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Anticoagulatory, Antiinflammatory, and Antioxidative Effects of Protocatechuic Acid in Diabetic Mice

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Content of protocatechuic acid (PA) in eight locally available fresh fruits was analyzed, and the protective effects of this compound in diabetic mice were examined. PA at 1%, 2%, and 4% was supplied to diabetic mice for 8 weeks. PA treatments significantly lowered plasma glucose and increased insulin levels. PA treatments at 2% and 4% significantly lowered plasminogen activator inhibitor-1 activity and fibrinogen level; increased plasma activity of antithrombin-III and protein C; decreased triglyceride content in plasma, heart, and liver; elevated glutathione level and the retention of glutathione peroxidase and catalase activities in heart and kidney. PA treatments at 2% and 4% also significantly lowered plasma C-reactive protein and von Willebrand factor levels and reduced interleukin-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1 levels in heart and kidney. These results support that protocatechuic acid could attenuate diabetic complications via its triglyceride-lowering, anticoagulatory, antioxidative, and antiinflammatory effects.

KEYWORDS: Protocatechuic acid; diabetes; monocyte chemoattractant protein-1; coagulation

INTRODUCTION

Protocatechuic acid (3,4-dihydroxybenzoic acid) is a phenolic compound found in many plant foods such as olives, *Hibiscus sabdariffa* (roselle), *Eucommia ulmoides* (du-zhong), and white grape wine (*1*, *2*). So far, less information is available regarding the content of this compound in fresh fruits. It has been documented that this compound possesses antioxidative, antibacterial, and antimutagenic activities (*3*−*5*). McCue et al. (*6*) reported that protocatechuic acid could be considered as an antihyperglycemic agent because it could inhibit porcine pancreatic amylase activity in vitro. Kwon et al. (*7*) observed that protocatechuic acid provided in vitro inhibitory activity on α-glucosidase and suggested that this compound might be able to manage diabetes and hypertension. However, the in vivo antidiabetic effects of this compound remain unknown.

Diabetic complications such as neuropathy, retinopathy, nephropathy, and atherosclerosis exacerbate the severity and mortality of this disease; the clinical characteristics of these complications include hyperglycemia, hyperlipidemia, oxidative stress, cytokine imbalance, and coagulation predomination (*8*−*10*). Hyperlipidemia including hypertriglyceridemia and hypercholesterolemia in diabetes is due to a variety of derangements, especially insulin deficiency, in the processes of metabolism and regulation, which increases the risk of premature atherosclerosis (*10*). In addition, it is well-known that disturbed balance between Th1 and Th2 cytokines and overproduced proinflammatory cytokines and chemokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein-1 (MCP-1) not only enhance systemic inflammatory stress in diabetic individuals but also promote the deterioration of diabetes associated cardiac and/or renal dysfunctions (*11*, *12*). Thus, any agent(s) with lipid-lowering and/or antiinflammatory activity may potentially prevent or delay the development of diabetic complications. On the other hand, the up-regulation of blood coagulation factors such as fibrinogen and down-regulation of anticoagulation factors such as antithrombin III occurred in diabetic individuals cause hypercoagulability and favor the occurrence of myocardial infarction and/or glomerulosclerosis (*13*, *14*). Thus, hemostatic imbalance also warrants attention in order to attenuate diabetic complications.

In this study, the content of protocatechuic acid in eight locally available fresh fruits was determined. The lipid-lowering, antioxidative, anticoagulatory, and antiinflammatory effects of this compound in diabetic mice were examined. These results will also elucidate the possible action modes from this compound against diabetic progression.

MATERIALS AND METHODS

Materials. Protocatechuic acid (PA, 99.5%) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used in these measurements were of the highest purity commercially available.

Determination of Protocatechuic Acid Content. Eight locally available fresh fruits were used to analyze the content of PA. These fruits included mulberry (*Mours alba* L.), carambola (*Averrhoa carambola*), waxapple (*Syzygium samarangensem*), mango (*Mangifera indica* L.), juiube (*Zizyphus mauritiana*), calamondin (*Citrus microcarpa* Bonge), guava (*Psidium guajava*), and loquat (*Eriobotrya japonica*). These fruits, harvested in summer of 2008, were purchased from five farms in central area of Taiwan. PA content in these fruits was analyzed by a high

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performance liquid chromatography (HPLC) method described bu Ma et al. (*15*).

