

# Oridonin Attenuates Diabetes-Induced Renal Fibrosis via Inhibition of the TXNIP/NLRP3 and NF- $\kappa$ B Pathway in Rats

**Gengzhen Huang**

Chengdu University of Traditional Chinese Medicine

**Yaodan Zhang**

Hospital of Chengdu University of Traditional Chinese Medicine

**Yingying Zhang**

Chengdu University of Traditional Chinese Medicine

**Xiaotao Zhou**

Chengdu University of Traditional Chinese Medicine

**Yuan Xu**

Chengdu University of Traditional Chinese Medicine

**Huiting Wei**

Chengdu University of Traditional Chinese Medicine

**Yuerong Ma** (✉ [mayr666@163.com](mailto:mayr666@163.com))

Chengdu University of Traditional Chinese Medicine

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## Research Article

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# Abstract

**Background:** Oxidative stress and its induced inflammation are important pathological processes of diabetic nephropathy (DN). Oridonin, a component isolated from *Rabdosia rubescens*, possesses remarkable anti-inflammatory, immunoregulatory properties, it is also a newly reported NLRP3 inhibitor. However, the renoprotective effects of Oridonin and the underlying molecular mechanisms have not been explored in DN. We hypothesized that Oridonin could reduce NLRP3 pathway and ameliorate diabetes-induced renal fibrosis.

**Methods:** We used STZ-induced diabetic rats combined high-fat diet to establish a T2DM animal model, and then treated with Oridonin (10, 20 mg/kg/day) for two weeks. Kidney function and renal fibrosis were assessed. In addition, the expression of inflammatory factors and fibrotic markers were analyzed by western blot.

**Results:** Oridonin treatment preserved kidney function and markedly limited the renal fibrosis size in diabetic rats. The renal fibrotic markers were inhibited in the 10 mg/kg/day group and 20 mg/kg/day group compared to the T2DM group. Moreover, the expression levels of TXNIP/NLRP3 and NF- $\kappa$ B pathway were decreased in the Oridonin treatment group compared to non-treated group.

**Conclusions:** The NLRP3-inflammasome inhibitor Oridonin reduces renal fibrosis and preserves kidney function in T2DM rat model, which indicates potential therapeutic effect of Oridonin on DN.

## Introduction

Diabetes has become a global health concern in recent years. Diabetic nephropathy (DN) has been identified as a major cause of persistent kidney disease and end-stage renal failure<sup>[1, 2]</sup>. The pathological symptoms of DN include the inflammatory infiltration of nephrocytes, damage of the renal tubular epithelial cells, glomerular sclerosis and apoptosis, and renal tubular interstitial fibrosis<sup>[3]</sup>.

Although DN has been considered primarily as a non-immune kidney disease in the past, recent theoretical and experimental studies have suggested that chronic inflammation and oxidative stress are two important contributors to the progression of DN<sup>[4, 5]</sup>. The diabetic environment leads to the production of advanced glycation end products and changes in circulation and hemodynamics, followed by the release of reactive oxygen species (ROS) and inflammatory mediators, resulting in impaired renal function and increased markers of fibrosis<sup>[6]</sup>. Evidence collected in recent years suggests that inflammatory processes promoted by innate immune responses are critical in the pathogenesis of DN<sup>[7, 8]</sup>.

TLR4 is a component of primary innate immune receptor-mediated inflammatory signaling pathway, which is distributed in glomerular mesangial cells and renal tubular epithelial cells in renal tissue<sup>[9, 10]</sup>. There is increasing evidence that TLR4 expression is significantly increased in renal tubular epithelial cells and mesangial cells in response to hyperglycemia and angiotensin II, which activates the downstream nuclear factor (NF- $\kappa$ B) pathway and accelerates secretion of pro-inflammatory cytokines.

Including interleukin-1  $\beta$  (IL-1 $\beta$ ), IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and monocyte chemoattractant protein 1 (MCP-1), which aggravate kidney injury<sup>[11, 12]</sup>.

Nod-like receptor protein-3 (NLRP3) inflammasome is an assembled molecular complex consisting of NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), and cysteinyl aspartate specific proteinase (caspase-1)<sup>[13, 14]</sup>. It plays an important role in the occurrence and development of various non-bacterial inflammatory diseases, especially those closely related to metabolic disorders and endogenous stimulation. In hyperglycemia, the body can produce a variety of dangerous related molecules, such as fatty acids and ROS, which can be recognized by the related pattern recognition receptor (PRR), thus initiating the activation of NLRP3 pathway. Upon the activation of inflammasomes, IL-1 $\beta$  and IL-18 are processed and activated, resulting in pro-inflammatory responses, which aggravate the inflammatory injury and decrease the kidney function. Studies have shown that direct or indirect inhibition of NLRP3 inflammasome activity is beneficial to reduce inflammatory injury and renal fibrosis<sup>[15]</sup>. NLRP3 inflammasome is a popular target in the treatment of diabetic nephropathy.

The Trx system is an important antioxidant system, which relieves oxidative stress by providing electrons to peroxides, thus enabling peroxides to effectively remove reactive oxygen species and nitrogen. Thioredoxin-interacting protein (TXNIP) play an important role in cell death and immune response through interaction with Trx system. TXNIP is reported as a key regulator of pancreatic  $\beta$ -cell biological function<sup>[16]</sup>. The excess production of reactive oxygen species in diabetes leads to the separation of TXNIP from its binding protein Trx, which binds to NLRP3, leading to the activation of NLRP3 inflammasome<sup>[17]</sup>. It has been reported that NADPH oxidase (NOX4)-derived ROS promotes the dissociation of TXNIP-Trx and increases the binding of TXNIP to NLRP3<sup>[18]</sup>. TXNIP might be the key to linking the hyperglycemic environment to inflammation by activating the NLRP3 inflammasome.

