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Moringa oleifera leaf extract ameliorates early stages of diabetic nephropathy in streptozotocin-induced diabetic rats

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ABSTRACT

Diabetic nephropathy (DN) is a long-term complication of diabetes mellitus. The characteristic of early-stage DN is glomerular hyperfiltration that has been linked to renal fibrosis. In this study, we investigated the effect of *Moringa oleifera* leaf extract (MOE) on DN in streptozotocin (STZ)-induced DN rats. Rats were injected with 50 mg/kg STZ to establish the DN model. Four weeks after receiving an injection of STZ, DN rats were administered distilled water, MOE (100 or 200 mg/kg body weight/day), dapagliflozin (1 mg/kg body weight/day), or combinations of these for further 8 weeks. DN rats exhibited significantly increased blood glucose (504.00 ± 28.41 mg/dl), proteinuria (192.85 ± 41.23 mg/24 hour), albuminuria (6.68 ± 1.54 mg/24 hours), blood urea nitrogen (47.14 ± 5.18 mg/dl), and creatinine clearance (5.64 ± 0.35 ml/minute) ($p < 0.05$) together with significantly increased malondialdehyde and decreased superoxide dismutase and catalase ($p < 0.05$). Administration of MOE could significantly reduce the high blood glucose, impaired renal function, and oxidative stress parameters of DN rats ($p < 0.05$). Histological examination of kidneys showed a thickening of the glomerular basement membrane and an increase in the mesangial matrix; all of these pathological changes were improved by MOE administration. The mRNA expression of transforming growth factor-beta 1 (TGF-β1) and collagen type IV were significantly increased in the kidney tissue of DN rats but were significantly downregulated in MOE -treated rats ($p < 0.05$). MOE could alleviate DN plausibly due to its activities in reducing blood glucose, oxidative stress, and fibrosis formation by downregulating the expression of TGF-β1 and collagen type IV genes. MOE may be useful as an alternative or supplementary medicine for treatment of DN.

INTRODUCTION

Diabetic nephropathy (DN) is a chronic decline of kidney function occurring in diabetic patients. As many as 40% of diabetic patients develop DN, which is a primary cause of end-stage renal disease (ESRD) (Abdelrahman *et al*., 2019). DN is also a serious microvascular complication of diabetes mellitus. Multiple mechanisms are involved in the pathogenesis and progression of DN including hyperglycemia, inflammation, fibrosis formation, and oxidative stress (Lin *et al*., 2018; Zeng *et al*., 2019). Early alterations in DN include glomerular hyperfiltration, expansion of the mesangial matrix, thickening of the glomerular basement membrane (GBM), and albuminuria. Finally, there is a decline of glomerular filtration rate and ESRD develops (Persson and Rossing, 2018).

An imbalance in control of pre- and post-glomerular arteriole tone is a potential mechanism leading to the development of glomerular hyperfiltration (Palatini, 2012; Tonneijck *et al*., 2017). It has been proposed that hyperglycemia induces the production of angiotensin II (Ang II) which causes vasoconstriction of the efferent arteriole resulting in increased glomerular capillary pressure (Patinha *et al*., 2013). The induction of oxidative stress by hyperglycemia plays an important role in the progression of DN (Jha *et al*., 2016). Several studies have revealed that an increased

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production of reactive oxygen species (ROS) in renal tissue can cause renal cell damage and dysfunction. Therefore, attenuation of this increase in ROS, by elevating cellular antioxidants such as catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD), could be useful in alleviating DN (Vodošek Hojs *et al*., 2020). In addition, the accumulation of extracellular matrix (ECM) in the glomerulus is one of the characteristics of DN (Kolset *et al*., 2012). This may be caused by hyperglycemia and the increased ROS that activates the production of fibrotic factors (Bhattacharjee *et al*., 2016) such as transforming growth factor-β1 (TGF-β1), which is well known to be associated with renal cell hypertrophy, and other fibrotic factors such as fibronectin, α-smooth muscle actin (α-SMA), and collagen type IV (Zeng *et al*., 2019). These ECM proteins accumulate in glomeruli and are deposited in the GBM, which is a hallmark of DN (Braga Gomes *et al*., 2014).

Moringa oleifera belongs to the family Moringaceae and is grown in many tropical and subtropical countries, including Thailand where it is called "Ma-Rum." *Moringa oleifera* is used in Thai traditional medicine as a cardiotonic, antihypertensive, hypocholesterolemic, and antipyretic agent and to detoxify poison (Vergara-Jimenez *et al*., 2017). It contains numerous bioactive compounds. These include vitamins, flavonoids, phenolic acid, isothiocyanate, and tannins (Leone *et al*., 2015). Some of the bioactive compounds in *M. oleifera* seed and leaf extract have been reported to reduce hyperglycemia (Jaiswal *et al*., 2013; Oh and Jun, 2014). Also, the antioxidant effects of extracts from all parts of *M. oleifera* have been demonstrated both *in vitro* and *in vivo* (Aekthammarat *et al*., 2019; Jaiswal *et al*., 2013). An anti-fibrotic effect of *M. oleifera* seed extract has been reported in CCl_4-induced liver fibrosis (Hamza, 2010). A low dose of *M. oleifera* seed extract has also been demonstrated to improve renal function markers and serum electrolytes, inhibit release of pro-inflammatory molecules, and attenuate oxidative stress in STZ-induced DN (Al-Malki and El Rabey, 2015). As mentioned above, many different interacting mechanisms are involved in DN development and progression, and the effect of *M. oleifera* leaf extract (MOE) on DN is still unexplored. In this study, we hypothesized that MOE can ameliorate diabetes-induced kidney dysfunction. Herein, we investigated the effect of MOE on the early stage of DN development using both physiological and histological approaches, and especially the molecular mechanism of action of MOE in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Moringa oleifera aqueous leaf extract (MOE) preparation

