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The Protective Effects and Mechanism of Mulberry Leaf Flavonoids on Type 2 Diabetic Nephropathy Mice

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Keywords

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Abstract

Purpose: This study investigated the protective effects and mechanism of mulberry leaf flavonoids on type 2 diabetic nephropathy (DN) in mice. **Methods:** The DPPH free radical scavenging activity of mulberry leaf flavonoids was measured *in vitro*. To establish a type 2 DN model, male ICR mice were induced with streptozotocin (STZ) after a high-fat diet regimen. The mice were then divided into groups: a normal control group, a DN model group (DM), a positive control group receiving 200 mg/kg metformin (Met group), and a treatment group receiving 1 g/kg mulberry leaf flavonoids (FM group). Both Met and FM groups received daily intragastric administration for 4 weeks, while the DM and control groups were given equivalent volumes of normal saline. Fasting blood glucose levels were recorded regularly during the intervention. After the treatment period, serum creatinine (CRE), blood urea nitrogen (BUN), superoxide dismutase (SOD) activity, malondialdehyde (MDA) levels, and renal glutathione peroxidase (GPx) activity were measured. Additionally, kidney histopathological changes and glycogen deposition were evaluated by HE and PAS staining, while Nrf2 and HO-1 expression in kidney tissue was assessed through immunohistochemistry. **Results:** Mulberry leaf flavonoids effectively scavenged free radicals, significantly reduced blood glucose levels, CRE, BUN, and MDA in serum and kidney tissues, and increased SOD and GPx activities. Histopathological improvements in kidney and pancreatic tissues were observed, alongside increased Nrf2 and HO-1 expression. **Conclusion:** Mulberry leaf flavonoids ameliorate renal damage in DN mice, potentially through oxidative stress reduction and activation of the Nrf2/HO-1 signaling pathway.

1 Introduction

Diabetic nephropathy (DN) is a major microvascular complication of type 2 diabetes and a leading cause of end-stage renal disease, affecting 30% to 40% of diabetic patients [1-3]. The growing prevalence of DN poses significant social and public health burdens [4]. Thus, developing safe, effective drugs for early DN prevention and late-stage treatment is essential. Traditional Chinese medicine has shown advantages in DN treatment and is a focal point in DN research [5-8].

The pathogenesis of DN is complex, with oxidative stress as a key factor. Prolonged hyperglycemia activates pathways like cyclooxygenase in renal tissue, where pro-oxidant enzymes generate excess reactive oxygen species (ROS), leading to oxidative stress, renal fibrosis, inflammation, and endothelial dysfunction [9].

Mulberry leaves (*Morus alba* L.) are traditionally used in Chinese medicine and contain bioactive compounds such as flavonoids, known for antioxidant, anti-inflammatory, hypoglycemic, and cardioprotective properties [10]. Studies have shown that mulberry leaf flavonoids possess antioxidative properties that mitigate oxidative stress and regulate glucose metabolism. Meng et al. [11] demonstrated that these flavonoids enhance skeletal muscle mitochondrial function via AMPK activation, supporting energy homeostasis and glucose metabolism. Cheng et al. [12] reported that mulberry leaf flavonoids activate brown adipose tissue and promote browning of white adipose tissue, enhancing glucose metabolism and insulin sensitivity. These findings support their multifaceted role in diabetes management and underscore their potential in treating DN. Huang et al. [13] and Su et al. [14] highlighted that flavonoids extracted under optimal conditions exhibit superior antioxidant activity, further supporting their therapeutic potential in oxidative stress-related conditions.

Oxidative stress and inflammation are central to DN progression, and natural products targeting these pathways have shown promise. The Nrf2/HO-1 pathway is a crucial regulator of cellular antioxidant responses and has been linked to DN progression [15]. Activation of this pathway mitigates oxidative damage and enhances cellular defense. Previous studies have shown that Nrf2 protects against DN by modulating oxidative stress and inflammation [9,16].

This study investigates the protective effects and mechanisms of mulberry leaf flavonoids on type 2 DN, focusing on their impact on oxidative stress, blood glucose regulation, and activation of the Nrf2/HO-1 pathway. Using a DN mouse model induced by streptozotocin (STZ) and a high-fat diet, this research aims to provide evidence for the therapeutic potential of mulberry leaf flavonoids in DN treatment.

2 Experimental materials

2.1 Equipment

UV-1600PC UV-Visible Spectrophotometer (MAPADA, Shanghai, China), SepctraMax M2 microplate reader (Molecular Devices, Shanghai, China), BH-200M optical microscope (Runxing Optics, Shenzhen, China), LBT80A blood glucose meter (Shanghai Mojin Medical Instrument Co., LTD., Shanghai, China), etc.

2.2 Reagents

Mulberry leaves (230901, Zhejiang Chinese Medical University of Chinese Medicine Decoction Co., LTD., Zhejiang, China) were identified as leaves of *Morus alba* L. by Guo'er Zhou, a director of the TCM pharmacist of Zhoushan Hospital of Traditional Chinese Medicine. Rutin standard product (purity ≥ 98%, SR8250) was purchased from Solarbio (Beijing, China). Other reagents were all analytical reagents, and the water was distilled water. Metformin (Met, EJ006532) and STZ (ST1668) were severally ordered from Coolbjc (Anhui, China) and Beyotime (Shanghai, China). Creatinine (CRE, G1203W), blood urea

nitrogen (BUN, G1201W), superoxide dismutase (SOD, G0101W), malondialdehyde (MDA, G0109W), and glutathione peroxidase (GPx, G0206W) test kits were procured from Grace Biotechnology (Suzhou, Jiangsu province, China). Primary antibodies against Nrf2 (ab62352) and HO-1 (ab13248) were sourced from Abcam (Cambridge, UK), and the secondary antibody (biotinylated anti-rabbit IgG, ab6721) was also obtained from Abcam.