Oridonin (Ori, Figure 1) is a famous diterpenoid isolated from *Rubescens rubescens*, which has many biological properties such as antioxidant, anti-inflammatory, immunomodulatory and anti-tumor<sup>[19]</sup>. Ori has been used to treat inflammatory diseases in China for hundreds of years and has become one of the most popular herbs clinically. Evidence reported that Ori efficiently increases survival, alleviates proteinuria, attenuates renal dysfunction and ameliorates the clinical manifestations of systemic lupus erythematosus (SLE) in mice<sup>[20]</sup>. Studies have also been reported the therapeutic effects of Ori in DN by reducing inflammatory cell infiltration, down-regulating TLR4 expression and inhibiting NF- $\kappa$ B and p38-MAPK activation with a type 2 diabetes mellitus (T2DM) rat model and HG-treated rat mesangial cells<sup>[21]</sup>. However, few studies have investigated the effects of Ori on TXNIP/NLRP3 in diabetic rats. Therefore, we aimed to investigate whether Ori protects against renal fibrosis in diabetic rats and further elucidate whether the anti-inflammatory mechanism that involves the TLR4/NF- $\kappa$ B and TXNIP/NLRP3 signaling pathways. Our findings may support the clinical application of Ori as a treatment for DN.

## Methods And Materials

## Materials

Oridonin was purchased from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). Streptozotocin (STZ) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Commercial assay kits for serum creatinine (Scr), blood urea nitrogen (BUN) and uric acid (UA) were purchased from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Antibodies for TGF- $\beta$ ,  $\alpha$ -SMA, Col-III, TLR4, p-p65, p65 were purchased from Cell Signaling Technology (MA, USA). Antibodies for TXNIP, NLRP3, caspase-1, IL-1 $\beta$ , GAPDH, goat anti-rabbit secondary antibody were purchased from Bioss (Beijing, China).

## Animals and treatment

Specific pathogen-free grade male Sprague Dawley rats (weighing 150-200 g) were purchased from the Charles River Laboratory Animal Technology Co. Ltd. (Beijing, China). The rats were maintained under a standard temperature (24 °C), humidity (55 %) and 12-h light/dark cycle.

Four groups (8 rats per group) were included: a normal control (NC), T2DM model (DM), DM + Ori-10 (10mg/kg/day), and DM + Ori-20 (20mg/kg/day). The DM and DM + Ori groups were fed a high-fat diet (D12492) for 4 weeks to establish an insulin resistance model. All the insulin resistance model rats were converted into DM rats by a single intraperitoneal injection of STZ (35 mg/kg dissolved in 0.1 mol/l citric acid buffer, pH 4.3). At 72 h after STZ injection, we measured random blood glucose levels to confirm the successful establishment of the diabetes model. A random blood glucose level of >16.7 mmol/l after STZ injection was indicative of the establishment of the model. The rats in DM + Ori-10 and DM + Ori-20 groups were intraperitoneally injected with 10 or 20 mg/kg/day Ori, while the rats in the NC and DM groups received the equivalent dose of normal saline. The injection lasted for 2 weeks. All the rats were sacrificed under sodium pentobarbital anesthesia. Serum was then separated by centrifugation and stored at -80 °C for subsequent experiments. Kidney tissues (cortex) were also excised, weighed, and stored in liquid nitrogen or fixed in 4% paraformaldehyde. The kidney weight-to-body weight ratio was calculated for each rat.

## Assessment of biochemical markers

Scr, BUN and UA concentrations were determined using assay kits, according to the protocols provided by the manufacturers (Nanjing Jiancheng Institute of Biotechnology).

## Histopathological examination of kidney tissues

Portions of the renal cortex fixed in 4% paraformaldehyde were embedded in paraffin and cut into 5- $\mu$ m-thick sections. The tissue sections were stained with hematoxylin-eosin (H&E) and MASSON for assessment under a light microscope (Olympus, Japan).

## Western blot analysis

Total protein was extracted from renal tissues, according to the manufacturer's protocol (Beyotime, China). Equal amounts of protein (50 µg) were separated by 8-10% sodium dodecyl sulfate (SDS) polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 1 h and then incubated with primary antibodies for the following proteins overnight at 4 °C. The membranes were then incubated with the appropriate secondary antibody, which was conjugated to horseradish peroxidase, for 1 h at room temperature. Protein bands were detected using an ECL Western Blotting Detection System (ImageQuant LAS, USA). GAPDH was used as a loading control.

