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Study of molecular mechanisms underlying the medicinal plant *Tripterygium wilfordii*-derived compound celastrol in treating diabetic nephropathy based on network pharmacology and molecular docking

Fengmei QIAN[1,2]; Peiyao REN[2]; Li ZHAO[2]; Danna ZHENG[2]; Wenfang HE[3]; Juan JIN[3,*]

[1] The Second School of Clinical Medicine, Zhejiang Chinese Medical University, Hangzhou, 310003, China
[2] Urology & Nephrology Center, Department of Nephrology, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, Hangzhou, 310014, China
[3] Department of Nephrology, The First Affiliated Hospital of Zhejiang Chinese Medical University (Zhejiang Provincial Hospital of Chinese Medicine), Hangzhou, 310000, China

Key words: Celastrol, Diabetic nephropathy, Network pharmacology, Molecular docking, Therapeutic mechanism

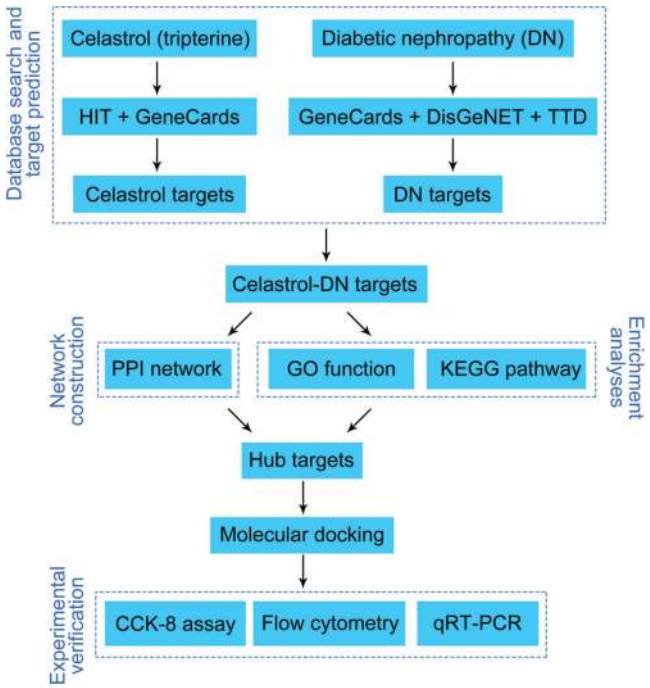
Abstract: Background: Diabetic nephropathy (DN) is a serious complication of diabetes with rising prevalence worldwide. We aimed to explore the anti-DN mechanisms of the compound celastrol derived from the medicinal plant *Tripterygium wilfordii*. **Methods:** Celastrol-related targets were obtained from Herbal Ingredients' Targets (HIT) and GeneCards databases. DN-related targets were retrieved from GeneCards, DisGeNET, and Therapeutic Targets Database (TTD). A Protein-protein interaction (PPI) network was established using the Search Tool for the Retrieval of Interacting Genes (STRING) database. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using ClusterProfiler. The cytoHubba plugin was used to select the top 10 hub targets. Molecular docking was performed employing PyMOL and AutoDock software. Cell counting kit-8 (CCK-8) and flow cytometry assays were used to detect the viability and apoptosis of NRK-52E cells, respectively. The mRNA expression levels of mitogen-activated protein kinase 3 (MAPK3), tumor necrosis factor (TNF), and AKT serine/threonine kinase 1 (AKT1) in NRK-52E cells were assessed using quantitative real-time polymerase chain reaction (qRT-PCR). **Results:** We obtained sixty-six overlapping targets of celastrol and DN. GO and KEGG analyses demonstrated that the core targets of celastrol and DN were mainly involved in the inflammatory and immune response, oxidative stress, advanced glycation end products (AGEs) and their receptors (RAGEs) (AGE-RAGE), TNF, interleukin 17 (IL-17), and MAPK signaling pathways. Finally, based on the good binding activity with celastrol, MAPK3, TNF, and AKT1 were identified as the foremost targets of celastrol. We observed that celastrol enhanced the viability of high glucose (HG)-treated NRK-52E cells and inhibited apoptosis in the *in vitro* assays. Moreover, celastrol decreased the mRNA expression levels of MAPK3, TNF, and AKT1 in high glucose (HG)-treated NRK-52E cells. **Conclusion:** Celastrol may treat DN by targeting APK3, TNF, and AKT1 and regulating inflammatory responses and oxidative stress through the AGE-RAGE, TNF, IL-17, and MAPK signaling pathways.

*Address correspondence to: Juan Jin, lang_018@163.com
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Graphical abstract



Introduction

Diabetic nephropathy (DN), also termed diabetic kidney disease (DKD), is a severe complication of diabetes and the leading cause of end-stage renal disease (ESRD). Its incidence is on the rise worldwide (Zhang *et al.*, 2018a; Khan *et al.*, 2020; Tang *et al.*, 2021). In 2019, approximately 463 million individuals were affected by DN worldwide, and this figure is predicted to rise to 700 million by 2045. DN is characterized by a gradual increase in urine albumin, elevated blood pressure, and decreased glomerular filtration rate (Lu *et al.*, 2021b). At present, the primary therapeutic strategy for DN focuses on the control of glucose and blood pressure (Mohsen *et al.*, 2021). Blocking the renin-angiotensin-aldosterone system (RAAS) remains the predominant approach of managing DN (Block *et al.*, 2022). Furthermore, some agents, such as sodium-glucose cotransporter type 2 (SGLT2) inhibitors (Shaffner *et al.*, 2021) and dipeptidyl peptidase-4 (DPP-4) inhibitors (Trakarnvanich *et al.*, 2021), reportedly have favorable therapeutic potential for DN. Despite improvements in the treatment of DN, current therapeutic approaches are insufficient to completely delay DN progression and numerous DN patients eventually progress into ESRD (Johnson and Spurney, 2015). Therefore, there is an urgent need to further understand the pathological mechanisms of DN, thereby developing novel and effective therapies to prevent its progression and occurrence.

