) of mice during the 12-week study period. Anthocyanins and ursolic acids have no impact on food intake. Values are mean ± SEM, $n = 8$. Food intakes by high-fat and normal diet control mice were significantly different (*, $P < 0.01$), and no difference was observed among the high-fat, anthocyanins, and ursolic acid treated mice.

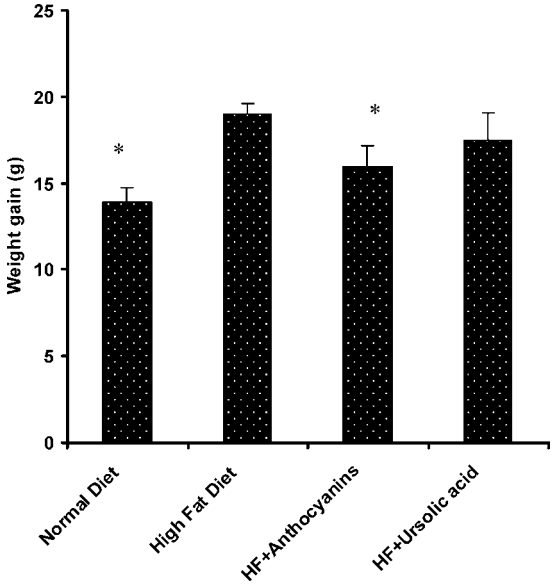


Figure 4. Weight gain in mice fed high-fat diet plus anthocyanins and ursolic acid. Body weights of mice are shown during the 12 weeks of feeding. The normal and high-fat diet controls received 10 and 60% kcal diets, respectively, throughout the experiment. Data represent mean ± SEM, $n = 8$ (*, $P < 0.05$).

by weight than mice on the high-fat diet. Caloric intake by the high-fat diet control and treatment groups was ∼14.6 kcal/day, whereas the mice on the normal diet consumed 13.3 kcal/day. After 12 weeks, the body weights of mice fed the normal and high-fat diets were significantly different, with average weights of 31.5 and 36.9 g, respectively. The animals fed a high-fat diet plus anthocyanins and high-fat diet plus ursolic acid for 8 weeks weighed 34.2 and 34.9 g, respectively. The average weight gains by mice fed normal diet, high-fat diet control, high-fat diet plus anthocyanin, and high-fat diet plus ursolic aid were 13.94, 18.98, 15.95, and 17.45 g, respectively, during the entire study period (**Figure 4**). These data show that anthocyanins and ursolic acids did not affect the food intake. Anthocyanins-fed mice had decreased weight gain, whereas the ursolic acid did not show any effect.

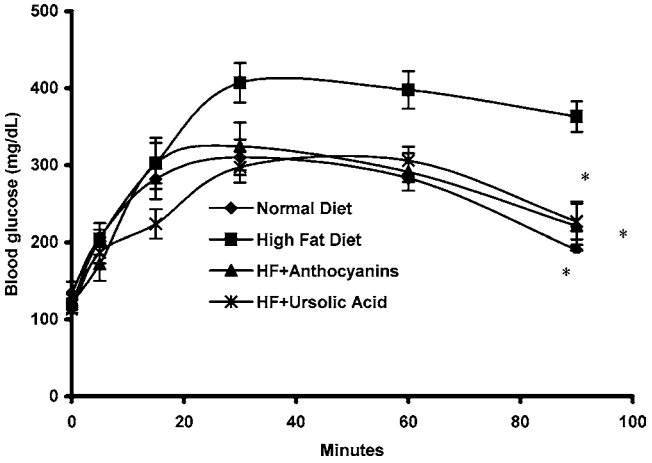


Figure 5. Anthocyanins and ursolic acids improved the glucose tolerance in C57BL/6 mice fed a high-fat diet. An intraperitoneal glucose tolerance test (2 g/kg) was performed on C57BL/6 mice fed normal diet, high-fat diet, or high-fat diet plus anthocyanins and ursolic acid. Values are mean ± SEM, $n = 5$, and significance (*, $P < 0.01$) was calculated for the data at 90 min.

