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Anti-angiogenesis and anti-inflammatory effects of *Moringa oleifera* leaf extract in the early stages of streptozotocin-induced diabetic nephropathy in rats

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ABSTRACT

Objective: To investigate the effect of *Moringa oleifera* leaf extract on angiogenesis and inflammatory process in a rat model of streptozotocin-induced diabetic nephropathy.

Methods: Four weeks after a single injection of 50 mg/kg streptozotocin, rats were treated with 100 or 200 mg/kg/day *Moringa oleifera* leaf extract, 1 mg/kg/day dapagliflozin, or a combination of *Moringa oleifera* leaf extract and dapagliflozin for further eight weeks. Renal function, kidney histology, and gene expression were evaluated at the end of the experiment.

Results: Renal function of diabetic rats was significantly impaired as evidenced by increased blood urea nitrogen, albuminuria, 24-h proteinuria, and high creatinine clearance which indicated glomerular hyperfiltration. In addition, diabetic rats showed an increase in gene expressions of vascular endothelial growth factor-A (VEGF-A), angiopoietin-2 (Ang2), the Ang2/Ang1 ratio, tumor necrosis factor-α, interleukin-1β and monocyte chemoattractant protein-1. Immunohistochemical staining demonstrated a significant increase in the density of glycoprotein CD34. *Moringa oleifera* leaf extract markedly improved all renal dysfunction markers and modulated the upregulated expression of angiogenic factors and inflammatory genes.

Conclusions: *Moringa oleifera* leaf extract could suppress abnormal angiogenesis and inflammatory processes possibly by downregulating gene expression of angiogenesis factors and proinflammatory cytokines.

KEYWORDS: *Moringa oleifera* leaf extract; Diabetic nephropathy; Angiogenesis; Angiopoietin; VEGF-A; Inflammation

1. Introduction

Globally, 783 million people are expected to suffer from diabetes by 2045[1]. Diabetic nephropathy (DN) is one of the major diabetic microvascular complications that affects approximately 30%-40% of types 1 and 2 diabetic patients[2]. Based on recent experimental and clinical studies, angiogenesis and inflammation seem to play major roles in both early development and progression of DN[3].

Angiogenesis is the process of forming a new blood vessel from preexisting ones[4]. Abnormal angiogenesis is associated with an increased glomerular filtration surface area, glomerular hypertrophy, and hyperfiltration in the early stage of DN[5]. Abnormal angiogenesis

Significance

Our previous study reported that *Moringa oleifera* leaf extract could attenuate the early stage of diabetic nephropathy in streptozotocin-induced diabetic rats *via* decreasing glomerular basement membrane fibrosis. This study provides additional evidence for the ameliorate effect of *Moringa oleifera* leaf extract on diabetic nephropathy. *Moringa oleifera* leaf extract can alleviate diabetic nephropathy *via* decreasing abnormal angiogenesis and inflammation of glomeruli.

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at this stage contributes to the progression of disease. Interestingly, several studies have revealed the beneficial effect of anti-angiogenic agents to ameliorate glomerular hypertrophy, glomerular hyperfiltration, and albuminuria in early stages of DN[6–8].

Vascular growth factors play an important role in maintaining the integrity of the glomerular filtration barrier. The processes of neovascularization involve interactions between several angiogenic growth factors and cytokines, such as vascular endothelial growth factor-A (VEGF-A), angiopoietin-1 and -2 (Ang1, Ang2), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1)[7]. A new abnormal blood vessel formed in DN is characterized by a thin wall at the basement membrane, while endothelial cells are swollen. Therefore, the new blood vessels are structurally immature, which leads to an increase in capillary permeability and enhanced leakage of albumin into the urine[4]. In patients with diabetic kidney disease, such abnormal vessels may occupy 1%-5% of glomerular capillary area[4].

VEGF-A, one of the growth factors for endothelial cells, is a crucial angiogenesis mediator which stimulates endothelial cell proliferation, migration, and tube formation[9]. Hyperglycemia stimulates both synthesis and secretion of VEGF-A. Increases in VEGF-A and its receptor have been reported in experimental models of DN[10]. A correlation between *VEGF-A* gene expression and glomerular neovascularization has been observed in DN patients[11]. Three types of angiopoietins, the other potential angiogenic mediators, have been identified: Ang1, Ang2, and Ang4. Ang1 and Ang4 are agonistic whereas Ang2 acts as a competitive antagonist for the Tie2-receptor (receptor tyrosine kinases). Ang1 is a physiological ligand that maintains the permeability of the mature vasculature[12] and promotes the formation of blood vessels. Ang2 has the opposing action to Ang1, inducing vasculature destabilization. In diabetic glomerulopathy, kidney Ang1 mRNA levels and protein levels do not change or diminish while those of Ang2 are elevated, resulting in an increase of Ang2/Ang1 ratio[6,13].

Activation of the immune system and chronic inflammation are involved in the pathogenesis of diabetes and also its complication, DN. Several stimulants from the diabetic pathological condition such as high blood glucose, increased renin angiotensin activity and oxidative stress all can activate the inflammatory process in kidney tissue leading to infiltration of monocytes and lymphocytes, which continue to produce inflammatory cytokines[14]. Recent studies have shown that the levels of inflammatory cytokines such as MCP-1, TNF-α, and IL-1β are increased in DN experimental models and also in DN patients[15–17]. In addition, inflammatory cytokines can enhance the upregulation of VEGF-A, which is involved in angiogenesis in DN and in several other chronic inflammatory diseases such as cardiovascular disease, rheumatoid arthritis and diabetic retinopathy[18].