Animals and Diets. Male Balb/cA mice, 3−4 weeks old, were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were housed on a 12 h light/dark schedule; water and mouse standard diet were consumed ad libitum. The use of mice was reviewed and approved by China Medical University Animal Care Committee (CMU-97-22-N). To induce diabetes, mice with body weight of 22.6±1.0 g were treated with a single iv dose (50 mg/kg) of streptozotocin dissolved in citrate buffer (pH 4.5) into the tail vein of 12 h fasted mice. Blood glucose level was monitored on days 5 and 10 from the tail vein using a one-touch blood glucose meter (Lifescan, Inc. Milpitas, CA). Mice with fasting blood glucose levels of ≥14.0 mmol/L were used for this study. After diabetes was induced, mice were divided into several groups (12 mice per group).

Experimental Design. PA at 1, 2, or 4 g was mixed with 99, 98, or 96 g of powder diet containing (g/100 g) 64 starch, 23 protein, 3.5 fat, 5 fiber, 1 vitamin mixture, and 3 salt mixture (PMI Nutrition International LLC, Brentwood, MO) and supplied to diabetic mice. All mice had free access to food and water at all times. Consumed water volume, feed, and body weight were recorded. Plasma levels of glucose and insulin were measured at weeks 1, 4, and 8. After 8 weeks of supplementation, mice were killed with carbon dioxide. Blood was collected, and plasma was separated from erythrocytes immediately. Heart, liver, and kidney were collected and weighed. Each organ at 0.1 g was homogenized on ice in 2 mL of phosphate buffered saline (PBS, pH 7.2), and the filtrate was collected. The protein concentration of plasma or organ filtrate was determined by the method of Lowry et al. (*16*) using bovine serum albumin as a standard. In all experiments, the sample was diluted to a final concentration of 1 g protein/L using PBS, pH 7.2.

Blood Glucose and Insulin Analyses. The plasma glucose level (mmol/L) was measured by a glucose kit (Sigma Chemical Co., St. Louis, MO). Plasma insulin level (nmol/L) was measured by using a rat insulin radioimmunoassay kit (SRI-13K, Linco Research Inc., St. Charles, MO).

Determination of Triglyceride and Cholesterol. Triglyceride (TG) and total cholesterol (TC) levels in plasma (g/L) were determined by triglycerides kit and cholesterol kit (Boehringer Mannheim, Germany), respectively. Total lipids were extracted from heart and liver, and TG concentration (mg/g wet tissue) was quantified by a colorimetric assay (*17*). TC content (mg/g wet tissue) was measured using *o*-phthalaldehyde (*18*).

Measurement of Coagulation and Anticoagulation Factors. Coagulation factors plasminogen activator inhibitor-1 (PAI-1) and fibrinogen and anticoagulation factors antithrombin III (AT-III) and protein C were measured in this study. Blood samples were anticoagulated using sodium citrate according to the protocols provided by the manufacturers. PAI-1 activity (kU/L) was assayed by a commercial kit (Trinity Biotech plc., Bray, C. Wicklow, Ireland). Fibrinogen level (g/L) was assayed on the basis of the principle of salting out by using a commercial kit (Iatroset Fbg, Iatron Laboratory, Tokyo, Japan). The activity (%) of AT-III and protein C in plasma was determined by chromogenic assays according to the manufacturer's instructions using commercial AT-III and protein C kits (Sigma Chemical Co., St. Louis, MO) and was shown as the ratio of those in normal human plasma.

Determination of Oxidative and Antioxidative Status. Lipid oxidation in organs was determined by measuring the level of malondialdehyde (MDA, μmol/L) via a HPLC method (*19*). Glutathione (GSH) and oxidized glutathione (GSSG) concentrations (nmol/mg protein) in organs were determined by commercial colorimetric GSH and GSSG assay kits (OxisResearch, Portland, OR). Glutathione peroxidase (GPX) and catalase activities (U/mg protein) in organs were determined by commercial assay kits (Calbiochem Inc., San Diego, CA).