Moringa oleifera leaves were harvested at Khon Kaen Province, Thailand. About 300 g of dried leaves powder were boiled two times in 5,000 mL of dH_2O for 40 minutes. After that, the extract was filtered with cotton and gauze. An aqueous extract of MOE was intensified using a rotary evaporator and freeze-dried using lyophilization to obtain dry powder. The percent yield, based on the dry weight of leaves, was 22.3% (w/w). The leaves were authenticated as belonging to *M. oleifera* by Assoc. Prof. Dr. Prathan Luecha, and a voucher specimen (number: PSKKU-PL-015) was deposited at Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Determination of phenolic compounds in MOE

Phenolic compounds were determined by HPLC-DAD method (Penarrieta *et al*., 2007). The quantities of phenolic components were evaluated by establishing the standard calibration curves using isoquercetin, rutin, tannic acid, quercetin, gallic acid, apigenin, and catechin.

Induction of diabetes in experimental animals

Male Sprague-Dawley rats (250–300 g) were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand. Animals were kept in the Northeast Laboratory Animal Center, Khon Kaen University. They were housed at a controlled room temperature (22°C ± 2°C) with a 12-hour light/dark cycle, fed a standard chow diet, and provided with water ad libitum. All procedures involving animals and their care were carried out in accordance with the guidelines of the Ethics of Animal Experimentation of the National Research Council of Thailand. The work was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (IACUC-KKU-NELAC 4/63).

After a week of acclimation, diabetes was induced in overnight fasting rats by a single intraperitoneal injection of STZ (50 mg/kg, in 0.01 M cold citrate buffer, pH 4.5). Four weeks later, rats with fasting blood glucose (FBG) concentration of 200 mg/dl or above were regarded as diabetic and included in further study. The rats were randomized into six groups, each of six animals: normal control group (NC), DN control group, two MOE-treated diabetic groups (100 or 200 mg/kg/day), dapagliflozin-treated diabetic group (Dapa 1 mg/kg/day, a standard antidiabetic drug), and diabetic group treated with combination of MOE 100 mg/kg and dapagliflozin 1 mg/kg (MOE + Dapa). All drugs were dissolved in distilled water and were orally administered once a day for eight consecutive weeks. At the end of the study, in week 12, rats were placed in metabolic cages for the collection of 24 hour urine for albuminuria, proteinuria, and creatinine assays. After that, all rats were euthanized with the combination of tiletamine, zolazepam, and xylazine given intraperitoneally. Blood was collected from the abdominal aorta, and both kidneys were removed and weighed. The longitudinal section of the left kidney tissue was used for histological examination and the rest of the kidney was kept at −80°C for oxidative stress examination and real-time polymerase chain reaction (PCR) analysis.

Examination of renal hypertrophy

Kidney index was calculated as [kidney weight (g)/Body weight (g)] × 100.

Biochemical analysis

Glucose levels in blood obtained from the tail vein, just before euthanizing, were measured using a glucometer (Accu-Chek Active, USA). After euthanizing, blood samples were withdrawn from abdominal aorta and separated by centrifugation at 4,500 rpm for 20 minutes. The serum was used for estimating the levels of serum creatinine (SCr) and blood urea nitrogen (BUN) using commercially available kits as per the manufacturer's instructions. The 24 h urine samples were centrifuged (10 minutes, 3,000 rpm) and the supernatant was collected to determine urinary creatinine (Erba^2 reagent, Mannheim, UK), urinary albumin

(ab235642 reagent, Abcam, UK), and total urinary protein (Bio-Rad reagent, Protein Assay Dye, USA). Creatinine clearance (CCr) was calculated using the following equation:

CCr (ml/minute): [urine creatinine (mg/dl) × urine volume (ml)]/[SCr(mg/dl) × 1440].

Measurement of oxidative stress markers and activity of antioxidant enzymes

The concentration of malondialdehyde (MDA), an indicator of lipid peroxidation, was measured as previously described (Kukongviriyapan *et al*., 2015). Briefly, MDA interacts with thiobarbituric acid to form a pink thiobarbituric-acid reaction product. The amount of MDA in serum or kidney tissue homogenate was calculated using a standard curve of 1,1,3,3 tetraethoxypropane and expressed as micromolar and micromolar per milligram kidney protein, respectively.

SOD and CAT activities were examined in both serum and kidney tissue. SOD activity was examined by quantifying the inhibition of nitroblue tetrazolium reduction in a xanthine-xanthine oxidase system as previously described (Sun *et al*., 1988). The calculation of SOD activity in percent inhibition, where one unit of SOD is defined as that amount of SOD causing half the maximum inhibition of nitroblue tetrazolium reduction. The activity of CAT was determined using formation of a stable complex of hydrogen peroxide with ammonium molybdate as previously described (Góth, 1991). The percentage of inhibition of formation of the yellow product was determined and compared with the standard (hepatic bovine CAT).

