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Research Note

# The Leaf of *Diospyros kaki* Thumb Ameliorates Renal Oxidative Damage in Mice with Type 2 Diabetes

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**ABSTRACT:** Diabetic kidney disease is the most common and severe chronic complication of diabetes. The leaf of *Diospyros kaki* Thumb (persimmon) has been commonly used for herbal tea and medicinal purposes to treat a variety of conditions, including hypertension and atherosclerosis. However, the effect of persimmon leaf on kidney failure has not been investigated. This study aimed to examine the role of persimmon leaf in protecting the diabetes-associated kidney damage in a mouse model of type 2 diabetes. Mice were fed either a normal chow diet with or without powered persimmon leaf (5%, w/w) for 5 weeks. In addition to kidney morphology and blood markers of kidney function, we assessed levels of oxidative stress markers as well as antioxidant enzymes activities and mRNA expression in the kidney. Supplementation of the diet with powered persimmon leaf not only decreased the concentration of blood urea nitrogen in the plasma but also improved glomerular hypertrophy. Furthermore, the persimmon leaf significantly decreased the levels of hydrogen peroxide and lipid peroxide in the kidney. The activities of superoxide dismutase, catalase, and glutathione peroxidase and the mRNA expression of their respective genes were also increased in the kidney of persimmon leaf-supplemented *db/db* mice. Taken together, these results suggest that supplementation with the persimmon leaf may have protective effects against type 2 diabetes-induced kidney dysfunction and oxidative stress.

**Keywords:** antioxidant activity, dietary herbal supplement, oxidative stress, renal impairments, type 2 diabetes

## INTRODUCTION

Oxidative stress plays a critical role in the development of complications associated with diabetes, and diabetic kidney disease is the most common and severe chronic complication of diabetes (1). Chronic kidney disease is the most frequent cause of death in patients with diabetes, and the incidence and prevalence of chronic kidney disease have increased worldwide (2,3). Previous studies showed that chronic kidney disease affects around 30% of patients with type 1 diabetes and 20% of patients with type 2 diabetes (4). Recent studies showed that approximately 50% of patients with type 2 diabetes worldwide have impaired kidney function (5). To date, however, few effective therapies are available to prevent or treat diabetic kidney disease (1). Therefore, development of novel therapeutic approaches is required for the prevention and treatment of diabetic kidney disease.

Oriental or Japanese *Diospyros kaki* Thumb (persimmon) is the most widely cultivated species of the genus *Diospyros* in the Korea, Japan, and China. The fruit of this plant is consumed as food, whereas the young leaf is commonly used for herbal tea and traditional medicines. The persimmon leaf contains various bioactive compounds, including proanthocyanidins (also called condensed tannins), flavonoids, and triterpenoids (6-8). Several studies have shown the beneficial effects of the persimmon leaf on hypertension, stroke, and atherosclerosis (9). Recently, Bae et al. (10), showed that the persimmon leaf extract ameliorates hyperglycemia and dyslipidemia through the inhibition of α-glucosidase and through the maintenance of functional β-cells. In addition, previously, we showed that powdered persimmon leaves exert antidiabetic effects in mice with type 2 diabetes by improving plasma insulin levels and regulating glucose and lipid metabolism (11). However, the effects of powdered persimmon leaf on kidney function and oxidative stress have not been completely elucidated to date in animal models of type 2 diabetes.

Thus, in this study, we investigated the protective ef-

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fects of powdered persimmon leaf on kidney function and morphology. Moreover, we examined the levels of lipid peroxide and hydrogen peroxide ($H_2O_2$) and the activities of antioxidant enzymes and mRNA expression of their respective genes in the kidney of mice with type 2 diabetes receiving persimmon leaf.

## MATERIALS AND METHODS

### Preparation of powdered persimmon leaf and feeding protocols

The persimmon leaf was harvested in Sangju (Korea). After the drying process, the persimmon leaf was ground into a fine powder and passed through 60-mesh sieves. The total content of fiber, phenols, and flavonoids were measured using the AOAC method, a modified Folin-Ciocalteu colorimetric method, and a method developed by Moreno et al., respectively (12-14). The total levels of fiber, phenols, and flavonoids in the powdered persimmon leaf were 630, 11.49, and 1.59 mg/g, respectively.