2.3 Animals

Specific pathogen-free (SPF) institute of cancer research (ICR) mice (male, 30-35 g), as experimental animals, were procured from Hangzhou Ziyuan Experimental Animal Co., LTD., with animal production license number SCXK (Zhejiang) 2019-0001. The mice were raised in the animal room of Zhejiang Ocean University, with free access to drinking water, a temperature of 23 ℃, and a light cycle of 12 h per day. The experiments were approved and reviewed by the Animal Experiment Ethics Committee of Zhejiang Ocean University (ethics number 2022018), and operated strictly as per the relevant regulations on Experimental animal Care and Use.

3 Methods

3.1 Extraction and purification of mulberry leaf flavonoids

An appropriate number of dried mulberry leaves were ground, soaked in 55% ethanol at a solid-liquid ratio of 1:30 (g/mL), and underwent ultrasonic water bath-assisted extraction at 40 ℃ for 30 min, followed by being concentrated under reduced pressure, filtered, and freeze-dried. The crude extract was purified by polyamide with D-101 macroporous resin, and the purified solution was freeze-dried. The yield was calculated twice.

3.2 Flavonoid content examination in mulberry leaves

With rutin as a control, the content of flavonoids in mulberry leaves after crude extraction and purification was determined by $Al(NO_3)_3$-$NaNO_2$ spectrophotometric colorimetry, and the twice extraction yields were calculated each time. Rutin at the standard gradient concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL was severally incubated with 0.5 mL 5% $NaNO_2$ solution and 0.5 mL 10% $Al(NO_3)_3$ both at room temperature for 5 min. Then, rutin was mixed with 4 mL 4% NaOH solution and diluted to 10 mL with distilled water for 15 min, and the UV absorbance of each tube was measured at 510 nm. 10 mg of freeze-dried mulberry leaf flavonoids after crude extraction and purification was separately collected, prepared into a solution with a concentration of 1 mg/mL using ethanol as the solvent, and stored for later use. 1 mL of mulberry leaf flavonoid sample solution each was precisely extracted and prepared into test solution according to the preparation method of the control. The absorbance of each sample was determined under the same conditions.

Extraction rate (%) = (C × V/m) × 100. (C: concentration; V: Dilution volume; m: the quality of the collected mulberry leaf flavonoid sample).

3.3 DPPH free radical scavenging rate measurement

DPPH with anhydrous ethanol was prepared to a concentration of 0.1 mg/mL. Appropriate number of freeze-dried mulberry leaf flavonoids solids after crude extraction and purification were dissolved in anhydrous ethanol to solution at concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25 mg/mL, respectively. 2 mL solution at the above concentrations was transferred into 10 mL volumetric flasks, and added with 2 mL DPPH solution to reach a constant volume. 30 min later, their absorbance at a wavelength of 517

nm was calculated and recorded as A_1. With anhydrous ethanol as the blank control group according to the same procedure as before, the absorbance value was recorded as A_2. Another 10 mL volumetric flask was used without mulberry leaf flavonoids sample solution but underwent the same procedure as before. The absorbance value was recorded as A_0. Clearance rate (%) = $(1 - (A_1 - A_2)/A_0) \times 100$.

3.4 Animal model construction, grouping and sampling

Male SPF ICR mice (30-35 g) were divided into a normal control group (Con, 10 mice) fed regular chow and administered normal saline, and a model control group (DM, 30 mice) fed a high-fat diet for 30 days and induced with DN via a single STZ injection (150 mg/kg). Mice with fasting blood glucose levels > 16.7 mmol/L after 72 hours were considered successfully modeled. These modeled mice were randomly assigned to the DM group (receiving normal saline), Met group (treated with 200 mg/kg metformin), or FM group (treated with 1 g/kg mulberry leaf flavonoids), with 10 mice per group. The dosage of 1 g/kg mulberry leaf flavonoids was selected based on prior studies indicating its effectiveness and safety in diabetic models [17]. Treatments were administered by gavage once daily for 4 weeks, totaling 28 treatments. At the end of the treatment period, mice were weighed, and blood samples were collected from the retro-orbital sinus, centrifuged at 2500 rpm for 10 min, and serum was stored at -80 °C. Mice were then euthanized, and kidneys were harvested, weighed, and processed—part fixed in 4% paraformaldehyde for histology, while the remainder was stored at -80 °C for biochemical assays. A 10% kidney tissue homogenate was prepared in cold saline for further analysis.

3.5 Fasting blood glucose determination in mice

During administration, the health status of mice was observed and recorded at the same time every day, and all mice were weighed every 2 days. Their tails were cut to measure fasting blood glucose levels.

3.6 Ratio of kidney mass to mouse body weight

The kidneys of mice were collected and weighed. The kidney to body ratio was calculated in each group. The ratio = kidney mass (g)/mouse body mass (g).