Determination of Ang II in serum

Serum Ang II was estimated using ELISA kits according to the manufacturer's instructions (RAB0010-1KT Sigma, USA).

Histopathological examination

Kidney tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 µm, and stained with periodic acid-Schiff (PAS) and Masson's trichrome. The sections were inspected under the light microscope at 400× magnification. Mesangial matrix fraction was calculated as the ratio of PAS-positive area to the total glomerular tuft area. Forty glomeruli from each rat were imaged and analyzed using ZEN 2 software (Blue edition) (Carl Zeiss Microscopy, Germany) to evaluate the glomerular volume (Gv) as described previously (Giribabu *et al*., 2017).

Real-time PCR analysis

Total RNA was extracted from the kidney using the TRIzol reagent (Invitrogen, Carlsbad, CA) and quantified by NanoDrop 2000/2000c spectrophotometer. Then, complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the iScript reverse transcription Supermix (Bio-Rad, USA), performed as instructed by the manufacturer.

The mRNA expression levels of TGF-β1 and collagen type IV were analyzed using an SYBR-Green qPCR kit. The real-time PCR program was as follows: a preincubation at 95°C for 3 minutes (1 cycle), amplification at 95°C for 15 seconds and at 60°C for 31 seconds (40 cycles), and a final step of melting curve at 95°C for 3 seconds and at 72°C for 5 minutes. Relative mRNA expression of genes was calculated based on the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin. The primers for TGF-β1, collagen type IV, and β-actin are listed in Table 1.

Statistical analysis

All results are displayed as mean ± SEM. The statistical analysis was done using SigmaPlot version 12.00 (Systat Software Inc., USA). One-way analysis of variance (ANOVA) followed by Student Newman–Keuls tests were performed to compare the multiple groups. Any p-values <0.05 were considered statistically significant.

RESULTS

Phenolic components of MOE

According to the HPLC-DAD method, MOE comprises large amounts of isoquercetin, rutin, tannic acid, quercetin, gallic acid, apigenin, and catechin (Table 2).

Effect of MOE on body weight, kidney index, and blood glucose in STZ-induced DN rat model

Decreasing in body weight is usually observed in STZ-injected animals. In this study, the body weight of DN rats was significantly lower ($p < 0.01$) than that of the NC group. However, no significant difference in body weight was observed between DN-treated groups and DN control group (Table 3). The kidney index was significantly greater in the DN control group than in the NC group ($p < 0.05$), indicating renal hypertrophy in the DN group. Treatment with MOE (100 or 200 mg/kg) and the combination of 100 mg MOE and 1 mg Dapa significantly reduced the relative kidney weight ($p < 0.05$) as compared with the DN control group.

At 4 weeks after the STZ injection, FBG levels in all diabetic groups were similar in the range of 391–458 mg/dl, but at the end of 8 weeks of treatment, DN control rats had a marked increase in FBG (Table 3). Treatment of diabetic rats with 100 or 200 mg/kg MOE, 1 mg/kg Dapa, or a combination of MOE and Dapa significantly ($p < 0.05$) decreased FBG relative to DN controls (Table 3).

Table 1. Primer sequences.

Primer name	Forward sequence	Reverse sequence	Product size (bp)
TGF-β1	GCGGACTACTACGCCAAAGA	TGCTTCCCGAATGTCTGACG	129
Collagen-IV	GGATCCAGCTTCCAAGGACC	CTCCTGTTGGGGCAAAGTCT	115
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG	150

Effect of MOE on renal function-related parameters in STZ-induced DN rat model

BUN, CCr, proteinuria, and albuminuria were significantly increased in the DN group compared to the NC rats, confirming that STZ-injected rats had developed DN (Fig. 1). Increased CCr is an indicator of glomerular hyperfiltration which has been well recognized as an early pathological change in DN. Treatment of DN rats with high dose of MOE or a combination of MOE and Dapa significantly reduced BUN, CCr, proteinuria, and albuminuria as compared to the DN controls. A lower dose of MOE could only ameliorate the proteinuria and albuminuria whereas Dapa alone ameliorated all changes except the increased BUN. These results indicated that MOE could improve kidney function of DN rats.

Effect of MOE on renal oxidative stress parameters in STZ-induced DN rat model

Free radicals-induced renal damage is one of the causes of DN. As we found that MOE could improve kidney function of DN rats, we explored the effects of MOE on oxidative stress in kidney tissue and serum by measuring levels of MDA, SOD, and CAT. When compared with the NC group, the DN control group exhibited a significant increase in serum and kidney tissue MDA levels. Treatment with MOE, Dapa, and the combination of these significantly decreased MDA levels in kidney tissue and serum of DN rats (Fig. 2A and B). Both SOD and CAT antioxidant enzyme activities were significantly ($p < 0.05$) lower in serum and kidney tissue of DN rats. Interestingly, treatment with MOE significantly enhanced activity of both of these enzymes in DN rats (Fig. 2C and D). Dapa and the combination treatment also significantly decreased MDA levels and increased SOD and CAT activities in serum and kidney tissue (Fig. 2).

Table 2. The phenolic components of MOE according to HPLC-DAD.