Measurement of Inflammation and Endothelial Injury Markers. Plasma levels of C-reactive protein (CRP) and von Willebrand factor (vWF) were measured as inflammation and endothelial injury markers. CRP (g/L) was determined with a commercial enzyme-link immunosorbent assay (ELISA) kit (Anogen, Ontario, Canada). vWF antigen level was measured by an ELISA method, using a rabbit antirat vWF polyclonal antibody (Dako, Glostrup, Denmark). The vWF level was expressed as relative percentage compared to normal pooled plasma.

Table 1. Content of Protocatechuic Acid (PA) in Eight Fruits: Mulberry, Carambola, Waxapple, Mango, Juiube, Calamondin, Guava, and Loquat[a]

	PA (mg/100 g dry weight)
mulberry	82 ± 12
carambola	51 ± 8
waxapple	22 ± 5
mango	43 ± 7
juiube	[b]
calamondin	109 ± 10
guava	29 ± 6
loquat	

[a] Data are the mean ± SD, n = 5. [b] Too low to be detected.

Table 2. Water Intake (WI, (mL/mouse)/d), Feed intake (FI, (g/mouse)/d), and Body Weight (BW, g/mouse) of Nondiabetic Mice (non-DM) and Diabetic Mice (DM) Consumed Normal Diet and 1%, 2%, 4% Protocatechuic Acid (PA) at Weeks 1, 4, and 8[a]

	non-DM	DM	DM + PA, 1%	DM + PA, 2%	DM + PA, 4%
			WI		
1	2.0 ± 0.8 a	3.5 ± 1.0 b	3.8 ± 1.2 b	3.4 ± 0.9 b	3.3 ± 1.1 b
4	2.3 ± 1.0 a	5.8 ± 1.4 c	5.5 ± 1.6 c	4.2 ± 0.7 b	4.0 ± 1.0 b
8	2.4 ± 0.9 a	7.0 ± 1.3 c	7.1 ± 1.0 c	5.6 ± 1.0 b	5.3 ± 1.2 b
			FI		
1	2.1 ± 0.6 a	2.6 ± 0.8 a	2.3 ± 0.5 a	2.7 ± 0.6 a	2.5 ± 0.9 a
4	2.9 ± 0.7 a	4.7 ± 1.0 c	4.5 ± 0.8 c	3.8 ± 0.9 b	3.6 ± 0.6 b
8	3.4 ± 1.0 a	6.4 ± 1.1 c	6.7 ± 1.2 c	5.0 ± 1.0	5.2 ± 0.8
			BW		
1	22.8 ± 1.3 b	20.8 ± 1.1 a	21.0 ± 0.9 a	20.6 ± 1.0 a	21.1 ± 0.7 a
4	26.0 ± 1.8 c	16.9 ± 1.2 a	17.3 ± 1.0 a	18.9 ± 1.1 b	19.1 ± 0.9 b
8	28.5 ± 2.1 c	13.1 ± 1.6 a	14.0 ± 0.9 a	17.6 ± 1.2 b	17.8 ± 1.3 b

[a] Data are the mean ± SD, n = 12. Mean values in a row without a common letter (a−c) differ, $P < 0.05$.

Cytokines Analyses. Tissue was homogenized in 10 mM Tris-HCl buffered solution (pH 7.4) containing 2 M NaCl, 1 mM ethylenediaminetetraacetic acid, 0.01% Tween-80, 1 mM phenylmethylsulfonyl fluoride and centrifuged at 9000*g* for 30 min at 4 °C. The resultant supernatant was used for cytokine determination. The levels of IL-1β, IL-6, TNF-α, and MCP-1 were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA). Samples were assayed in duplicates according to manufacturer's instructions.

Statistical Analysis. All data were expressed as the mean ± standard deviation (SD). Statistical analysis was done using one-way analysis of variance (ANOVA), and post hoc comparisons were carried out using Dunnett's t test. Statistical significance is defined as $P < 0.05$.

