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Gomisin D alleviates podocyte injury in diabetic kidney disease by inhibiting oxidative stress *via* activation of the GSK3 β (Ser9)/Nrf2/ARE signaling pathwayMing Xiang^{1,2}, Hai-Ying Chen^{3✉}, Xiang-Dong Lin^{4✉}¹Provincial Key Laboratory of TCM Diagnostics, Hunan University of Chinese Medicine, Changsha, Hunan, 410208, China²Key Laboratory of TCM Heart and Lung Syndrome Differentiation & Medicated Diet and Dietotherapy, Hunan University of Chinese Medicine, Changsha, Hunan, 410208, China³Department of Nephrology, The First Hospital of Hunan University of Chinese Medicine, Changsha, Hunan, 410007, China⁴Department of Endocrinology, The First Hospital of Hunan University of Chinese Medicine, Changsha, Hunan, 410007, China

ABSTRACT

Objective: To explore whether gomisin D, a lignan derived from *Schisandra chinensis*, protects against podocyte injury in diabetic kidney disease and to elucidate its underlying mechanisms.

Methods: Network pharmacology was employed to analyze the potential interactions between gomisin D and podocyte injury-related targets in diabetic nephropathy. A high glucose-induced injury model of mouse podocyte MPC5 cells was established, and the effect of gomisin D on podocyte injury was subsequently evaluated by measurement of reactive oxygen species levels and biochemical markers and Western blotting. Molecular docking and further molecular biology assays were conducted to explore the interaction between gomisin D and the target protein.

Results: Gomisin D inhibited high glucose-induced oxidative stress, improved cell viability, and preserved the expression of podocin and synaptopodin. Network pharmacology identified glycogen synthase kinase-3 β (GSK3 β) as a key target. Molecular docking predicted a potential interaction between gomisin D and GSK3 β , which was further validated by drug affinity responsive target stability and cellular thermal shift assay, confirming direct binding in live cells. Mechanistically, gomisin D enhanced the phosphorylation of GSK3 β at Ser9, promoted the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), and upregulated levels of downstream antioxidant proteins heme oxygenase-1 and NAD(P)H quinone oxidoreductase 1. Furthermore, forced expression of the constitutively active GSK3 β mutant S9A decreased GSK3 β (Ser9) phosphorylation and reversed gomisin D-induced upregulation of these antioxidant markers.

Conclusions: Gomisin D mitigates high glucose-induced podocyte injury *in vitro* by inhibiting oxidative stress *via* activation of the GSK3 β (Ser9)/Nrf2/ARE signaling pathway.

KEYWORDS: Gomisin D; Diabetic kidney disease; Podocyte injury; GSK3 β ; Nrf2/ARE pathway; Oxidative stress

1. Introduction

Diabetic kidney disease (DKD) is a microvascular complication, posing a significant challenge to global healthcare systems owing

Summary

Question: Does gomisin D protect against podocyte injury in diabetic kidney disease by modulating the GSK3 β (Ser9)/Nrf2/ARE signaling pathway?

Findings: Gomisin D alleviates high glucose-induced podocyte injury *in vitro* by restoring podocin and synaptopodin expression, inhibiting oxidative stress, and activating the Nrf2/ARE pathway through enhanced GSK3 β phosphorylation, thereby upregulating HO-1 and NQO1.

Meaning: Gomisin D shows great potential to alleviate podocyte injury in diabetic kidney disease.

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to its high morbidity and mortality[1]. Although current therapeutic strategies focus on inhibiting the renin-angiotensin system and controlling blood glucose and blood pressure, their efficacy in halting the progression of DKD is limited[2,3]. The glomerular filtration barrier, consisting of podocytes, endothelial cells, and the glomerular basement membrane, is crucial for maintaining normal renal function. Podocyte hypertrophy, shedding, and apoptosis damage the integrity of the glomerular filtration barrier, leading to increased proteinuria, abnormal glomerular filtration rate, and increased creatinine level[4]. Consequently, recent research has increasingly focused on the role of podocyte injury in DKD, with growing efforts to develop therapies targeting this pathological process. Accumulating evidence suggests that multiple factors contribute to podocyte damage, including lipotoxicity, hemodynamic abnormalities, oxidative stress, and impaired autophagy[4]. Podocyte detachment and death, along with compensatory hypertrophy and fusion of surviving cells, constitute the most critical pathological alterations in early DKD and are a key cause of proteinuria, thereby driving disease progression[5,6].

Traditional Chinese Medicine (TCM) has gained increasing recognition in treating diabetic nephropathy due to its holistic approach and multi-target therapeutic potential[7,8]. Wenyang Zhenshuai Granules, a TCM formulation clinically used for chronic heart failure[9] and sepsis[10], consists of seven herbal components in specific proportions: prepared aconite root (10 parts), dried ginger (10 parts), poria cocos (15 parts), red ginseng (6 parts), *Ophiopogon* tuber (15 parts), *Schisandra chinensis* (*S. chinensis*) (10 parts), and licorice root (10 parts). Among these, *S. chinensis*, a dioecious plant, has attracted increasing attention due to its lignan constituents and potential medicinal applications[11]. Previous research demonstrates that *S. chinensis* fruit extract significantly reduced urinary protein excretion, alleviated glomerulosclerosis, and protected against podocyte loss in mice with streptozotocin-induced diabetic nephropathy[12]. These beneficial effects are potentially attributed to the inhibition of epithelial-mesenchymal transition and the preservation of podocyte integrity[12].

Gomisin D, a lignan isolated from *S. chinensis*, has demonstrated significant hypoglycemic activity[13] and has been suggested to hold therapeutic potential for both diabetes and Alzheimer's disease[14]. Therefore, we established a diabetic-like MPC5 injury model using high glucose (HG) combined with inflammatory cytokines and insulin to mimic the type 2 diabetic microenvironment. Using this model in conjunction with network pharmacology, we investigated the protective effect of gomisin D against DKD-associated podocyte injury and its underlying mechanisms (*e.g.*, inhibition of oxidative stress). These findings are expected to provide novel insights for understanding the pharmacodynamic material basis of TCM formulations and developing targeted therapeutic agents.

2. Methods

2.1. Cell culture

Mouse podocyte clone-5 (MPC5) cells (Abiowell, AW-CNM109, China) were cultured at 37°C with 10% fetal bovine serum and 1% penicillin-streptomycin in RPMI 1640 medium. To induce differentiation and restore the cells to a near-normal, mature, quiescent podocyte phenotype, MPC5 cells were maintained at 37°C in interferon- γ free medium for 10-14 d before being used in subsequent experiments[15].

To assess the cytotoxicity of gomisin D under normal conditions, MPC5 cells were treated with various concentrations of gomisin D (10, 20, 40, 60, 80, and 100 μ M) for 48 h. The differentiated podocytes were assigned to the following experimental groups: The control group was cultured in medium containing 5 mM glucose and 20 mM mannitol as an osmotic control. To simulate the inflammatory and hyperinsulinemic microenvironment characteristic of type 2 diabetes, the HG group was treated with medium containing 25 mM D-glucose, 1 ng/mL tumor necrosis factor- α (TNF- α ; HY-P7058A, MCE, USA), 1 ng/mL interleukin (IL)-6 (HY-P78854, MCE), and 100 nM insulin (HY-P0035, MCE) for 48 h[16]. For the HG + gomisin D group, cells were pretreated for 1 h with different concentrations of gomisin D (10, 20, and 40 μ M; HY-N2413, MCE)[17], followed by 48 h of incubation in the same HG medium.