Phenolic compounds	Contain (mg/kg dry extract)
Isoquercetin	2,672
Rutin	1,182
Tannic acid	1,127
Quercetin	1,034
Gallic acid	1,032
Apigenin	307.66
Catechin	166.37

Effect of MOE on Ang II levels in STZ-induced DN rat model

Ang II has been proposed to play an important role in glomerular hyperfiltration in DN, so we examined the level of this hormone in DN rats. Ang II levels in the DN control group were significantly ($p < 0.05$) greater than those in normal controls (Fig. 3). However, MOE and the combination of MOE and Dapa did not significantly reduce the Ang II levels. In the Dapa-treated group, the level of Ang II was comparable to that in the DN control group.

Effect of MOE on renal morphological changes in rats with STZ-induced DN

Structural changes occurring in DN involve thickening of the GBM and expansion of the mesangial matrix. Rats were sacrificed at the end of the experiment and sections of their kidneys were stained with PAS dye to highlight basement membranes of glomerular capillary loops and the accumulation of mesangial matrix in glomeruli. PAS staining showed that DN rats had glomerular hypertrophy and mesangial matrix expansion and that the proximal tubules had more vacuolar degeneration than NC rats (Fig. 4A and B). Treatment with MOE, Dapa, or the combination of MOE and Dapa improved these histological changes (Fig. 4C–F).

Forty glomeruli from each rat were analyzed using ZEN version 2 software program to calculate the glomerular volume and using Image-Pro Plus software to measure the PAS-positive area (deep red color) of mesangial matrix. Glomerular volume was significantly increased, and mesangial matrix was significantly expanded in DN rats (Fig. 4G and H). Treatment with MOE, Dapa, or the combination of these significantly decreased ($p < 0.05$) glomerular volume and extent of mesangial matrix expansion in diabetic rats (Fig. 4G and H).

Renal fibrosis is one of the pathological characteristics of DN. Masson's trichrome staining was used to identify sites of collagen deposition. Collagen fibers were stained blue, cytoplasm was stained red, and nuclei were stained brown. In the NC group,

Table 3. Body weight, relative kidney weight, and blood glucose level of rats in the different groups at the end of the experiment.

Group	Body weight (g)	Relative kidney weight	Blood glucose (mg/dl)
NC	700.13 ± 19.70	0.63 ± 0.02	84.75 ± 2.06
DN	293.13 ± 12.54*	1.42 ± 0.01*	504.00 ± 28.41*
DN + 100 mg MOE	304.50 ± 15.52	1.23 ± 0.04#	251.80 ± 34.44#
DN + 200 mg MOE	307.33 ± 8.58#	1.10 ± 0.05#	398.00 ± 32.24#
DN + 1 mg Dapa	336.50 ± 15.08	1.33 ± 0.05	208.17 ± 28.74#
DN + 100 mg MOE + 1 mg Dapa	324.00 ± 30.65	1.25 ± 0.05#	238.67 ± 20.02#

NC: Normal control group, DN: Diabetic nephropathy control group receiving distilled water, MOE: *M. oleifera* extract, and Dapa: dapagliflozin. Shown are means ± SEM ($n = 6$).
*$p < 0.05$ versus NC.
#$p < 0.05$ versus DN, using one-way ANOVA with Student–Newman–Keuls *post-hoc* tests.

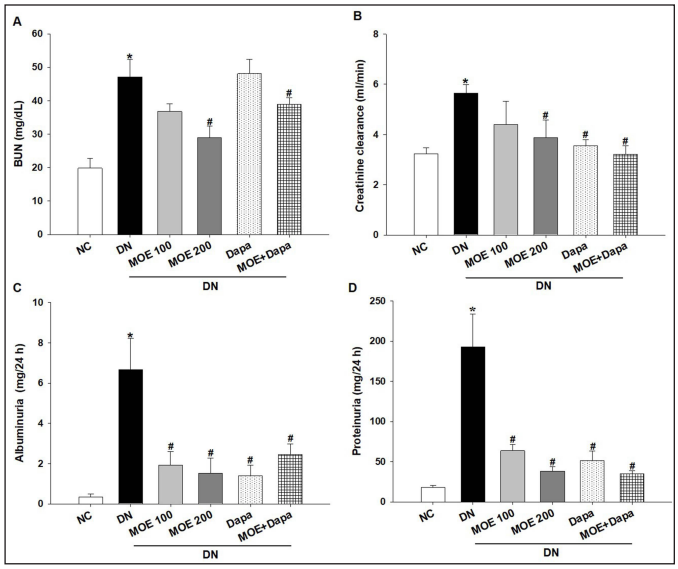

Figure 1. Effects of MOE on renal function parameters in rats with STZ-induced DN. BUN (A), CCr (B), Albuminuria (C), and Proteinuria (D). Shown are means ± SEM ($n = 6$). $^{*}p < 0.05$ versus NC; $^{\#}p<0.05$ versus DN, using one-way ANOVA with Student–Newman–Keuls *post-hoc* tests.