RESULTS

The content of PA in eight fruits is shown in **Table 1**. Besides juiube and loquat, this compound was detectable in six other test fruits with a range of 22−109 mg/100 g dry weight. The highest content was presented in calamondin. Feed intake, water intake, and body weight of diabetic mice at weeks 1, 4, and 8 are presented in **Table 2**. Compared with diabetic control group, mice with 2% and 4% PA treatments had significantly lower water intake, lower feed intake, and higher body weight at 4 and 8 weeks. Plasma levels of glucose and insulin at weeks 1, 4, and 8 are presented in **Figure 1**. Compared with diabetic control group, PA treatments dose-dependently lowered glucose level, but insulin level was increased only in 2% and 4% PA treated mice. TG content and TC content in plasma, heart, and liver at week 8 are shown in **Table 3**. Diabetic mice with 2% and 4% PA

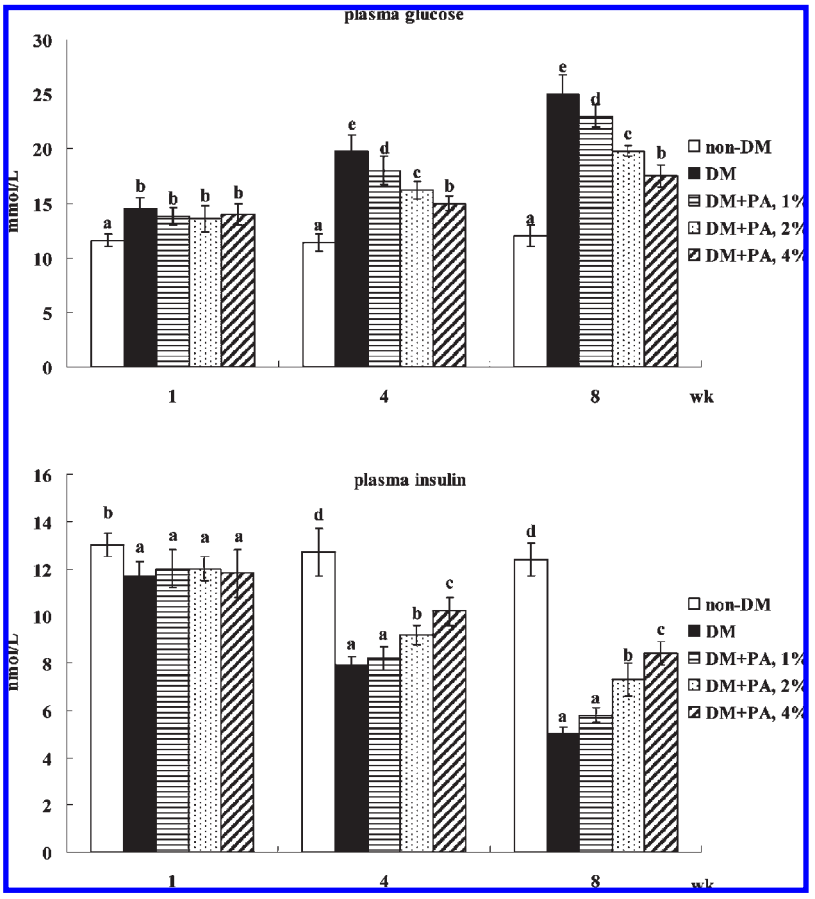

Figure 1. Plasma levels of glucose (mmol/L) and insulin (nmol/L) of nondiabetic mice (non-DM) and diabetic mice consumed normal diet (DM) or 1%, 2%, 4% protocatechuic acid (PA) at weeks 1, 4, and 8. Data are the mean ± SD, $n = 12$. (a−e) Mean values among bars without a common letter differ, $P < 0.05$.

Table 3. Level of Triglyceride (TG) and Total Cholesterol (TC) in Plasma (g/L), Heart, and Liver (mg/g wet tissue) from Nondiabetic Mice (non-DM) and diabetic mice (DM) Consumed Normal Diet and 1%, 2%, 4% Protocatechuic Acid (PA) at 8 Weeks[a]