3.7 Pathological observation of pancreas and kidneys

Mouse pancreas and kidney tissues experienced hematoxylin eosin (HE) staining for histomorphological observation. After being fixed in 4% paraformaldehyde for 24 h, the samples underwent dehydration, transparentization, and paraffin embedment. The samples were sliced (4-6 μm), placed on a glass slide, put into a staining box, stained with alkaline hematoxylin-eosin solution, dehydrated with ethanol and sealed with transparent agent. The glass slide was covered with a cover slip and sealed with edge sealant to create a histopathological section. The section was sealed with neutral gel, followed by observation and imaging under an optical microscope.

3.8 Periodic acid-schiff staining of mouse kidneys

The mouse kidney tissues were subjected to periodic acid-schiff staining. Briefly, the tissues were embedded in paraffin, sliced, deparaffinized, and washed with distilled water for 2 min. Then, the tissues were added with periodic acid solution dropwise for 5-min oxidization, washed by distilled water for 10 min, immersed in schiff reagent (dropwise) for 10-20 min. Following the removal of schiff reagent and rinse with running water for 10 min, the tissues were soaked in hematoxylin staining solution (dropwise) for 1-2-min nucleus staining, rinsed with running water for 5 min, differentiated with acid ethanol differentiation solution, and washed with Scott repeatedly for 3 min, followed by conventional transparentization, sealing and

observation under a light microscope.

3.9 CRE and BUN content testing

According to the kit instructions, the contents of CRE and BUN were detected in all groups of mice.

3.10 Measurement of relevant oxidative markers in serum and kidneys

In light of the kit instructions, the SOD activity and MDA content in the serum, as well as the SOD, GPx activity and MDA content in the kidneys were measured in each group of mice.

3.11 Immunohistochemical observation of Nrf2/HO-1 pathway-related protein expressions in the kidney

Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm. After deparaffinization, rehydration, and antigen retrieval in citrate buffer (pH 6.0), sections were blocked with 3% hydrogen peroxide for 10 min. Primary antibodies against Nrf2 (1:200) and HO-1 (1:100) were applied overnight at 4 °C, followed by incubation with a biotinylated secondary antibody (1:500) for 30 min at room temperature. Visualization was achieved using diaminobenzidine (DAB) staining, with hematoxylin counterstaining. Sections were observed under a light microscope, and protein expression was analyzed using ImageJ software.

3.12 Statistical analysis

All experiments were conducted in triplicate to ensure reproducibility and reliability of the results. Data analyses were completed using SPSS 26.0 and GraphPad software, and the experimental data were delineated by mean ± standard deviation (SD). For between-group differences, one-way ANOVA was used for statistical analysis, where $p < 0.05$ implied statistical difference, $p < 0.01$ reflected significant statistical difference, and $p < 0.001$ signified extremely significant statistical difference.

4.Results

4.1 Mulberry leaf flavonoid preparation and DPPH free radical scavenging rate

With rutin as the control, the total flavonoid content of mulberry leaves was calculated. The linear regression curve equation of the control of rutin is: $Y = 0.43964x + 0.0011$, and $R^2 = 0.9986$ (Figure 1A). The concentration of flavonoids crudely extracted from mulberry leaves was 0.043 mg/mL, with a yield of 4.3%. The concentration of purified mulberry leaf flavonoids was 0.82 mg/mL, with a yield of 82%. The final yield of mulberry leaf flavonoids was 3.53%.

The results of DPPH free radical scavenging rate (Figure 1B) revealed that the DPPH free radical scavenging ability of mulberry leaf flavonoids after crude extraction and purification was continuously enhanced with the increase of flavonoid concentration, and tended to stable at a concentration of around 0.03 mg/mL. The purified mulberry leaf flavonoids were superior to crudely extracted mulberry leaf flavonoids in DPPH free radical scavenging ability. The purified mulberry leaf flavonoids at 0.04 mg/mL and about 0.05 mg/mL had a 90% and 97.5% DPPH free radical scavenging rates, respectively. These results indicated that mulberry leaf flavonoids had a good *in vitro* free radical scavenging ability.

4.2 The intervention effects of mulberry leaf flavonoids on DN mice

During the experiment, the weight of the DN model mice showed a decreasing trend (Figure 2A), with a weight loss range of Met group < DM group < FM group. The results reflected that the weight loss of DN mice was decelerated in Met group, and was more apparent in FM group than DM group. According to Figure 2B, the blood glucose levels of the three groups of mice were all higher than 16.7 mmol/L, indicating successful modeling. After 4 weeks of administration, the degree of blood glucose decline among the three

groups was FM group > Met group > DM group. The blood glucose levels in the FM group were declined markedly and fluctuated greatly. The degree of blood glucose decline in the Met group was not as good as that in the FM group, but the decrease was stable. These data proved that both Met and mulberry leaf flavonoids had a certain effect on reducing blood glucose, with mulberry leaf flavonoids having more evident effects.

The pathological examination of pancreatic tissues showed (Figure 2C) that pancreatic islet cells were evenly distributed in the pancreatic tissues of Con group, with clear layer and structure, as well as the uniformly stained and circular or oval nuclei in pancreatic islets and exocrine tissues. Compared with the Con group, the number of pancreatic islet cells in the DM group was reduced, with uneven cell distribution, varying degrees of damage, atrophy and degeneration, deformation and rupture of pancreatic islet structure, and abnormal proliferation and structural disorder in pancreatic exocrine tissues, inflammatory cell infiltration and fibrosis, and uneven staining of pancreatic islet cell nuclei. Relative to the DM group, the pancreatic tissues of Met and FM groups showed increased pancreatic islet cells and even cell distribution, with no obvious abnormal proliferation or apoptosis, clear and distinguishable structure of pancreatic islets and exocrine tissues, decreased inflammatory cells and the degree of fibrosis, uniformly stained and circular or oval nuclei of pancreatic islets and exocrine tissues, and no obvious nuclear cytoplasmic abnormalities or atypia. These results denoted that mulberry leaf flavonoids can mitigate pancreatic damage in type 2 DN mice to some extent.