Moringa oleifera (*M. oleifera*), a medicinal plant belonging to the Moringaceae family, is a native of Asia[19]. Leaf extract of *M. oleifera* contains numerous bioactive components including flavonoids, phenolic acids, alkaloids, carotenoids, isothiocyanates, glucosinolates, tannins, saponins, oxalates, and phytates[20]. An oil extracted from *M. oleifera* seed has been reported to have nephroprotective effects on gentamicin-induced nephrotoxicity in rats, possibly *via* anti-inflammatory pathways[21]. Recent studies have revealed anti-angiogenic activity of *M. oleifera* leaf extract *via* inhibiting VEGF-A in streptozotocin (STZ)-induced diabetic retinopathy and breast cancer, in both *in vitro* and *in vivo* models[22,23]. However, the effect of aqueous extracts of *M. oleifera* leaves on angiogenesis and inflammatory processes in DN is still unclear and requires elucidation. Our preliminary experiment showed that *M. oleifera* leaf extract could reduce high blood glucose, suppress renal oxidative stress, and diminish fibrosis in the early stage of DN induced by STZ in rats. Therefore, for further understanding the mechanism of action of *M. oleifera* leaf extract in alleviation of DN, this study aimed to evaluate the effect of *M. oleifera* leaf extract on angiogenesis and inflammatory processes in rats with STZ-induced DN, with an emphasis on the molecular mechanism involved.

2. Materials and methods

2.1. Reagents and drug

STZ and 3,3'-diaminobenzidine (D5637) were purchased from Sigma-Aldrich (MO, USA). Blood urea nitrogen test kit and creatinine test kit were purchased from Erba® reagent (Mannheim, UK). Dapagliflozin was obtained from AstraZeneca Pharmaceuticals (Indiana, USA). Rabbit monoclonal IgG antibody CD34 (Abcam81289) and biotinylated goat anti-rabbit IgG (Abcam64256) were purchased from Abcam (Cambridge, MA, UK). TRIzol® reagent was obtained from Invitrogen (OR, USA). iScript Reverse Transcription Supermix was obtained from BIO-RAD (CA, USA). SYBR-Green/Fluorescein qPCR kit was purchased from biotechrabbit CAPITAL™ (Berlin, Germany).

2.2. M. oleifera leaf extraction

Fresh *M. oleifera* leaves were collected in May 2019 from Khon Kaen Province, Thailand, and were taxonomically identified by Assoc. Prof. Dr. Prathan Luecha. A representative specimen of this plant (PSKKU-PL-015) was deposited at the herbarium of the Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. The leaves were air-dried for 2 d and powdered. This powder (300 g) was boiled twice in 5 L distilled water for 40 min and filtered through cotton and gauze. The filtered solution was evaporated using a rotary evaporator and a freeze-drying machine to obtain a powder (*M. oleifera* leaf extract). About 22.3 g of *M. oleifera* leaf extract was obtained from 300 g of leaves.

Some of the phenolic components in the extraction of *M. oleifera* leaf were analyzed using an HPLC-DAD-based assay. *M. oleifera* leaf extract consisted of isoquercetin (2 672 mg/kg of dry extract), rutin (1 182 mg/kg of dry extract), tannic acid (1 127 mg/kg of dry extract), quercetin (1 034 mg/kg of dry extract), gallic acid (1 032 mg/kg of dry extract), apigenin (307.66 mg/kg of dry extract), and catechin (166.37 mg/kg of dry extract).

2.3. Experimental animals and induction of DN

Male Sprague-Dawley rats (250-300 g) were provided from Nomura Siam International Co., Ltd. Bangkok, Thailand. All rats were housed under controlled temperature [(22 ± 2) ℃] with a 12 h day/night cycle and had free access to food and water. After a week of acclimation, the rats fasted for 16 h, and after that, rats were injected intraperitoneally with a single dose of 50 mg/kg STZ dissolved in 0.1 M sodium citrate buffer at pH 4.5. Four weeks after STZ injection, fasting blood glucose (FBG) was measured using Accu-Chek Active (Roche Diagnostic, Germany). Rats with FBG equivalent to or greater than 200 mg/dL were included in the experiment.

2.4. Experimental design

The experimental groups were divided into 6 groups with 6 rats in each group as follows: Group 1: normal rats receiving distilled water (normal control rats, NC); Group 2: DN rats receiving distilled water (DN control); Group 3: DN rats receiving 100 mg/kg/day *M. oleifera* leaf extract (DN+100 mg/kg *M. oleifera* leaf extract)[24]; Group 4: DN rats receiving 200 mg/kg/day *M. oleifera* leaf extract (DN+200 mg/kg *M. oleifera* leaf extract)[24]; Group 5: DN rats receiving 1 mg/kg/day dapagliflozin (DN+Dapa); Group 6: DN rats receiving 100 mg/kg/day *M. oleifera* leaf extract+1 mg/kg/day Dapa (DN+*M. oleifera* leaf extract+Dapa).

M. oleifera leaf extract and dapagliflozin were dissolved in distilled water. All the treatments were given by gavage daily for eight weeks. At the end of treatment period, animals were placed in metabolic cages to collect 24-h urine samples for determination of urinary metabolites. Afterward, rats were euthanized with an intraperitoneal injection of a combination of tiletamine (25 mg/kg) and zolazepam (25 mg/kg) together with xylazine (5 mg/kg). Blood was collected from the abdominal aorta, centrifuged at 4 500 rpm for 20 min, and the serum was stored at −80 ℃ for further analysis. Kidneys were quickly excised and weighted. The left kidney tissue was fixed in 10% neutral-buffered formalin for histopathological and immunohistochemical analysis. The right kidney was washed with ice-cold normal saline solution and stored at −80 ℃ for molecular analysis.