	non-DM	DM	DM + PA, 1%	DM + PA, 2%	DM + PA, 4%
Plasma					
TG	2.17 ± 0.16 a	4.11 ± 0.36 d	4.02 ± 0.24 d	3.67 ± 0.21 c	3.21 ± 0.25 b
TC	1.29 ± 0.25 a	2.98 ± 0.30 c	2.90 ± 0.27 c	2.85 ± 0.18 c	2.37 ± 0.20 b
Heart					
TG	22.7 ± 1.3 a	40.7 ± 3.3 d	38.8 ± 4.1 d	32.6 ± 3.0 c	27.4 ± 2.2 b
TC	2.8 ± 0.6 a	4.5 ± 1.3 c	4.2 ± 0.9 c	4.3 ± 0.8 c	3.7 ± 0.5 b
Liver					
TG	29.8 ± 2.0 a	51.2 ± 4.0 a	49.3 ± 3.5 d	43.1 ± 3.2 c	37.7 ± 2.6 b
TC	3.6 ± 0.8 a	5.6 ± 1.4 c	5.4 ± 1.2 c	5.1 ± 0.9 c	4.5 ± 0.7 b

[a] Data are the mean ± SD, $n = 12$. Mean values in a row without a common letter (a−d) differ, $P < 0.05$.

treatments had significantly lower TG content in plasma, heart, and liver, and there was a dose-dependent effect between 2% and 4% treatments. TC content in plasma and two organs was reduced only in 4% PA treated mice. Effects of PA on plasma coagulation and anticoagulation factors are shown in **Table 4**. PA treatments from 2% and 4% significantly lowered PAI-1 activity and fibrinogen level; however, there was no dose-dependent effect between 2% and 4% treatments ($P > 0.05$). PA treatments at 2% and 4% dose-dependently increased AT-III and protein C activities. Treatment from 1% PA failed to affect levels of TG, TC, coagulation, and anticoagulation factors in blood and/or organs of diabetic mice ($P > 0.05$).

As shown in **Table 5**, PA treatments dose-dependently decreased cardiac and renal MDA levels. However, only 2% and 4% treatments significantly increased GSH level, decreased GSSG formation, and enhanced the retention of GPX and

catalase activities in these two organs, and there was dose-dependent effect between 2% and 4% treatments. Plasma levels of CRP and vWF at weeks 1, 4, and 8 are presented in **Figure 2**. At week 4, plasma CRP and vWF levels were significantly decreased in mice with 2% and 4% PA treatments. However, at 8 weeks, PA treatments from 1% to 4% dose-dependently reduced plasma CRP and vWF levels. Cardiac and renal levels of inflammatory factors are presented in **Table 6**. PA treatments dose-dependently reduced IL-6 and TNF-α levels in heart and kidney. IL-1β and MCP-1 levels in these two organs were decreased only in 2% and 4% PA treated mice, and there was a dose-dependent effect between 2% and 4% treatments.

DISCUSSION

Protocatechuic acid is a phenolic compound presented in plant foods such as olives, roselle, du-zhong, and white grape wine (*1*, *2*). The results of our present study further indicated that mulberry, carambola, waxapple, mango, calamondin, and guava also contained this compound. On the other hand, the in vitro antihyperglycemic effect of protocatechuic acid has been reported (*6*, *7*). Our present study provided in vivo data to elucidate the antidiabetic effects of this compound. We found that the dietary supplement of protocatechuic acid improved glycemic control, lowered cardiac and hepatic triglyceride content, decreased oxidative and inflammatory stress in heart and kidney, and attenuated hemostatic disorder in diabetic mice. These findings support that this compound is a potent antidiabetic agent.

Diabetes is a thrombosis-prone condition because hyperglycemia-induced reactive oxygen species causes platelet dysfunction, and insulin deficiency diminishes the release of thrombolytic enzymes such as tissue plasminogen activators (*20*). Fibrinogen is a precursor for fibrin formation and a cofactor in platelet aggregation; PAI-1 is the primary physiologic inhibitor of fibrinolysis (*21*). Thus, the elevated plasma fibrinogen level and PAI-1 activity favored the progression of thrombosis. On the other hand, activated AT-III and protein C are important anticoagulation factors because AT-III inhibits the activity of a number of proteases in the coagulation cascade, and protein C inactivates coagulation factors such as factors Va and VIIIa (*14*). Thus, the enhanced AT-III and protein C activities promote fibrinolytic reactions. The results of our present study found that protocatechuic acid treatments markedly lowered PAI-1 activity and fibrinogen level and elevated AT-III and protein C activities in these diabetic mice. These findings supported that this compound could alleviate aggregation and enhance thrombolysis, which subsequently reduced the risk of diabetes associated atherogenesis and thrombosis. On the other hand, we also found that protocatechuic acid supplement decreased lipid accumulation in blood and organs of diabetic mice. These results indicated that this compound could attenuate diabetes associated hyperlipidemia, which may also benefit hemostatic balance.

