

Gallic Acid Alleviates Diabetic Glomerulopathy in Diabetic Mice through Suppression of p47phox and TGF- β 1

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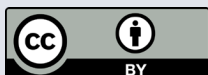
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ABSTRACT

OBJECTIVE This study aims to evaluate whether gallic acid (GA) alleviates diabetic glomerulopathy in a type 2 diabetic mouse model.

METHODS ICR mice were fed a high-fat diet (60% fat) and providing a 25% (w/v) fructose solution for 10 weeks and given multiple intraperitoneal injections of streptozotocin (STZ) (35 mg/kg) to induce type 2 diabetes (T2D) and kidney injury. After diabetes onset, mice were treated with GA at 30 or 60 mg/kg/day for four weeks. Fasting glucose levels, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), renal malondialdehyde (MDA) levels, renal superoxide anion radicals ($O_2^{\cdot-}$) formation, glomerular histology, and the expression of p47phox, a key regulator of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and transforming growth factor beta 1 (TGF- β 1), were assessed.

RESULTS Diabetic mice exhibited marked hyperglycemia, increased HOMA-IR, and elevated renal oxidative stress, as demonstrated by higher MDA levels, enhanced $O_2^{\cdot-}$, and increased p47phox expression ($p < 0.05$ vs. controls). Histological analysis showed glomerular hypertrophy, enlarged glomerular volume, expanded Bowman's space, thickening of the glomerular basement membrane, and raised TGF- β expression ($p < 0.05$ vs. controls). Notably, GA treatment significantly reduced these pathological changes, with the 60 mg/kg/day dose being more effective than the 30 mg/kg/day dose.

CONCLUSION GA effectively reduces oxidative stress and mitigates structural kidney alterations in a diabetic mouse model, highlighting its potential as an adjunct therapy for diabetes-induced kidney injury.

KEYWORDS gallic acid, diabetic glomerulopathy, type 2 diabetic mice, oxidative stress

INTRODUCTION

Diabetic kidney disease (DKD), a primary microvascular complication of type 2 diabetes (T2D), is driven by chronic hyperglycemia (1). This sustained hyperglycemic state accelerates glomerulopathy by promoting glomerular hyperfiltration, oxidative stress, inflammation, and fibrosis within the kidney (2). These pathological processes lead

to structural changes, including glomerular basement membrane (GBM) thickening, expanded Bowman's space, and increased glomerular volume, ultimately impairing glomerular filtration and permeability (3, 4).

At the molecular level, the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and the upregulation of transforming

growth factor-beta 1 (TGF- β 1) are recognized as key mediators of diabetic glomerulopathy (5, 6). The NOX complex, whose activation is critically dependent on its regulatory subunit p47phox, generates an excess of superoxide anions ($O_2^{\cdot-}$). This surge in reactive oxygen species drives oxidative stress and inflammation, disrupting glomerular homeostasis. Concurrently, the cytokine TGF- β 1 promotes fibrosis by recruiting inflammatory cells, inducing epithelial-mesenchymal transition (EMT), and stimulating extracellular matrix accumulation within the glomerulus (7, 8).

Gallic acid (GA; 3,4,5-trihydroxyl-benzoic acid) is a natural phenolic compound found in numerous fruits and vegetables. As a potent anti-oxidant, GA has demonstrated pharmacological effects in animal studies, including blood glucose reduction, decreased albuminuria, and kidney protection under diabetic conditions (9, 10). However, despite these promising results, data on its specific effects in diabetic glomerulopathy remain limited, and the underlying mechanisms of its protective action are still unknown.

To investigate T2D-associated kidney injury, a well-established mouse model combining a high-fat diet (HFD) with multiple low-dose injections of streptozotocin (STZ) was used. This dual approach effectively mimics the human condition: the HFD induces peripheral insulin resistance, while subsequent STZ injections partially destroy pancreatic β -cells, leading to impaired insulin secretion. The model successfully recapitulates the progression of T2D and key pathological alterations of DKD, such as glomerular hypertrophy and renal fibrosis, making it a valuable tool for studying the pharmacological effects of GA.

This study aims to examine the protective effect of GA on glomerulopathy in HFD/STZ-induced T2D mouse model.