Traditional Chinese medicine has attracted a lot of attention worldwide due to its good efficacy and safety in the treatment of various disorders, such as malignant tumors and inflammatory diseases. Some active ingredients of Chinese herbs, such as geniposide (Li *et al.*, 2020), catalpol (Chen *et al.*, 2020), tripterygium glycosides (Guo *et al.*, 2021a), and quercetin (Hu *et al.*, 2022), have exhibited their beneficial effects in the treatment of DN. Celastrol, also named tripterine, is a natural bioactive ingredient isolated from the root extract of *Tripterygium wilfordii* (a traditional Chinese herb) (Wagh *et al.*, 2021; Guo *et al.*, 2021b; Zhang *et al.*, 2022). Furthermore, it has been reported that celastrol can also be synthesized (Lu *et al.*, 2021a). Celastrol is well-known for its multiple pharmacological effects, including anti-inflammatory, anti-autoimmune, anti-cancer, anti-oxidative, and neuroprotective functions (Wagh *et al.*, 2021). This has made celastrol an appealing therapeutic drug for treating cancer, inflammatory conditions, and autoimmune disorders. For instance, celastrol has been indicated to inhibit inflammation by suppressing the reactive oxygen species (ROS)/nuclear factor kappa B subunit 1(NF-κB)/NLR family pyrin domain containing 3 (NLRP3) axis to mitigate rheumatoid arthritis (Jing *et al.*, 2021). Notably, a previous study revealed that celastrol could suppress inflammation and alleviate renal injury to protect the kidney of diabetic rats by blocking the mitogen-activated protein kinase (MAPK)/NF-κB pathway (Zhang *et al.*, 2019). Further, celastrol has been recently demonstrated to mitigate renal injury, suppress glomerular basement membrane thickening, and assist podocyte homeostasis to delay the progression of early DN in rats (Nie *et al.*, 2020). Another study revealed the inhibition of proteinuria and the kidney-protective effects of celastrol in the treatment of DN (Liu *et al.*, 2021). Despite these findings, the specific mechanisms underlying the anti-DN role of celastrol have not yet been fully illustrated. This prompted us to further investigate the efficacy of celastrol against DN and its associated mechanisms.

In recent years, network pharmacology has been widely employed in the investigation and development of traditional Chinese medicines. Network pharmacology aims to systematically exhibit the mechanism of drug intervention in disease networks by integrating biology, computer science, and bioinformatics, to name a few (Lin *et al.*, 2021).

In this study, we investigated the therapeutic effects of celastrol on DN and related mechanisms. We also conducted *in vitro* experiments to verify the efficacy of celastrol and its mechanisms in the treatment of DN. We aimed to uncover the pharmacological mechanisms of the anti-DN role of celastrol through network pharmacology and molecular docking, thereby providing novel targets for treating DN.

Materials and Methods

Screening of celastrol-related targets

Herbal Ingredients' Targets (HIT) database (version 2.0; http://hit2.badd-cao.net) is a comprehensive database to offer integrated information about medicinal herbs, herb-active compounds, and protein targets under different experimental conditions (Yan et al., 2022; Ye et al., 2011). GeneCards database (version 3.0; https://www.genecards.

org/) is another integrative platform to provide annotated and predicted human gene information (Safran et al., 2010). The target genes of celastrol were acquired from HIT and GeneCards databases employing the keywords "Celastrol" and "Tripterine", and only "Homo sapiens" target genes were selected. Celastrol-related targets were standardized in the UniProt database (https://www.uniprot.org/) and the duplications were removed.

Screening of diabetic nephropathy-related targets

DisGeNET (version 7.0; http://www.disgenet.org/) is a resource platform covering massive data about genes and variants related to human diseases. Therapeutic Targets Database (TTD; https://idrblab.org/ttd/) is focused on providing data about the known and investigated therapeutic protein targets, the targeted disease, the pathway, and the corresponding drugs/ligands directed at each of the targets (Chen *et al.*, 2002; Zhou *et al.*, 2022). Information about the DN-related target genes was collected using the term "Diabetic nephropathy" as queries from GeneCards, DisGeNET, and TTD platforms, with the species set to "*Homo sapiens*". DN-related targets were standardized in the UniProt database and the duplications were removed.

Identification of potential targets of celastrol in the treatment of diabetic nephropathy

VennDiagram (version 1.7.3; http://bioinfogp.cnb.csic.es/tools/venny/index.html) is a widely applied tool in biological analysis that presents the differences between gene lists from distinct differential analyses (Wang *et al.*, 2021). Screened celastrol-related and DN-related were imported into VennDiagram and intersection targets were obtained for further analysis. The network of intersection targets was visualized using Cytoscape software (version 3.8.2).

Construction of the protein-protein interaction network

PPI network was constructed by importing the celastrol-DN intersection genes into the STRING database (https://string-db.org/), which covers nearly all the interactions between proteins (Szklarczyk *et al.*, 2021). Protein interactions were analyzed using ggraph (version 2.0.5) and igraph (version 1.3.1) R packages. The nodes of the PPI network denote proteins, and the edges denote the interactions between two proteins. The organism was limited to "*Homo sapiens*", and the confidence score was set to ">0.4" (Ye *et al.*, 2021; Khan *et al.*, 2022). The other parameters were set to default (network type, full STRING network; size cutoff, no more than 10 interactors). Additionally, the cytoHubba (version 0.1) plugin was used to identify the hub nodes (top 10) in the PPI network. The following parameters of cytoHubba plugin were applied: ranking method, maximal clique centrality (MCC); Hubba nodes, and the top 10 nodes ranked by MCC (default).

Gene ontology functional and kyoto encyclopedia of genes and genomes pathway enrichment analyses

The GO database (http://geneontology.org) was used for gene function analysis including biological process (BP), cellular component (CC), and molecular function (MF) analyses. The KEGG database (https://www.kegg.jp) was utilized to determine biological functions and candidate targets. In this research study, GO functional annotation and KEGG pathway analyses were performed using ClusterProfiler (https://bioconductor.org/packages/release/clusterProfiler.html) in the R package. Furthermore, a hypergeometric test was used to evaluate the association of a specific gene ontology term or biological pathway with the query genes. The hypergeometric distribution formula applied is as follows:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the total number of genes; M is the number of annotated genes in GO and KEGG pathways; n is the number of explored target genes of celastrol; k is the number of common genes of celastrol and annotated genes. A p value < 0.05 was considered to be significantly associated.

Screening of hub targets

The cytoHubba plugin was used to screen out the top 10 hub genes obtained from the GO functional annotation and KEGG pathway analyses. The parameters of the cytoHubba plugin were set to "ranking method: MCC; Hubba nodes: top 10 nodes ranked by MCC (default)". The circlize (version 0.4.15) R package (Gu *et al.*, 2014) was utilized to visualize the network of the top 10 hub genes.

Molecular docking

Molecular docking was conducted to find out the underlying interactions between celastrol and DN. Firstly, the crystal structures of the target proteins were downloaded from RCSB Protein Data Bank (PDB) (https://www.rcsb.org/), while the structure of celastrol in the mol2format was obtained from ZINC (https://zinc.docking.org/) (Ye *et al.*, 2021). Subsequently, PyMOL (version 2.5) and AutoDockTools (version 1.5.6) software were used to remove water, eliminate the original ligands of the active center, hydrogenate, repair broken chains, optimize amino acids, and calculate the charges of the target proteins (Seeliger and de Groot, 2010). All the structures were saved in PDBQT format using Open Babel GUI software. All the flexible bonds of the small molecule ligands were set to be rotatable, whereas the receptors were set to be rigid. AutoDock Vina is a novel optimized molecular docking and virtual screening software, which uses a complicated gradient optimization approach to enhance the accuracy and speed of molecular docking in the course of the local optimization (Shang *et al.*, 2023). We used AutoDock Vina for molecular docking and PyMOL for the visualization of docking results. The binding receptor-ligand activity was evaluated by the value of the binding energy. The lower the binding energy is, the more stable is the receptor-ligand binding as per reports (He *et al.*, 2021). In this study, docking pairs that simultaneously met the criteria of binding energy <−5.0 kcal/mol and the formation of hydrogen bonds (Feng *et al.*, 2021) were considered effective docking and reserved for further analysis.