Male C57BL/KsJ-*db/db* (*db/db*) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a 12-h light/dark cycle and a temperature of 22±2°C. They were fed a normal chow diet for acclimation for 2 weeks after delivery. At 7 weeks of age, they were randomly divided into 2 groups (n=10) and fed a standard semisynthetic diet (AIN-76) with or without powered persimmon leaf (5 g/100 g diet, w/w) for 5 weeks. The mice had free access to food and water. At the end of the experimental period, the mice were anesthetized with ketamine after withholding food for 12 h, and blood samples were withdrawn for determination of plasma blood urea nitrogen (BUN) levels. The kidney was removed after collecting the blood, then rinsed with a physiological saline solution, and immediately stored at −70°C. All animal procedures were in compliance and approved by the animal ethics committee of Kyungpook National University (Approval No. KNU-2011-28).

### Analysis of plasma BUN levels

At the end of the experimental period, blood samples were taken from the inferior vena cava in a heparin-coated tube and centrifuged at 1,000 *g* for 15 min at 4°C. The plasma BUN concentration was determined using a commercial kit (Sigma, St. Louis, MO, USA).

### Preparation of tissue samples

Kidney enzymes were prepared as follows: the kidney and adipose tissues were homogenized in a 0.25 M sucrose buffer and centrifuged at 600 *g* for 10 min at 4°C to discard any cell debris, and then, the supernatant was centrifuged at 10,000 *g* for 20 min at 4°C to remove the mitochondrial pellet. The resulting mitochondrial pellets were then redissolved in 0.8 mL of homogenization buffer. Finally, the supernatant was further ultracentrifuged at 105,000 *g* for 60 min at 4°C to obtain the cytosolic supernatant. The amount of protein in the mitochondrial and cytosolic fractions was determined using the Bradford's method (15).

### Enzyme analyses

The activity of superoxide dismutase (SOD) was spectrophotometrically measured using a modified version of the method developed by Marklund and Marklund (16). Briefly, SOD activity was detected on the basis of its ability to inhibit superoxide-mediated reduction, and one unit was defined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. Catalase (CAT) activity was measured using the Aebi's method (17) with a slight modification, in which the disappearance of $H_2O_2$ was monitored at 240 nm for 5 min using a spectrophotometer. The activity was defined as a decrease in μmoL of $H_2O_2$/min/mg of protein. The activity of glutathione peroxidase (GPx) was measured using the spectrophotometric assay at 25°C as described previously by Paglia and Valentine (18) with slight modifications. The reaction mixture contained 1 mM glutathione, 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 0.24 units of glutathione reductase in a 0.1 M Tris-HCl (pH 7.2) buffer. The reaction was initiated by adding 0.25 mM $H_2O_2$, and the absorbance was measured at 340 nm for 5 min. The activity was expressed as nmoL of oxidized NADPH/min/mg of protein.

### Determination of $H_2O_2$ and lipid peroxidation in the kidney

The $H_2O_2$ level in the kidney was measured using the method of Wolff (19). The ferrous oxidation with xylenol orange (FOX 1) reagent was prepared with 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM $H_2SO_4$. Then 50 μL of the test sample was added to 950 μL FOX 1 reagent, vortexed, and incubated at room temperature for a minimum of 30 min at which time color development was virtually complete. The absorbance was read at 560 nm, and the standard was linear in the 0∼5 μM concentration range. One unit of activity was expressed as μmoL/g of protein.

The concentration of kidney thiobarbituric acid-reactive substances (TBARS) was measured, as a marker of lipid peroxidation, spectrophotometrically using the method of Ohkawa et al. (20). Briefly, the kidney homogenate (20%, w/v) was well mixed with 0.2 mL of 8.1% (w/v) sodium dodecyl sulfate and 0.6 mL of distilled water. The reaction mixture was heated at 80°C for 90 min after the addition of 1.5 mL of 20% (w/v) acetic acid and 1.5 mL of freshly prepared 0.8% (w/v) thiobarbituric acid. After cooling the mixture, the organic

mixture was added to 1.0 mL of distilled water and 5.0 mL of *n*-butanol-pyridine (15:1 v/v) and then centrifuged at 1,000 *g* for 10 min. The resulting colored layer was measured at 532 nm using 1,1,3,3-tetramethoxypropane as the standard.