METHODS

Animals and treatment protocols

Male ICR mice, aged five weeks and weighing 25 to 30 grams, were obtained from and housed in the Northeastern Laboratory Animal Center, Khon Kaen University, Thailand. The animals were housed in a room with an HVAC (Heating, Ventilation, and Air-Conditioning) system and were maintained on a 12-hour light/dark cycle. The mice had unrestricted (*ad libitum*) access to

standard drinking water and were provided with a standard chow diet from Charoen Pokphand Co. Ltd., Thailand. All animal procedures were approved by the Khon Kaen University Institutional Animal Ethics Committee (IACUC-KKU-25/66).

Forty mice were randomly assigned to one of five groups: a control group (group 1, $n = 8$) that received deionized water (5 mL/kg BW/day, *i.g.*), and four diabetic groups. Diabetes was induced in the diabetic groups by feeding a 60% HFD and providing a 25% (w/v) fructose solution for 10 weeks, with a STZ injection at week 4. Diabetic mice (fasting blood glucose > 200 mg/dL 72 hours post-STZ) were divided into four subgroups at week 7 and received daily oral gavage treatments for 4 weeks: group 2 (DM, $n = 8$) received deionized water (5 mL/kg BW/day), group 3 (DM+ GA30, $n = 8$) received GA (30 mg/kg BW/day), group 4 (DM+ GA60, $n = 8$) received GA (60 mg/kg BW/day), and group 5 (DM+ Met200, $n = 8$) received metformin (200 mg/kg BW/day), with all treatments dissolved in deionized water.

Measurement of body weight and blood glucose profile

Body weight was measured weekly and fasting blood glucose every two weeks. An intraperitoneal glucose tolerance test (IPGTT) was performed in weeks 0, 4, 6, and 10. For the IPGTT, overnight-fasted mice were injected with glucose (2 g/kg, *i.p.*), and blood glucose was monitored at 15-, 30-, 60-, and 120-minutes post-injection using a glucometer (ACCU-CHEK®, Roche, Germany).

At the end of the study, blood was collected via cardiac puncture to obtain plasma. Insulin concentrations were quantified using a commercial sandwich ELISA kit (Millipore Corp., MA, USA) according to the manufacturer's protocol. Insulin resistance was calculated using the homeostasis model assessment (HOMA-IR) formula: [Fasting Glucose (mmol/L) $\times$ Fasting Insulin (μ IU/mL)]/22.5.

Assay of production rate of $O_2^{\cdot-}$ in kidney tissues

$O_2^{\cdot-}$ production in kidney tissue was quantified using a lucigenin-enhanced chemiluminescence assay (11). Excised kidney sections were incubated with lucigenin (60 μ M), and the resulting signal was measured with a luminometer. Results were normalized to tissue dry weight and expressed as relative light units (RLU)/mg/min.

Measurement of MDA in kidney tissues

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) levels using a thiobarbituric acid reactive substances (TBARS) assay, following established procedures (11, 12). Briefly, 150 μ L of kidney homogenate was mixed with 10% trichloroacetic acid, 5 mM EDTA, 8% SDS, and 0.5 μ g/mL butylated hydroxytoluene and incubated at room temperature. Subsequently, 0.6% thiobarbituric acid was added, and the mixture was heated at 95°C for 30 minutes. After cooling, the samples were centrifuged to pellet the precipitate, and the absorbance of the supernatant was measured at 532 nm. MDA concentrations were calculated from a standard curve generated with 1,1,3,3-tetraethoxypropane (0.3–10 μ M).

Assessment of histomorphometry of glomeruli

The upper portion of the kidney was fixed in 4% phosphate-buffered paraformaldehyde for 48 hours, then dehydrated in graded ethanol (70%, 80%, 95%, and 100%, 1 hour each). Afterward, the tissue was cleared with xylene (three 1-hour changes) and infiltrated with xylene–paraffin mixtures (2:1, 1:1, and 1:2 ratios, 1 hour each), followed by pure paraffin overnight. Finally, the specimens were embedded in paraffin blocks, sectioned at 2 μ m, and stained with Hematoxylin and Eosin (H&E) for morphological and glomerular volume examination.