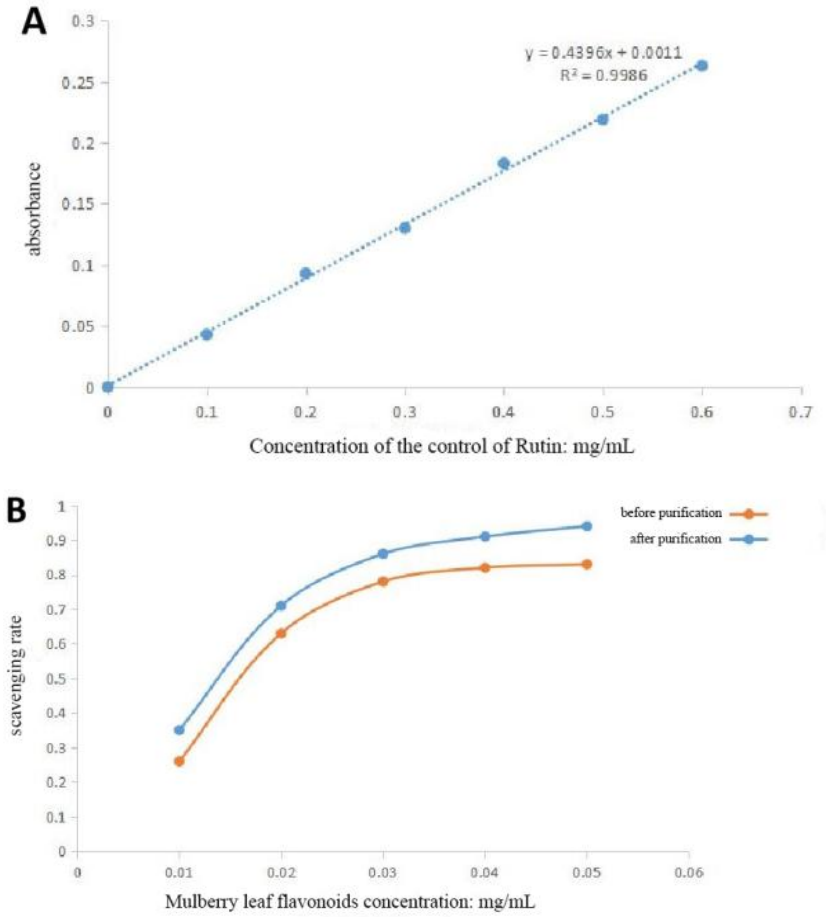

Figure 1 Regression curve of rutin' control (A) and DPPH free radical scavenging ability of mulberry leaf flavonoids before and after purification (B) (n = 3).