### Gene expression analysis

Total RNA was isolated from the kidney using the guanidine thiocyanate-phenol method of Chomzynski and Sacchi (21). The total RNA (20 μg) was electrophoresed on a 0.9% agarose gel containing 2.2 M formaldehyde and transferred to Nytran-Plus membranes (Schleicher & Schuell BioScience GmbH, Dassel, Germany). The blotted membranes were then hybridized with a [$^{32}$P]-labeled cDNA probe. After washing, the membranes were exposed to an X-ray film with an intensifying screen at −70°C. The DNA probes were prepared from the mouse kidney RNA using reverse transcription polymerase chain reaction (RT-PCR) with the following primers: for SOD, 5'-AGG ATT AAC TGA AGG CGA GCA T-3' and 5'-TCT ACA GTT AGC AGG CCA GCA G-3'; for CAT, 5'-ACG AGA TGG CAC ACT TTG ACA G-3' and 5'-TGG GTT TCT CTT CTG GCT ATG G-3'; for GPx, 5'-AAG GTG CTG CTC ATT GAG AAT G-3' and 5'-CGT CTG GAC CTA CCA GGA ACT T-3'; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TTG AAG GGT GGA GCC AAA CG-3' and 5'-AGT GGG AGT TGC TGT TGA AGT CG-3'. The intensities of the mRNA bands were quantified using a Bio Image Whole Band Analyzer (50S, BI Systems Corporation, Ann Arbor, MI, USA) and subsequently normalized based on the intensity of the respective GAPDH mRNA bands.

### Kidney morphology

The kidneys were removed from the mice and fixed in a buffer solution of 10% formalin. Fixed tissues were processed routinely for paraffin embedding, and 3-μm sections were prepared and stained with hematoxylin eosin (H&E); the stained areas were viewed using an optical microscope with a 400× magnification.

### Statistical analysis

All data were presented as the mean±standard error (SE). Statistical analyses were performed using the statistical package for the social science software (SPSS) program (version 11.0, SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to assess the differences between the groups. Statistical significance was considered at $P<0.05$.