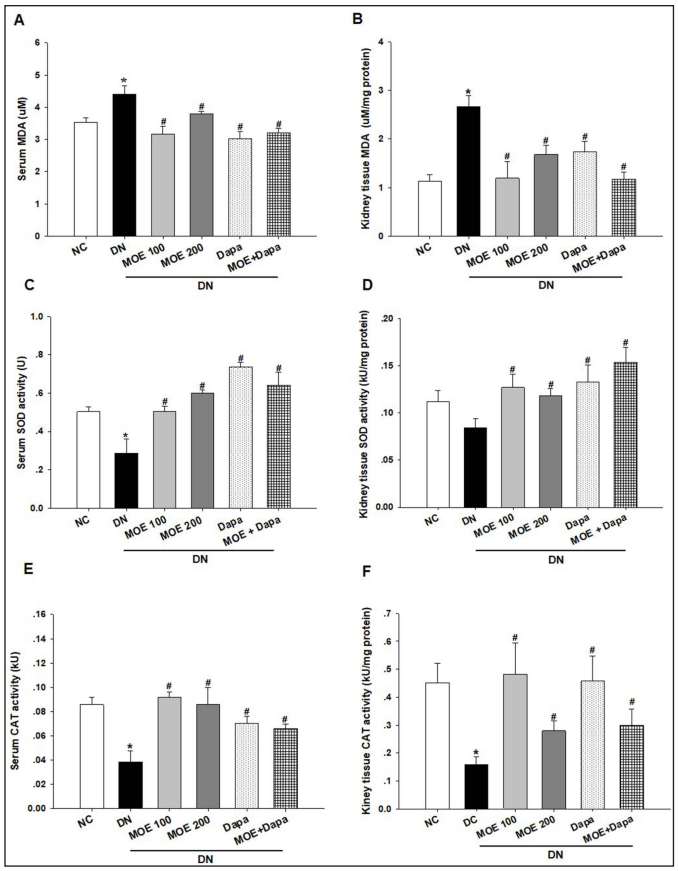

Figure 2. Effects of MOE on markers of oxidative stress in rats with STZ-induced DN. MDA levels in serum and kidney tissue, respectively (A and B), SOD activity in serum and kidney tissue (C and D), and CAT activity in serum and kidney tissue (E and F). Shown are means ± SEM ($n = 6$). $^{*}p < 0.05$ versus NC; $^{\#}p<0.05$ versus DN, using one-way ANOVA with Student–Newman–Keuls *post-hoc* tests.

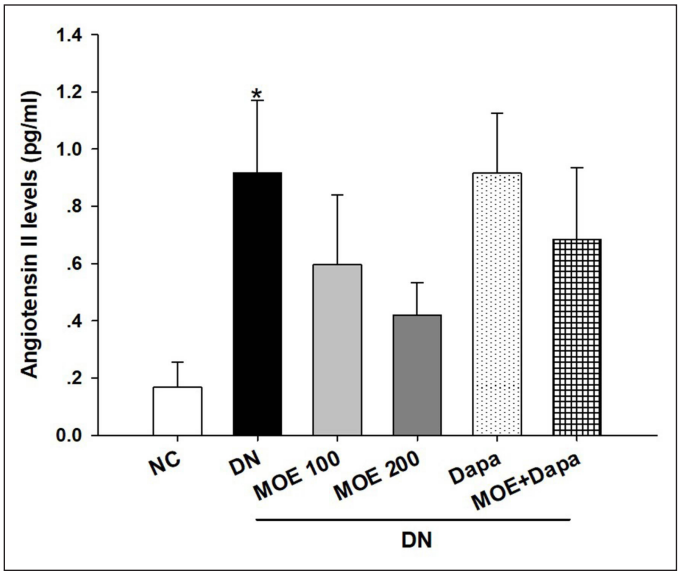

Figure 3. Effect of MOE on serum Ang II levels in rats with STZ-induced DN. Shown are means ± SEM ($n = 6$). $^{*}p<0.05$ versus NC; $^{\#}p<0.05$ versus DN, using one-way ANOVA with Student–Newman–Keuls *post-hoc* tests.

no collagen fibers were observed (Fig. 5A), but in the DN control group, collagen fibers were found around the glomerular, renal tubular, and renal interstitial areas (Fig. 5B). After treatment with MOE, Dapa, and the combination of these, collagen fiber staining in these areas was lessened (Fig. 5C–F).

Effect of MOE on TGF-β1 and collagen type IV gene expression in kidney tissue

TGF-β1 is a key cytokine in the development of renal inflammation and fibrosis, which is a common finding in DN and is characterized by accumulation of ECM, mainly comprising collagen types I and III and basement membrane collagen type IV. We, therefore, investigated mRNA expression of TGF-β1 and collagen type IV in kidney tissue. Interestingly, we found high expression levels of both TGF-β1 and collagen type IV mRNA in kidney tissue of DN rats as compared to normal rats (Fig. 6A and B). All treatments led to decreased gene expression of both TGF-β1 (Fig. 6A) and collagen type IV (Fig. 6B) as compared to DN control rats.

DISCUSSION

Extracts of *M. oleifera* leaves have long been used in Asian traditional medicine to treat hypertension and dyslipidemia (Mbikay, 2012). In this experiment, we found that MOE significantly reduced FBG and improved the kidney functional impairment. In addition, MOE inhibited lipid peroxidation, enhanced antioxidant enzyme activity, and decreased GBM fibrosis possibly via downregulation of the expression of fibrosis formation genes: TGF-β1 and collagen type IV genes.