TABLE 1

Primers used in this study

Gene	Sequence (5′ to 3′)
Mitogen-activated protein kinase 3 (MAPK3) (rat)	Forward: TCAAACCTACTGTCAGCGCA Reverse: GGTGCTCTGAGGATGTCTCG
Tumor necrosis factor (TNF) (rat)	Forward: GATCGGTCCCAACAAGGAGG Reverse: TCCCTCAGGGGTGTCCTTAG
AKT serine/threonine kinase 1 (AKT1) (rat)	Forward: CTCATTCCAGACCCACGACC Reverse: CTCCGTTCACTGTCCACACA
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rat)	Forward: GCTGAGAATGGGAAGCTGGT Reverse: CTCGTGGTTCACACCCATCA

Cell culture, diabetic nephropathy induction and celastrol treatment

The rat renal tubular epithelial cell line NRK-52E, was purchased from the American Type Culture Collection (Manassas, VA, USA). NRK-52E cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture at 37°C in 5% CO_2. High glucose (HG) in the concentration of 30 mmol/L was used to induce a cell model of DN. Cells treated with 5.5 mmol/L glucose served as controls. Subsequently, the Celastrol (purity ≥ 98%; #IC0220) was purchased from Solarbio (Beijing, China). HG-induced cells were treated with different doses of celastrol (0.5, 1, and 2 μM). The doses of celastrol used were based on previous research (Fang and Chang, 2021).

Cell counting kit-8 assay

A CCK-8 kit (#C0037, Beyotime, Shanghai, China) was used to probe the NRK-52E cell viability according to the manufacturer's instructions. Briefly, 100 μL of NRK-52E cells were seeded in 96-well plates (2,000 cells/well), treated with various concentrations of celastrol, and cultured at 37°C in 5% CO_2 for 24 h. The cells were then incubated with 10 μL of CCK-8 solution for another 2 h. Cell viability was evaluated using a microplate reader (DR-3518G, Hiwell Diatek, Wuxi, China).

Flow cytometry

Annexin V-fluorescein isothiocyanate (FITC) Cell Apoptosis Detection Kit (#C1062S, Beyotime) was used to assess NRK-52E cell apoptosis. After washing with phosphate-buffered saline (PBS) twice, cells were suspended in a binding buffer (300 μL). The cells were then stained with Annexin V-FITC (5 μL) for 15 min and propidium iodide (PI; 10 μL) for 10 min at 25°C in the dark. Cell apoptosis was assessed using a CytoFLEX S flow cytometer (Beckman, FL, USA) and then quantified using Cell Quest software (BD Biosciences, NJ, USA).

Quantitative real-time polymerase chain reaction

Total RNA was isolated from NRK-52E cells using the TRIzol reagent (#15596018, Invitrogen, CA, USA). Reverse transcription was performed for cDNA synthesis using the FastKing-RT SuperMix (#KR118-02, Tiangen, Beijing, China). qRT-PCR analysis was then carried out with the SYBR Green PCR Master Mix (#4364344; Thermo Fisher Scientific, MA, USA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA). The following reaction program was applied: 95°C for 3 min, followed by 95°C for 12 s, and then 62°C for 40 s; 40 cycles. The gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Primers used in this study are listed in Table 1.

Statistical analysis

Data were exhibited as mean ± standard deviation and analyzed using GraphPad 7.0 software (La Jolla, CA, USA). We applied one-way analysis of variance (ANOVA) followed by Tukey's tests to compare differences between groups. $p < 0.05$ indicated statistical significance.

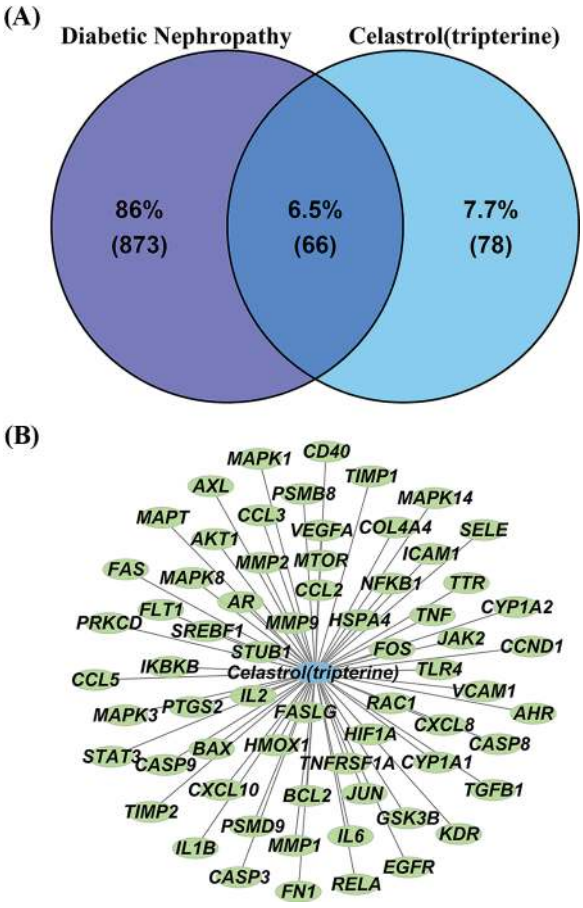


FIGURE 1. Venn diagram (A) and network (B) of celastrol-DN intersection targets. Key: DN, diabetic nephropathy.

Results

Selection of celastrol-diabetic nephropathy intersection targets

As outlined above, HIT and GeneCards databases were used for screening the potential celastrol-DN-related targets. A total of 144 target genes linked with celastrol were acquired after UniProt standardization and duplication deletion (Suppl. Table S1). Moreover, we obtained 939 DN-related target genes after eliminating nonstandard and duplicate targets (Suppl. Table S2). Finally, 66 intersection targets were screened out from the identified celastrol-related and DN-related targets (Fig. 1). The 66 candidate targets are listed in Suppl. Table S3.

Protein-protein Interaction network analysis of celastrol-diabetic nephropathy intersection targets

To probe the interactions among the identified targets (n = 66), the PPI network was constructed using the STRING database. The obtained PPI network contained 66 nodes (proteins) and 1,168 edges (interaction pairs). The top 10 core network proteins (red nodes) were acquired using the cytoHubba plugin. These ten genes included interleukin 6 (IL6), Jun proto-oncogene (JUN), caspase 3 (CASP3), prostaglandin-endoperoxide synthase 2 (PTGS2), matrix metallopeptidase 9 (MMP9), signal transducer and activator of transcription 3 (STAT3), interleukin 1 beta (IL1B), mitogen-activated protein kinase 3 (MAPK3), tumor necrosis factor (TNF), and AKT serine/threonine kinase 1 (AKT1). Furthermore, some of these candidate genes such as JUN and STAT3 are transcriptional regulatory factors involved in cell growth (Fig. 2).