Oxidative stress is an important factor responsible for diabetic cardiomyopathy and nephropathy (*22*, *23*). Our present study found that protocatechuic acid supplement effectively lowered cardiac and renal oxidative stress via decreasing the formation of MDA and GSSG and enhanced antioxidant defense via restoring the activity of two antioxidant enzymes and increasing GSH retention in these organs. The in vitro antioxidative activity of this compound such as scavenging free radicals has been reported (*3*). Masella et al. (*24*) indicated that protocatechuic acid could increase glutathione-related enzymes protein levels in murine macrophage-like cells by activating their mRNA transcription. It seems that protocatechuic acid could offer antioxidative protection via both nonenzymatic and enzymatic actions. However, Cao et al. (*25*) found that protocatechuic acid could be metabolized to catechol methylated metabolite, acyl−coenzyme thioester, and glycine conjugation in rat liver and heart.

Table 4. Coagulatory Factors PAI-1 Activity (kU/L) and Fibrinogen Level (g/L) and Anticoagulatory Factors AT-III Activity (%) and Protein C Activity (%) in Plasma from Nondiabetic Mice (non-DM) and Diabetic Mice Consumed Normal Diet (DM) and 1%, 2%, 4% Protocatechuic Acid (PA) at 8 Weeks[a]

	non-DM	DM	DM + PA, 1%	DM + PA, 2%	DM + PA, 4%
Coagulatory Factors					
PAI-1	7.9 ± 0.7 a	18.2 ± 1.4 c	17.6 ± 1.0 c	15.5 ± 0.9 b	14.8 ± 1.1 b
fibrinogen	2.27 ± 0.18 a	4.66 ± 0.23 c	4.57 ± 0.19 b	4.06 ± 0.15 b	3.89 ± 0.20 b
Anticoagulatory Factors					
AT-III	132 ± 13 c	74 ± 5 c	82 ± 6 a	100 ± 8 b	105 ± 7 c
protein C	98 ± 7 c	63 ± 4 a	65 ± 4 a	77 ± 6 b	82 ± 5 b

[a] Data are the mean ± SD, $n = 12$. Mean values in a row without a common letter (a−c) differ, $P < 0.05$.

Table 5. Levels of MDA (μmol/L), GSSG (nmol/mg Protein), GSH (nmol/mg Protein), and Activity (U/mg Protein) of Catalase, GPX in Heart and Kidney from Nondiabetic Mice (non-DM) and Diabetic Mice (DM) Consumed Normal Diet, and 1%, 2%, 4% Protocatechuic Acid (PA) at 8 Weeks[a]

Heart					
	non-DM	DM	DM + PA, 1%	DM + PA, 2%	DM + PA, 4%
MDA	0.95 ± 0.14 a	3.71 ± 0.32 e	3.26 ± 0.20 d	2.65 ± 0.21 c	2.17 ± 0.19 b
GSSG	0.27 ± 0.07 a	1.16 ± 0.12 d	1.08 ± 0.10 d	0.78 ± 0.09 c	0.56 ± 0.08 b
GSH	18.9 ± 1.8 d	10.6 ± 1.0 a	11.1 ± 0.9 a	13.4 ± 1.1 b	15.7 ± 1.2 c
catalase	27.3 ± 2.0 d	14.0 ± 0.9 a	14.6 ± 1.0 a	17.8 ± 1.1 b	20.7 ± 1.3 c
GPX	35.4 ± 1.6 d	17.6 ± 1.2 a	18.4 ± 1.3 a	20.6 ± 1.5 b	24.9 ± 1.0 c
Kidney					
	non-DM	DM	DM+PA, 1%	DM+PA, 2%	DM+PA, 4%
MDA	1.08 ± 0.08 a	4.03 ± 0.36 e	3.66 ± 0.25 d	2.94 ± 0.23 c	2.41 ± 0.21 b
GSSG	0.35 ± 0.09 a	1.31 ± 0.18 d	1.28 ± 0.15 d	0.98 ± 0.12 c	0.74 ± 0.10 b
GSH	11.3 ± 1.3 d	4.9 ± 0.9 a	5.2 ± 1.1 d	7.0 ± 1.4 b	8.5 ± 1.3 c
catalase	17.0 ± 1.7 d	8.9 ± 0.9 a	8.5 ± 1.1 a	10.7 ± 1.2 b	12.9 ± 1.4 c
GPX	19.6 ± 2.0 d	11.2 ± 1.1 a	10.8 ± 1.3 a	13.5 ± 1.7 b	15.1 ± 1.3 c