### Statistical analysis

SPSS 22.0 software was used to analyze the data, which are presented as the mean ± SD. Differences between the groups were assessed using one-way analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

## Results

### 1. Ori alleviated blood glucose and improved renal function in DM

An increased kidney weight/body weight ratio is a sign of swelling and damage to the kidneys. As shown in Fig. 2B, the kidney-to-body weight ratio in the DM group was significantly higher than that in the normal and Ori control groups ( $P < 0.05$ ); Diabetic rats treated with Ori (10 and 20 mg/kg/day) for 4 weeks had a much lower kidney weight-to-body weight ratio than untreated diabetic rats ( $P < 0.05$ ). Besides, there were significant differences in blood glucose levels between the DM and DM + Ori groups ( $P < 0.05$ , Fig. 2C). Increases in the indicated renal functional parameters (Scr, BUN, UA) are considered hallmarks of the progression of renal disease. Scr, BUN, and UA concentrations in the DM group were higher than those in the normal and Ori control groups (Fig. 2D,E,F  $P < 0.05$ ). Remarkably, those parameters were significantly decreased by treatment with Ori in the DM + Ori groups compared with those in the DM group, with a dose-dependent manner. These data showed that Ori protects the kidney in diabetic rats to some certain extent.

### 2. Ori attenuated renal histopathological injury in DM

The changes in renal histopathology in the different groups were shown in Fig. 3A. Sections from normal group showed normal renal structures. H&E and MASSON staining showed that the sections from the DM group displayed glomerular hypertrophy, mesangial matrix expansion, tubular dilation and interstitial inflammation in DM. However, Ori-10 and Ori-20 treatment significantly attenuated these diabetic histopathological alterations.

### 3. Ori inhibited renal fibrosis in DM

MASSON images (representative images in Fig. 3A) showed that the normal group had almost no blue staining, indicating that the area of fibrosis was very small, while the renal tissue of DM rats had renal tubulointerstitial and glomerular fibrosis. Masson's trichrome staining revealed significantly diminished fibrosis in Oridonin-treated rats than in saline-treated controls. The protein expression levels of TGF- $\beta$ ,  $\alpha$ -SMA and Collagen-III indicated that Oridonin (10 and 20 mg/kg/day) treatment reduced renal fibrosis in DM rats (Fig. 3B,C,D,E) ( $P < 0.05$ ). These data showed that Ori protects the kidney from the progression of fibrosis in diabetic rats with a dose-dependent manner.

### 4. Ori inhibited TLR4/NF- $\kappa$ B activation in DM

TLR4/NF- $\kappa$ B is a key innate immune pathway involved in inflammatory processes. To assess the inhibitory effects of Ori on the inflammatory response, we examined the effects of Ori (10 and 20 mg/kg/day) on TLR4/NF- $\kappa$ B activity in renal tissues by western blot. As shown in Fig. 4A, TLR4 and NF- $\kappa$ B (p-p65) protein expression levels in the DM group were significantly higher than those in the normal and Ori-10 and Ori-20 groups ( $P < 0.05$ ). The increase in NF- $\kappa$ B protein expression was attenuated by Ori treatment (Fig. 4B,C  $P < 0.05$ ). These data showed that Ori protects the kidney from the activation of TLR4/NF- $\kappa$ B in diabetic rats with a dose-dependent manner.

### 5. Ori inhibited TXNIP/NLRP3 pathway in DM

To determine how Oridonin reduces renal fibrosis, we evaluated the expression of inflammatory factors known to promote fibrosis. TXNIP/NLRP3 has been shown to be involved in the progression of diabetic renal fibrosis. As previously reported, Ori is a covalent NLRP3 inhibitor<sup>[19]</sup>. As shown in Fig. 5A, TXNIP/NLRP3 pathway protein expression levels in the DM group were significantly higher than those in the normal ( $P < 0.05$ ). Treatment with Ori (10 and 20 mg/kg/day) had reduced ( $P < 0.05$ ) protein expression levels of TXNIP (Fig. 5B), NLRP3 (Fig. 5C), cleaved-caspase-1 (Fig. 5D), and cleaved-IL-1 $\beta$  (Fig. 5E). Given the mechanistic role of cleaved IL-1 $\beta$  in promoting fibrosis, these findings also suggest that Ori reduces renal fibrosis by inhibiting NLRP3 and cleaved IL-1 $\beta$  release. These data showed that Ori protects the kidney from the activation of TXNIP/NLRP3 in diabetic rats with a dose-dependent manner for the first time.

## Discussion

Renal fibrosis is the key pathological change of diabetic nephropathy (DKD), and significantly increases the mortality of patients with advanced DKD. Different signaling pathways are involved in renal fibrosis, including the TGF- $\beta$ , MAPK, PI3K/Akt, JAK/STAT, Wnt/ $\beta$ -catenin, and Notch pathways<sup>[3, 4]</sup>. These pathways all play important roles in the accumulation of ECM, the expression of collagen and fibronectin,

and the secretion of other related proteins. In addition, more and more new therapies are being investigated in clinical trials, and many efforts have been made to delay or even attempt to reverse the progression of renal fibrosis<sup>[22-24]</sup>.

Evidence accumulated in recent years indicates that inflammation plays an important role in the occurrence and aggravation of DN kidney injury<sup>[7, 8]</sup>. Increasing numbers of studies have shown that increases in inflammatory marker levels are related to the anti-DN effects of some renoprotective molecules<sup>[2]</sup>. However, the mechanisms behind these phenomena are not fully understood. Therefore, further study on the mechanism of renal immunity and inflammation and search for drugs to inhibit immune inflammatory response may find new targets for DN anti-inflammatory therapy. Natural anti-inflammatory products are a safe alternative to traditional methods for regulating inflammatory diseases<sup>[25-28]</sup>.