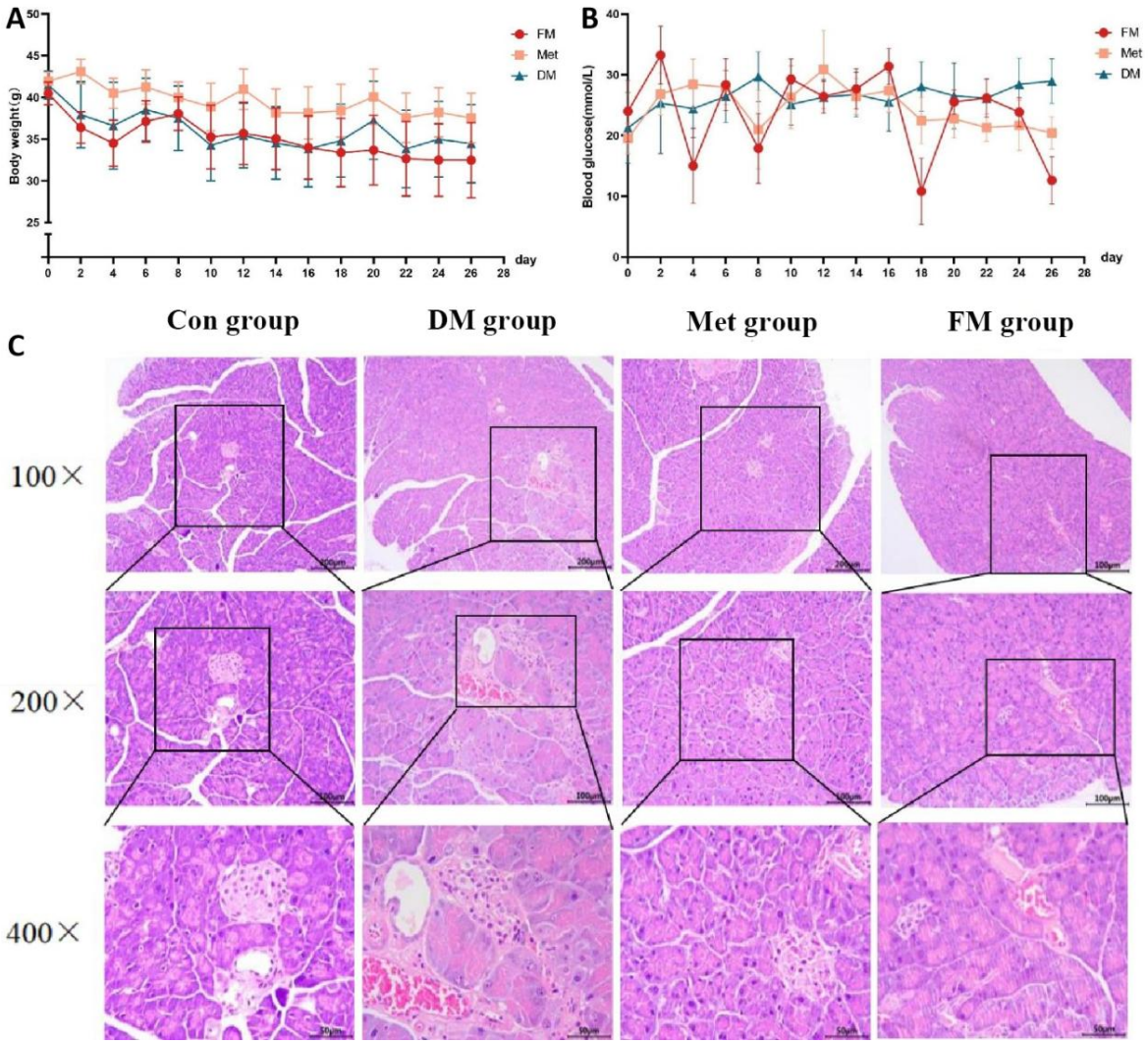

Figure 2 The intervention effects of mulberry leaf flavonoids on DN mice. A: the weight of mice in all groups; B: the blood glucose levels of mice in all groups; C: the representative image of pancreatic tissues (HE staining).

4.3 The protective effects of mulberry leaf flavonoids on kidneys of DN mice

As exhibited in Figure 3A, in contrast to Con group, there was a significant statistical difference ($p < 0.001$) in the kidney to body ratio of the DM group, and the kidneys were larger in DN mice than normal mice and showed edema. Compared with the DM group, the kidney to body ratio of Met and FM groups was remarkably reduced ($p < 0.01$, $p < 0.05$). These data suggested that mulberry leaf flavonoids can improve DN-induced renal edema. The renal function test results (Figure 3B,3C) revealed that the serum levels of CRE and BUN were higher in DM group than Con group ($p < 0.001$), and lower in FM group than DM group ($p < 0.01$, $p < 0.001$), indicating that mulberry leaf flavonoids had a protective effect on renal function in DN mice.

The histopathological examination results (Figure 3D) showed a good boundary between the cortex and medulla of the kidney tissue, even distribution of glomeruli, regular morphology of glomeruli, even number of cells and matrix, neat and regular arrangement of renal tubular epithelial cells, round and full morphology, no obvious proliferation of renal interstitium, and no apparent pathological changes in the Con group. The DM group presented renal structural disorder, glomerular atrophy, increased extracellular matrix, renal tubular swelling and vacuolar-like degeneration, mesangial proliferation, and significant pathological changes of DN. In Met

group, the structure of the glomeruli was improved, and the structures of the distal and proximal tubules were vaguely distinguishable; however, in this experiment, Met did not significantly improve the renal structure. The FM group showed prominent improvement in renal tissue lesions, clear renal corpuscle structure, and distinguishable structures of distal and proximal tubules, hinting that mulberry leaf flavonoids had a good protective effect on the kidneys.

The periodic acid-schiff staining of kidney tissues (Figure 3E) revealed that glycogen polysaccharides appeared purplish red. In the DM group, glomerular deformation was disrupted, with much more purplish red color compared to the Con group, indicating glycogen deposition. In the Met group, the glomeruli tended to be normal, and there was less glycogen precipitation in the surrounding area. Almost no glycogen precipitation was detected in the FM group, with clear boundaries similar to the Con group. These findings proved that mulberry leaf flavonoids can improve renal glycogen deposition resulting from DN.