[a] Data are the mean ± SD, $n = 12$. Mean values in a row without a common letter (a−e) differ, $P < 0.05$.

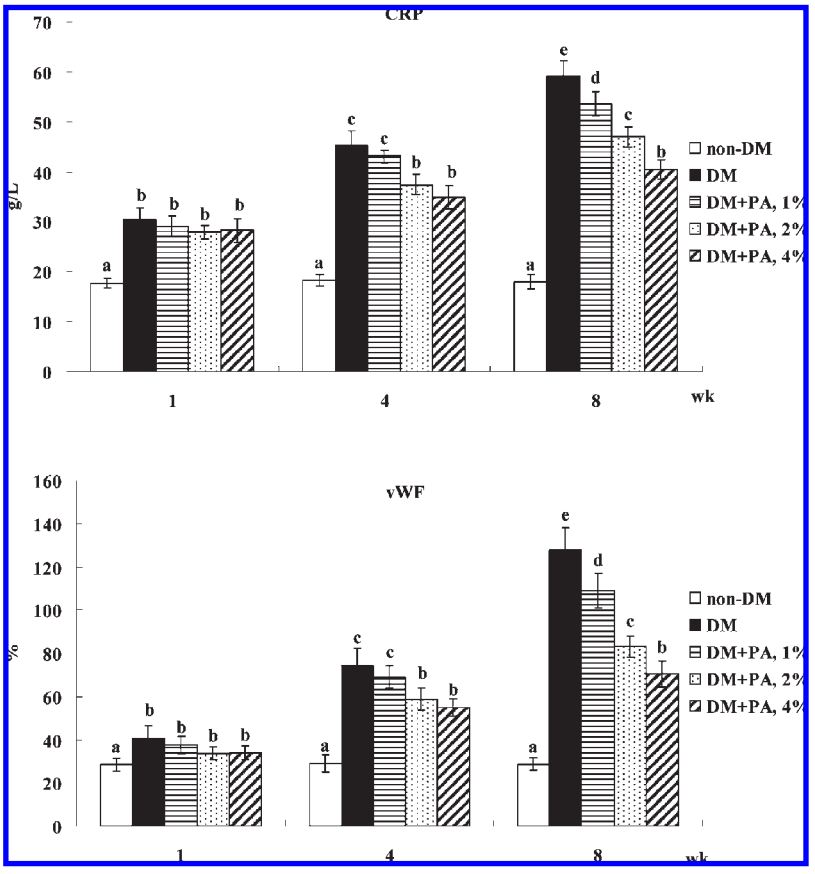

Figure 2. Plasma levels of CRP and vWF in nondiabetic mice (non-DM) and diabetic mice consumed normal diet (DM) or 1%, 2%, 4% protocatechuic acid (PA) at weeks 1, 4, and 8. Data are the mean ± SD, $n = 12$. (a−e) Mean values among bars without a common letter differ, $P < 0.05$.