Assessment of ultrastructure of glomeruli by transmission electron microscopy

The kidneys were quickly harvested and cut into cubic millimeter sized pieces. The tissues were collected and fixed in 2.5% glutaraldehyde at 4°C, washed with PBS, ethanol, and acetone, and then embedded. Ultrathin sections were prepared and post-stained with a 2% saturated solution of uranyl acetate and lead citrate. Ultrastructural changes in the GBM and podocytes were observed using transmission electron microscopy (TEM) at 15,000 $\times$ magnification. Measurements and calculations were performed to determine GBM and podocyte by using the ImageJ software (NIH).

Western blot analysis of renal p47phox and TGF- β expression

Protein expressions of p47phox and TGF- β 1 in kidney homogenates were quantified by Western

blot, adapted from Sangartit et al. (13). Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon®, Germany), and probed with p47phox (Cell Signaling Technology, Danvers, MA, USA) and TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies and an HRP-conjugated secondary antibody. Bands were visualized using an ECL substrate (Super Signal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA), and their intensities were captured and normalized to β -actin as a loading control.

Statistical analysis

Statistical analysis was performed to compare the results among the different groups. The data are presented as the mean $\pm$ standard error of the mean (SEM). To determine whether there were significant differences among the groups, a one-way analysis of variance (ANOVA) was conducted. If the ANOVA revealed a significant difference, a post-hoc Tukey test was performed to identify which specific groups differed from one another. The level of statistical significance was set at a p -value of less than 0.05, indicating that any observed differences with a p -value below this threshold were considered statistically significant.

RESULTS

Effect of GA on body weight and blood glucose in diabetic mice

Mice were successfully induced with T2D after administration of a HFD combined with multiple doses of STZ injection, as demonstrated by significantly increased body weight and elevated fasting blood glucose levels in the diabetic group compared to controls ($p < 0.05$, Figure 1A, 1B). Treatment with GA at both 30 mg/kg and 60 mg/kg doses effectively reduced blood glucose levels to levels comparable with metformin treatment ($p < 0.05$, Figure 1B), though body weight remained unchanged across all treatment groups (Figure 1A).

The IPGTT revealed impaired glucose clearance in the diabetic mice, with prolonged elevated plasma glucose levels compared to controls ($p < 0.05$, Figure 1C, 1D). Both GA treatment groups and Met significantly improved glucose clearance capacity ($p < 0.05$, Figure 1C, 1D).

Insulin resistance assessment using HOMA-IR showed markedly elevated levels in the diabetic group versus controls ($p < 0.05$; Table 1). GA treatment dose-dependently reduced insulin resistance, with HOMA-IR values of 1.78 for the 30 mg/kg group and 1.19 for the 60 mg/kg group (Table 1). Met treatment achieved similar improvement with a HOMA-IR. These results indicate that GA treatment, particularly at 60 mg/kg/day, effec-

tively alleviates insulin resistance in diabetic mice comparable to Met therapy.

Effect of GA on histomorphological changes of glomeruli in diabetic mice

H&E staining of kidney tissue revealed that persistent hyperglycemia in the DM group led to significant structural damage, indicative of kidney injury. Compared to controls, DM mice exhibited