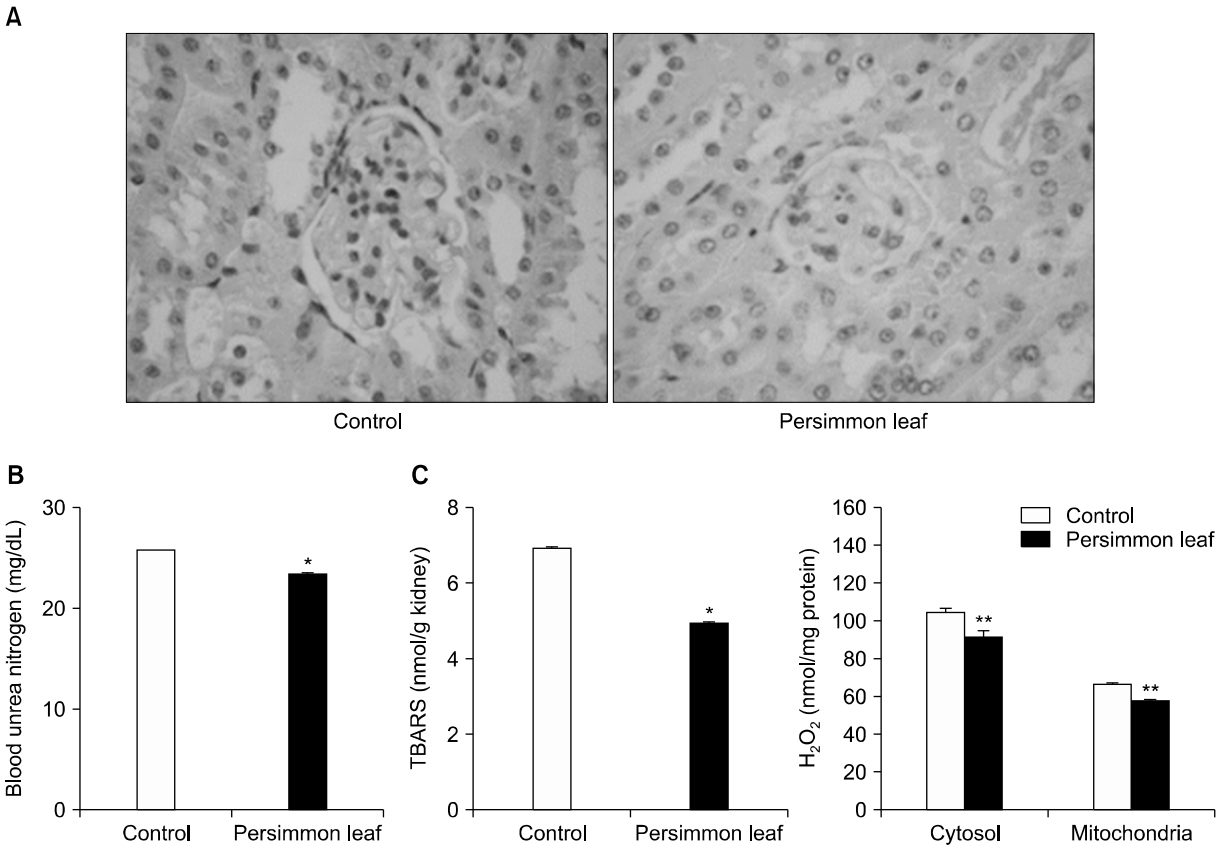


**Fig. 1.** Effect of persimmon leaf on kidney morphology. Histological sections of kidneys (magnification ×400) (A), plasma blood urea nitrogen (B), and oxidative stress markers levels (C) in C57BL/KsJ-*db/db* mice. Values are the mean±SE (n=10). $^{*}P<0.05$ and $^{**}P<0.01$.

## RESULTS AND DISCUSSION

Our results showed that supplementation of the diet with persimmon leaf ameliorated kidney dysfunction as well as oxidative stress in *db/db* mice with type 2 diabetes. Diabetic kidney disease is a progressive kidney disease caused by damage to the capillaries in the glomeruli of the kidney, and it is characterized by renal hypertrophy, glomerular enlargement, and a decline in renal function (22). The *db/db* mouse, a widely used genetic animal model of type 2 diabetes, exhibits most of the characteristics similar to those of humans with diabetic kidney disease (23,24). The kidneys of the *db/db* mice show a high glomerular surface area at the onset of diabetes (24). In the present study, persimmon leaf reduced renal injuries such as glomerular expansion, in the kidney of *db/db* mice (Fig. 1A).

In addition, the concentration of BUN, which is a useful diagnostic and prognostic marker of kidney disease (25), was significantly lowered in the plasma of persimmon leaf-supplemented *db/db* mice (Fig. 1B). The BUN levels indicate the amount of urea nitrogen, a waste product of protein metabolism, in the blood (25). Urea nitrogen is normally filtered by the kidneys, and usually, a small but stable amount of urea nitrogen is present in the blood (25). However, kidney dysfunction because of disease or damage causes an increase in the urea nitrogen in the blood because impaired kidneys cannot filter wastes out of the blood (25). The concentration of BUN is higher in rats with diabetic nephropathy than that in normal rats (26). *db/db* mice also showed higher BUN compared to the *db/m* littermates (23). Taken together, our results indicate that the persimmon leaf improved the renal functional and histological abnormalities in the kidney of *db/db* mice. It is possible that the beneficial effects of persimmon leaf could be due to the high content of fiber in persimmon leaf, because dietary fiber supplementation significantly reduced serum urea in individuals with chronic kidney disease (27). Fujii et al. (28), also demonstrated the association between dietary fiber intake and lower prevalence of chronic kidney disease in Japanese patients with type 2 diabetes.