2.5. Kidney organ coefficient calculation

Kidney organ coefficient, which is an indicator of kidney hypertrophy, was calculated as a ratio of the wet weight of the kidney to the body weight. Kidney organ coefficient = [kidney weight (g)/ body weight (g)] × 100.

2.6. Urine and blood sample analysis

After centrifugation at 3 000 rpm for 10 min, the 24-h urine sample was taken to measure the levels of creatinine (Erba Mannheim, UK), urinary albumin (ab235642 reagent, Abcam, UK), and total urinary protein (Bio-Rad reagent, Protein assay dry, USA). Serum was taken to examine the amount of blood urea nitrogen (BUN) and creatinine using diagnostic kits (Erba Mannheim, UK).

Creatinine clearance (CCr) was calculated on the basis of urinary creatinine, urine volume and serum creatinine. CCr (mL/min) = [urine creatinine (mg/dL) × 24 h urine volume (mL)]/[serum creatinine (mg/dL) × 1 440].

2.7. Renal histopathological examination

The kidneys of rats were fixed in a 10% neutral phosphate-buffered formalin solution. Fixed samples were processed through the conventional paraffin embedding technique. Transverse sections of 4 μm thickness were cut and stained with hematoxylin and eosin (H&E). Histological changes were examined under the light microscope and photographed using a Nikon microscope (Nikon, ECLIPSE Ni-U Melville, NY, USA).

2.8. Immunohistochemical analysis of glycoprotein CD34

CD34 is a surface marker of a variety of vascular endothelial beds and is used in investigation of neovascularization[25,26]. Kidney tissue sections of 5 μm thickness were incubated at 60 ℃ for 30 min for deparaffinization, then rehydrated through an ethanol concentration series (100%, 95%, 90%, 80%, 70%, and 50%) for 1 min at each concentration. To retrieve antigens, kidney tissue sections were boiled in Tris-EDTA plus 0.05% tween 20, pH 9 for 10 min, then quenched with 0.3% hydrogen peroxide solution for 10 min and washed twice for 5 min in Tris-buffered saline (TBS) plus 0.025% Triton X-100. After that, sections were blocked using 10% normal goat serum in TBS plus 0.025% Triton X-100 for 2 h. Rabbit monoclonal-IgG antibody against CD34 (Abcam81289, Cambridge, UK) was applied to slides and incubated overnight at 4 ℃ (dilution 1:2 500). Later, slides were washed twice with TBS plus 0.025% Triton X-100 and further incubated with biotinylated goat anti-rabbit IgG secondary antibody for 1 h (Abcam64256, Cambridge, MA, USA), and sites of the antigen-antibody reaction were visualized using the ABC kit (Vector Laboratories, Burlingame, CA, USA). Finally, immunostaining was visualized using 3,3'-diaminobenzidine (D5637, Sigma-Aldrich®, MO, USA). Photographs of thirty glomeruli from each kidney section were randomly captured using a light microscope at a magnification of 400× (Nikon ECLIPSE Ni-U

Melville, NY, USA). The presence and extent of positive signal (brown color) was analyzed using a quantitative image-analysis system (Image-Pro Plus).

2.9. Real–time polymerase chain reaction (RT–PCR) analysis

Expression levels of *VEGF–A*, *Ang1*, *Ang2*, *TNF-α*, *MCP–1*, and *IL-1β* genes were evaluated using real-time RT-PCR. Total mRNA was extracted from frozen kidneys using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's introduction. Complementary DNA (cDNA) was synthesized from 1 μg of total mRNA using the iScript reverse transcription Supermix (Bio-Rad, USA) at 25 ℃ for 5 min, 42 ℃ for 30 min, and 85 ℃ for 5 min in a C1000 Thermal Cycler (BioRAD, Hercules, CA, USA). Then, the real-time RT-PCR was performed in an Applied Biosystems™ QuantStudio 6 Flex using an SYBR-Green/Fluorescein qPCR kit (biotechrabbit CAPITAL™, Berlin, Germany) detection system. Thermal cycling conditions included pre-incubation at 95 ℃ for 3 min followed by 40 cycles of amplification at 95 ℃ for 15 s and 60 ℃ for 31 s, finally a melting curve at 95 ℃ for 3 s, 72 ℃ for 5 min, and 97 ℃ for 15 s. The specific primers are listed in Table 1. Relative mRNA expression of genes was calculated based on the $2^{-\Delta\Delta Ct}$ method. ΔCt represents the differences in the cycle threshold number between the target gene and *β-actin*. ΔΔCt represents the relative change in the differences between the control and treatment groups.

2.10. Statistical analysis

Statistical analysis was performed using SigmaPlot 12.00 (Systat Software Inc.). For multiple comparisons, one-way ANOVA was followed by Student-Newman-Keuls *post–hoc* tests. The results were presented as mean ± standard error of mean (SEM), and $P<0.05$ was considered to be statistically significant.

2.11. Ethical statement

All procedures were approved by the Institutional Animal Care and Use Committee of Khon Kaen University (IACUC-KKU-NELAC 4/63).

3. Results

3.1. M. oleifera leaf extract lowered FBG and decreased renal hypertrophy in rats with STZ–induced DN

At the end of the experiment, the DN control group showed a marked increase in FBG when compared with normal control rats ($P<0.05$) (Figure 1A). However, DN rats treated with 100 and 200 mg/kg *M. oleifera* leaf extract had significantly lower FBG levels ($P<0.05$) (Figure 1A). As well, treatment with 1 mg/kg dapagliflozin or a combination of *M. oleifera* leaf extract and dapagliflozin

Table 1. Primer sequences.