Li et al. showed that Ori exerts protective effect in diabetes-induced renal injury through the TLR4/NF- $\kappa$ B signaling pathways<sup>[21]</sup>. Lin et al. demonstrated that Ori reduced proteinuria and attenuated renal damage in a spontaneous SLE mouse model by regulating the inflammatory responses<sup>[20]</sup>. These results suggested that Ori attenuates proteinuria and protects the kidney from injury. Moreover, Ori also acts on a variety of cells, including immune cells, hepatocytes and vascular endothelial cells, to exert its protective effect. Bohanon et al. reported that Ori inhibited hepatic stellate cell proliferation and fibrogenesis by suppressing endogenous and TGF- $\beta$ 1-induced ECM proteins<sup>[29]</sup>. Current study demonstrated that oridonin inhibits collagen deposition and inflammation to attenuate CCl<sub>4</sub>-induced liver fibrosis in mice through inhibition of the NLRP3 inflammasome<sup>[30]</sup>. However, to date, whether Ori suppresses NLRP3 pathway and thus exerts beneficial effects on diabetes-induced renal fibrosis has not been explored. To confirm these effects, we investigated the inhibitory effects of Ori on the inflammatory response and fibrosis in a diabetic rat model. In our study, compared with rats in the control groups, rats in the DM group displayed increased plasma glucose levels, as well as an increased kidney weight-to-body weight ratio, which is indicative of renal injury. Significantly increased BUN, Scr, and UA concentrations were also noted in rats in the DM group. However, treatment with Ori effectively reversed these changes, as it lowered BUN, Scr, UA concentrations, and the kidney weight-to-body weight ratio. Previous studies have shown that Ori hardly affected plasma glucose levels. The discrepancy may be due to difference of the duration of the experiment. Previous experiment lasted for 12 weeks and we conducted for 6 weeks<sup>[21]</sup>.

The typical pathological changes of DN are mesangial cell proliferation, dilatation of renal tubules with accumulation of extracellular matrix and thickening of glomerular capillary wall, accompanied by nodular sclerosis, and eventually progressed to complete diabetic nephropathy. In this study, Ori treatment significantly ameliorated these diabetes-induced histopathological alterations. These data indicated that Ori improves renal function, ameliorates diabetes-induced renal injury and delays progressive nephrotoxicity in rats with DM.

The significance of the Trx-TXNIP signaling system is increasingly recognized<sup>[16]</sup>. Recent studies have shown a complex thiol-dependent interaction between TXNIP and the inflammation-related pathway of progressive diabetic nephropathy, the interaction of NLRP3 and TXNIP may be a significant signal of the formation of NOX4-derived NLRP3 inflammation in hyperhomocysteinemia-induced glomerular damage<sup>[31]</sup>. To verify whether the TXNIP-NLRP3 axis plays an important role in the therapeutic effects of Ori, we investigated the expression of NLRP3, TXNIP, and IL-1 $\beta$ . Our findings confirm the previous view that hyperglycemia-induced mitochondrial dysfunction plays an important role in the occurrence of DN, and also confirm to some extent that Ori can regulate TXNIP-NLRP3 axis to affect ROS levels to ameliorate mitochondrial damage.

In DN, the NLRP3 inflammasome is an intracellular platform that converts pro-IL-1 $\beta$  into active forms (IL-1 $\beta$  p17) responding to danger signals and triggers inflammatory programmed cell death. In recent studies, MCC950 (inhibitor of NLRP3) reduced liver inflammation and fibrosis by suppression collagen I,  $\alpha$ -SMA and hepatic connective tissue growth factor expression in a mouse model of non-alcoholic steatohepatitis<sup>[32]</sup>. TGF- $\beta$ 1 is a major cytokine secreted by mesangial cells that mediates the development of DN<sup>[33]</sup>. TGF- $\beta$ 1 is a key cytokine mediating collagen deposition in kidney, including promoting the production of ECM, inhibiting the degradation of ECM and participating in renal fibrosis<sup>[34, 35]</sup>. ECM of DN patients is produced by mesangial cells and mainly consists of fibronectin, type IV collagen and a small amount of type I collagen.  $\alpha$ -SMA was weakly expressed in normal mesangial cells, but was significantly increased under high glucose stimulation. IL-1 $\beta$  has been demonstrated to stimulate production of TGF- $\beta$ 1, fibronectin, collagen I and mesangial proliferation. Our experimental results showed that Ori has a good inhibitory effect on these indicators promoting renal fibrosis.

In conclusion, our present study suggests that oridonin inhibits collagen deposition and inflammation, thereby alleviating diabetic induced renal injury and fibrosis in rats. However, the experiments conducted in this study covered only a narrow and superficial scope of pharmacological identification. Therefore, the renal protective effect of Ori remains to be further studied. These results should also be compared with current first-line drugs for renal fibrosis.

## **Declarations**

### **Ethics approval and consent to participate**

Animal experiments were carried out in accordance with the National Animal Protection and Use Guidelines and approved by the Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine.

### **Consent for publication**

Authors are responsible for correctness of the statements provided in the manuscript.

### **Availability of data and materials**

All authors have confirmed that all data and materials support their published claims and comply with field standards.

### **Competing interests**

The authors declare no conflict of interests.

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### **Authors' contributions**

Gengzhen Huang and Yuerong Ma conceived the idea. Gengzhen Huang performed the experiments and wrote the manuscript. Yaodan Zhang, Yingying Zhang, Xiaotao Zhou, Yuan Xu, Huiting Wei analyzed data.