Gene ontology functional and kyoto encyclopedia of genes and genomes pathway enrichment analyses

To investigate the potential functions and mechanisms of the 66 targets obtained in DN, the GO functional annotation and KEGG pathway analyses were performed. Based on GO function analysis, a total of 1860 BP, 95 MF, and 33 CC terms were obtained (p value < 0.05). As shown in Fig. 3A, the top 10 enriched GO functions were respectively selected from the BP, MF, and CC terms. Meanwhile, 164 enriched pathways were acquired through KEGG pathway analysis (c < 0.05). As displayed in Fig. 3B, the top 30 enriched pathways were then screened (p value < 0.05). KEGG results revealed that the 66 candidate targets were mainly correlated with AGE-RAGE, TNF, IL-17, and MAPK signaling pathways. Detailed information about the critical GO functions and KEGG pathways is provided in Tables 2 and 3, respectively.

Determination of hub genes based on Gene Ontology functional and kyoto encyclopedia of genes and genomes pathway enrichment analyses

To further ascertain which target plays a pivotal role in the 30 vitally enriched biological functions and pathways, the cytoHubbaplugin was used to screen the hub targets. The top 10 GO function-related targets were selected according to MCC scores. These were mitogen-activated protein kinase 1 (MAPK1), MAPK3, TNF, Janus kinase 2 (JAK2), Fas ligand (FASLG), mitogen-activated protein kinase 14 (MAPK14), transforming growth factor beta 1 (TGFB1), IL6, C-C motif chemokine ligand (CCL3), and AKT1 (MCC score ≥ 12) (Fig. 4A). Additionally, the top 10 KEGG pathway-related targets were also screened based on MCC scores, including nuclear factor kappa B subunit 1 (NFKB1), nuclear factor kappa B subunit p65 (RELA), AKT1, inhibitor of nuclear factor kappa B kinase subunit beta (IKBKB), mitogen-activated protein kinase 8 (MAPK8), MAPK3, MAPK1, TNF, MAPK14, and JUN (MCC score ≥ 12) (Fig. 4B).

Determination of potential targets and molecular docking verification

To further assess the vital candidate targets, molecular docking was conducted to evaluate the reliability of the anti-DN targets of celastrol. Three intersection targets (MAPK3, TNF, and AKT1) were obtained from the hub target networks of PPI, GO, and KEGG (Fig. 5A). A binding energy value lower

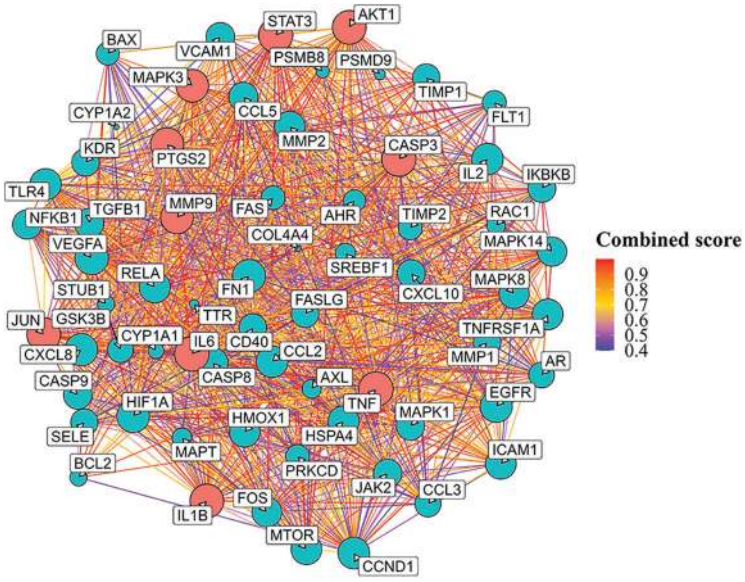


FIGURE 2. PPI network analysis of celastrol-DN intersection targets using STRING database. Red nodes represent the top 10 hub genes. Key: PPI, protein-protein interaction; DN, diabetic nephropathy.

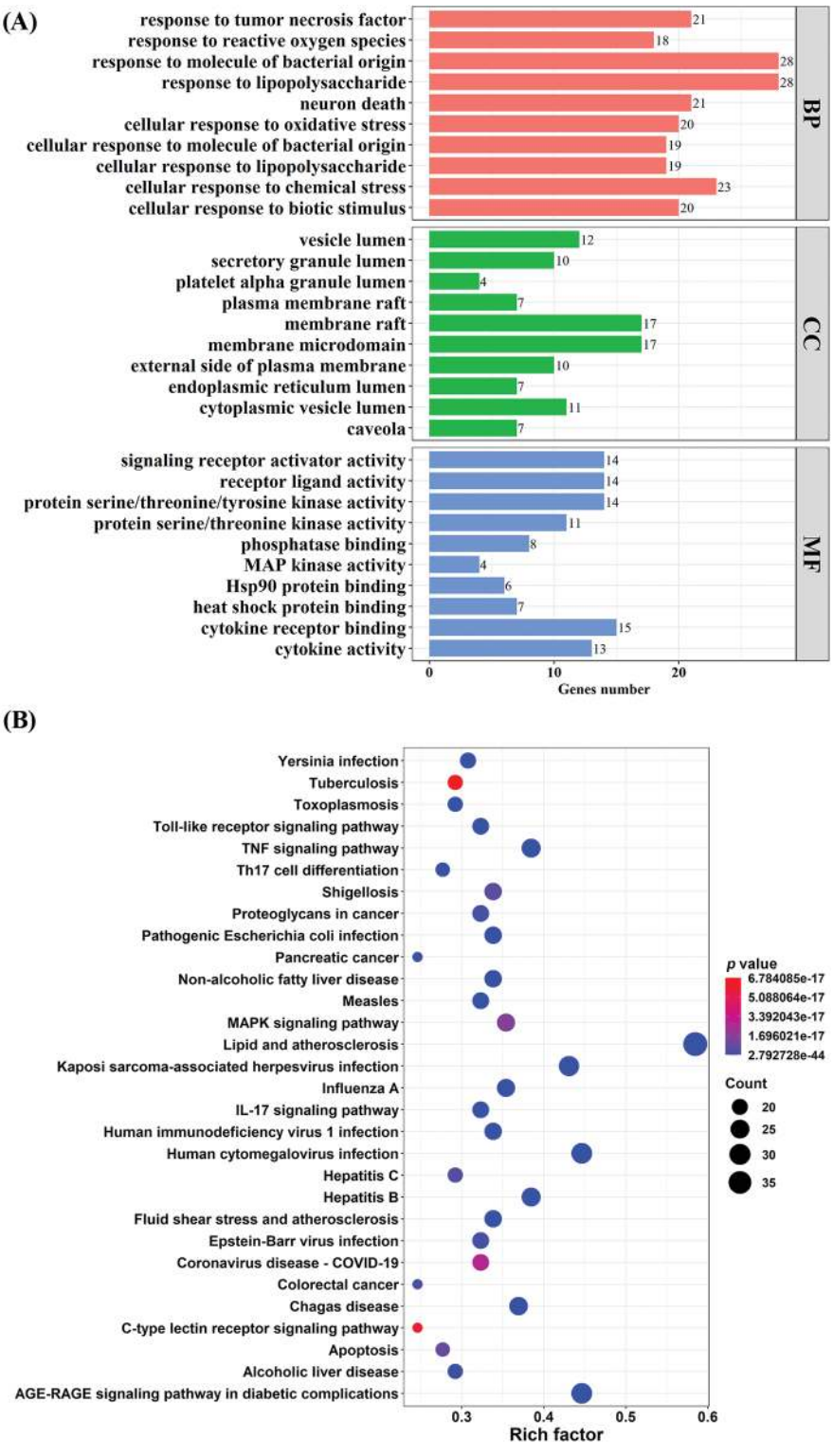


FIGURE 3. GO functional and KEGG pathway enrichment analyses. (A) Top 10 enriched BP, CC, and MF terms from GO functional enrichment analysis. (B) Top 30 enriched pathways from KEGG pathway enrichment analysis. Key: GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