Primer	Forward sequence	Reverse sequence	Product size (bp)
VEGF–A	GCTGCAATGATGAAGCCCTG	GCTGGCTTTGGTGAGGTTTG	90
Ang1	CCACGCTGAACGGTTACAC	ACTGCTTGTTTGACGCTCTC	200
Ang2	GCACCGCTAACCAACCAAAG	AATGCATGCTGTCCCTGTGA	97
TNF-α	GTAGCCCACGTCGTAGCAAAC	ACCACCAGTTGGTTGTCTTTGA	113
MCP–1	TGTCTCAGCCAGATGCAGTTAAT	CCGACTCATTGGGATCATCTT	77
IL-1β	AGGCTGACAGACCCCAAAAG	CTCCACGGGCAAGACATAGG	178
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG	150

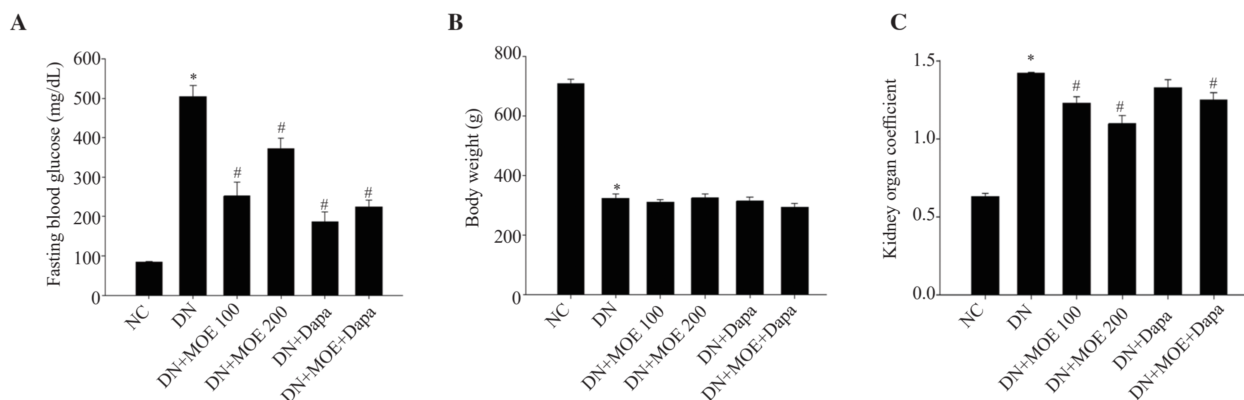

Figure 1. Effects of *Moringa oleifera* leaf extract (MOE) in rats with streptozotocin (STZ)-induced diabetic nephropathy. Fasting blood glucose (A), body weight (B), and kidney organ coefficient (C). Data are shown as mean ± SEM (n=6). $^{*}P<0.05$ *vs.* NC, $^{\#}P<0.05$ *vs.* DN, using one-way ANOVA with Student-Newman-Keuls *post–hoc* tests. NC: normal controls; DN: diabetic nephropathy controls; MOE 100 or 200: *Moringa oleifera* leaf extract 100 or 200 mg/kg; Dapa: 1 mg/kg dapagliflozin.

produced a significant decrease in FBG (P<0.05).

The final body weight of DN control rats was significantly lower (P<0.01) than that of normal control rats (Figure 1B). However, the kidney coefficient of DN control rats was significantly increased, suggesting development of renal hypertrophy in DN rats (Figure 1C). Body weight was not significantly changed in any treatment group relative to the DN control group. Interestingly, treatment with 100 and 200 mg/kg *M. oleifera* leaf extract or a combination of *M. oleifera* leaf extract and dapagliflozin significantly decreased the kidney organ coefficient value in DN rats (P<0.05).

3.2. M. oleifera leaf extract improved renal functions in rats with STZ-induced DN

DN control rats showed increased levels of BUN, CCr, albuminuria, and proteinuria (P<0.05), indicating renal dysfunction (Figure 2). After eight weeks of treatment, the high dose of *M. oleifera* and a combination of *M. oleifera* and dapagliflozin significantly reduced CCr. Moreover, either dose of *M. oleifera* leaf extract or a combination of *M. oleifera* leaf extract and dapagliflozin significantly decreased albuminuria and proteinuria (P<0.05).

However, only the high dose of *M. oleifera* leaf extract and the combination treatment significantly reduced the increased BUN (P<0.05). These data suggested that *M. oleifera* leaf extract could improve renal function in DN rats.

3.3. M. oleifera leaf extract attenuated the histological changes in kidneys of rats with STZ-induced DN

The histological extent of kidney injury was examined in sections stained with H&E. Diabetic rats developed the pathological characteristics of DN including noticeable enlargement of the glomerulus, desquamation of tubular epithelial cells, and vacuolar degeneration of tubular epithelium (Figure 3B). After treatment with *M. oleifera* leaf extract or dapagliflozin, or with the combination of both, all these lesions were reduced (Figures 3C-F). Moreover, the DN control group showed a significant increase in glomerular area (P<0.05), indicating that DN rats developed hypertrophy of the glomerular tuft (Figure 3G). Treatment with *M. oleifera* leaf extract or dapagliflozin or with the combination remarkably lessened glomerular area (P<0.05), which indicated a reduction of glomerular hypertrophy (Figure 3G).