GTT were performed on fed mice after 6 weeks of treatment with anthocyanins or ursolic acid (**Figure 5**). The average blood glucose levels prior to the GTT for control and high-fat diet fed mice were 133.8 and 119.8 mg/dL, respectively. Similarly, the average blood glucose levels for mice treated with anthocyanins and ursolic acid were 123.4 and 113.5 mg/dL, respectively. Mice fed a high-fat diet were found to be glucose intolerant when compared to mice fed the normal diet (**Figure 5**). Thus, the average blood glucose levels of mice fed normal and high-fat diets after 90 min of ip injection of glucose (2 g/kg) were 190 and 363 mg/dL, respectively. In contrast, mice fed a high-fat diet containing the anthocyanin or ursolic acid had improved glucose tolerance as indicated by the blood glucose levels of 221 and 227 mg/dL at 90 min, respectively. These data show that anthocyanin and ursolic acid improved glucose tolerance in high-fat-fed mice.

After 8 weeks of anthocyanins or ursolic acid treatment, mice were sacrificed and tissue and blood were collected. The week prior to sacrifice, the fasting blood glucose levels of control and high-fat-fed mice were 127 and 125 mg/dL, respectively. The insulin levels measured by radioimmunoassay for control animals fed normal and high-fat diets were 0.47 ± 0.14 and 0.41 ± 0.1 ng/mL, respectively, whereas the animals fed diets containing anthocyanins and ursolic acid showed 567.98 ± 32.36 and 52.25 ± 8.84 ng/mL of insulin, respectively. This large elevation in plasma insulin levels was independently confirmed by ELISA. Because of the marked elevation in insulin levels in mice fed with anthocyanins and ursolic acid, pancreatic sections were stained for insulin. Islets from C57BL/6 mice fed the high-fat diet were enlarged and had diffused staining compared to mice fed a low-fat diet (**Figure 6**). In contrast, islets from the mice fed the high-fat diet plus anthocyanins or ursolic acid were similar in size and stained intensely for insulin in a manner similar to low-fat diet mouse islets.

Next the effects of anthocyanins or ursolic acid on lipid accumulation in liver was determined by Oil Red O staining. High-fat-fed mice stained for intense lipid accumulation in the liver (**Figure 7**). In contrast, mice fed a high-fat diet plus anthocyanins or ursolic acid reversed the effect and stained similar to normal diet fed mice. Because diets high in fat lead to TAG accumulation in the liver, we next tested whether TAG levels were affected by anthocyanins and ursolic acid. The TAG

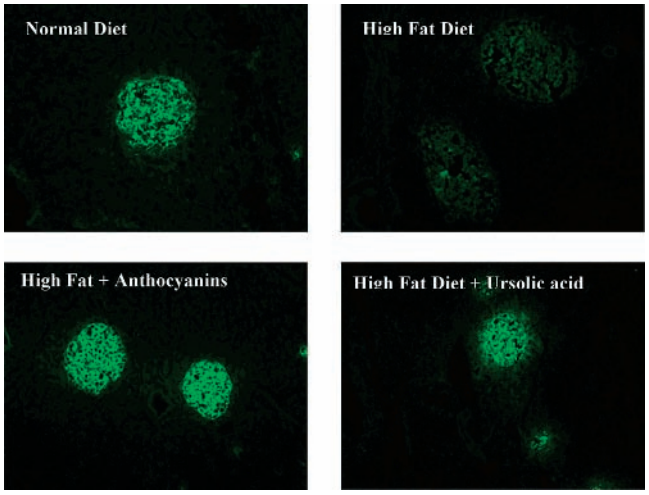


Figure 6. Anthocyanins and ursolic acids preserve islet structure and insulin content in C57BL/6 mice fed a high-fat diet. Pancreatic sections mice fed a normal, high-fat, or high-fat diet with anthocyanins and ursolic acid were immunofluorescently stained for insulin. Representative sections are from four animals per dietary group.