To obtain podocytes expressing high levels of glycogen synthase kinase-3 β (GSK3 β), MPC5 cells were transfected with 2 μ g plasmid DNA encoding a hemagglutinin (HA)-tagged constitutively active mutant of GSK3 β (S9A) using Lipofectamine 2000 reagent (11668019, Invitrogen, USA)[16]. The S9A plasmid (HG-MO019827S9A) and the empty vector control (HG-VPH2035), both carrying a C-terminal HA tag, were obtained from HonorGene (Changsha, China). The sequence of the S9A mutant is provided in Supplementary File. The empty vector contains no GSK3 β insert and served as the negative control (NC). In the HG + gomisin D + S9A group, MPC5 cells were transfected with this plasmid for 48 h, pretreated with 40 μ M gomisin D for 1 h, and then exposed to HG medium for an additional 48 h. As a control, MPC5 cells transfected with an empty vector were used in the HG + gomisin D + NC group.

2.2. Cell Counting Kit-8 (CCK-8)

Cells were plated in 96-well plates at 5×10^3 cells/well in 100 μ L of culture medium, with three replicates per group. Subsequently, each well was treated with 100 μ L of 10% CCK-8 working solution. The optical density (450 nm) was recorded after 4 h of incubation.

2.3. Western blot

Cells were washed once with ice-cold phosphate buffered

saline (PBS) and lysed with 200 μ L RIPA lysis buffer (P0013B, Beyotime, China) containing protease and phosphatase inhibitors. The cell lysates were collected using a cell scraper, followed by ultrasonication for 1.5 min on ice. After lysis for 10 min on ice, the samples were centrifuged at 12000 rpm for 15 min at 4°C. The supernatants were transferred to new tubes for further analysis. For nuclear and cytoplasmic fractionation, cells were processed using a commercial nuclear/cytoplasmic extraction kit (Beyotime, P0027, China) according to the manufacturer's protocol. Protein concentrations were determined using the BCA protein assay kit (P0010, Beyotime, China). Subsequently, 25 μ L of each sample or standard was added to a 96-well plate, followed by 200 μ L of BCA working solution. After incubation at 37°C for 30 min, the absorbance at 550 nm was measured. Protein concentrations were calculated from a standard curve.

Equal amounts of protein (10 μ g per lane) were separated by 10% SDS-PAGE. The proteins were transferred onto PVDF membranes at 300 mA constant current. The membranes were blocked with 5% non-fat milk in PBST for 90 min at room temperature, followed by overnight incubation at 4°C with primary antibodies (Table 1). After washing three times with PBST (15 min each), the membranes were incubated with HRP-conjugated secondary antibodies for 90 min at room temperature, followed by three washes with PBST.

β -actin was used as the internal loading control for total and cytoplasmic proteins, whereas PCNA was used as the loading control for nuclear proteins. The purity of subcellular fractions was validated by Western blotting using β -actin (cytoplasmic marker) and PCNA (nuclear marker) (Supplementary Figure A). Band density was quantified using a chemiluminescence imaging system (ChemiScope6100, Cline Science Instruments Co., Ltd, China) and analyzed by ImageJ software.

2.4. Reactive oxygen species (ROS) detection

ROS levels were measured using a ROS detection kit (Beyotime Biotechnology, S0033S, China). Briefly, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) stock solution (10 mM) was diluted 1:1000 in serum-free medium to a final concentration of 10 μ M. This diluted

solution was added to the cells to ensure complete coverage, followed by incubation for 20 min at 37°C. ROS levels were then analyzed using a flow cytometer (Beckman Coulter, CytoFLEX, USA) with excitation at 488 nm and emission at 525 nm.

2.5. Biochemical test

The cellular levels of glutathione (GSH; A006-2-1), superoxide dismutase (SOD; A001-3-2), and malondialdehyde (MDA; A003-1-2) were measured using assay kits from the Nanjing Jiancheng Bioengineering Institute (China). In addition, the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) was measured using a commercial kit (A061-1, Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

2.6. Network pharmacology

The SMILES ID of gomisin D was obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and then imported into the SEA database (<http://sea.bkslab.org/>), SwissTargetPrediction database (<https://swisstargetprediction.ch/>), PharmMapper database (<http://www.lilab-ecust.cn/pharmmapper/>), GeneCards database (<https://www.genecards.org/>), and HERB database (<http://herb.ac.cn/>) for compound target prediction. For SwissTargetPrediction, targets with a probability > 0 were included. For PharmMapper, targets with a normalized fit score > 0.5 were included. All predicted targets were normalized and deduplicated using the UniProt database (<https://www.uniprot.org/>), yielding 199 unique targets.

Human genes associated with DKD and podocyte injury were retrieved from the GeneCards, NCBI, OMIM, and CTD databases using the keywords “diabetic nephropathy” and “podocyte injury”. For the CTD database, targets were filtered by the median score to select more relevant genes. After merging and removing duplicates, 7517 DKD-related genes and 408 podocyte injury-related genes were obtained.

The 22 overlapping targets between gomisin D and DKD/podocyte injury were submitted to the STRING database (<https://string-db.org/>) to construct a PPI network (confidence score > 0.4, species

Table 1. Antibodies used in Western blotting assays.

Name	Dilution rate	Cat number	Company	Country
Podocin	1:1000	AWA10004	Abiowell	China
Synaptopodin	1:2000	21064-1-AP	Proteintech	USA
GSK3 β	1:2000	AWA10357	Abiowell	China
p-GSK3 β	1:3000	AWA10356	Abiowell	China
Nrf2	1:8000	66504-1-Ig	Proteintech	USA
HO-1	1:3000	66743-1-Ig	Proteintech	USA
NQO1	1:5000	67240-1-Ig	Proteintech	USA
PCNA	1:5000	10205-1-AP	Proteintech	USA
β -actin	1:5000	66009-1-Ig	Proteintech	USA
HRP goat anti-mouse IgG	1:5000	SA00001-1	Proteintech	USA
HRP goat anti-rabbit IgG	1:6000	SA00001-2	Proteintech	USA

“*Homo sapiens*”). The PPI network was then imported into Cytoscape 3.8.0 for topological analysis using the NetworkAnalyzer tool. Key targets were prioritized based on degree values. Additionally, MCODE clustering analysis was performed to identify hub gene clusters.

2.7. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses

GO enrichment analysis, including biological process, molecular function, and cell component, was conducted on shared drug-disease targets using the STRING database. Subsequently, an enrichment analysis of KEGG pathways was performed to uncover biological pathways associated with the proteins of interest, focusing on those with a corrected P -value below 0.05. A histogram was constructed to visualize the data using R 4.1.2 and related packages (clusterProfiler, enrichplot, ggplot2).