Table 6. Cardiac and Renal Levels (pg/mg Protein) of Inflammatory Cytokines (IL-1β, IL-6, TNF-α, and MCP-1) in Nondiabetic Mice (non-DM) and Diabetic Mice (DM) Consumed Normal Diet and 1%, 2%, 4% Protocatechuic Acid (PA) at 8 Weeks[a]

Heart					
	non-DM	DM	DM + PA, 1%	DM + PA, 2%	DM + PA, 4%
IL-1βa	20 ± 3 a	230 ± 29 d	223 ± 23 d	183 ± 17 c	147 ± 20 b
IL-6	14 ± 4 a	321 ± 30 e	276 ± 21 d	232 ± 20 c	192 ± 15 b
TNF-α	19 ± 2 a	252 ± 22 e	215 ± 17 d	176 ± 19 c	132 ± 14 b
MCP-1	15 ± 3 a	199 ± 24 d	184 ± 20 d	141 ± 13 c	106 ± 12 b
Kidney					
	non-DM	DM	DM + PA, 1%	DM + PA, 2%	DM + PA, 4%
IL-1β	17 ± 2 a	216 ± 25 d	197 ± 18 d	156 ± 20 c	113 ± 14 b
IL-6	21 ± 4 a	245 ± 28 e	216 ± 21 d	178 ± 16 c	139 ± 10 b
TNF-α	16 ± 3 a	228 ± 19 e	190 ± 13 d	148 ± 17 c	105 ± 11 b
MCP-1	14 ± 4 a	230 ± 23 d	219 ± 20 d	183 ± 15 c	141 ± 12 b

[a] Data are the mean ± SD, $n = 12$. Mean values in a row without a common letter (a−e) differ, $P < 0.05$.

Therefore, it is also possible that the antioxidative protection in heart and kidney of protocatechuic acid treated diabetic mice as we observed was partially due to the intact form of protocatechuic acid and partially due to these metabolites.

IL-1β, IL-6, and TNF-α are central mediators for the production of several inflammatory biomarkers such as CRP and vWF, which consequently facilitates the progression of inflammation, endothelial dysfunction, and coagulation and finally exacerbates the severity of diabetes (*26*, *27*). Furthermore, TNF-α induces neutrophil accumulation and activation at sites of tissue injury (*28*). Thus, the decline on these cytokines could retard or alleviate inflammation and improve endothelial functions. Our present study found that protocatechuic acid supplement substantially decreased IL-1β, IL-6, and TNF-α levels in heart and kidney. Thus, the lower plasma levels of CRP and vWF in protocatechuic acid treated mice could be explained. These results supported that this compound was a potent agent against diabetes-associated cardiac and renal inflammatory injury. It has been reported that elevated serum MCP-1 level could serve as an inflammatory marker in patients at risk for atherosclerotic vascular diseases because MCP-1 is a chemotactic factor for activating monocytes and macrophages and could recruit

monocytes to the sites of injury (*29*). In our present study, the increased cardiac and renal MCP-1 levels revealed that these organs were injured, and these diabetic mice were at risk for further cardiac or renal complications. Meanwhile, we found that the supplement of protocatechuic acid markedly decreased MCP-1 levels in these organs. These findings implied that this compound could also protect heart and kidney against inflammation via diminishing the activation of monocytes and macrophages and lowering the recruitment of monocytes.

Protocatechuic acid content in roselle calyx and du-zhong leaves was 3.8 and 17.2 mg/g fresh weight, respectively (*30*,*31*). Obviously, the six test fruits in the present study contained less protocatechuic acid than roselle calyx and du-zhong. So far, the protection from this compound against neurotoxicity, hepatotoxicity, and diabetes has been observed in other studies (*3*, *32*) and our present study. Therefore, this compound could be considered as a nutraceutical agent with multiple benefits. However, Nakamura et al. (*33*) reported that overdoses of protocatechuic acid caused significant hepatic and renal GSH depletion and disturbed the detoxification of electrophilic toxicants. Thus, this compound needs further study to prove its safety before it is applied to humans.

In summary, protocatechuic acid was presented in six examined fruits with the range of 22−109 mg/100 g dry weight, and this compound provided triglyceride-lowering, anticoagulatory, antioxidative, and antiinflammatory protection for diabetic mice. Protocatechuic acid not only improved glycemic control but also lowered the formation of MDA and GSSG and restored the activity of catalase and GPX in heart and kidney. Furthermore, this compound attenuated hemostatic disorder and decreased the production of inflammatory cytokines in heart and kidney. Therefore, the supplement of this agent or foods rich in this compound might be helpful for the prevention or alleviation of diabetic complications.

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