3.4. M. oleifera leaf extract reduced the expression of CD34 in glomerulus

The expression of CD34 in endothelial cells was detected using immunohistochemical staining to evaluate the angiogenesis of glomerular capillaries. The results showed that the normal control rats had a low density of CD34 in glomerular capillaries (Figure 4A), while DN control rats had a high density of CD34 which indicated some angiogenesis (Figure 4B). Treatment with 100 and 200 mg/kg *M. oleifera* leaf extract decreased the expression of CD34 (Figure 4C-D). DN rats treated with dapagliflozin or a combination of *M. oleifera* leaf extract and dapagliflozin also showed significantly reduced expression of CD34 (Figure 4E-F). Quantitative results are shown in Figure 4G. The result of this investigation indicated that *M. oleifera* leaf extract could inhibit angiogenesis of glomerular capillaries in DN rats.

3.5. M. oleifera leaf extract downregulated the mRNA expression levels of angiogenesis-associated genes in rats with STZ-induced DN

The effect of *M. oleifera* leaf extract on the expression of mRNA of angiogenesis-associated genes including *VEGF-A*, *Ang1*, and *Ang2* was determined by real-time qPCR. The mRNA expression of *Ang1* was significantly reduced in DN control rats (P<0.05). In contrast, *M. oleifera* leaf extract at both doses caused a significant upregulation of *Ang1* gene expression compared with DN control rats (P<0.05) (Figure 5A).

Moreover, mRNA expression levels of *VEGF-A* and *Ang2* (acting as an Ang1-antagonist contributing a vascular-disrupting property) were markedly increased in DN control rats as compared to normal control, which was significantly downregulated by administration of *M. oleifera* leaf extract (P<0.05) (Figure 5B and C). In addition, the ratio of *Ang2*/*Ang1* expression was increased in DN rats compared with normal rats (Figure 5D). However, treatment with 100 and 200 mg/kg of *M. oleifera* leaf extract significantly decreased the *Ang2*/*Ang1* ratio (P<0.05). These results indicated that *M. oleifera* leaf extract administration downregulated the expression of genes involved in abnormal angiogenesis in kidney tissue. Dapagliflozin and the combination treatment also reversed the DN-induced changes in these gene expressions.

3.6. M. oleifera leaf extract downregulated the mRNA expression levels of inflammatory mediator genes in rats with STZ-induced DN

As shown in Figure 6, compared with normal rats, the mRNA expression levels of *TNF-α*, *MCP-1*, and *IL-1β* were significantly increased in DN control rats (P<0.05). Treatment with 100 or 200 mg/kg *M. oleifera* leaf extract showed significant suppression of *TNF-α*, *MCP-1*, and *IL-1β* expression (P<0.05). Dapagliflozin, or a combination of *M. oleifera* leaf extract and dapagliflozin, also decreased the mRNA expression of inflammatory mediators. These results demonstrated that *M. oleifera* leaf extract could ameliorate the inflammatory process in the kidney during the chronic diabetic condition.

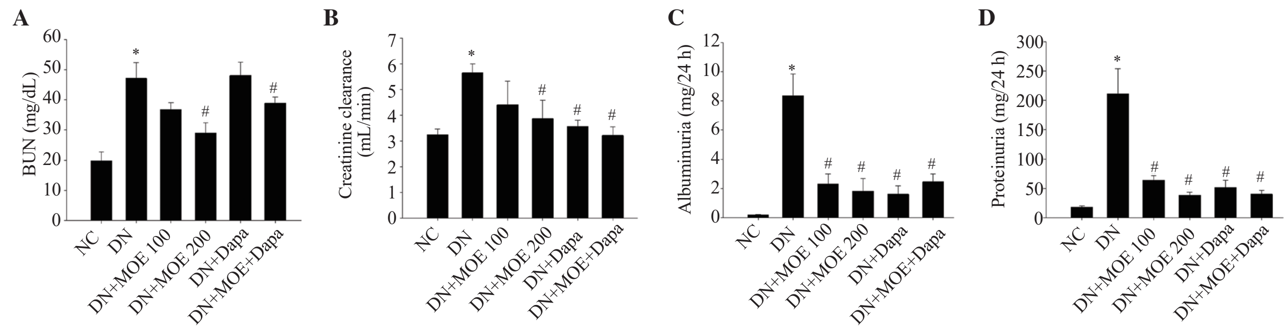

Figure 2. Effects of MOE on renal function in rats with STZ-induced diabetic nephropathy. BUN (A), creatinine clearance (B), albuminuria (C), and proteinuria (D). Data are shown as mean ± SEM (n=6). $^{*}P$<0.05 *vs.* NC, $^{\#}P$<0.05 *vs.* DN, using one-way ANOVA with Student-Newman-Keuls *post–hoc* tests. BUN: blood urea nitrogen.