2.8. Molecular docking studies

VINA 1.1.2 software was used for molecular docking to study compound-protein interactions. PyMOL software was used for further visualization.

2.9. Drug–protein interaction analysis using drug affinity responsive target stability

Gomisin D at concentrations of 5, 10, and 20 μ M was mixed and incubated with GSK3 β protein for 2 h. Then, 5 μ g/mL pronase was incorporated. The mixture was further incubated for 15 min, and then a loading buffer was added to terminate the reaction. Finally, Western blot analysis was carried out.

2.10. Cellular thermal shift assay

Cellular thermal shift assay was performed as previously described[18]. For thermal stability curve analysis, MPC5 cells were lysed with RIPA buffer, and equal amounts of protein lysates were incubated with either dimethyl sulfoxide or gomisin D (40 μ M) for 24 h at room temperature. The samples were then heated at temperatures ranging from 37 °C to 72 °C, centrifuged at 12000 rpm for 15 min, and the supernatants were collected for Western blot analysis of GSK3 β . For concentration-dependent analysis, equal amounts of protein lysates were incubated with increasing concentrations of gomisin D (2, 5, 10, 20, 50, 100, 200, and 300 μ M) for 24 h at room temperature, heated at 65 °C, and then processed as described above.

2.11. Statistical analysis

Results are presented as mean $\pm$ standard deviation. All experiments were performed with five biological replicates. Normality was checked using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene’s test. One-way ANOVA followed by Tukey’s *post hoc* test was used for multiple group comparisons, and Student’s *t*-test was used for two-group comparisons. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism software 8.0 (GraphPad Software, USA).

3. Results

3.1. Gomisin D inhibits HG-induced ROS production and oxidative stress

To investigate whether gomisin D protects podocytes against oxidative injury under HG conditions, we first assessed its effects on cell viability. As shown in Supplementary Figure B, gomisin D at concentrations up to 40 μ M did not significantly affect normal MPC5 cell viability, whereas concentrations of 60 μ M and above significantly reduced cell viability ($P < 0.05$). HG exposure significantly reduced MPC5 cell viability compared with the control group ($P < 0.05$) (Figure 1A). Pretreatment with gomisin D at 10, 20, and 40 μ M attenuated this reduction, with the 40 μ M dose showing the most pronounced effect ($P < 0.05$) (Figure 1A). We next examined the expression of podocyte-specific structural proteins. Western blot analysis revealed that HG treatment downregulated the expression of podocin and synaptopodin ($P < 0.05$) (Figure 1B). Gomisin D treatment upregulated these protein expressions in a dose-dependent manner ($P < 0.05$) (Figure 1B). To evaluate intracellular oxidative stress, ROS levels were measured by flow cytometry. HG stimulation significantly increased ROS accumulation ($P < 0.05$), which was suppressed by gomisin D pretreatment ($P < 0.05$) (Figure 1C). Consistent with these findings, SOD and GSH activities were decreased ($P < 0.05$) under HG conditions, while MDA levels were elevated ($P < 0.05$) (Figure 1D). Gomisin D pretreatment restored SOD and GSH activities and reduced MDA content, with the most pronounced effect found at 40 μ M ($P < 0.05$) (Figure 1D). Furthermore, the GSH/GSSG ratio was significantly reduced in HG-treated cells, and this effect was reversed by gomisin D pretreatment ($P < 0.05$) (Figure 1D). Collectively, these results indicate that gomisin D alleviated HG-induced podocyte damage by improving cell viability, preserving structural integrity, and reducing oxidative stress, with the 40 μ M dose being most effective. Therefore, this concentration (40 μ M) was selected for subsequent mechanistic studies.

3.2. Analysis of interactions between gomisin D and podocyte injury targets in DKD based on network pharmacology

To identify potential molecular targets of gomisin D in DKD-associated podocyte injury, we performed a network pharmacology analysis. Initial screening by constructing a Venn diagram identified 22 overlapping targets shared between gomisin D and DKD-associated podocyte injury (Figure 2A). Subsequent topological analysis of these common targets revealed five key proteins ranked in order of importance: peroxisome proliferator-activated receptor gamma (PPARG), mechanistic target of rapamycin (mTOR), matrix metalloproteinase 9 (MMP9), cyclin D1 (CCND1), and GSK3 β , suggesting their central roles in the potential therapeutic network (Figure 2B). To further understand the biological functions of these overlapping targets, GO enrichment analysis was performed (Figure 2C). The results indicate significant enrichment in biological processes related to “response to oxygen levels” and “cell cycle G₁/S phase transition”. Cellular component analysis showed enrichment

in “serine/threonine protein kinase complex” and “protein kinase complex”. Molecular function analysis revealed enrichment in “protein serine/threonine kinase activity”, “p53 binding”, and “cyclin-dependent protein kinase activity”. These findings link gomisin D’s action to oxidative stress regulation, cell cycle modulation, and kinase activity in podocytes. Furthermore, KEGG pathway analysis was conducted to map the potential signaling pathways involved. As shown in Figure 2D, significantly enriched pathways relevant to podocyte function and DKD pathogenesis included phosphatidylinositol 3-kinase-protein kinase B (PI3K-Akt) signaling, hypoxia-inducible factor 1 (HIF-1) signaling, forkhead box O (FoxO) signaling, cellular senescence, and cell cycle regulation. Overall, network pharmacology analysis revealed that gomisin D may target key proteins, including PPARG, mTOR, MMP9, CCND1, and GSK3 β , suggesting its potential role in modulating oxidative stress, cell cycle, and metabolic signaling pathways.