Administration of STZ causes diabetes by damaging the pancreatic β cells, leading to a deficit in insulin secretion and hyperglycemia (Lenzen, 2008). Hyperglycemia is the major factor that induces the development and progression of DN. In this study, DN rats showed a massive increase in FBG levels, as has been noted by others (Mestry *et al*., 2017; Tuorkey, 2016; Yadav *et al*.,

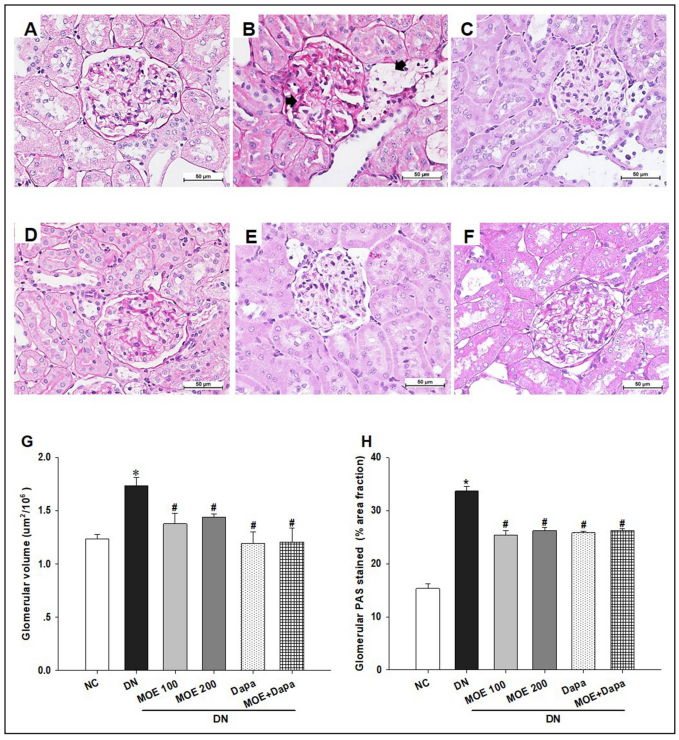

Figure 4. Effects of MOE on glomerular volume and ECM accumulation in rats with STZ-induced DN. PAS-stained section of a normal control rat (A), rat with DN (B, left arrow shows mesangial matrix accumulation and right arrow shows vacuolar degeneration in a renal tubule), 100 mg MOE (C), 200 mg MOE (D), 1 mg Dapa (E), and MOE + Dapa-treated rats (F) with original magnification of 400×. The glomerular volumes (G) and area of mesangial matrix (H) are shown. Shown are means ± SEM (n = 6). *p < 0.05 versus NC; #p < 0.05 versus DN, using one-way ANOVA with Student–Newman–Keuls *post-hoc* tests.

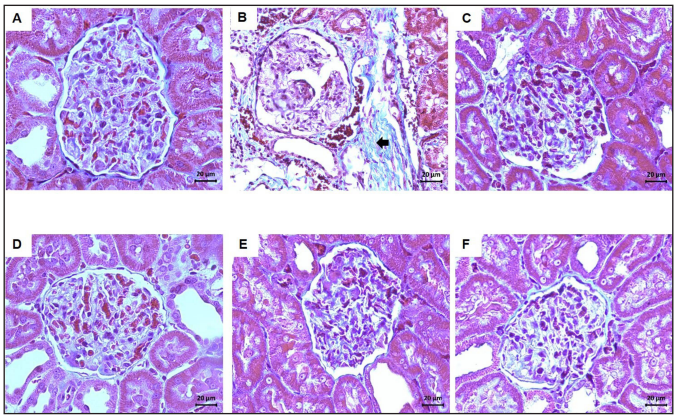

Figure 5. Masson's trichrome staining for collagen deposition (black arrow). Normal control rat (NC) (A), DN rat (B), 100 mg MOE (C), 200 mg MOE (D), 1 mg Dapa (E), and MOE + Dapa (F).

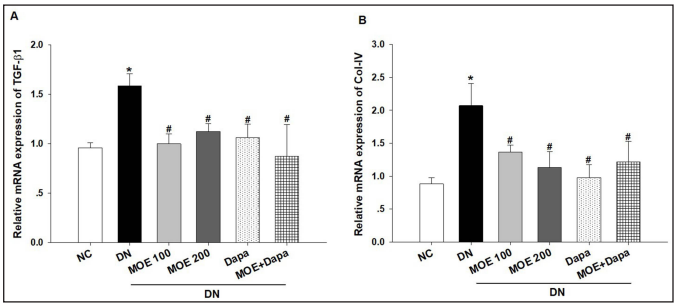

Figure 6. Effects of MOE on the expression of TGF-ß1 (A) and collagen type IV mRNA (B) in rats with STZ-induced DN. Relative gene expression values were normalized to β-actin levels and expressed as fold change compared with the NC group. Shown are means ± SEM (n = 6). *p < 0.05 versus NC; #p < 0.05 versus DN using one-way ANOVA with Student–Newman–Keuls *post-hoc* tests. Original magnification, 400×.

2013; Yassa and Tohamy, 2014), and MOE could reduce levels of blood glucose. It is possible that MOE contains several kinds of flavonoids, such as quercetin and kaempferol, which have been reported to reduce blood glucose by decreasing the intestinal absorption of glucose by competitive inhibition of the sodium-glucose linked transporter type 1 (SGLT 1) (Ndong *et al*., 2007). Moreover, quercetin can inhibit glucose transporter 2 (GLUT2) which is involved in the glucose absorption at small intestine basolateral membrane (Vargas-Sánchez *et al*., 2019).