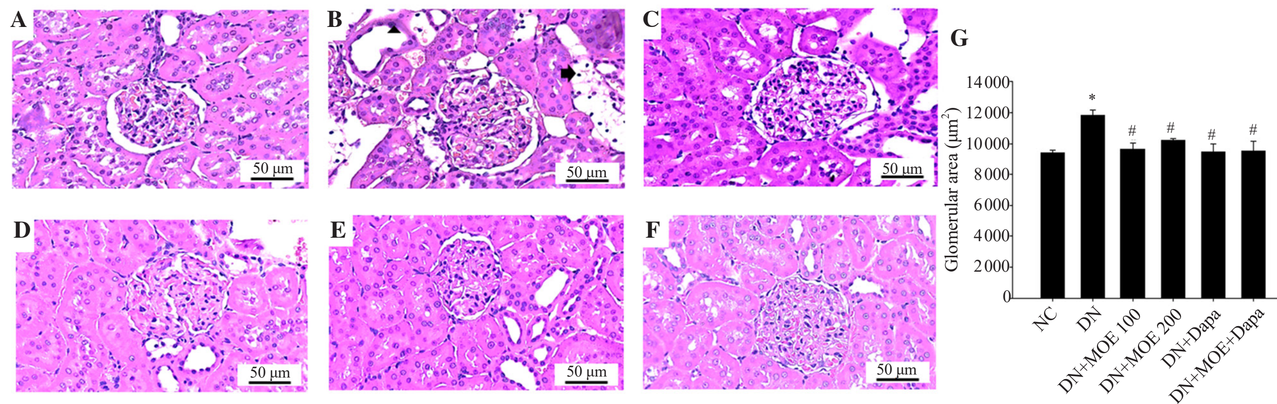

Figure 3. Effects of MOE on histological changes in kidneys of rats with STZ-induced diabetic nephropathy. H&E staining of the kidney sections from each group of rats (400× magnification). Normal control rats (NC) (A); Diabetic nephropathy control rats (DN) showing desquamation of tubular epithelial cells (arrowhead) and vacuolar degeneration of tubular epithelium (arrow) (B); DN treated with 100 mg/kg MOE (C); 200 mg/kg MOE (D); 1 mg/kg dapagliflozin (E) and MOE+dapagliflozin (F). Quantitative analysis of glomerular area of each group (G). Data are shown as mean ± SEM (n=6). $^{*}P$<0.05 *vs.* NC, $^{\#}P$<0.05 *vs.* DN, using one-way ANOVA with Student-Newman-Keuls *post–hoc* tests.

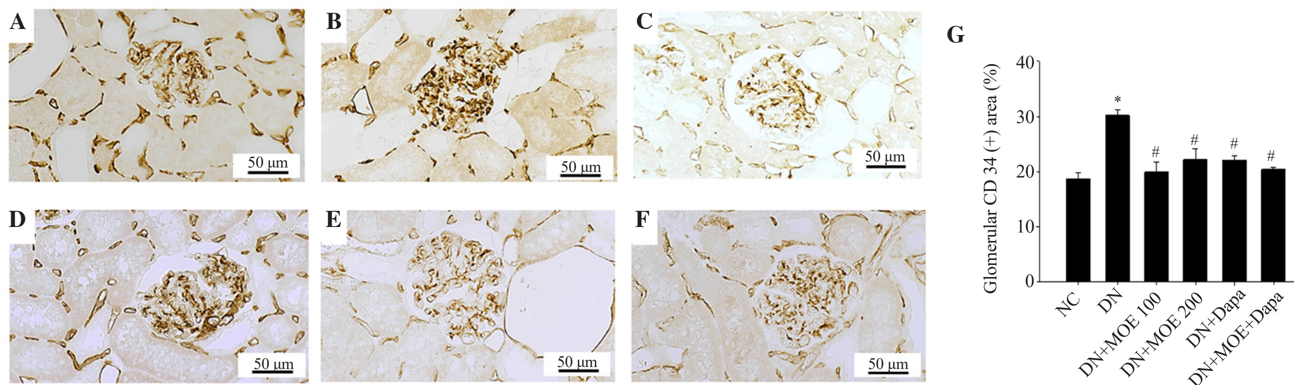

Figure 4. Immunohistochemical staining of CD34, an endothelial-cell marker. Brown color is positive staining. Normal control rats (NC) (A); Diabetic nephropathy control rats (DN) (B); DN rats treated with 100 mg/kg (C) and 200 mg/kg MOE (D), 1 mg/kg dapagliflozin (E), 100 mg/kg MOE and 1 mg/kg dapagliflozin (F). Glomerular CD34 (+) endothelial area (G). Data are shown as mean ± SEM (n=6). $^{*}P$<0.05 *vs.* NC, $^{\#}P$<0.05 *vs.* DN, using one-way ANOVA with Student-Newman-Keuls *post–hoc* tests.

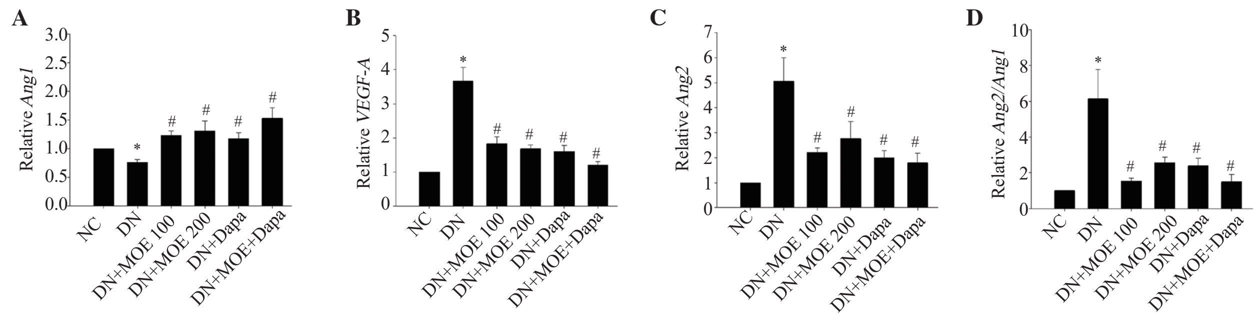

Figure 5. Effects of MOE on the expression of angiogenesis-associated genes in rats with STZ-induced diabetic nephropathy. Angiopoietin-1 (*Ang1*) (A), *VEGF–A* (B), angiopoietin-2 (*Ang2*) (C), and *Ang2/Ang1* ratio (D). Data are shown as mean ± SEM. Relative gene expression was normalized to *β-actin* levels and expressed in arbitrary units as fold change compared with normal control. $^{*}P<0.05$ *vs.* NC, $^{\#}P<0.05$ *vs.* DN, using one-way ANOVA with Student-Newman-Keuls *post–hoc* tests.