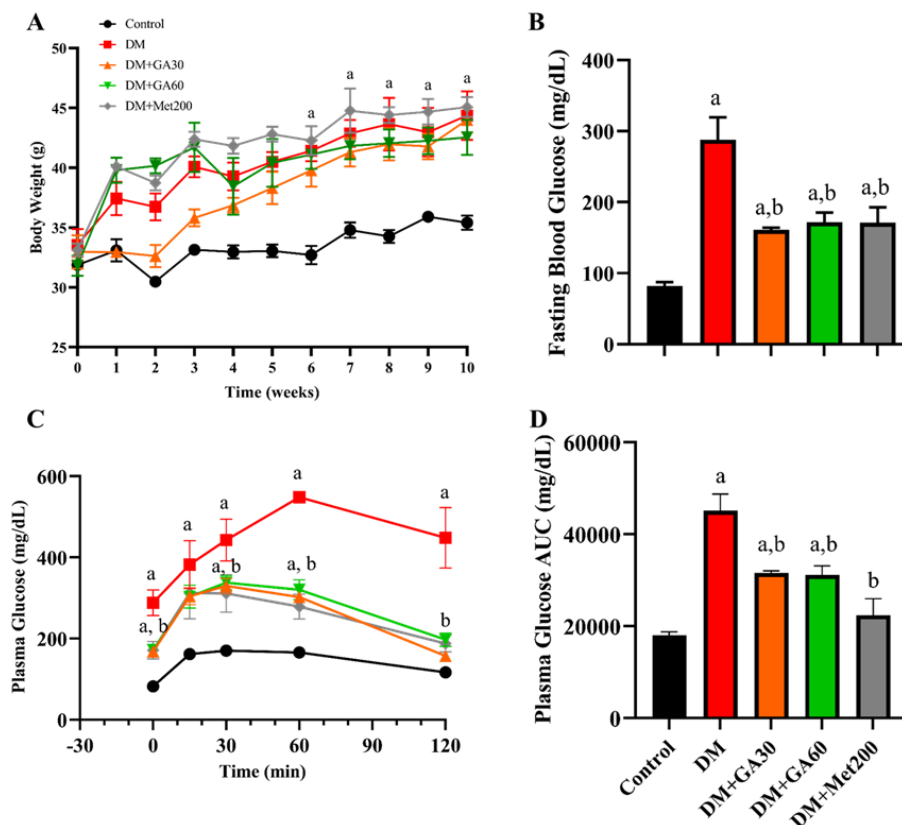


Figure 1. Effect of GA on body weight and blood glucose in diabetic mice. This figure displays changes in body weight over the study duration (A), fasting blood glucose levels at week 10 (B), and glucose progression during an IPGTT at week 10 (C), with the corresponding area under the curve (AUC) depicted in (D). Data are presented as means $\pm$ SEM. Statistical comparisons are indicated as follows: ^a $p < 0.05$ compared to the control group; ^b $p < 0.05$ compared to the DM group.

Table 1. Effect of GA on fasting insulin, fasting glucose and HOMA-IR in diabetic mice at week 10

Group	Fasting insulin (μ U/mL)	Fasting glucose (mg/dL)	HOMA-IR
Control	1.52 $\pm$ 0.23	90.34 $\pm$ 7.30	0.33 $\pm$ 0.08
DM	5.10 $\pm$ 1.93 ^a	251.57 $\pm$ 11.58 ^a	2.47 $\pm$ 0.76 ^a
DM+GA30	4.30 $\pm$ 0.87 ^{a,b}	184.53 $\pm$ 14.22 ^{a,b}	1.78 $\pm$ 0.21 ^{a,b}
DM+GA60	3.21 $\pm$ 0.14 ^{a,b,c}	170.92 $\pm$ 9.68 ^{a,b}	1.19 $\pm$ 0.06 ^{a,b,c}
DM+Met200	3.42 $\pm$ 0.60 ^{a,b,c}	168.56 $\pm$ 8.37 ^{a,b,c}	1.17 $\pm$ 0.05 ^{a,b,c}

Data is presented as means $\pm$ SEM; Statistical significance is indicated as follows; ^a $p < 0.05$ compared to the control group, ^b $p < 0.05$ compared to the DM group, and ^c $p < 0.05$ compared to DM+GA30 group.

marked glomerular hypertrophy, characterized by significant increases in glomerular area, glomerular volume, and Bowman's space area ($p < 0.05$, Figure 2A, 2C-2E). Crucially, these pathological changes were counteracted by therapeutic intervention. GA administration (60 mg/kg/day) effectively normalized the renal morphology (Figure 2C-2E), while Met also provided significant protection by alleviating these structural alterations ($p < 0.05$). This demonstrates that both GA and Met can protect against or reverse the detrimental morphological effects of diabetes on the kidney.

Effect of GA on ultrastructural changes of glomeruli in diabetic mice

TEM at 15,000x magnification was used to examine ultrastructural changes in renal tissues, focusing on the GBM, podocytes, and filtration slit pores (Figure 3A).

The diabetic group demonstrated significant GBM thickening and increased podocyte base width compared to controls ($p < 0.05$, Figure 3C, E). Additionally, filtration slit pore numbers were notably reduced in diabetic mice (Figure 3D, $p < 0.05$ vs. controls), indicating impaired glomerular filtration structure.

Treatment with GA at both 30 mg/kg and 60 mg/kg doses effectively reversed these structural alterations, with improvements comparable to Met treatment ($p < 0.05$). Both GA and Met successfully restored normal GBM thickness, podocyte morphology, and filtration slit pore density in diabetic mice (Figure 3C-3E).