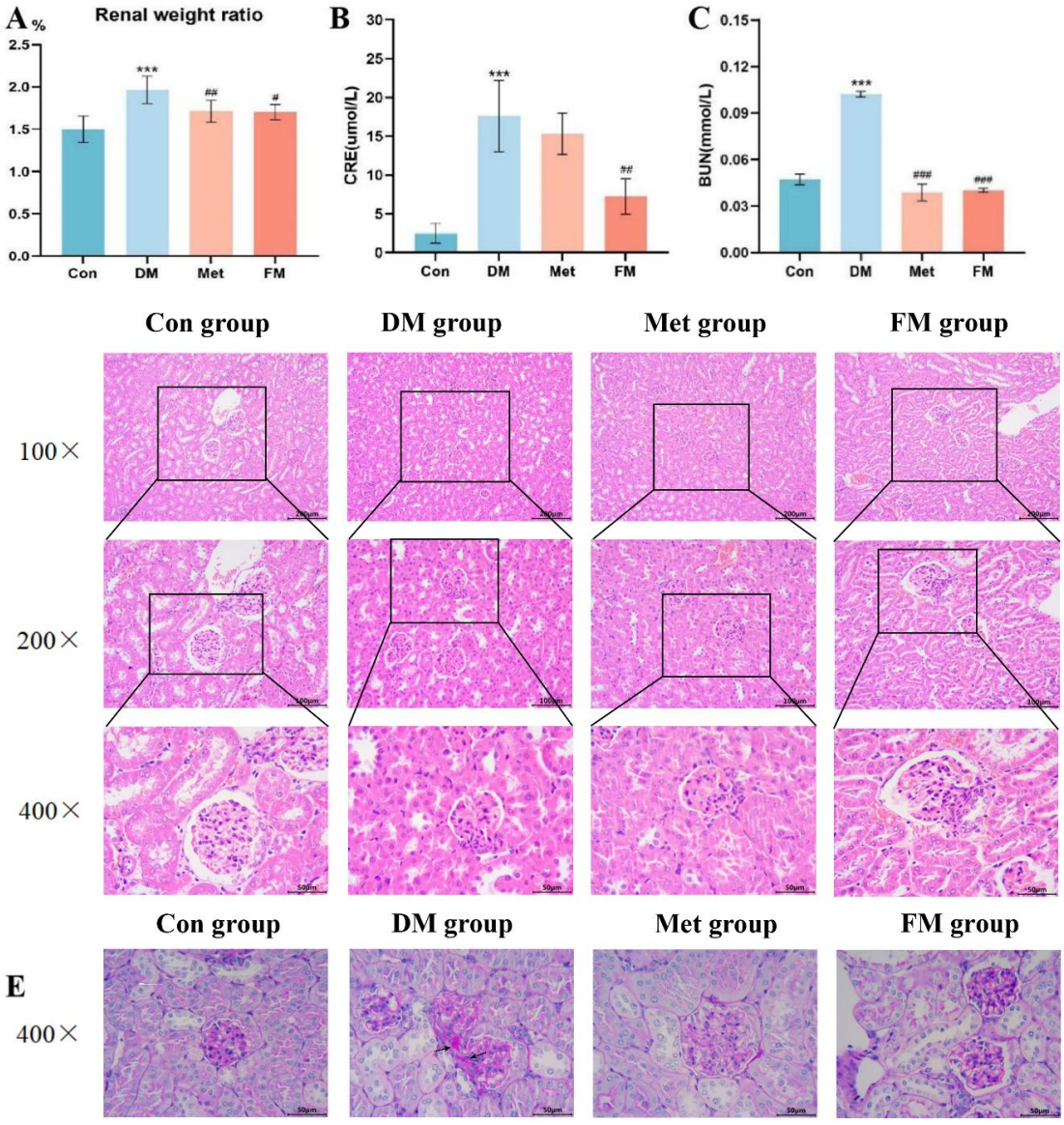

Figure 3 The protective effects of mulberry leaf flavonoids on kidneys of DN mice. A: kidney to body ratio; B: the serum levels of CRE and C: BUN; D: the representative histopathological image of kidney tissues; E: the representative images of periodic acid-schiff staining of kidney tissues. (Note: *** $p < 0.001$ vs. Con group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. DM group, (n = 3)).

4.4 Mulberry leaf flavonoids had anti-oxidant effects

According to the detection results of anti-oxidant index (Figure 4A,4B), compared with the Con group, the serum SOD activity in the DM group was reduced ($p < 0.001$), while the MDA content was increased ($p < 0.01$). Relative to the DM group, the serum SOD activity in FM and Met groups was signally enhanced ($p < 0.001$), and the MDA content was significantly decreased ($p < 0.001$). Figure 4C-4E showed that in contrast to the Con group, the activities of SOD and GPx in the kidneys of DM group were inhibited ($p < 0.001$), and the content of MDA was elevated ($p < 0.001$). The GPx content was higher ($p < 0.05$) and the MDA content ($p < 0.001$) was lower in Met group than DM group. The SOD and GPx activities in the FM group were markedly increased ($p < 0.001$, $p < 0.01$), while the MDA content was dramatically decreased ($p < 0.001$). These results hinted that mulberry leaf flavonoids can strengthen the anti-oxidant capacity of the kidneys and protect the kidneys of DN mice.

4.5 Mulberry leaf flavonoids elevated Nrf2 and HO-1 expression levels in kidneys of DN mice

The immunohistochemical results (Figure 5) showed that the positive expressions of Nrf2 (Figure 5A-E) and HO-1 (Figure 5F-J) in the kidneys of mice were lower in the DM group than the Con group ($p < 0.01$). Relative to the DM group, the Met group and FM group presented remarkable upregulation of Nrf2 and HO-1 ($p < 0.01$). These data implied that the expressions of Nrf2/HO-1 pathway node proteins were changed in type 2 DN mice, and mulberry leaf flavonoids can effectively upregulate the expressions of Nrf2 and HO-1 proteins to improve kidney injury in DN mice.