Characteristics of early-stage DN include the development of glomerular hyperfiltration, increased albuminuria, and histological changes such as glomerular basement-membrane thickening, ECM accumulation in the mesangium and interstitium, and effacement of podocyte foot processes (Lin *et al*., 2018). Glomerular hyperfiltration is likely due to increased intraglomerular pressure or glomerular barrier damage, or a combination of these. Several studies have revealed that glomerular hyperfiltration is associated with initiation and progression of renal injury in DN (Palatini, 2012; Patinha *et al*., 2013; Tonneijck *et al*., 2017). The increased vasoactive effect of humoral mediators, which controls pre- and post-glomerular arteriole tone, leads to glomerular hyperfiltration (Tonneijck *et al*., 2017). Hyperglycemia causes an increase in sodium reabsorption through SGLT 2 at proximal tubules, resulting in decreasing Na^+ delivery to the macula densa, which then activates tubuloglomerular feedback (TGF). The activation of the TGF signal can activate the renin–angiotensin system (RAS) (Takenaka *et al*., 2015). The role of RAS, one of the hormonal mediator systems, is associated with renal hemodynamics. Ang II interacts with the angiotensin 1 receptor on vascular smooth muscle causing an increase in efferent arteriole resistance leading to increased intraglomerular pressure and GFR, which is called glomerular hyperfiltration (Patinha *et al*., 2013). We also found that rats with STZ-induced DN had a significantly elevated level of serum Ang II and increased CCr, which also indicated glomerular hyperfiltration. Although MOE did not modify the production of Ang II, administration of MOE did improve the glomerular hyperfiltration, as indicated by the decrease in CCr when MOE was administered. Remarkably, albuminuria and proteinuria were improved by MOE treatment. Therefore, MOE can improve kidney function in early-stage DN.

Oxidative stress plays a crucial role in the pathogenesis of DN. Several metabolic pathways, including activation of protein kinase C, increased activity of the mitochondrial electron transport chain, and formation of advanced glycation end products, are associated with hyperglycemia-induced production of ROS (Singh *et al*., 2011). Increased ROS generation can subsequently induce auto-oxidation in amino acids, DNA, and lipids. Moreover, the overproduction of ROS could trigger podocyte damage through the Wnt/β-catenin pathway, leading to their dysfunction and albuminuria (Zhou *et al*., 2019). Several studies of DN have