Effect of GA on renal oxidative stress in diabetic mice

The evaluation of oxidative stress through MDA measurement demonstrated significantly elevated levels in renal tissues of diabetic mice compared to controls ($p < 0.05$, Figure 4A). This increase in MDA levels indicates heightened lipid peroxidation and oxidative damage in diabetic conditions.

Treatment with GA at both 30 mg/kg and 60 mg/kg doses effectively reduced elevated MDA levels ($p < 0.05$ vs. DM; Figure 4A). Met treatment produced comparable results, significantly decreasing MDA levels in renal tissues ($p < 0.05$ vs. DM; Figure 4A). These findings demonstrate that both GA and metformin effectively mitigate diabetes-associated oxidative stress.

Analysis of superoxide anion production in kidney tissue revealed significantly increased

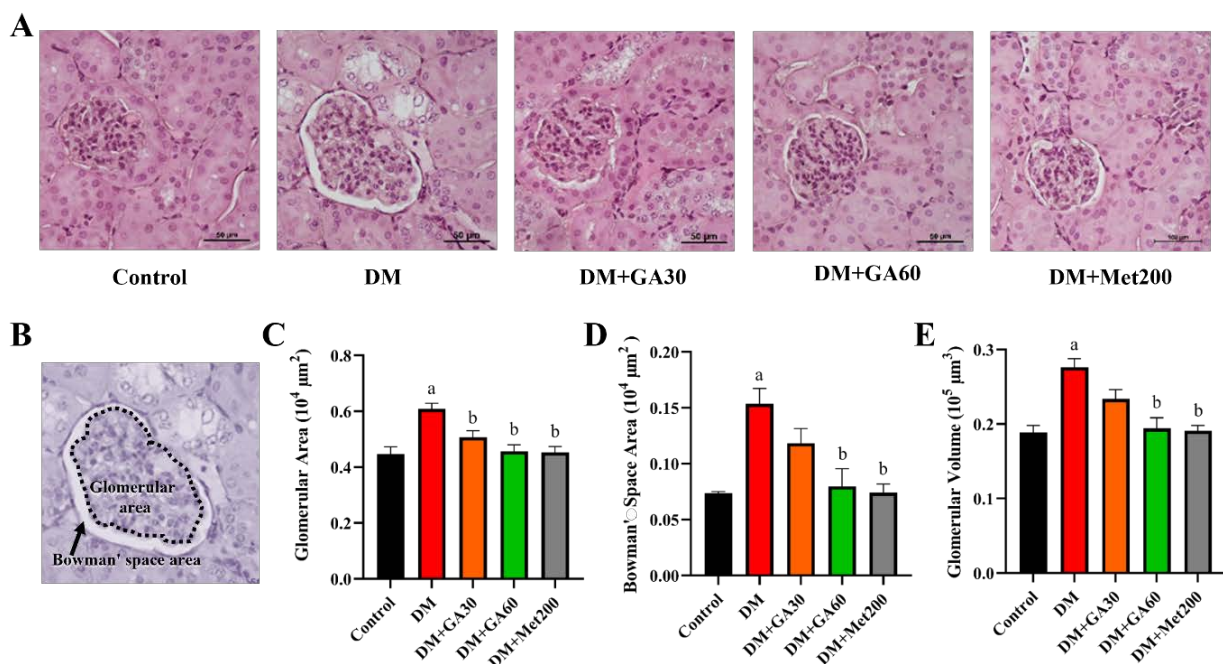


Figure 2. Effect of GA on histomorphological changes of glomeruli in diabetic mice. Representative glomerular sections (H&E) from $n = 3$ animals per group were photographed at $400\times$ (A). Glomerular area is outlined by the dotted line; Bowman's space is the clear area outside the outline and is indicated by the black arrow (B). Quantification of glomerular area (C), Bowman's space area (D), and glomerular volume (E) are shown. Data are means $\pm$ SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