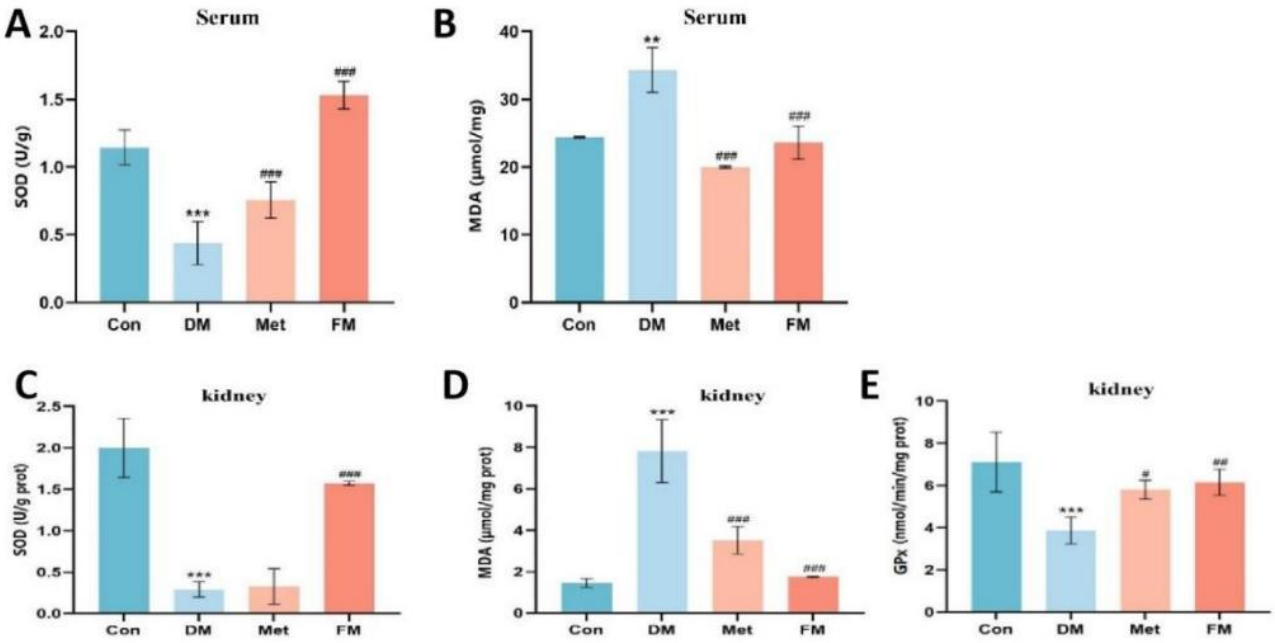

Figure 4 Mulberry leaf flavonoids had anti-oxidant effects on DN mice. A: serum SOD activity; B: MDA content; C: SOD activity in the kidneys; D: MDA content; E: GPx activity. (Note: ** $p < 0.01$, *** $p < 0.001$ vs. Con group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. DM group, (n = 3)).

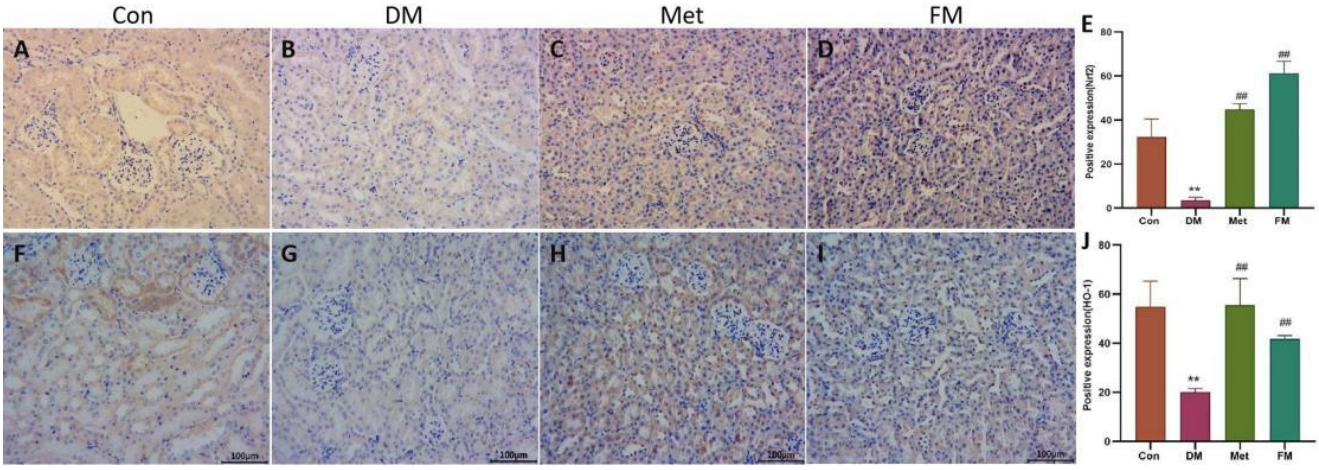

Figure 5 Nrf2 (A~E) and HO-1 (F~J) expressions in kidneys of all groups of mice (DAB staining, ×200). (Note: compared to Con group, ** $p < 0.01$; compared to DM group, $^{\#\#}$ $p < 0.01$).

5 Discussion

Huang et al. [13] confirmed through orthogonal experiments that the optimal extraction conditions of mulberry leaf flavonoids are 60% ethanol, 1:30 solid-liquid ratio, and 35-min ultrasonic extraction at 40 ℃. Su et al. [14] unveiled, through single factor experiments and response surface analysis, that the optimized process conditions for total flavonoids in mulberry leaves are 80% ethanol, 1:30 solid-liquid ratio, and 75-min ultrasonic extraction at 40 ℃. Zhong et al. [16] conducted single factor experiments and orthogonal experiments, and confirmed that the optimal conditions for simultaneous ultrasonic extraction of total flavonoids in mulberry leaves and tannins are 75% ethanol, 1:30 solid-liquid ratio, and 10-min ultrasonic extraction at 80 ℃. Herein, the final conditions for extracting flavonoids from mulberry leaves were proved to be 55% ethanol, 1:30 solid-liquid ratio, and 30-min ultrasonic extraction at 40 ℃.