TABLE 2

Data of the top 10 enriched biological process (BP), cellular component (CC), and molecular function (MF) terms from gene ontology (GO) functional enrichment analysis

ONTOLOGY	ID	Description	Count
BP	GO:0032496	Response to lipopolysaccharide	28
BP	GO:0002237	Response to molecule of bacterial origin	28
BP	GO:0062197	Cellular response to chemical stress	23

Table 2 (continued)

ONTOLOGY	ID	Description	Count
BP	GO:0034612	Response to tumor necrosis factor	21
BP	GO:0071216	Cellular response to biotic stimulus	20
BP	GO:0071222	Cellular response to lipopolysaccharide	19
BP	GO:0071219	Cellular response to molecule of bacterial origin	19
BP	GO:0034599	Cellular response to oxidative stress	20
BP	GO:0000302	Response to reactive oxygen species	18
BP	GO:0070997	Neuron death	21
CC	GO:0045121	Membrane raft	17
CC	GO:0098857	Membrane microdomain	17
CC	GO:0031983	Vesicle lumen	12
CC	GO:0060205	Cytoplasmic vesicle lumen	11
CC	GO:0005901	Caveola	7
CC	GO:0044853	Plasma membrane raft	7
CC	GO:0034774	Secretory granule lumen	10
CC	GO:0009897	External side of plasma membrane	10
CC	GO:0031093	Platelet alpha granule lumen	4
CC	GO:0005788	Endoplasmic reticulum lumen	7
MF	GO:0005126	Cytokine receptor binding	15
MF	GO:0005125	Cytokine activity	13
MF	GO:0004712	Protein serine/threonine/tyrosine kinase activity	14
MF	GO:0048018	Receptor ligand activity	14
MF	GO:0030546	Signaling receptor activator activity	14
MF	GO:0051879	Hsp90 protein binding	6
MF	GO:0004707	MAP kinase activity	4
MF	GO:0031072	Heat shock protein binding	7
MF	GO:0004674	Protein serine/threonine kinase activity	11
MF	GO:0019902	Phosphatase binding	8

TABLE 3

Data of the top 30 enriched terms from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

ID	Description	*p* value	Count
hsa05417	Lipid and atherosclerosis	2.79273E-44	38
hsa04933	AGE-RAGE signaling pathway in diabetic Complications	8.05873E-40	29
hsa04668	TNF signaling pathway	6.98077E-31	25
hsa05142	Chagas disease	3.24433E-30	24
hsa05167	Kaposi sarcoma-associated herpesvirus infection	4.47717E-29	28
hsa05163	Human cytomegalovirus infection	1.05545E-28	29
hsa05161	Hepatitis B	1.43757E-26	25
hsa04657	IL-17 signaling pathway	7.63083E-26	21
hsa04620	Toll-like receptor signaling pathway	7.77174E-25	21
hsa05418	Fluid shear stress and atherosclerosis	1.50382E-23	22
hsa05164	Influenza A	6.00699E-23	23
hsa04932	Non-alcoholic fatty liver disease	1.84131E-22	22

(Continued)

Table 3 (continued)

ID	Description	*p* value	Count
hsa05162	Measles	5.08797E-22	21
hsa05145	Toxoplasmosis	6.34288E-21	19
hsa05135	Yersinia infection	1.17894E-20	20
hsa05130	Pathogenic *Escherichia coli* infection	4.0978E-20	22
hsa04659	Th17 cell differentiation	1.09688E-19	18
hsa05170	Human immunodeficiency virus 1 infection	2.08327E-19	22
hsa05212	Pancreatic cancer	2.79889E-19	16
hsa04936	Alcoholic liver disease	7.00228E-19	19
hsa05169	Epstein-Barr virus infection	1.56667E-18	21
hsa05205	Proteoglycans in cancer	2.13616E-18	21
hsa05210	Colorectal cancer	2.35929E-18	16
hsa05160	Hepatitis C	4.92939E-18	19
hsa05131	Shigellosis	5.90738E-18	22
hsa04210	Apoptosis	8.22658E-18	18
hsa04010	MAPK signaling pathway	1.61954E-17	23
hsa05171	Coronavirus disease—COVID-19	2.82667E-17	21
hsa04625	C-type lectin receptor signaling pathway	5.84233E-17	16
hsa05152	Tuberculosis	6.78409E-17	19

than −5 kcal/mol denoted good binding activity while a binding energy value lower than −7 kcal/mol denoted strong activity (Gai *et al.*, 2022). The molecular docking results revealed that celastrol had a favorable binding ability with MAPK3 (binding energy = −7.03 kcal/mol), TNF (binding energy = −7.15 kcal/mol), and AKT1 (binding energy = −7.97 kcal/mol) (Table 4 and Fig. 5B).

In vitro anti-DN effects of celastrol

To verify the therapeutic effects of celastrol on DN *in vitro*, 30 mmol/L HG was used to induce a cell model of DN, and cells were then treated with celastrol at different doses (0.5, 1, and 2 μM). Results of the CCK-8 assay showed that HG treatment significantly inhibited NRK-52E cell viability ($p < 0.01$), which was rescued by celastrol in a dose-dependent manner ($p < 0.05$; Fig. 6A). According to flow cytometry analysis, while HG markedly promoted the apoptosis of NRK-52E cells ($p < 0.01$), celastrol reversed this HG-induced cell apoptosis in a dose-dependent manner ($p < 0.05$; Fig. 6B). Further, qRT-PCR results showed that the mRNA expression levels of MAPK3, TNF, and AKT1 in NRK-52E cells were observably elevated by HG treatment ($p < 0.01$; Fig. 6C). Similarly, celastrol was found to decrease the mRNA expression levels of MAPK3, TNF, and AKT1 in HG-treated NRK-52E cells in a dose-dependent manner ($p < 0.05$; Fig. 6C).