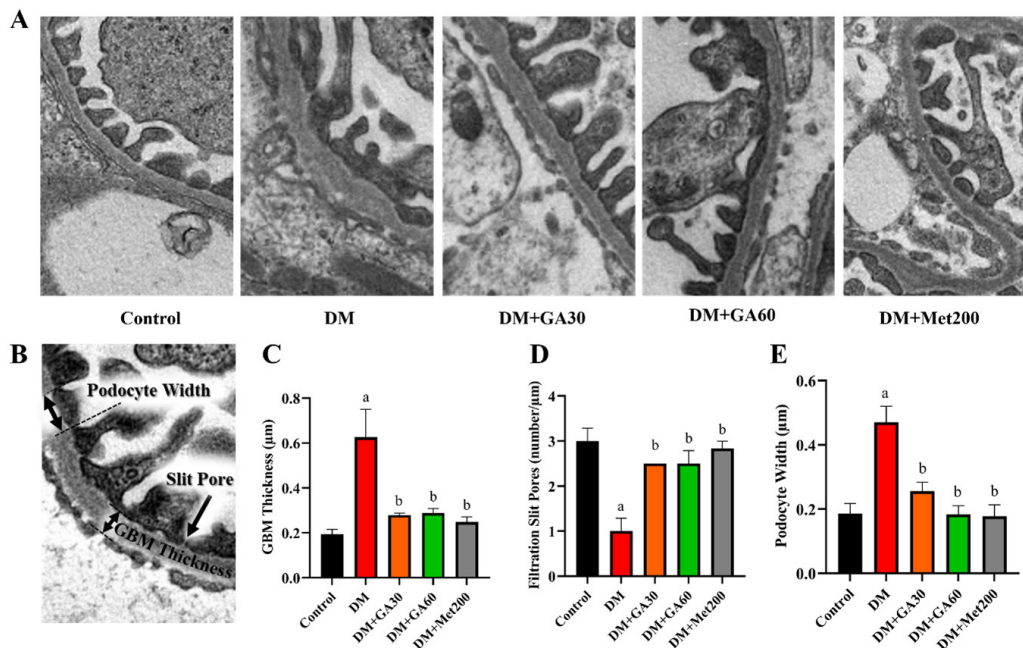


Figure 3. Effect of GA on ultrastructural changes of glomeruli in diabetic mice. The ultrastructure of the glomeruli was examined with TEM at 15,000× magnification (A). In panel (B), GBM thickness, filtration slit pore, and podocyte width are illustrated. Quantitative analyses of GBM thickness (C), filtration slit pore density per area (D), and podocyte width (E) were performed on three samples from each group using ImageJ (NIH). Data are means ± SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

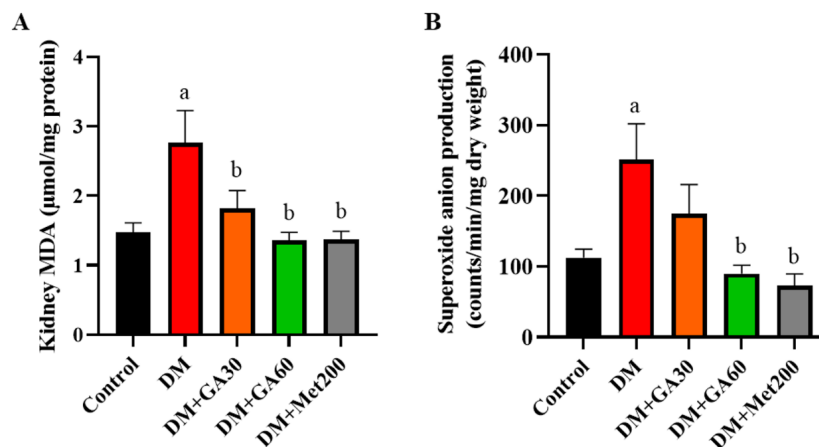


Figure 4. Effect of GA on renal oxidative stress in diabetic mice. Levels of MDA (A) and the rate of superoxide anion production (B) were measured in the kidney tissue of all animals ($n=8$ per group). Data are presented as means ± SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

generation in the diabetic group compared to controls ($p < 0.05$, Figure 4B). Treatment with GA at 60 mg/kg effectively attenuated superoxide production, while Met treatment similarly contributed to the reduction of oxidative stress in diabetic mice ($p < 0.05$).