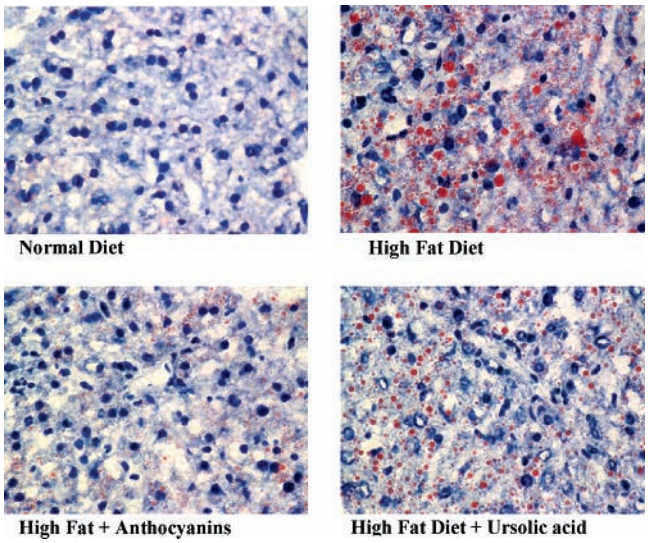


Figure 7. Anthocyanins and ursolic acid decrease lipid accumulation in livers of C57BL/6 mice fed a high-fat diet. Oil Red O was used to stain liver sections of mice treated with normal and high-fat diets and high-fat diet containing anthocyanin and ursolic acid. Sections were stained with 0.2% Oil Red O solution for 30 min. Representative sections are from three mice from dietary group.

levels in liver of normal and high-fat-fed animals were 63.7 and 82.5 mg/mg of protein, respectively (**Figure 8**). The concentrations of TAG in the liver of animals fed on the high-fat diet containing anthocyanins and ursolic acid were 61.0 and 74.3 mg/mg of protein, respectively. The plasma cholesterol levels of normal and high-fat diet control animals were 120.5 and 156.4 mg/dL, respectively. The plasma cholesterol level of anthocyanins-treated animals was 123.2 mg/dL. Due to lack of plasma collected, the plasma cholesterol level of ursolic acid treated animals was not determined.

DISCUSSSION

Several species of *Cornus* are widely grown as ornamental plants throughout the United States. The dried fruits of *Cornus officinalis*, called Hachimi-Gan, were used to treat diabetes in China (*21*). A previous study on the alcoholic extract of *C.*

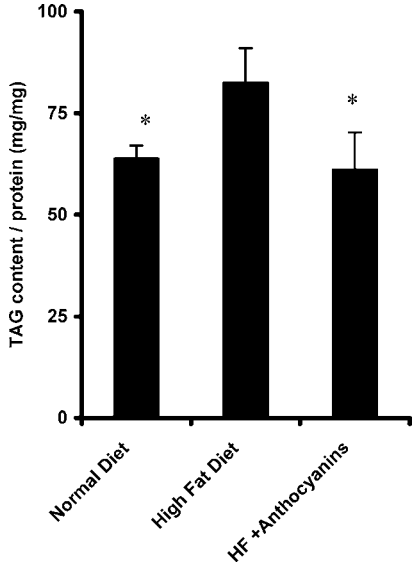


Figure 8. Quantitative measurement of liver lipids in mice treated with normal and high-fat diets and high-fat diet containing anthocyanin and ursolic acid. The *y*-axis represents milligrams of TG (triglycerides) content per milligram of protein. The values are mean ± SD for $n = 8$ (*, $P < 0.05$).

officinalis showed that this extract enhanced GLUT 4 mRNA expression in streptozotocin-treated rats (*21*). In the present study, we have isolated anthocyanins and ursolic acid as major compounds from the fruits of *C. mas*, a species closely related to *C. officinalis*, and tested these compounds to attenuate metabolic changes associated with high-fat diets.

The anthocyanins and ursolic acid (**Figure 1**) were tested for their efficacy to attenuate insulin resistance in high-fat-fed C57BL/6 mice. The doses of compounds used in our study were determined on the basis of the quantity of these compounds present in *C. mas* fruits and its average human consumption (~25 fresh fruits per day). The control animals on normal diet consumed more food than the animals on the high-fat diet. However, anthocyanins and ursolic acid did not affect the food intake of the animals. The caloric intake by animals fed on high-fat diet control, high-fat diet containing test compounds, and normal diet did not vary much. The dietary anthocyanins led to a 24% decrease in body weight, and this change is independent of food intake (**Figure 4**). A similar trend was also observed for mice fed ursolic acid containing diet, but the reduction in body weight was not significant compared to mice fed on high-fat diet alone (**Figure 4**). These observations suggest that anthocyanins, pigments found in fruits and vegetables, have the potential to reduce weight gain along with their other health benefits such as antioxidant and anti-inflammatory activities (*25*−*27*).