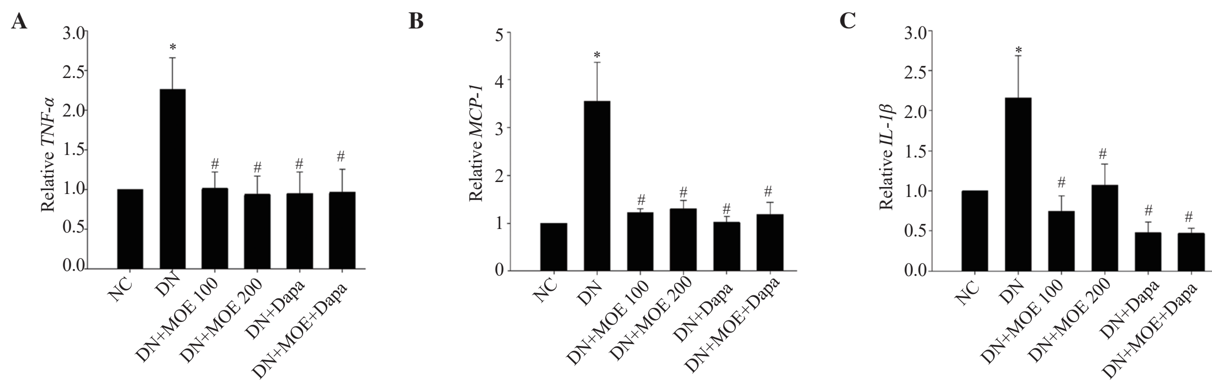

Figure 6. Effects of MOE on the gene expression of inflammatory mediators in rats with STZ-induced diabetic nephropathy. *TNF-α* (A), *MCP-1* (B) and *IL-1β* (C). Data are shown as mean ± SEM. Relative gene expression levels were normalized to *β-actin* levels and expressed in arbitrary units as fold change compared with normal control. $^{*}P<0.05$ *vs.* NC, $^{\#}P<0.05$ *vs.* DN, using one-way ANOVA with Student-Newman-Keuls *post–hoc* tests.

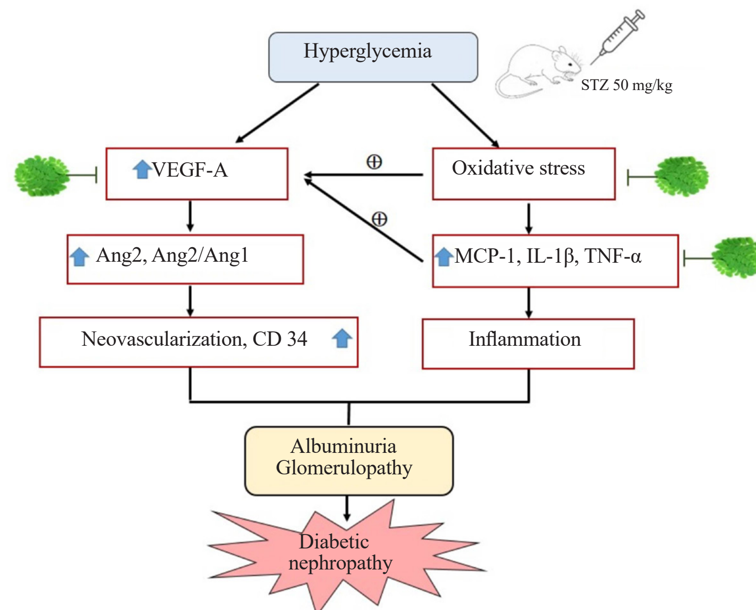

Figure 7. The proposed mechanism of MOE in attenuation of STZ-induced diabetic nephropathy.

4. Discussion

In this study, an STZ-induced diabetic nephropathy model was used to evaluate the nephroprotective effect of *M. oleifera* leaf extract. We found that *M. oleifera* leaf extract significantly reduced FBG and improved renal function parameters including BUN, CCr, albuminuria, and proteinuria. *M. oleifera* leaf extract treatment could reverse the enlargement of glomeruli. Moreover, an immunohistochemical assay showed that CD34 was obviously decreased in angiogenesis of glomerular capillaries. Interestingly, *M. oleifera* leaf extract suppressed the expression of key molecules related to abnormal angiogenesis: VEGF-A, Ang2 and also lowered the Ang2/Ang1 ratio. Our study also showed that *M. oleifera* leaf extract inhibited the inflammatory processes *via* downregulation of the expression of inflammatory mediators: TNF-α, MCP-1, and IL-1β.

Abnormal angiogenesis is associated with an increased glomerular filtration surface leading to glomerular hypertrophy and hyperfiltration in the early stage of DN. Multiple steps are required for the formation of a new blood vessel including 1) the degradation of the vascular basement membrane matrix by protease, 2) migration and proliferation of endothelial cells, 3) endothelial tube formation, 4) recruitment and attachment of mesenchymal cells to the tube, and 5) maturation of blood vessel[27]. Morphological changes of capillaries in abnormal angiogenesis, such as elongation and increased number, contribute to glomerular hypertrophy and are recognized in the early stage of DN[4]. The involvement of pro-angiogenic factors including VEGF-A, Ang1, and Ang2 in the development of neovascularization in DN has been reported[7,28]. The upregulation of VEGF-A and its receptor in the kidney have been observed in the early stage of DN[10]. In the kidneys of DN patients, the degree of neovascularization is significantly increased and correlated with the expression of *VEGF–A* mRNA[11]. Excess angiogenesis would contribute to structural immaturity and high permeability leading to plasma-protein extravasation[4,29]. Moreover, an increased glomerular filtration surface is associated with elevation of CCr which may lead to further severe glomerular dysfunction, such as a rapid progression of the decline of glomerular filtration and increased albuminuria. In our study, an increase of CD34 in the glomerular endothelial area and an upregulation of *VEGF–A* gene expression were found in the kidney tissue of DN rats, which is consistent with previous studies[7,8]. Interestingly, *M. oleifera* leaf extract could decrease the formation of CD34 in glomerular endothelial cells and the expression of *VEGF–A* gene in DN rats, which indicates the suppressive effect of *M. oleifera* leaf extract on the formation of angiogenesis in glomeruli of DN animals. Previously, an ethanolic extract of *M. oleifera* leaves and seeds has also been reported to reduce the expression of VEGF-A in xenograft breast-tumor mice[22].