Discussion

DN is one of the most common microvascular complications of diabetes mellitus, manifesting as thickened glomerular basement membrane, enhanced extracellular matrix formation, and podocyte loss (Han *et al.*, 2017). DN tends to develop into ESRD which has high morbidity and mortality rates (Zhu *et al.*, 2021; Noor *et al.*, 2021). Natural bioactive compounds such as terpenoids have exhibited their potential to treat DN. For example, paeoniflorin could inhibit TLR4-mediated inflammation and ameliorate the clinical symptoms and the severity of DN (Shao *et al.*, 2019). In another study, catalpol could mitigate endothelial dysfunction and inflammation to impede the progression of DN (Shu *et al.*, 2021). Celastrol, a quinone methide triterpene, extracted from *Tripterygium wilfordii* has been shown to possess various pharmacological properties, including anti-inflammatory, anti-autoimmune, anti-cancer, and neuroprotective activities (Wagh *et al.*, 2021; Lim *et al.*, 2021; Gu *et al.*, 2018). Further, celastrol has been extensively used in the treatment of autoimmune diseases, such as rheumatoid arthritis (Jing *et al.*, 2021) and systemic lupus erythematosus (Song *et al.*, 2020). An increasing number of studies indicated that celastrol could alleviate the pathological injury in DN (Nie *et al.*, 2020; Zhan *et al.*, 2018). Moreover, a recent study has elaborated that CN suppressed proteinuria, and showed anti-inflammatory and kidney-protective functions celastrol in DN (Liu *et al.*, 2021). However, the mechanisms underlying the anti-DN effects of celastrol have not yet been fully elucidated. Therefore, it is essential to further explore the therapeutic effects of celastrol and its mechanisms in the treatment of DN.

In this study, sixty-six intersection targets of celastrol and DN were screened based on public databases. In the PPI network of 66 targets, IL6, JUN, CASP3, PTGS2, MMP9, STAT3, IL1B, MAPK3, TNF, and AKT1 were identified as

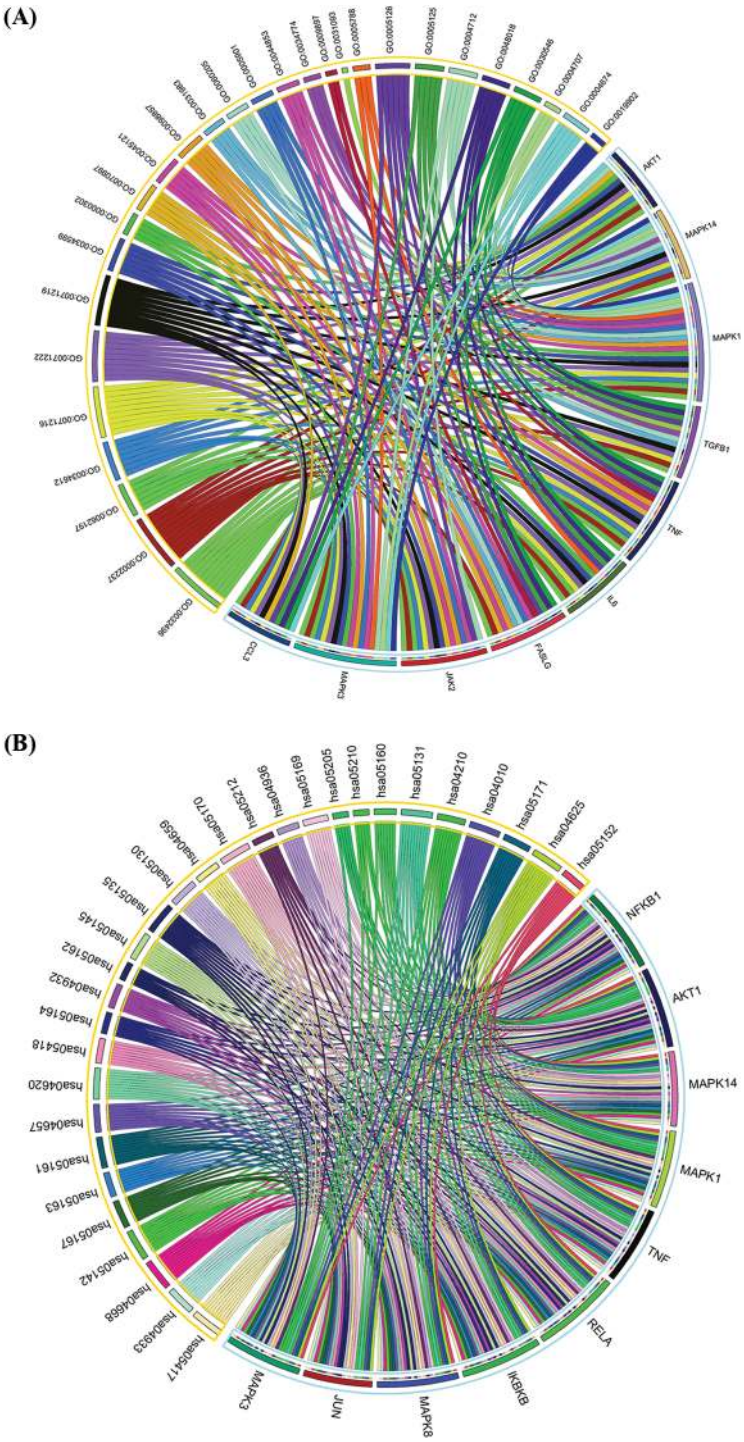


FIGURE 4. Determination of hub targets originating from GO functional (A) and KEGG pathway (B) enrichment analyses. Key: GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

the top 10 core targets for celastrol in the treatment of DN. The hub targets, such as IL6, IL1B, and TNF, were mainly associated with inflammatory and immune responses. Chronic low-grade inflammation has been a crucial characteristic of DN and a failure in addressing inflammation is a primary contributing factor in the constant development of DN (Wu *et al.*, 2021). A previous study demonstrated that celastrol could reduce levels of IL6, IL1B, and TNF to attenuate oxidative damage of type 2 diabetes (Cascão *et al.*, 2017). Further evidence indicated that down-regulation of IL6, IL1, and TNF can alleviate the pathological injury of DN (Wu *et al.*, 2020; Sun *et al.*, 2020). Another study showed that the inhibition of the STAT3 pathway-mediated inflammation can attenuate DN (Zhang *et al.*, 2021). Further, celastrol was found to effectively inhibit the secretion of pro-inflammatory cytokines, including IL6, IL1B, and TNF-α, thus mitigating DN (Zhan *et al.*, 2018). This accounts for the key role of IL6, IL1B, and TNF in treating DN as revealed in our study. Collectively, these results suggest that the anti-DN effects of celastrol can be partly attributed to its potent anti-inflammatory activity.