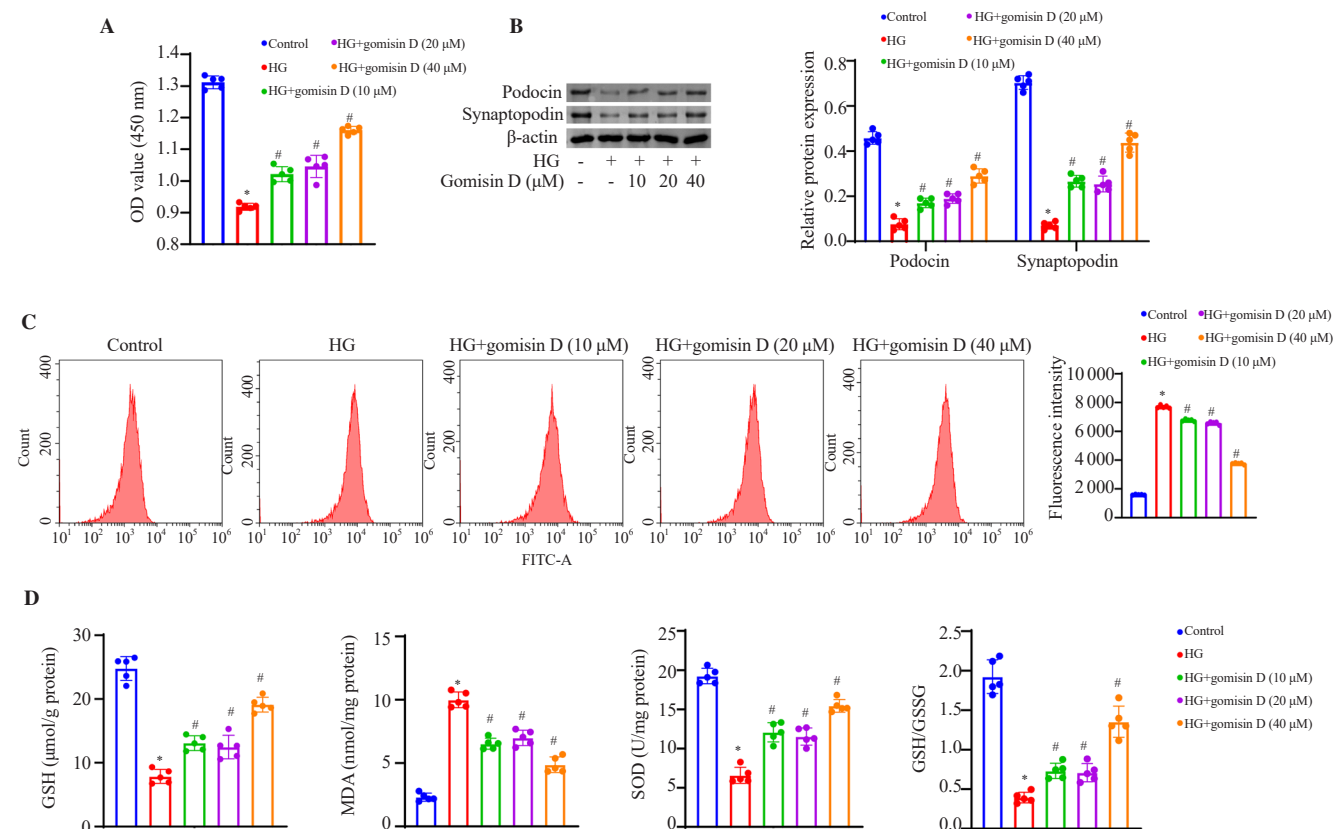


Figure 1. Gomisin D inhibits high glucose (HG)-induced ROS production and oxidative stress. MPC5 cells were treated with 25 mM HG for 48 h with or without 1 h pretreatment with gomisin D (10, 20, or 40 μ M). (A) MPC5 cell viability was assessed by CCK-8 assay. (B) Western blot analysis of podocin and synaptopodin in MPC5 cells. (C) Intracellular ROS levels measured by flow cytometry in MPC5 cells. (D) Measurement of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and GSH/GSSG ratio in cell culture supernatant. The data are expressed as mean $\pm$ standard deviation (SD) and analyzed by one-way ANOVA followed by Tukey’s *post hoc* test ($n = 5$). * $P < 0.05$ vs. Control; # $P < 0.05$ vs. the HG group. GSSG: glutathione disulfide; ROS: reactive oxygen species.

3.3. Molecular docking validation of the binding between gomisin D and podocyte injury-related targets in DKD

To further evaluate whether gomisin D could directly interact with the key targets identified from network pharmacology, molecular docking analysis was conducted. Gomisin D displayed favorable binding affinities to all five hub proteins, with binding energies of -7.7 kcal/mol for PPARG, -7.8 kcal/mol for mTOR, -7.7 kcal/mol for MMP9, -6.8 kcal/mol for CCND1, and -8.2 kcal/mol for GSK3 β (Figure 3A). GSK3 β was subsequently prioritized for experimental validation due to its highest binding affinity with gomisin D in molecular docking. Further analysis of the predicted interaction modes suggests that gomisin D may engage with the target proteins

through van der Waals forces, conventional hydrogen bonds, and carbon-hydrogen bonds. Specific potential interactions also included π -alkyl and π -cation contacts in certain complexes. In the gomisin D-GSK3 β complex, the docking simulation predicted that gomisin D could bind within the protein cavity, with possible hydrogen bond formation involving residues glutamine 151 (chain C), arginine 209, threonine 235, serine 237, and proline 331, as well as potential hydrophobic interactions with lysine 150 (chain B). These computational predictions suggest a potential binding mode between gomisin D and GSK3 β . To experimentally verify this interaction, the drug affinity responsive target stability assay was conducted to assess the protective effect of gomisin D on GSK3 β protein against protease digestion. Pronase treatment completely degraded GSK3 β