Ma et al. [18] performed chromatographic purification of flavonoid solution using AB-8 macroporous adsorption resin, and determined that the purity of total flavonoids in mulberry leaves in the ethanol eluate reaches 63.2%. The treatment with AB-8 macroporous resin results in increased total flavonoids of mulberry leaves from 12.3% to 33.8% [14]. Herein, we applied macroporous resin column and polyamide column to perform chromatographic purification of mulberry leaf flavonoids and revealed that the yield reached 82% after purification, providing certain theoretical basis for industrial production.

Mulberry leaf flavonoids had a high DPPH free radical scavenging rate at low concentrations, indicating their good anti-oxidant capacity *in vitro*. In the *in vivo* anti-oxidant experiments, compared with the Con group, mulberry leaf flavonoids can effectively increase SOD activity in the serum, SOD in the kidneys and GPx activity of DN mice, while reducing the content of MDA in the serum and kidneys of DN mice, eliminate excess free radicals produced in the body, have good anti-oxidant effects, improve kidney damage, and protect the kidneys of DN mice. In the FM group, relative to the DM group, mulberry leaf flavonoids can apparently decline body weight of DN mice, and improve DN-caused kidney edema to some extent.

In this study, DN mice showed structural disorder in the kidneys, glomerular atrophy, increased extracellular matrix, renal tubular swelling and vacuolar-like degeneration, mesangial proliferation, and evident DN pathological changes, indicating

successful modeling. The number of pancreatic islet cells in the DM group was reduced, with uneven cell distribution, varying degrees of damage, atrophy and degeneration, deformation and rupture of pancreatic islet structure, and abnormal proliferation and structural disorder in pancreatic exocrine tissues, inflammatory cell infiltration and fibrosis, and uneven staining of pancreatic islet cell nuclei. Compared to DM group, mulberry leaf flavonoids can pronouncedly improve renal tissue lesions, with clear renal corpuscle structure and distinguishable structures of distal and proximal tubules, protect kidneys and improve pancreatic injury in type 2 DN mice to a certain extent.

The periodic acid-schiff staining results unraveled that compared to DM group, mulberry leaf flavonoids can ameliorate DN-induced renal glycogen deposition to mitigate kidney damage. CRE and BUN are sensitive indexes to evaluate DN, mainly reflecting the impairment condition of glomerular filtration function [19,20]. Herein, in type 2 DN model mice, mulberry leaf flavonoids explicitly reduced serum CRE and BUN contents. Nrf2 is a key factor in regulating oxidative stress, and HO-1 has anti-oxidant effects. Nrf2 regulates downstream HO-1 expression, and the Nrf2/HO-1 signaling pathway is a vital pathway for mediating oxidative stress, inflammation, and cell apoptosis [15]. In this study, we confirmed mulberry leaf flavonoids and Met impacted Nrf2/HO-1 pathway-related proteins to varying degrees, thereby improving kidney damage in type 2 DN mice. The Nrf2/HO-1 pathway has been widely studied in diabetic nephropathy due to its role in regulating oxidative stress and inflammation. Studies have shown that activation of Nrf2 can significantly reduce renal oxidative damage and inflammatory response, thereby protecting kidney tissue from hyperglycemia-induced injury [21]. For instance, Bai et al. [22] demonstrated that Nrf2 activation reduces oxidative stress in diabetic nephropathy models, providing a potential target for DN therapy. Furthermore, Nrf2 activators like sulforaphane and cinnamic aldehyde have shown promise in DN treatment by enhancing Nrf2 function and reducing renal injury [23,24]. Our study aligns with these findings, suggesting that mulberry leaf flavonoids may upregulate Nrf2 and HO-1 expressions, contributing to renal protection in DN. However, this study did not perform further validation using inhibitors or agonists of the Nrf2/HO-1 signaling pathway, which is a limitation that future studies should address to confirm the role of this pathway in the observed effects.

In this study, a single concentration of 1 g/kg mulberry leaf flavonoids was administered to DN model mice, demonstrating significant protective effects on renal function and antioxidative stress. However, it is acknowledged that using multiple concentrations could help determine the optimal dose with the most effective therapeutic impact. Future studies should consider a dose-response analysis with varying concentrations of mulberry leaf flavonoids to establish a dose that maximizes therapeutic efficacy while minimizing potential side effects. Such an approach would allow for a more precise understanding of the pharmacodynamics and optimal therapeutic window of mulberry leaf flavonoids in treating diabetic nephropathy.

In conclusion, mulberry leaf flavonoids have good anti-oxidative effects *in vivo* and *in vitro*, effectively suppress the upregulation of CRE and BUN contents, improve pathological structure of renal tissue in DN mice and protects kidneys to some extent, the mechanism of which may be related to reduction of oxidative stress and regulation of Nrf2/HO-1 signaling pathway.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

H.H. and Y.G.: Investigation; H.H.: Formal analysis and Writing - original draft; L.Z.: Conceptualization; K.L.: Methodology and Writing - review & editing.

Ethics Approval and Consent to Participate

The experiments were approved and reviewed by the Animal Experiment Ethics Committee of Zhejiang Ocean University (ethics number 2022018), and operated strictly as per the relevant regulations on Experimental animal Care and Use.

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Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Supplementary Materials

Not applicable.

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