According to the GO functional enrichment analysis, the potential targets of celastrol against DN were mainly concentrated in BPs, such as response to lipopolysaccharide,

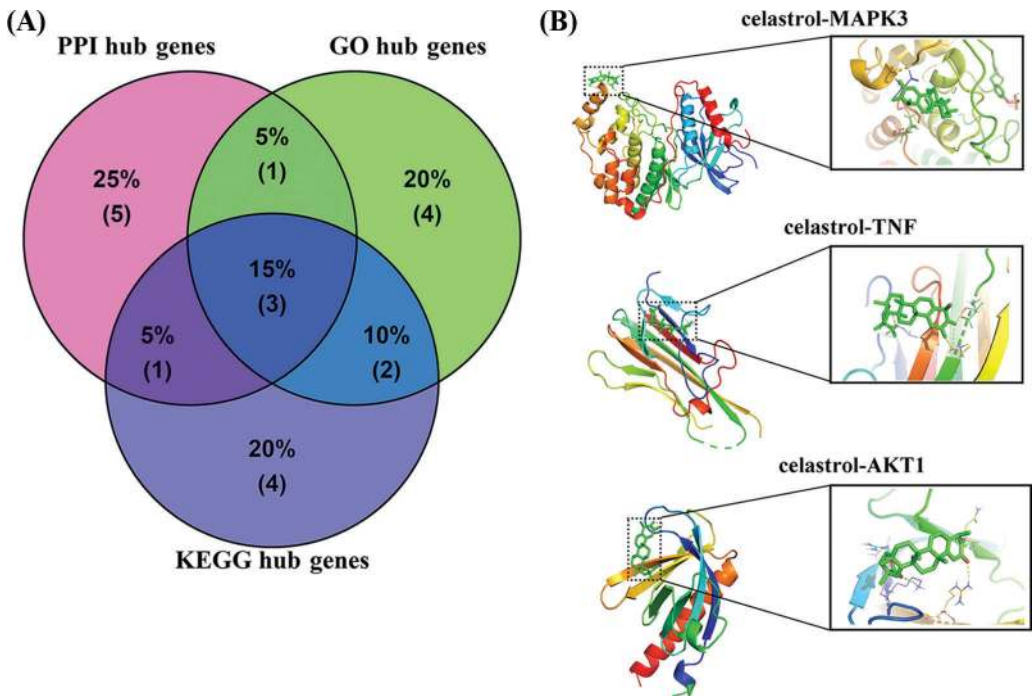


FIGURE 5. Determination of hub targets and molecular docking assesment. (A) Acquisition of intersection targets from PPI, GO, and KEGG hub target networks. (B) Molecular models of the binding of celastrol with MAPK3, TNF, and AKT1. Key: PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK3, mitogen-activated protein kinase 3; TNF, tumor necrosis factor; AKT1, AKT serine/threonine kinase 1.

TABLE 4

Results of molecular docking

Component	Target	Binding energy (kcal/mol)
Celastrol (tripterine)	Mitogen-activated protein kinase 3 (MAPK3)	−7.03
	Tumor necrosis factor (TNF)	−7.15
	AKT serine/threonine kinase 1 (AKT1)	−7.97

response to molecules of bacterial origin, and oxidative stress. Relevant findings have been shown in other reports. For example the development of DN was associated with lipopolysaccharide-induced inflammation (Jiang *et al.*, 2020), gut microbiota dysbiosis (Fernandes *et al.*, 2019), and oxidative stress (Gerardo Yanowsky-Escatell *et al.*, 2020), which were partially in line with our findings. Notably, a previous study indicated the crosstalk between inflammation and oxidative stress mechanisms in DN pathogenesis (Samsu, 2021). Another study found that celastrol could inhibit oxidative stress to ameliorate the pathological damage of the chronic complications of diabetes (Guan *et al.*, 2016). The core targets related to GO functions included MAPK1, MAPK3, TNF, JAK2, FASLG, MAPK14, TGFB1, IL6, CCL3, and AKT1, which mainly participate in the regulation of inflammatory responses. For instance, MAPK signaling pathways are vital for the inflammatory process, including the secretion of pro-inflammatory cytokines induced by ROS (Galganska *et al.*, 2021). CCL3 is a chemokine that triggers various pro-inflammatory reactions such as leukocyte chemotaxis and pushes T cells into the inflammatory tissue area from blood circulation (Zhang *et al.*, 2018b). Consistent with our data, previous data has documented the involvement of CCL3 in DN development (Araújo *et al.*, 2020). To summarize, our findings further indicate the complexity of the pathological mechanisms of DN, including inflammatory responses, response to lipopolysaccharide, response to molecules of bacterial origin, and oxidative stress.

KEGG pathway enrichment analysis showed that celastrol could address DN primarily through AGE-RAGE, TNF, IL-17, and MAPK signaling pathways. Evidence has indicated that the increase in end products of advanced glycosylation (AGEs) and its receptor (RAGE) can contribute to the onset and development of DN and nephron cellular injury (Barocio-Pantoja *et al.*, 2021). Notably, a recent study has demonstrated that inhibiting AGE-RAGE-mediated inflammation signaling pathways could prevent the development and progression of DN (Chen *et al.*, 2022). Further, accumulating evidence showed that the pro-inflammatory cytokines, TNF and IL-17, play a vital part in the development and progression of DN (Park *et al.*, 2019; Kim *et al.*, 2021). Additionally, blocking the TNF/IL-17 axis has been proven to alleviate pathological injury of DN (Ma *et al.*, 2019). In terms of the MAPK signaling pathway, it was found to be associated with multiple pathological mechanisms of DN, including inflammation and oxidative stress (Song *et al.*, 2018; Zhu *et al.*, 2020; Ma *et al.*, 2021). Interestingly, a previous study observed that celastrol-loaded nanomicelles could ameliorate inflammation, lipid accumulation, and insulin resistance in obese mice (Zhao *et al.*, 2019). Thus, it is reasonable to