Effect of GA on TGF-β1 and p47phox protein expression in kidney of diabetic mice

To further explore the underlying causes of structural changes in the kidneys, we analyzed

the expression of TGF-β1, an inflammatory marker associated with extracellular matrix deposition, using Western blotting (Figure 5A). The DM group exhibited increased TGF-β1 expression compared to the control group ($p < 0.05$). Notably, both treatment with GA and Met significantly inhibited this elevated expression, indicating that both treatments may provide protective effects against renal structural changes ($p < 0.05$).

Additionally, to identify the source of superoxide anion production in the kidney, we assessed

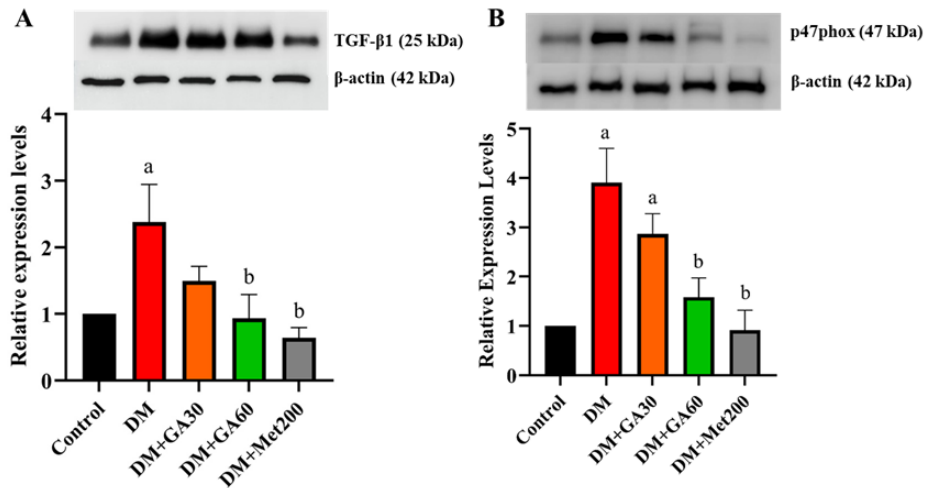


Figure 5. Effect of GA on TGF-β1 and p47phox protein expression in kidneys of diabetic mice. The figure shows the protein expression and quantitative band analysis of TGF-β1 (A) and p47phox (B) in kidney tissue. Representative images were obtained from three specimens per group. Data are presented as means ± SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

p47phox protein expression in renal tissues. This analysis revealed increased expression levels in the DM group ($p < 0.05$) (Figure 5B). Importantly, administration of GA at a dose of 60 mg/kg/day significantly reduced oxidative stress in the DM group, further supporting its protective role in mitigating kidney injury ($p < 0.05$).

DISCUSSION

This investigation demonstrates that GA effectively prevents diabetic kidney injury through a comprehensive therapeutic approach that addresses both metabolic dysfunction and glomerulopathy. The study utilized a clinically relevant T2D model, combining HFD with multiple low-dose STZ administration to create insulin resistance and pancreatic beta-cell dysfunction that closely mirrors human diabetic pathophysiology.

The experimental model successfully induced characteristic diabetic complications, including hyperglycemia, oxidative stress, and significant kidney damage manifested through glomerular hypertrophy, increased Bowman's space, and thickened basement membranes. These structural alterations were accompanied by markedly elevated MDA levels and overexpression of inflammatory mediators p47phox and TGF-β1, indicating considerable oxidative stress and inflammatory pathway activation within renal tissue (14). These findings are consistent with a well-established body of research demonstrating the central role of oxidative stress and inflammation in the pathogenesis

of DKD. For instance, chronic hyperglycemia is known to trigger the overproduction of ROS through various metabolic pathways, leading to oxidative damage to renal cells. This process, in turn, activates inflammatory cascades, including the upregulation of cytokines like TGF-β1, which promotes fibrosis and extracellular matrix deposition, ultimately causing the structural kidney damage observed in this model (15, 16). Thus, the model effectively replicates key pathological mechanisms of DKD.

GA supplementation at 30 mg/kg for 4 weeks produced significant therapeutic benefits by reducing blood glucose levels, oxidative stress, and inflammation while preventing further progression of renal damage. The 60 mg/kg/day dose demonstrated superior efficacy, suggesting that optimal therapeutic concentration maximizes the compound's antioxidant and anti-inflammatory properties. This dose-dependent response indicates that GA achieves its protective effects through concentration-dependent mechanisms that require adequate bioavailability for maximum therapeutic impact.