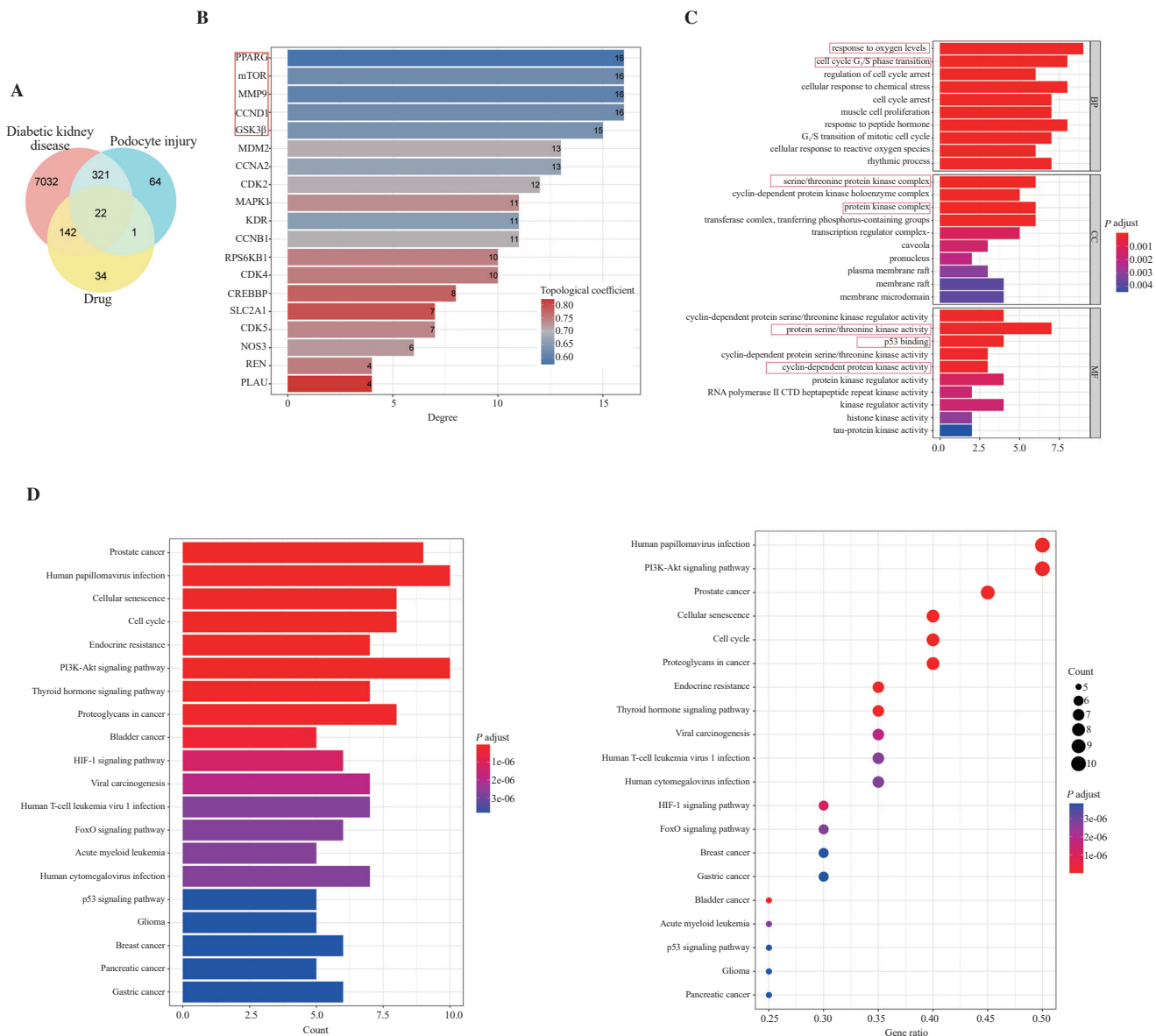


Figure 2. Analysis of interactions between gomisin D and podocyte injury-associated targets in diabetic kidney disease based on network pharmacology. (A) Venn diagram showing the overlapping targets among gomisin D, diabetic kidney disease, and podocyte injury. (B) Topological network of core targets. (C-D) GO and KEGG pathway enrichment analyses of common targets.

protein, whereas pretreatment with gomisin D at concentrations of 5, 10, and 20 μM conferred concentration-dependent protection against proteolytic degradation (Figure 3B). As shown in Figure 3C and D, gomisin D treatment increased the thermal stability of GSK3 β in a concentration-dependent manner ($P < 0.05$), indicating direct binding between gomisin D and GSK3 β in live cells. Collectively, these results identified GSK3 β , which exhibited robust binding affinity for gomisin D, as the principal hub target mediating the protective effects against podocyte injury.

3.4. Gomisin D regulates ROS production and oxidative stress via the GSK3 β /nuclear factor erythroid 2–related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway

Based on the observed binding of gomisin D to GSK3 β and its antioxidative role, we further examined whether it alleviates podocyte injury by regulating the GSK3 β /Nrf2/ARE signaling pathway under HG conditions. Western blot analysis revealed

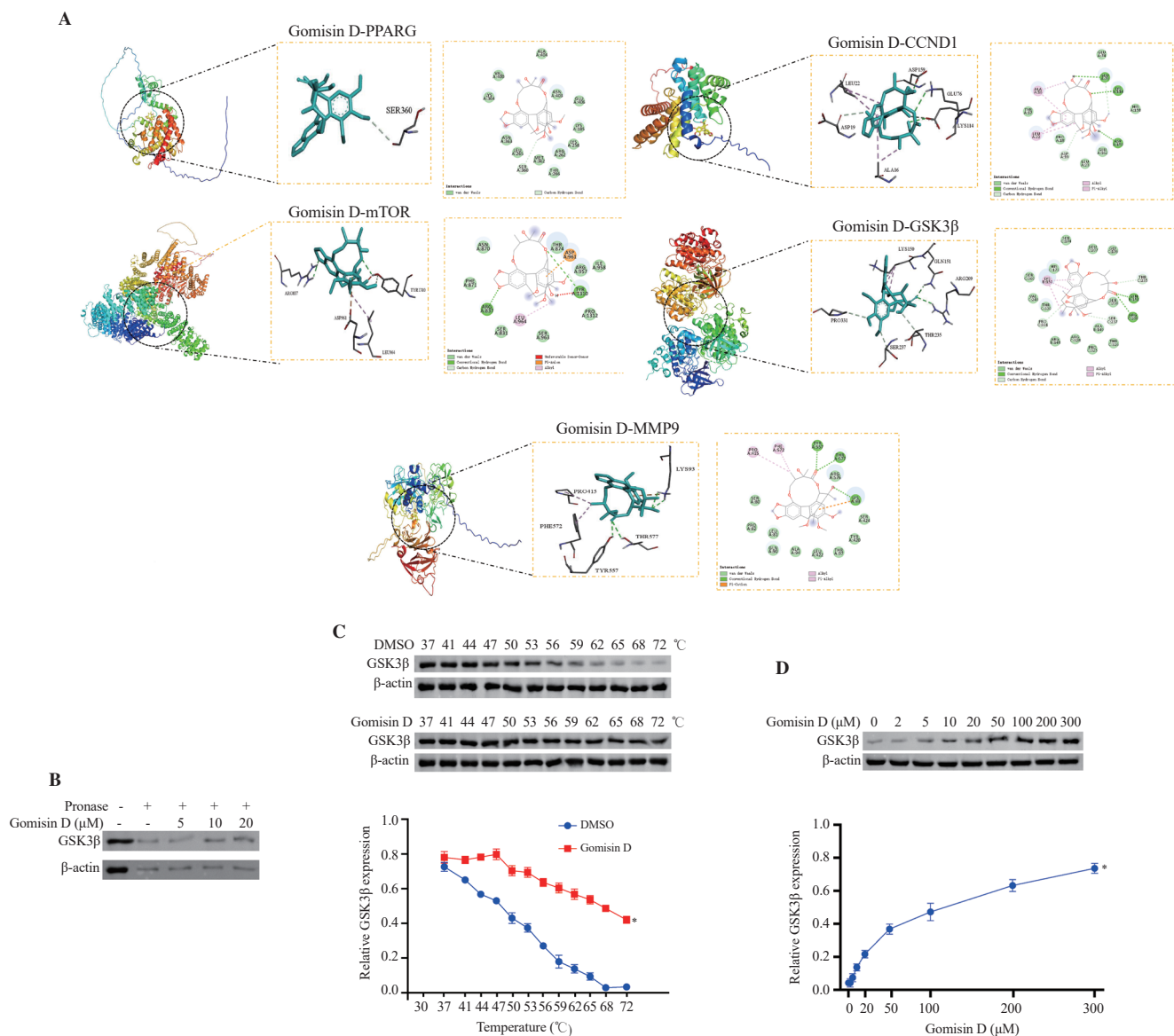


Figure 3. (A) Molecular docking validation of the binding between gomisin D and podocyte injury-related targets (PPARG, mTOR, MMP9, CCND1, and GSK3 β) in diabetic kidney disease. (B) Validation of the gomisin D-GSK3 β interaction by drug affinity responsive target stability assay. (C) Cellular thermal shift assay of GSK3 β thermal stability in live cells treated with gomisin D across a temperature gradient. The data are expressed as mean $\pm$ SD and analyzed by Student's t -test ($n = 5$). * $P < 0.05$ vs. the DMSO group. (D) Cellular thermal shift assay of GSK3 β thermal stability in live cells treated with increasing concentrations of gomisin D at 65°C. The data are expressed as mean $\pm$ SD and analyzed by one-way ANOVA followed by Tukey's *post hoc* test ($n = 5$). * $P < 0.05$ vs. 0 μM gomisin D group.