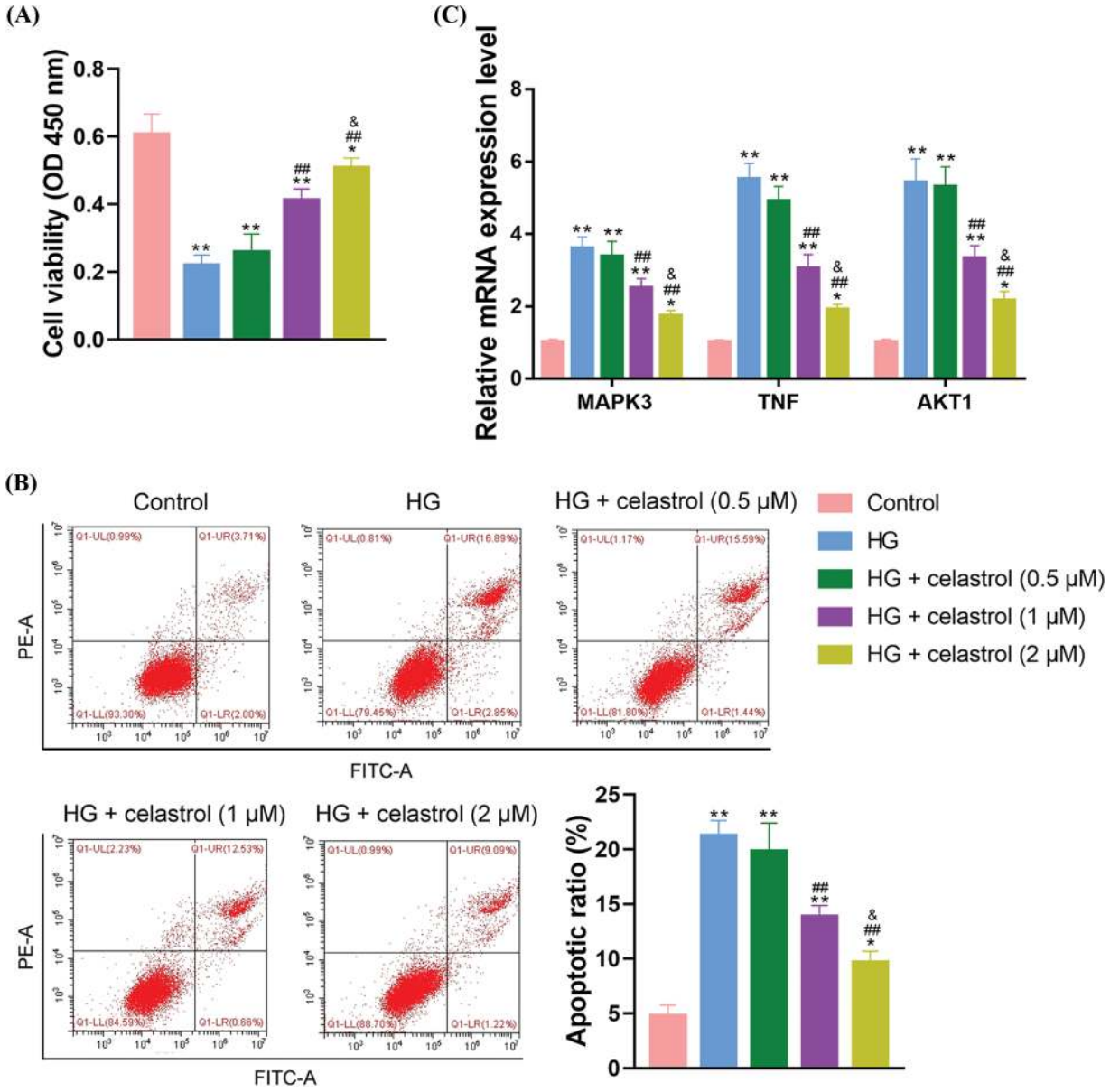


FIGURE 6. *In vitro* therapeutic effects of celastrol on DN. (A) Detection of NRK-52E cell viability using CCK-8. (B) Detection of NRK-52E cell apoptosis using flow cytometry. (C) Detection of the mRNA expression levels of MAPK3, TNF, and AKT1 in NRK-52E cells using qRT-PCR. Data were exhibited as mean ± standard deviation. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* Control group; $^{\#\#}p < 0.01$ *vs.* HG group; $^{\&}p < 0.05$ *vs.* HG + celastrol (1 μM) group. Key: DN, diabetic nephropathy; CCK-8, cell counting kit-8; MAPK3, mitogen-activated protein kinase 3; TNF, tumor necrosis factor; AKT1, AKT serine/threonine kinase 1; qRT-PCR, quantitative real-time polymerase chain reaction.

hypothesize the positive effects of celastrol on inflammation, lipid homeostasis, and insulin resistance in preventing DN progression. Besides, our data showed that NFKB1, RELA, AKT1, IKBKB, MAPK8, MAPK3, MAPK1, TNF, MAPK14, and JUN were the key celastrol targets in the KEGG analysis. Similarly, these targets, such as MAPK8, MAPK3, MAPK1, TNF, and MAPK14, are mainly involved in inflammatory responses. Collectively, these data further demonstrate that celastrol inhibits DN progression mainly by regulating inflammatory responses through the AGE-RAGE, TNF, IL-17, and MAPK signaling pathways.

In the present study, we finally obtained three overlapping targets (MAPK3, TNF, and AKT1) from the PPI, GO, and KEGG networks of the hub targets. Molecular docking showed that celastrol exhibited good binding abilities with MAPK3, TNF, and AKT1.

An increasing number of studies demonstrates that cell apoptosis is one of the major mechanisms in the development and progression of DN. Elevated apoptosis has been found in the kidneys of DN patients and suppression of the cell apoptosis in DN mice could mitigate DN symptoms (Fan *et al.*, 2022). It was previously revealed that celastrol pre-treatment reversed HG-induced cell apoptosis to ameliorate the pathological injury of DN (Zhan *et al.*, 2018). Consistent with this report, our data also showed that celastrol significantly inhibited the apoptosis of HG-treated NRK-52E cells. Additionally, we found that the viability of HG-treated NRK-52E cells was suppressed compared with control cells, which was however reversed by celastrol treatment.

MAPK3, also known as ERK1, participates in a wide range of cellular processes including proliferation,

inflammation, and cellular metabolism (Chen *et al*., 2019; Kassouf and Sumara, 2020). Further, MAPK3/MAPK1 signaling could enhance pancreatic β-cell mass and insulin production (Kassouf and Sumara, 2020). TNF, which is a common pro-inflammatory cytokine acts as a switch in immunity (Fischer *et al*., 2020). Therefore, TNF inhibitors have been developed and clinically applied to treat inflammatory and autoimmune disorders (Fischer *et al*., 2020). AKT1 is a key mediator of cellular survival and growth, which is involved in inflammatory responses (Zhang *et al*., 2021; Vergadi *et al*., 2017). An animal model-based investigation indicated that celastrol could suppress the expression of pro-inflammatory cytokines (e.g., TNF-α) to mitigate neuropathic pain in rats (Jin *et al*., 2022). Furthermore, a recent study has demonstrated that downregulation of the levels of MAPK3 and TNF by blocking AKT1 signaling could prevent the progression of DN (Li and Xu, 2022). We observed that celastrol effectively decreased the expression of MAPK3, TNF, and AKT1 in HG-treated NRK-52E cells. This was similar to that reported in research (Jin *et al*., 2022; Li and Xu, 2022). Collectively, these findings indicate that celastrol can be used to treat DN where MAPK3, TNF, and AKT1 are the pivotal therapeutic targets in its action.

Our study has some limitations. First, the data used in this study were from public databases that need to be updated in real-time. This makes it possible that some other vital targets or pathways associated with DN might have been overlooked. Second, we only verified the *in vitro* therapeutic effects of celastrol on DN without *in vivo* validation. Third, the crosstalk between the core mechanisms (e.g., inflammation and oxidative stress) revealed in this study remains to be further elucidated. These limitations will be perfected and addressed in our subsequent studies.

Conclusions

This study reveals that celastrol exerts therapeutic effects on DN through multiple pathways and mechanisms, including AGE-RAGE, TNF, IL-17, and MAPK signaling pathways, the inflammatory and immune response, and oxidative stress. MAPK3, TNF, and AKT1 are the foremost targets of celastrol against DN. Additionally, the anti-DN effects of celastrol and its therapeutic targets were confirmed *in vitro*. Our study provides a direction for follow-up animal and clinical studies, deepens our insights into the mechanisms related to DN, and furnishes a reference for developing celastrol as a novel drug for the treatment of DN.

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Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

TABLE S1. Celastrol-related targets
TABLE S2. DN-related targets. DN, diabetic nephropathy
TABLE S3. Celastrol-DN intersection targets. DN, diabetic nephropathy