Ang1 and Ang2 that are other angiogenesis-associated factors bind to the same receptor, the TEK tyrosine-kinase (Tie-2) receptor, which is expressed in endothelial cells, but they exert opposing actions. An imbalance of Ang1 and Ang2 could contribute to the abnormal angiogenesis in DN[30]. Ang1 is a major physiological ligand and is important in maintaining the permeability of mature vasculature[31]. Ang2, the natural antagonist of Ang1, promotes angiogenesis and induces destabilization of blood-vessel walls, resulting in enhancement of vascular permeability[32]. Ang2 can be upregulated by VEGF and Ang2 can enhance VEGF-A-mediated angiogenesis[33,34]. In the early stage of DN, the expression level of Ang1 in the kidney is diminished or not changed while that of Ang2 is increased in rats with STZ-induced DN[13,35]. The findings of the present study are consistent with those of the previous studies. Thus, *M. oleifera* leaf extract has significant ameliorative effects on abnormal angiogenesis. Moreover, we further examined CD34 (a marker protein in endothelial cells) to confirm the increased abnormal angiogenesis in DN rats using immunohistochemical staining and found an increase of CD34 in the glomerular endothelial area in DN rats, which was suppressed by treatment with *M. oleifera* leaf extract. This result further confirms that *M. oleifera* leaf extract alleviated abnormal angiogenesis.

Activation of the immune system and chronic inflammation are involved in the pathogenesis of diabetes and DN. Therefore, we evaluated the effect of *M. oleifera* leaf extract on production of inflammatory cytokines in DN rats. Consistent with other reports[36], our results showed that mRNA expression levels of *TNF-α*, *MCP–1*, and *IL-1β* were elevated in the kidneys of DN rats. All of these cytokines are well-known to recruit macrophages to the sites of inflammation, and to have an important role in the development and progression of DN[37]. It is proposed that the persistence of hyperglycemia, renin angiotensin system, oxidative stress, and NF-κB signaling may induce the expression of the inflammatory chemokine MCP-1 in kidney cells[38]. The elevation of MCP-1 level may upregulate cytokine production such as IL-1, IL-6, and TNF-α *via* the recruitment of macrophages and monocytes to the site of inflammation[38]. TNF-α directly causes cytotoxicity to renal cells, triggering renal cell injury, apoptosis, and necrotic cell death. Previous studies revealed that the expression of TNF-α was increased both in urine and renal tissue in STZ-induced diabetic rats[39]. In DN patients, an elevation of serum TNF-α is implicated in the initiation and progression of microalbuminuria[40]. Additionally, high levels of IL-1β have been found in serum and urine of experimental models and DN patients, which has been related to increased albuminuria[41,42]. Interestingly, in the present study, we found that *M. oleifera* leaf extract could suppress the mRNA expression of *MCP–1*, *TNF-α*, and *IL-1β* in rats with STZ-induced DN. These findings indicate that *M. oleifera* leaf extract may ameliorate the chronic inflammatory process which is involved in the pathogenesis of DN.

Dapagliflozin, a sodium-glucose co-transporter 2 inhibitor, has an

impact on improving renal hemodynamics and protecting against DN. Several preclinical studies have demonstrated that a sodium-glucose co-transporter 2 inhibitor attenuates etiological factors responsible for the development of diabetic kidney disease including oxidative stress[43], inflammation, and fibrosis[44]. In a clinical trial, dapagliflozin treatment reduced albuminuria in diabetic patients[45]. In our study, treatment of DN rats with dapagliflozin also improved renal function. Additionally, the gene expressions of angiogenesis-associated factors as well as inflammatory cytokines were suppressed by administration of dapagliflozin. However, the combination treatment (100 mg/kg *M. oleifera* leaf extract and 1 mg/kg dapagliflozin) did not enhance the DN-ameliorative activity of *M. oleifera* leaf extract or dapagliflozin alone.

The limitation of this study is that the angiogenesis in the early stages of DN in type 1 diabetes was established in an animal model using STZ-induced hyperglycemia in which morphological changes in the kidney of this animal model might not represent the changes in humans with type 2 diabetes. Moreover, the efficacy of this plant extract needs to be further verified in future investigations.

In conclusion, *M. oleifera* leaf extract may ameliorate DN in rats with STZ-induced diabetes by decreasing FBG, improving renal function, and suppressing the mRNA expressions of angiogenesis-associated factors and inflammatory cytokines (Figure 7). *M. oleifera* leaf extract may have therapeutic effects against the early stage of DN.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

PP, PT and RT developed conception and design of the work; LS and WH supervised molecular investigation and immunostaining procedures; RT performed the experiments and data collection; PP, PT, RT, LS and WH contributed the data analysis and interpretation; RT wrote a first draft of the article; PP made a critical revision of the article; PP, PT, RT, LS and WH gave the final approval of the version to be published.

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