Pelargonidin 3-*O*-rhamnoside isolated from *Ficus bengalensis* has also been shown to improve glucose tolerance in diabetic rats (*16*). The improvement in glucose tolerance was associated with its ability to enhance insulin secretion in vitro. In our study with a mixture of pure delphinidin, cyanidin, and pelargonidin 3-*O*-galactosides from *C. mas*, we have observed that the glucose tolerance in animals was improved and thus similar to the control group fed a low-fat diet. Although ursolic acid did not significantly decrease the body weight, all of the tested animals in this group corrected the glucose levels. In the case of high-fat-fed animals, the blood glucose concentration reached the maximum at 30 min and stayed steady, showing that these animals were insulin resistant (**Figure 5**). The blood glucose concentration reached the maximum at 30 min for all groups except ursolic acid treated mice. It may be that ursolic acid treatment is delaying the absorption of glucose into the blood. The above data suggest that the anthocyanins and ursolic acid are increasing either insulin sensitivity and/or insulin secretion.

In type-2 diabetes, an increase of TAG levels in skeletal, muscle, heart, pancreatic β-cells, and liver tissues is generally observed (*28*). The C57BL/6 mice are susceptible to diet-induced obesity, type-2 diabetes, and atheroscelorosis. Animals fed on high-fat diet showed massive lipid accumulation in their livers. In this study, the consumption of anthocyanins by C57BL/6 mice dramatically decreased the lipid accumulation in the liver (**Figure 7**). The lipid accumulation and TAG concentration in anthocyanin-treated mice were similar to those of low-fat diet controls. Ursolic acid also caused a moderate decrease in the liver lipid depositions (**Figure 7**). A similar effect was observed with plasma cholesterol level of anthocyanins-treated mice. The mechanism for the plasma cholesterol is unknown; however, it has recently been reported that anthocyanins induce reverse-cholesterol efflux from macrophages (*29*). Thus, the consumption of anthocyanins and ursolic acid might regulate lipid metabolism by affecting hepatic lipid oxidation and lipogenesis.

The transition from diet-induced obesity toward type-2 diabetes in rodent models is initially associated with extensive β-cell hyperplasia and loss of islet architecture and, eventually, by loss of β-cell mass (*30*). In the present study, anthocyanins and ursolic acid protected the islet architecture and elevated the levels of insulin. This extremely large increase in plasma insulin levels was verified with two independent method, that is, RIA and ELISA. These observations suggest that the anthocyanins consumption may be affecting the insulin secretion and biosynthesis or insulin clearance. Insulin immunostaining revealed that high-fat-fed mice had enlarged islets with diffuse staining and irregular structure when compared to mice fed on normal control diet (**Figure 6**). In addition, the high-fat diet also caused an apparent increase in the number of small islets. In contrast, islets from mice fed the high-fat diet plus anthocyanin were similar in size and structure as islets from mice fed on normal diet. Moreover, islets from mice fed anthocyanins and high-fat diet stained more intensely than islets from mice fed on normal or high-fat diets. Although ursolic acid preserved islet structure and insulin staining, it was less effective than anthocyanins. In the present study, the data revealed that anthocyanins and ursolic acid protected islets from metabolic insults associated with high-fat diets and enhanced insulin secretion. These findings show that the large increase in plasma insulin levels that occurred in response to anthocyanins or ursolic acid was probably from enhanced islet function and not due to marked degranulation of islets. Nevertheless, it is possible that these compounds might affect insulin clearance and thereby protect the β-cells, "β-cells exhaustion".

In conclusion, the C57BL/6 mice fed the high-fat diet containing anthocyanins showed a decrease in body weight, normalized glucose intolerance, preserved islet architecture, an extremely elevated level of circulating insulin, and a dramatic decrease in liver lipid. Treatment of mice with ursolic acid led to similar changes. Our results suggest that consumption of Cornelian cherry or other fruits containing anthocyanins and ursolic acid has the potential to reduce the risk of diabetes and obesity. At this stage the mechanism of action of these effects in vivo is unclear. However, our results along with published results on anthocyanins show that these dietary polyphenols in general have the potential to prevent obesity and hyperglycemia.

An understanding of the mechanism of action of these compounds in vivo may lead to a safer preventive agent for obesity and type-2 diabetes.

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Received for review August 18, 2005. Revised manuscript received November 2, 2005. Accepted November 3, 2005. This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, Grant 2003-35504-13618.

JF0520342