Next, we examined the effects of persimmon leaf on kidney oxidative stress because cellular metabolic changes during chronic kidney disease may increase production of reactive oxygen species (ROS), which are involved in the abnormalities of kidney structure and function (29). ROS are highly active molecules that may oxidize proteins, lipids, and nucleic acids and consequently damage cells and tissues (1). In particular, the kidney is one of the organs highly vulnerable to damage induced by ROS, partly because of the high levels of long-chain polyunsaturated fatty acids in the composition of renal lipids (1). Although ROS are produced as normal products of

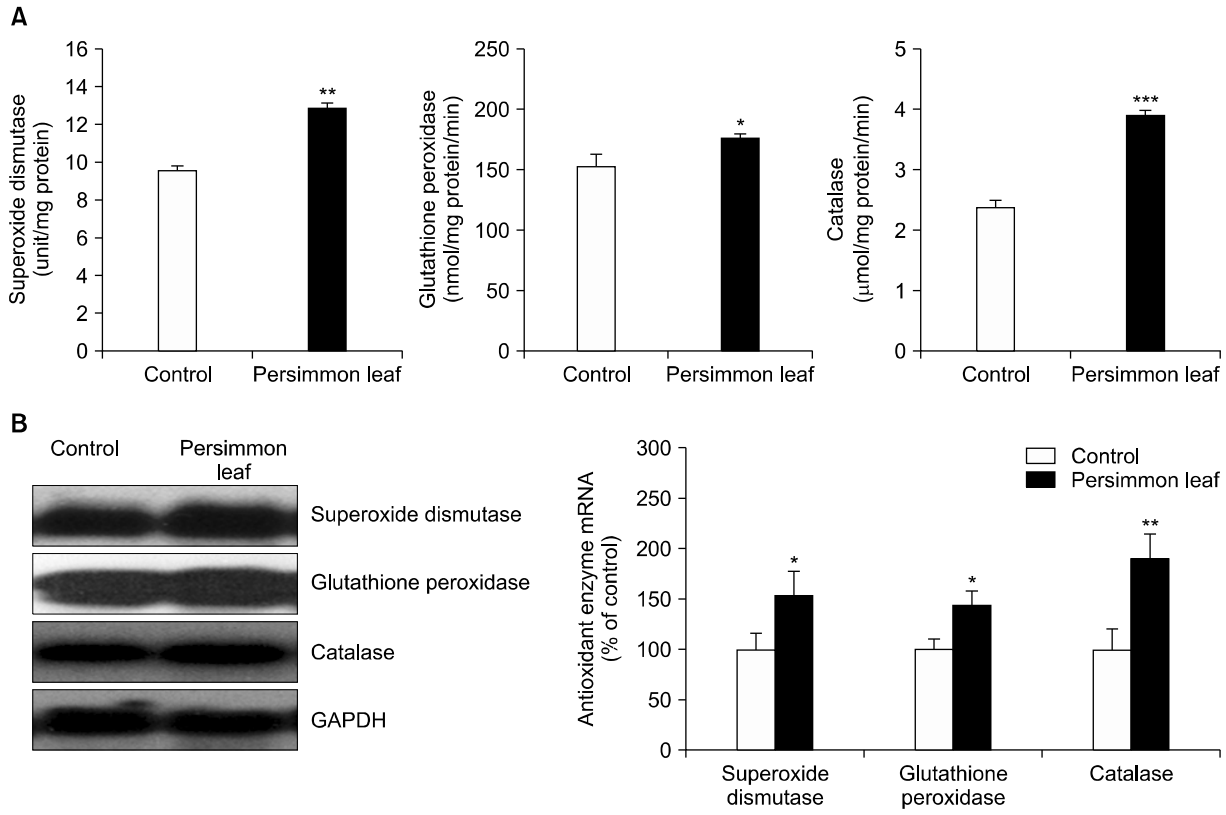


**Fig. 2.** Effect of persimmon leaf on the activities (A) and mRNA expression (B) of antioxidant enzymes in the kidney of C57BL/KsJ-*db/db* mice. Values are the mean±SE (n=10). $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

cellular metabolism, they are completely inactivated by the antioxidant defense system (1). However, the delicate balance between ROS generation and the antioxidant defense system leads to the damage of cells and tissues (1). In this context, a decrease in antioxidant capacity could play an important role in diabetic kidney damage, in which the resulting oxidative insult eventually could cause kidney damage (30).