GA significantly improved insulin sensitivity in diabetic mice, as indicated by a marked reduction in HOMA-IR. This improvement is likely due to GA's capacity to reduce hepatic lipid deposition and mitigate oxidative stress and inflammation, which disrupt insulin signaling pathways (17). These combined effects lead to enhanced glucose tolerance and metabolic control (17). This result

is consistent with previous findings where GA reversed HFD-induced changes in GLUT-4 and IRS-1 expression, activated key metabolic regulators like AMPK and SIRT1, reduced oxidative stress, and preserved pancreatic β -cell function, thereby improving glucose handling (18).

GA effectively mitigated renal oxidative stress and inflammation, evidenced by suppressed rate of renal $O_2^{\cdot -}$ production and reduced expression of p47phox and TGF- β 1. Protective molecular mechanisms involve sophisticated regulation of inflammatory and fibrotic signaling. GA suppresses p47phox, a key NADPH oxidase subunit, through direct free radical scavenging and by inhibiting NF- κ B activation and its nuclear translocation, thereby reducing NADPH oxidase activity (19, 20). Crucially, this suppression of NADPH oxidase-derived oxidative stress may also contribute to the downregulation of TGF- β 1, as reactive oxygen species are known to activate this pro-fibrotic cytokine. Additionally, GA directly inhibits fibrotic signaling by suppressing the Smad and MAPK pathways downstream of TGF- β 1, preventing the excessive collagen production and extracellular matrix accumulation that drive basement membrane thickening and glomerular hypertrophy (21). The therapeutic actions observed in our study are strongly supported by evidence from other systems. Consistent with our findings, GA has been shown to reduce inflammation and oxidative stress in lung disease by modulating Nrf2-HO-1-NF- κ B signaling (22). Furthermore, its protective role in the central nervous system has been confirmed, where it reduces inflammation and apoptosis in the hippocampus of neurodegenerating rats by targeting TNF- α (21). Collectively, these studies underscore GA's versatile and potent ability to counteract disease pathology across multiple organ systems.

Regarding renal protection, GA demonstrated remarkable efficacy in preserving glomerular structure. The compound successfully mitigated the characteristic glomerulopathy, including increased glomerular area, Bowman's space expansion, basement membrane thickening, podocyte fusion, and reduced filtration slits. These protective effects were achieved through GA's targeted intervention in key pathological pathways that drive diabetic nephropathy progression.

While the present study focused on morphological outcomes, assessment of kidney function is limited in this study. However, previous investigations have shown that GA improves functional parameters by decreasing serum creatinine levels and reducing the urinary albumin-to-creatinine ratio. Moreover, combined treatment with metformin and GA (30 mg/kg daily) yielded superior improvements compared to either monotherapy, although those studies did not assess glomerular morphology (23). The current findings thus establish a crucial mechanistic link, suggesting that GA's documented functional benefits are directly tied to its profound ability to preserve glomerular architecture.

The study's findings confirm GA's dual therapeutic approach, which simultaneously addresses metabolic dysfunction and prevents structural renal damage. This comprehensive mechanism resulted in marked improvements in glomerular architecture, including reduced basement membrane thickening, preserved podocyte integrity, and maintained filtration slit architecture. The compound's ability to normalize oxidative stress markers, including MDA and superoxide anion levels, further demonstrates its potent antioxidant properties and protective role against inflammatory renal injury.

CONCLUSIONS

In conclusion, this research establishes GA as a promising therapeutic candidate for diabetic nephropathy management through its unique ability to target multiple pathological processes simultaneously. The compound's comprehensive approach to addressing both metabolic dysfunction and structural renal damage positions it as a valuable adjunctive therapy for diabetes-related complications. The evidence presented supports advancing GA toward clinical evaluation, with significant potential for improving patient outcomes through targeted prevention and management of DKD.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding this manuscript.

AUTHOR CONTRIBUTIONS

P.B.: data curation, investigation, methodology, writing – original draft; P.W.: visualization, validation; K.S.: methodology, validation; W.S.: conceptualization, supervision, project administration, funding acquisition, methodology, validation, writing – review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand. (IACUC-KKU-25/66; approved March 21, 2023).

INFORMED CONSENT STATEMENT:

Not applicable. This study did not involve human subjects.

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