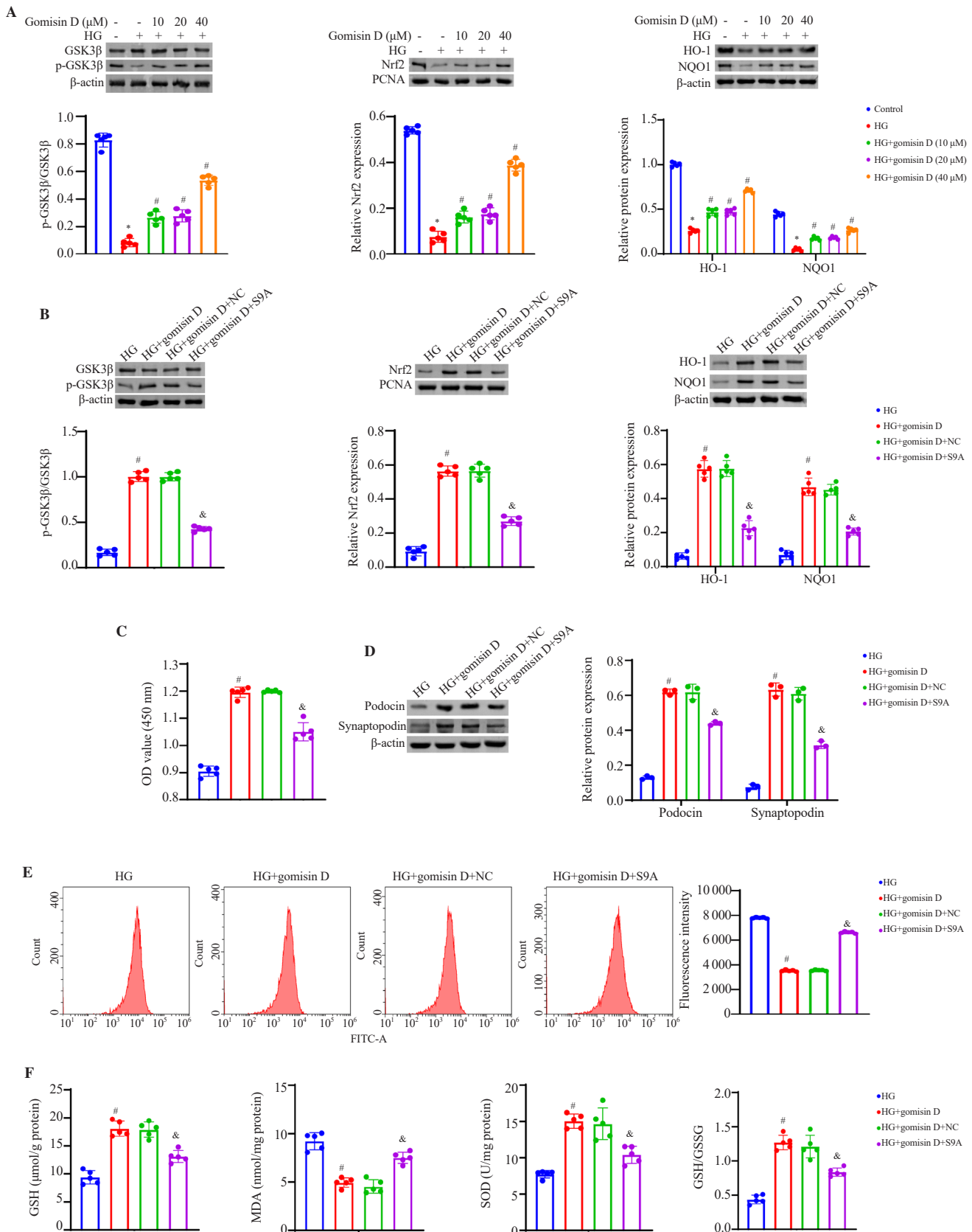


Figure 4. Gomisin D regulates ROS production and oxidative stress *via* modulating the GSK3 β /Nrf2/ARE signaling pathway. (A) Western blot analysis of p-GSK3 β (Ser9), total GSK3 β , nuclear Nrf2, and cytoplasmic HO1 and NQO1 in MPC5 cells treated with different concentrations of gomisin D. PCNA was used as the loading control for nuclear proteins, and β -actin for cytoplasmic proteins. (B) Western blot analysis of p-GSK3 β (Ser9), total GSK3 β , nuclear Nrf2, and cytoplasmic HO1 and NQO1 in MPC5 cells after transfection with a plasmid vector encoding the constitutively active GSK3 β mutant (S9A). PCNA was used as the loading control for nuclear proteins, and β -actin for cytoplasmic proteins. (C) MPC5 cell viability was assessed by CCK-8 assay. (D) Western blot analysis of podocin and synaptopodin in MPC5 cells. (E) Intracellular ROS levels measured by flow cytometry in MPC5 cells. (F) Measurement of MDA, GSH, SOD, and GSH/GSSG ratio in cell culture supernatant. The data are expressed as mean $\pm$ SD and analyzed by one-way ANOVA followed by Tukey's *post hoc* test ($n = 5$). * $P < 0.05$ vs. Control; # $P < 0.05$ vs. the HG group; & $P < 0.05$ vs. the HG +gomisin D + NC group.

that HG decreased the level of phosphorylated GSK3 β at Ser9 compared with the control group. Gomisin D upregulated p-GSK3 β (Ser9) levels, with the most significant effect observed at 40 μ M ($P < 0.05$) (Figure 4A). Correspondingly, HG exposure markedly reduced nuclear accumulation of Nrf2, which was significantly reversed by gomisin D pretreatment, especially at the high doses ($P < 0.05$) (Figure 4A). Furthermore, the expression of the downstream antioxidant proteins heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) in the cytoplasm was significantly downregulated under HG conditions ($P < 0.05$). Gomisin D treatment effectively restored their expression in a dose-dependent manner ($P < 0.05$) (Figure 4A).

To determine whether the protective effect of gomisin D relies on the modulation of GSK3 β activity, MPC5 cells were transfected with a plasmid vector encoding the constitutively active GSK3 β mutant (S9A). As shown in Supplementary Figure C, GSK3 β expression was markedly increased in S9A-transfected cells compared with the NC group, while p-GSK3 β (Ser9) levels were decreased ($P < 0.05$), confirming successful transfection and the expected inhibition of GSK3 β Ser9 phosphorylation by the S9A mutant. Compared with the HG group, the HG + gomisin D group exhibited increased phosphorylation of GSK3 β (Ser9) ($P < 0.05$) (Figure 4B). However, in the HG + gomisin D + S9A group, p-GSK3 β (Ser9) levels were significantly lower than in the HG + gomisin D + NC group ($P < 0.05$), confirming the inhibition of GSK3 β activity (Figure 4B). Correspondingly, compared with the HG group, the HG + gomisin D group showed significantly higher nuclear Nrf2 accumulation and expression of its downstream targets HO-1 and NQO1 ($P < 0.05$) (Figure 4B). In contrast, the effects of gomisin D were markedly attenuated in cells transfected with S9A ($P < 0.05$), with nuclear Nrf2 and HO-1/NQO1 expression remaining at low levels (Figure 4B). CCK-8 assays indicated that the improvement in cell viability mediated by gomisin D was significantly diminished under forced S9A expression ($P < 0.05$) (Figure 4C). Western blot further revealed reduced expression of the key podocyte structural proteins podocin and synaptopodin in the HG + gomisin D + S9A group compared with the HG + gomisin D + NC group ($P < 0.05$) (Figure 4D). Flow cytometry analysis of intracellular ROS levels demonstrated that ROS accumulation was significantly increased in the HG + gomisin D + S9A group compared with the HG + gomisin D + NC group ($P < 0.05$) (Figure 4E). Moreover, significantly elevated MDA levels and decreased SOD and GSH activities were observed in the HG + gomisin D + S9A group compared with the HG + gomisin D + NC group ($P < 0.05$), indicating that the modulatory effects of gomisin D on these parameters were markedly limited under forced S9A expression (Figure 4F). Similarly, gomisin D treatment increased the GSH/GSSG ratio ($P < 0.05$); however, this effect was significantly attenuated in cells forced to express the S9A mutant ($P < 0.05$) (Figure 4F). These results demonstrate that reducing GSK3 β activity by forced S9A expression abated the protective effects of gomisin D