### **Acknowledgements**

Not Applicable

### **Authors' information**

Not Applicable

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## Figures

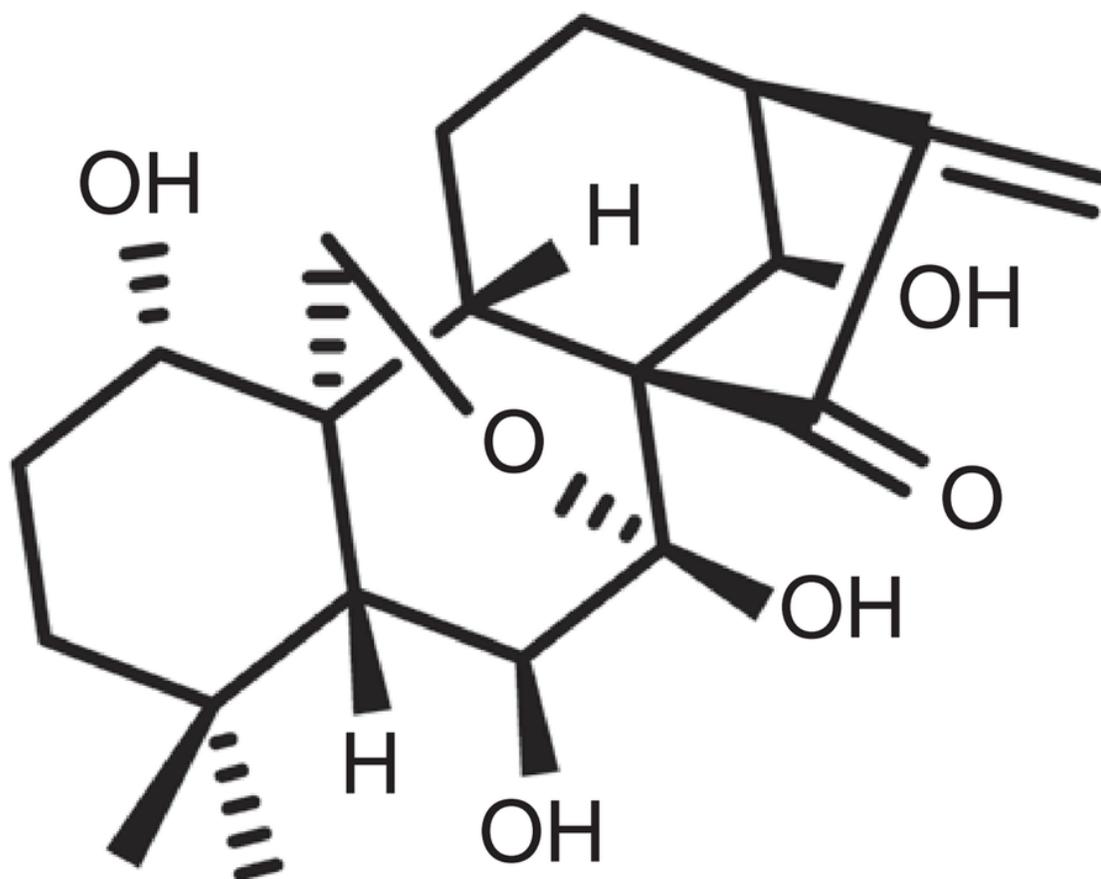
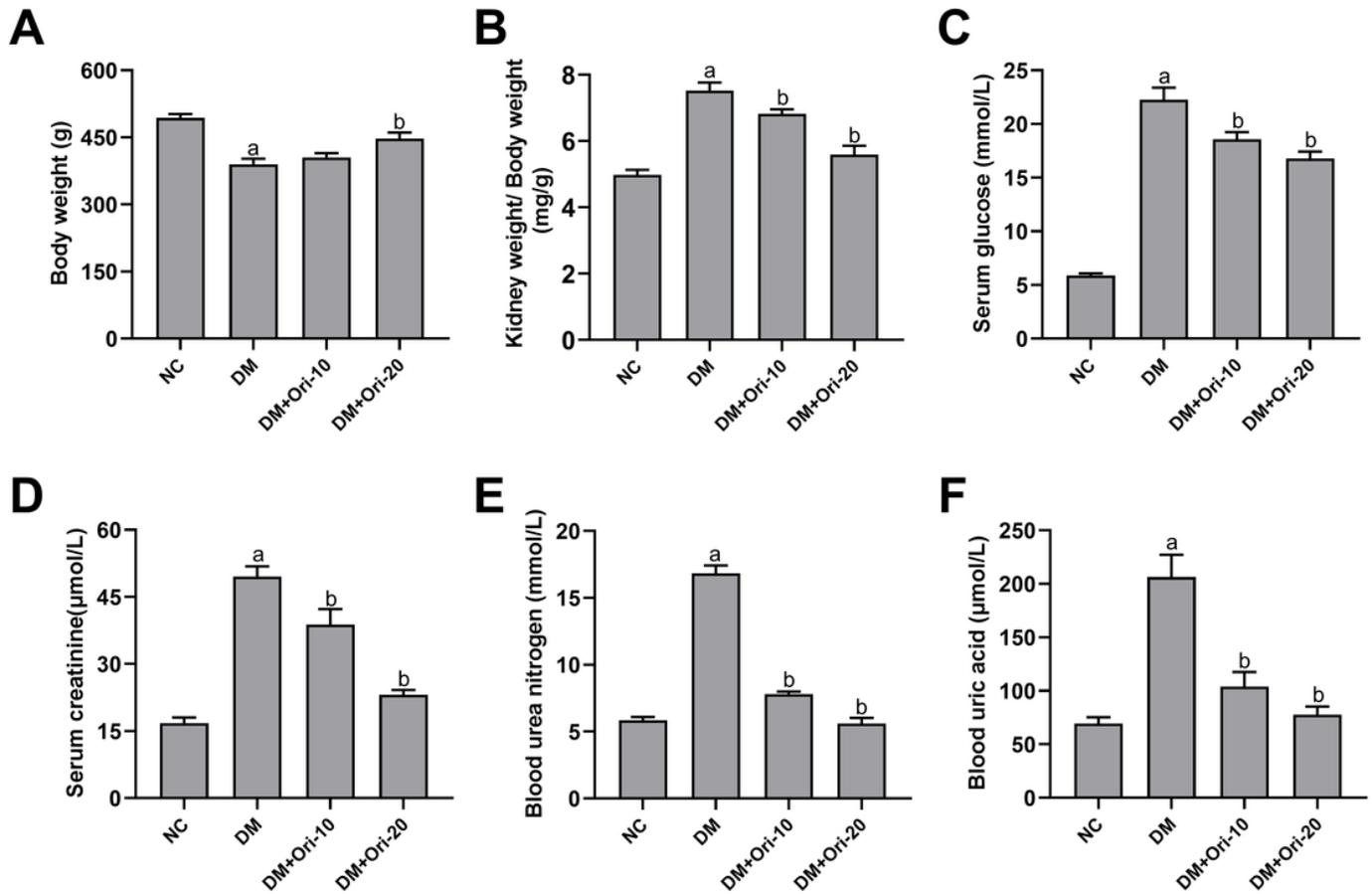