In the present study, the persimmon leaf significantly decreased the levels of oxidative stress markers such as lipid peroxide and $H_2O_2$ in the kidney of *db/db* mice (Fig. 1C). In addition, persimmon leaf significantly increased the activities and mRNA expression of the 3 antioxidant enzymes (SOD, CAT, and GPx) in the kidney (Fig. 2). SOD is the major antioxidant enzyme for superoxide removal, which converts superoxide into $H_2O_2$ (31). The $H_2O_2$ is further detoxified to water by 2 different enzymes, CAT and GPx (32,33). The levels of antioxidant enzymes such as SOD, CAT, and GPx are decreased in the kidney of animals with diabetes (34-36). The type 2 diabetic *db/db* mice also showed increased levels of ROS and decreased activities of the major protective antioxidant enzymes such as SOD, CAT, and GPx in the kidney compared to *db/+* mice (37). Down-regulation of renal SOD plays a key role in the pathogenesis of diabetic nephropathy (38), and diabetic renal injury is accelerated in SOD-knockout mice (39), whereas overexpression of SOD has beneficial effects in preventing and attenuating diabetic kidney disease in mice (40). In addition, overexpression of CAT appears to have protective effects against kidney damage in diabetic mice (41), and a recent study showed an association between allelic variations of the GPx gene and the risk of kidney complications in patients with type 1 diabetes (42). Therefore, our data suggests that the combined activation of CAT and GPx together with SOD in persimmon leaf-supplemented *db/db* mice may contribute to the protective effects against ROS, thereby preventing the formation of lipid peroxidation as well as $H_2O_2$ in mitochondrial and cytosolic fractions of the kidney and exerting beneficial effects in diabetic kidney disease.

In conclusion, our findings suggest that, in *db/db* mice with type 2 diabetes, dietary supplementation with persimmon leaf improved functional and histological abnormalities in the diabetic kidney, possibly by attenuating the oxidative stress through scavenging of ROS (Fig. 3). Taken together, our findings suggest that persimmon leaf could be a good alternative to help prevent or decrease diabetic kidney disease. Therefore, further studies are required to elucidate whether persimmon leaf may be effective for the prevention and/or treatment of kidney dysfunction in patients with diabetes.

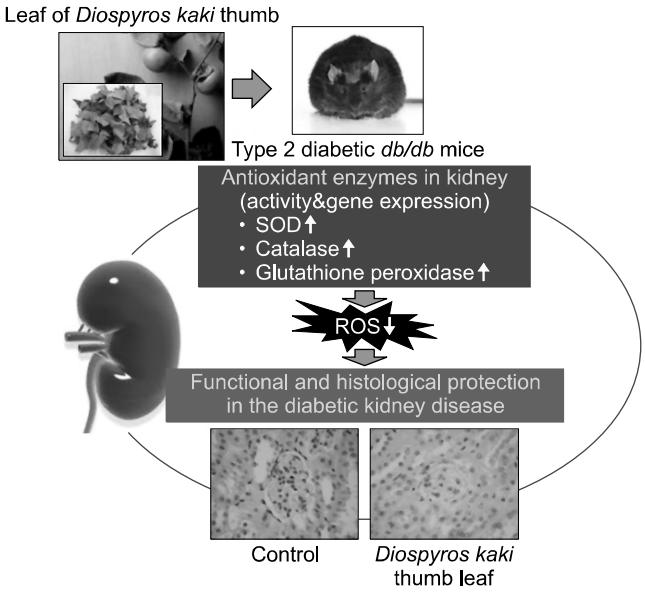


**Fig. 3.** Summary of persimmon leaf supplementation effects on diabetic kidney dysfunction.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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