against HG-induced oxidative stress and podocyte injury, confirming that gomisin D regulated ROS production and oxidative stress *via* the GSK3 β /Nrf2/ARE signaling axis.

4. Discussion

DKD is characterized by progressive renal damage that often leads to end-stage renal disease[19,20]. Podocyte injury driven by oxidative stress is a critical event in the pathogenesis of DKD, yet targeted therapies remain limited[21,22]. In this study, we demonstrated that gomisin D, a bioactive lignan compound identified from the TCM formula Wenyang Zhenshuai Granules, effectively attenuated podocyte injury induced by a diabetic-like environment mimicking type 2 diabetes. This protective effect was achieved by suppressing oxidative stress *via* modulation of the GSK3 β /Nrf2/ARE signaling pathway.

GSK3 β acts as a signaling hub that integrates the Wnt/ β -catenin, NF- κ B, Nrf2, and PI3K/Akt pathways, and plays a key role in the progression of chronic kidney disease[23]. A study clearly demonstrated that GSK3 β knockdown enhances the Nrf2-mediated antioxidant response and inhibits oxidative stress, leading to the amelioration of podocyte injury and senescence[16]. The Nrf2/ARE signaling pathway is a central regulator of the cellular antioxidant response and has become a key target for the treatment of diabetic nephropathy[24]. Nrf2 activation leads to the upregulation of antioxidant and detoxifying enzymes, which can counteract oxidative stress[25,26]. Our results showed that gomisin D restored the inhibitory phosphorylation of GSK3 β at Ser9 and enhanced Nrf2 nuclear translocation under HG conditions, providing direct pharmacological evidence from a natural product perspective. The observation that forced expression of the constitutively active GSK3 β mutant (S9A) abolished the protective effects of gomisin D confirms the causal role of GSK3 β inhibition in its mechanism of action.

Mammalian GSK3 exists as two isoforms, GSK3 α and GSK3 β , which share 97% homology in their catalytic domains but are not functionally interchangeable[23]. In the kidney, GSK3 β is predominantly expressed and particularly enriched in podocytes and renal tubular cells, whereas GSK3 α expression is more restricted[27]. Notably, combined knockout of both GSK3 α and GSK3 β specifically in podocytes causes severe podocyte injury and glomerulosclerosis, whereas knockout of either isoform alone does not, suggesting functional compensation between the two isoforms[28]. The antibodies used in this study detect total GSK3 β without distinguishing between isoforms. Therefore, whether gomisin D exhibits selectivity for GSK3 β over GSK3 α remains to be determined. Interestingly, recent natural compound screening studies have identified isoform-specific GSK-3 inhibitors. For example, psoralidin shows greater potency against GSK-3 α (IC_{50} =2.26 μ M)

than against GSK-3 β (IC₅₀=4.23 μ M), whereas rosmarinic acid exhibits the opposite selectivity[29]. Whether gomisin D possesses similar isoform selectivity, and whether such selectivity would confer therapeutic advantages or disadvantages in podocyte protection, awaits future investigation.

Similar findings have been reported for other natural compounds. Protocatechuic aldehyde was shown to protect HG-induced podocytes by attenuating endoplasmic reticulum stress through the GSK3 β /Nrf2 pathway[30]. These independent lines of evidence collectively support the GSK3 β /Nrf2 axis as a credible pathway involved in podocyte protection against DKD. Recent studies have identified natural products with nephroprotective effects in DKD, such as orientin, which ameliorates diabetic nephropathy in rats by reducing inflammation and oxidative stress[31]. This growing body of evidence underscores the therapeutic potential of natural compounds in DKD, although the underlying mechanisms may vary. Furthermore, our study aligns with the broader principle in TCM-based DKD research, where multi-target intervention is a common strategy. Beyond our focused axis, emerging evidence highlights that complex TCM formulas, such as Jinlida[32] and Liuwei Dihuang pills[33], exert renal protection partly by inhibiting the pro-fibrotic transforming growth factor- β /Smad pathway and modulating the gut-kidney axis. Furthermore, gut microbiota and their metabolites (*i.e.*, the “gut-kidney axis”) also play a key role in DKD[34,35]. *Phellodendron* polysaccharides can exert renal protective effects by modulating the PI3K/GSK3 β /Nrf2/TGF- β /Smad pathway and regulating gut microbiota[36]. These studies reveal the complex network through which TCM compounds exert multitarget therapeutic advantages by modulating the host-microbiota symbiotic system. Future research could further explore whether the monomer compound gomisin D is associated with the broader “gut-kidney axis”, which would significantly enrich the research foundation for its potential as a therapeutic candidate for DKD. Additionally, targeting renal lipid accumulation represents another promising strategy for DKD treatment, as demonstrated by the finding that *Alpinia oxyphylla* Fructus ameliorates renal lipid accumulation *via* activation of PPAR α [37].

Beyond oxidative stress and the gut-kidney axis, the pathological landscape of DKD has expanded to include other regulated cell death pathways, notably ferroptosis[38]. A critical link to our findings is that Nrf2 serves as a master regulator against ferroptosis. Nrf2 activation transcriptionally upregulates a network of genes involved in glutathione synthesis, iron metabolism, and lipid antioxidant defense, thereby suppressing ferroptotic cell death[39,40]. Given our results that gomisin D potentially activates the Nrf2 pathway, it is plausible that this mechanism may concurrently confer resistance to ferroptosis in podocytes under diabetic conditions. This hypothesis is supported by independent studies showing that pharmacological modulation of the GSK3 β /Nrf2 axis can inhibit ferroptosis in renal cells. In DKD, the natural product swietenine was recently demonstrated to protect

HG-induced MPC-5 cells from ferroptosis by activating the Akt/GSK-3 β /Nrf2 pathway[41]. In cisplatin-induced acute kidney injury models, the compound bergenin was shown to inhibit ferroptosis in HK-2 cells through activation of the p-GSK3 β /Nrf2/peroxisome proliferator-activated receptor gamma pathway[42]. Furthermore, the clinically used drug Empagliflozin alleviates ferroptosis and renal fibrosis in diabetic models by activating the AMPK/GSK3 β /Nrf2 pathway in tubular epithelial cells[43]. Therefore, investigating the effect of gomisin D on established ferroptosis markers in podocytes, such as glutathione peroxidase 4 expression, acyl-CoA synthetase long-chain family member 4 levels, and lipid ROS accumulation, represents a compelling future direction to more comprehensively define its cytoprotective profile against multifaceted DKD injury.