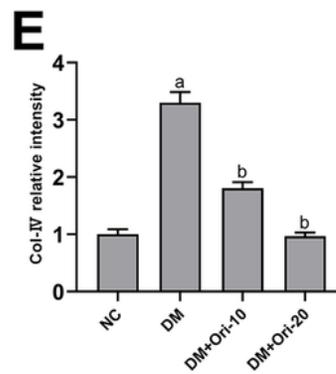
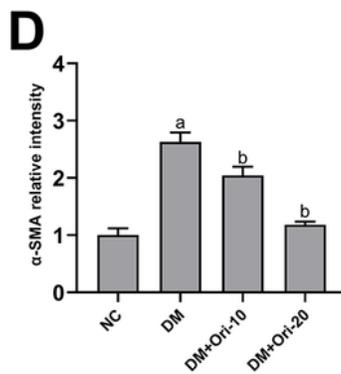
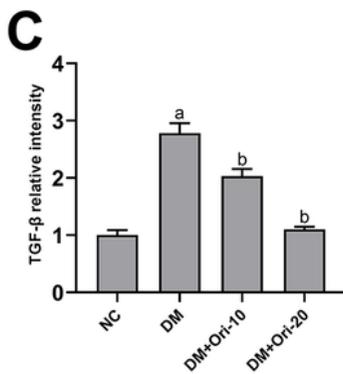
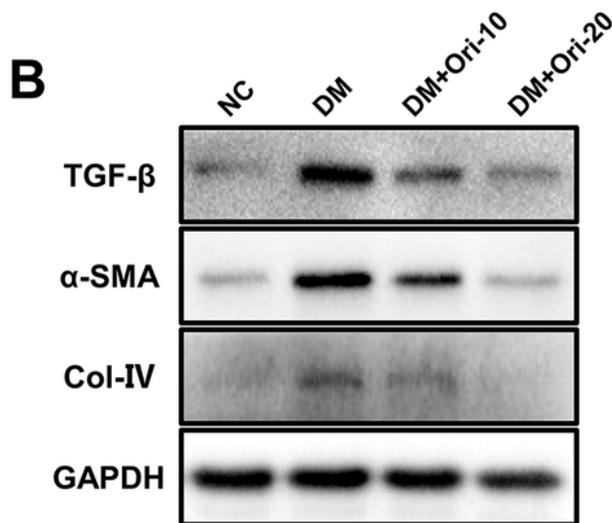
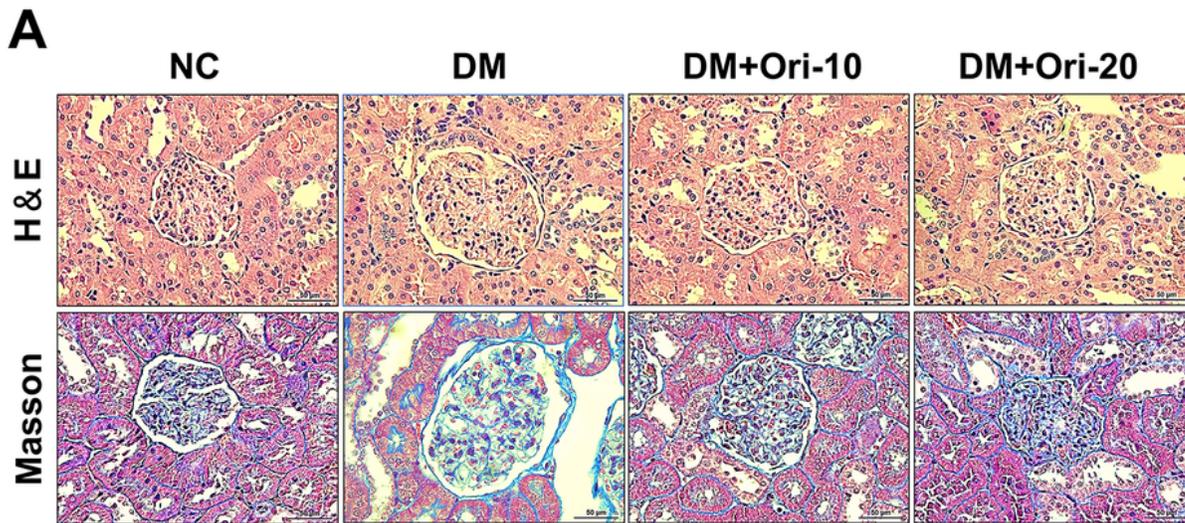
Figure 1