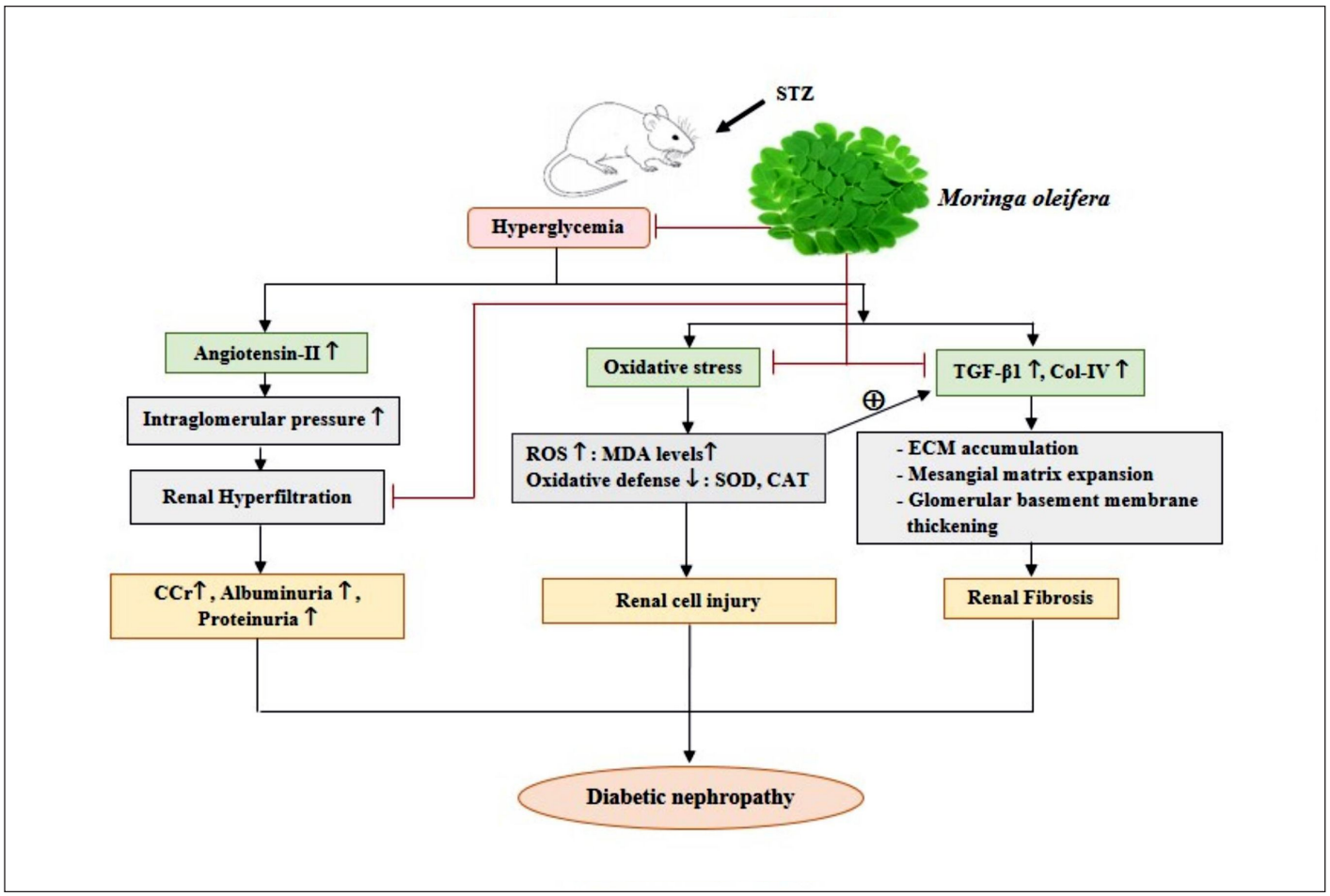

Figure 7. A proposed schematic diagram of the nephropathic protective effect of MOE in rats with STZ-induced DN. MOE could improve renal function including hyperfiltration and slow down the progression of DN by reducing oxidative stress both systemically and in kidney tissue (MDA↓, SOD↑, and CAT↑) and decreasing fibrosis formation by downregulating the expression of TGF-β1 and collagen type IV genes.

revealed the depletion of the main antioxidant defenses in the cells, including GSH, SOD, and CAT (Forbes *et al*., 2008; Jha *et al*., 2016). In our study, the level of MDA, an indicator of lipid peroxidation, was significantly increased, and SOD and CAT levels were significantly decreased in rats with STZ-induced DN. Treatment with MOE diminished the MDA levels both in serum and kidney tissue and enhanced the activity of SOD and CAT in serum and kidney tissue of DN rats. According to our previous report, MOE similarly showed anti-oxidant effects in L-NAME hypertensive rats by ameliorating MDA levels and enhancing antioxidant activities. Moreover, *in vitro*, MOE exerts free-radical scavenging properties (Aekthammarat *et al*., 2019). The antioxidant activity of MOE in DN animal model in this study also corresponds with other reports (Al-Bayumi *et al*., 2021; Al-Malki and El Rabey, 2015; Wen *et al*., 2022). Thus the antioxidant activity of MOE may contribute to kidney injury lessening effect and especially ameliorate the consequences of the pathological changes in DN.

Glomerular hyperfiltration is usually accompanied by glomerular hypertrophy caused by an increase in accumulation of ECM component in the mesangium (Kroustrup *et al*., 1977; Malatiali *et al*., 2008) and thickening of the GBM. Hyperglycemia-induced oxidative stress plays a critical role in the induction of the expression of TGF-β1. The involvement of TGF-β1 in the pathogenesis of DN is well known: increased production of downstream molecules comprising of fibronectin, laminin, and collagen type IV, leading to increased thickness of GBM, expansion of mesangial cells, and interstitial fibrosis (Braga Gomes *et al*., 2014; Tervaert *et al*., 2010). Consistent with previous studies on the role of TGF-β1, we found increased PAS and Masson's trichrome-positive areas in sections of kidney tissue and increased glomerular volume associated with increased expression of both TGF-β1 and collagen type IV genes in DN rats. Interestingly, MOE treatment could ameliorate these histological changes and downregulate the expression of TGF-β1 and collagen type IV genes in DN rats. The downregulation of the expression of TGF-β1 and collagen type IV genes may be one of mechanisms of action of MOE in improving kidney function in DN. Since free radicals can induce the expression of TGF-β1, the antioxidant activity of MOE may also play some part in suppressing TGF-β1 expression. Moreover, the previous study reported the anti-renal fibrosis activities of *M. oleifera* seeds extract by activating glycogen synthase kinase-3 beta (GSK-3β) pathway (Wen *et al*., 2022).

Dapagliflozin, an antidiabetic drug, inhibits SGLT2 in the kidney resulting in decreasing of blood glucose, and its renoprotective effect has been demonstrated (Huang *et al*., 2019; Oraby *et al*., 2019). In our study, treatment with dapagliflozin in rats with STZ-induced DN also led to significant improvement of renal function. Moreover, dapagliflozin suppressed the production of oxidative-stress markers and expression of the fibrotic cytokine TGF-β1 and collagen type IV genes, as well as having an improved histological picture consistent with previous studies (Abdel-Wahab *et al*., 2018; Oraby *et al*., 2019). However, the combination treatment (100 mg MOE and 1 mg Dapa) did not seem to enhance the DN amelioration activity of MOE or Dapa alone.

CONCLUSION

In conclusion, our results suggested that MOE exhibits renoprotective effects in rats with STZ-induced DN. MOE can significantly improve renal function and slow down the progression of DN. The pharmacological efficacy of MOE treatment is plausibly due to its activities in (a) reducing oxidative stress both systemically and in kidney tissue and (b) decreasing fibrosis formation by downregulating the expression of TGF-β1 and collagen type IV genes (Fig. 7). This novel finding supports the development of MOE as a natural alternative medicine in the treatment of early-stage DN.

AUTHORS' CONTRIBUTIONS

P. T., P. P., and R. T. developed the conception of this work and designed the experiment; L. S., K. B., and S. S. supervised molecular technique and histological investigation; R. T. performed the experiments and data collection. All the authors contributed to the data analysis and interpretation; the manuscript text was mainly written by R. T. and P. T.; all the authors gave the final approval of the version to be published.

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CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

ETHICAL APPROVALS

The research protocol for the animal study was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (IACUC-KKU-NELAC 4/63).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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