This study has several limitations. First, the conclusions are based solely on an *in vitro* MPC5 cell model, and the pharmacokinetics, bioavailability, and safety of gomisin D have not been assessed. Whether the concentrations used *in vitro* (10–40 μ M) are achievable or safe *in vivo* remains unknown. Thus, further *in vivo* studies are required to extend these findings. Second, while we focused on the GSK3 β /Nrf2 axis, other potential targets predicted by network pharmacology (*e.g.*, mTOR, PPARG) require further exploration. Additionally, while network pharmacology predicted several potential targets (*e.g.*, mTOR, PPARG) and identified enriched pathways relevant to podocyte biology, the present study did not experimentally validate these additional targets. Their potential contributions to the protective effects of gomisin D remain to be explored in future studies. Notably, while we used the GSK3 β S9A gain-of-function mutant to assess pathway dependency, complementary loss-of-function approaches (*e.g.*, GSK3 β inhibitor or Nrf2 siRNA) were not included in this study. Further investigations employing such approaches would help to further elucidate the role of the GSK3 β /Nrf2/ARE signaling axis in mediating the protective effects of gomisin D.

In conclusion, gomisin D effectively mitigated podocyte injury by modulating the GSK3 β /Nrf2/ARE signaling pathway. Specifically, gomisin D enhanced the phosphorylation of GSK3 β at Ser9, promoted the expression of nuclear Nrf2 and cytoplasmic HO-1 and NQO1, thereby reducing intracellular ROS accumulation and oxidative stress. Notably, the protective effects of gomisin D were largely abolished by the forced expression of a constitutively active GSK3 β mutant (S9A). Collectively, these results provided experimental and mechanistic evidence supporting great potential of gomisin D in treating podocyte injury in DKD.

Conflict of interest statement

The authors declare no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

HYC designed the study. MX, XDL, and HYC performed experiments. MX analyzed the data. XDL and HYC wrote the manuscript. All authors contributed to the editorial changes in the manuscript. All authors have read and approved the final manuscript.

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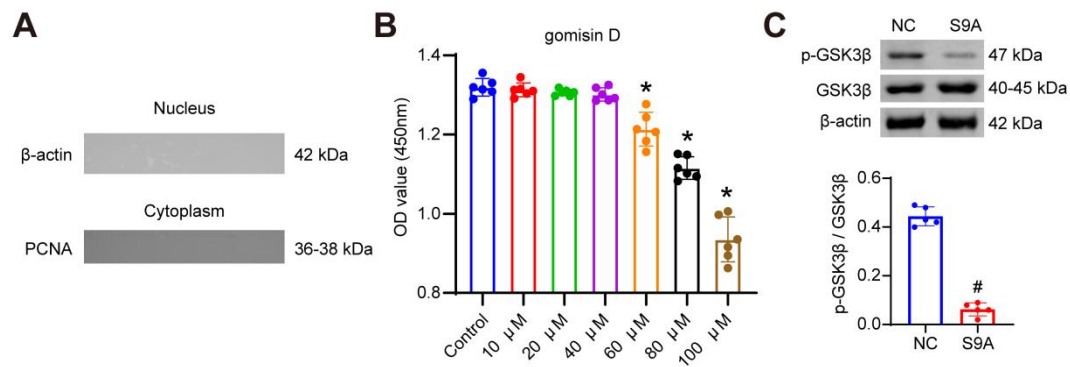
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Supplementary File. Sequence of GSK3 β S9A mutant.

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atgtc ggggcgaccg agaaccaccg ctttgcgga
1561 gagctgcaag ccagtgcagc agccttcagc ttttgtagc atgaaagta gcagagataa
1621 agatggcagc aaggtaacca cagtagtggc aactcctggc cagggctcctg acaggccaca
1681 ggaagtcagt tatacagaca cgaaagtgat tggaaatgga tcatttgggtg tggatatca
1741 agccaaactt tgtgattctg gagaactggt tgccatcaag aaagttctac aggacaagcg
1801 atttaagaac cgagagctcc agatcatgag aaagctagac cactgtaaca tagtccgact
1861 gcggtatttc ttctactcga gtggtgagaa gaaagatgag gtctacctta acctggtgct
1921 ggactatgtt ccggagacag tgtacagagt cgccagacac tatagtcgag ccaagcagac
1981 actccctgtg atctatgtca agttgtatat gtatcagctg ttcagaagtc tagcctatat
2041 ccattccttt ggaatctgcc atcgagacat taaaccacag aacctcttgt tggatcctga
2101 tacagctgta ttaaaactct gtgactttgg aagtgc aaag cagctggtcc gaggagagcc
2161 caatgtttca tatactgtt ctcggtacta cagggcacca gaggtagctt ttggagccac
2221 tgattacagc tccagtatag atgtatggc tgcaggctgt gtgttggctg aattgttgc
2281 aggacaacca atatttctg gggacagtgg tgtggatcag ttggtggaaa taataaaggt
2341 cctaggaaca ccaacaaggg agcaaattag agaaatgaac ccaaattata cagaattcaa
2401 attccctcaa atcaaggcac atccttgac aaaggtcttc cggccccgaa ctccaccaga
2461 ggcaattgca ctgtgcagcc gtctgctgga gtacacacct accgcccggc taacaccact
2521 ggaagcttgt gcacattcat tttcgatga attgcgggac ccaaagtca aactacaaa
2581 tgggcgagac acacctgcac tctcaactt taccactcaa gaactgtcaa gtaaccccc
2641 tctggccacc atccttatcc ctccatgc tcggattcag gccgtgctt caccgcctgc
2701 caacgccaca gcagcctcag atactaatgc tggagaccgt ggacagacca ataacgcgc
2761 ttctgcatca gttccaact ccactga
```

Note: The wild-type GSK3 β sequence at codon 9 is ACCACCTCC (encoding serine), which was mutated to ACCACCGCC (encoding alanine) in the S9A construct. The empty vector (NC, HG-VPH2035) contains no GSK3 β insert.



Supplementary Figure. (A) Western blot validation of subcellular fractionation purity. No β-actin signal was detected in the nuclear fraction, and no PCNA signal was detected in the cytoplasmic fraction. (B) Cytotoxicity of gomisin D in normal MPC5 cells. Cells were treated with increasing concentrations of gomisin D (10-100 μM) for 48 h, and cell viability was assessed by CCK-8 assay. The data are expressed as mean ± SD and analyzed by Student's *t*-test ($n = 6$). * $P < 0.05$ vs. the control group. (C) GSK3β and p-GSK3β (Ser9) levels were detected by Western blot. The data are expressed as mean ± SD and analyzed by Student's *t*-test ($n = 5$). # $P < 0.05$ vs. the NC group.