The chemical structure of Oridonin



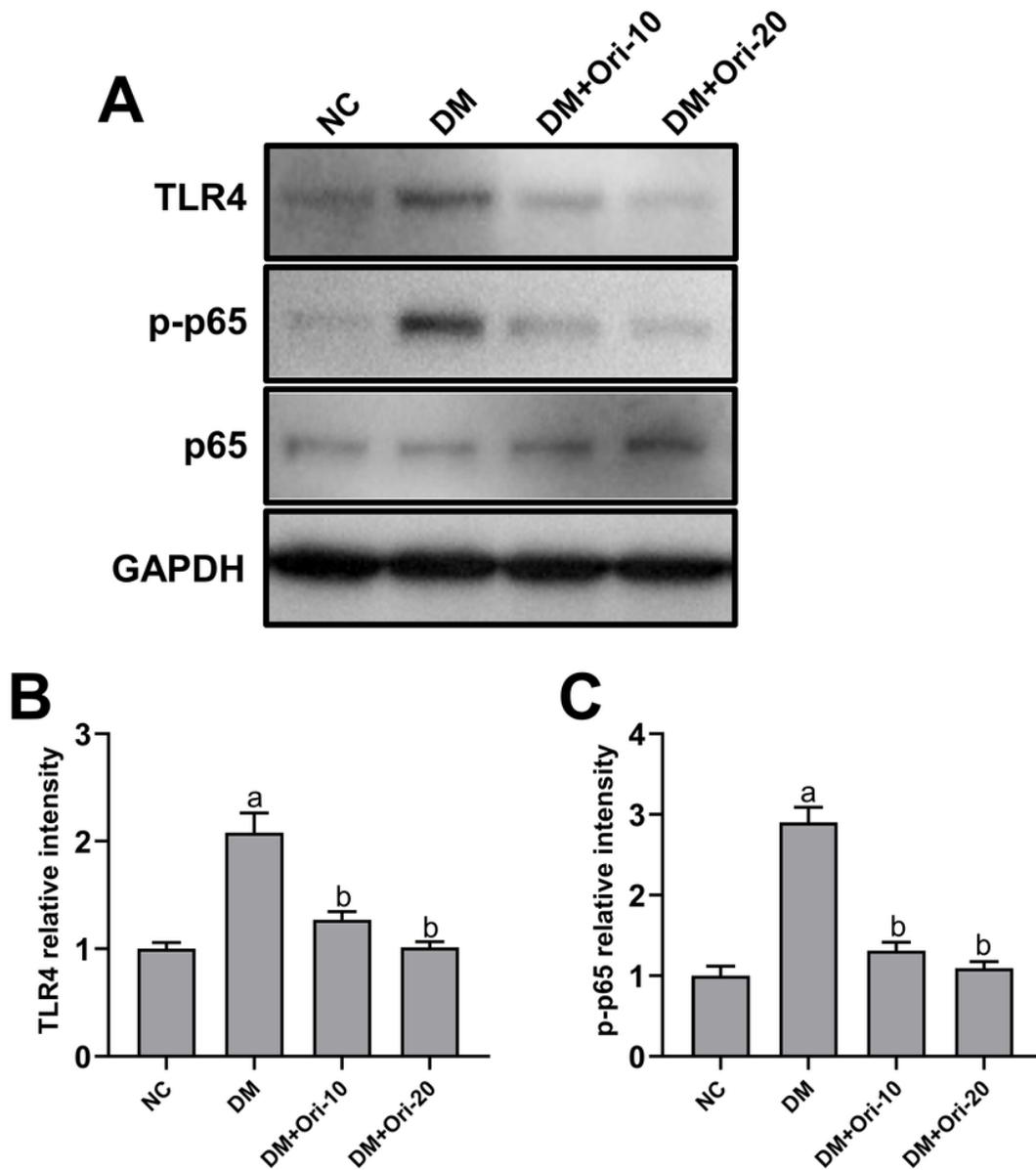
**Figure 2**

Effect of Oridonin (Ori) on renal function in type 2 diabetic rats. A normal control (NC) group, T2DM model (DM) group, DM+Ori-10 (10mg/kg/day) group, and DM+Ori-20 (20mg/kg/day) group were included, (A)body weight, (B) renal index, (C) fasting serum glucose, (D) serum creatinine, (E)blood urea nitrogen and (F)uric acid were tested. Data are presented as means  $\pm$  SD. aP < 0.05 (DM vs. NC), bP < 0.05 (DM+Ori vs. DM).



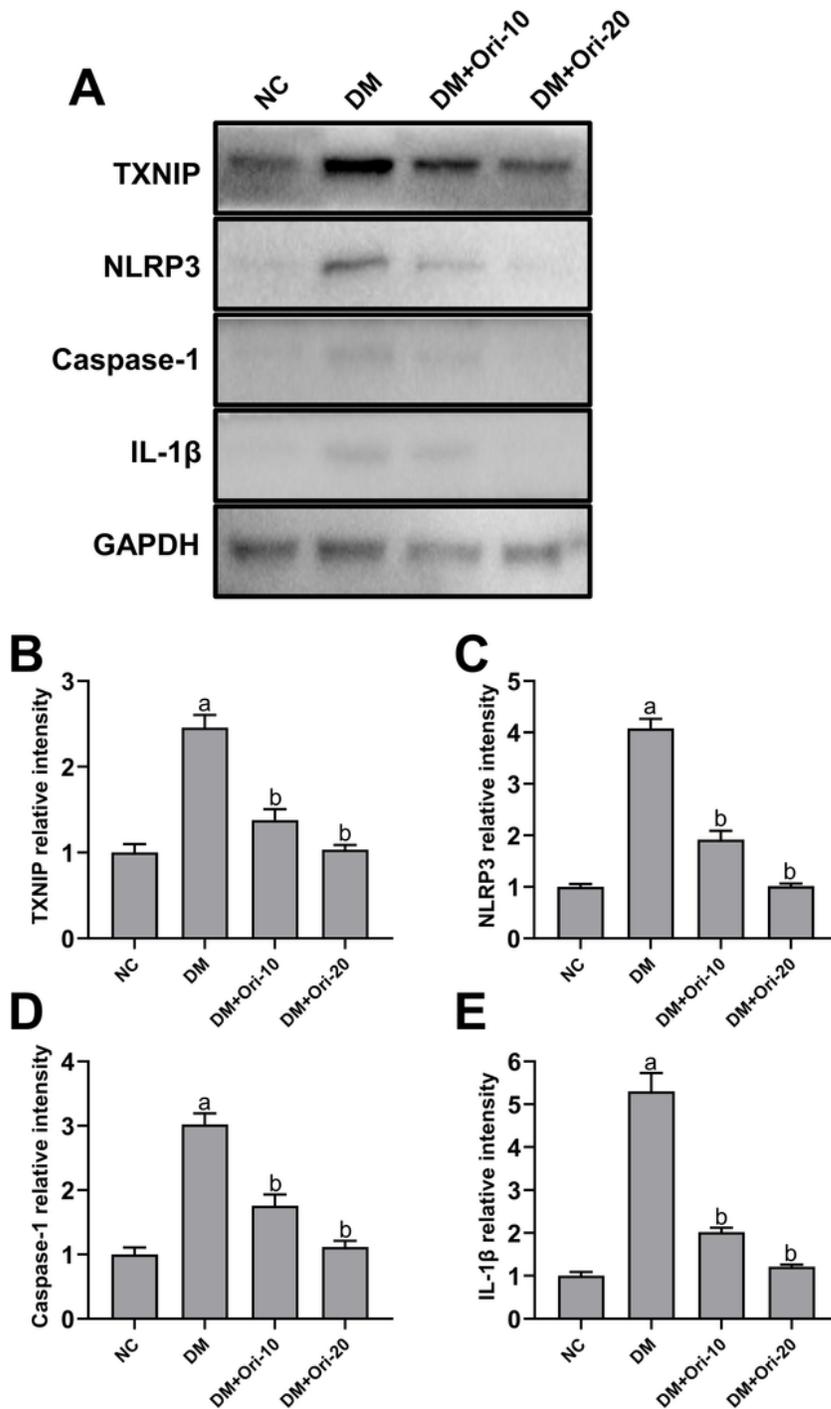
**Figure 3**

Effect of Oridonin (Ori) on renal fibrosis in type 2 diabetic rats. NC, DM, DM+Ori-10 group, and DM+Ori-20 were included. (A) Representative photographs of H&E and MASSON staining in groups were presented. (C) TGF- $\beta$ , (D)  $\alpha$ -SMA, (E) Col-IV expression in the kidney tissues were determined with (B) western blot. Data are presented as means  $\pm$  SD. aP < 0.05 (DM vs. NC), bP < 0.05 (DM+Ori vs. DM).



**Figure 4**

Changes of TLR4, p-p65 expression in the kidney tissues. NC, DM, DM+Ori-10 group, and DM+Ori-20 were included. (A)Western blot was used to measure the protein expression of TLR4 (B) and p-p65(C) in the kidney tissues. Data are presented as means  $\pm$  SD. aP < 0.05 (DM vs. NC), bP < 0.05 (DM+Ori vs. DM).



**Figure 5**

Changes of TXNIP, NLRP3, Caspase-1 and IL-1 $\beta$  expression in the kidney tissues. NC, DM, DM+Ori-10 group, and DM+Ori-20 were included. (A) Western blot was used to measure the protein expression of (B) TXNIP, (C) NLRP3, (D) Caspase-1, and (E) IL-1 $\beta$  in the kidney tissues. Data are presented as means  $\pm$  SD.  $aP < 0.05$  (DM vs. NC),  $bP < 0.05$  (DM+Ori vs. DM).