

Hedyotis diffusa Willd extract protects rat renal tubular epithelial cells from high glucose-induced injury by inhibiting PI3K/AKT signaling pathway

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Received 2 Nov 2021, Accepted 24 Feb 2022

Available online 25 May 2022

ABSTRACT: The effects of *Hedyotis diffusa* Willd on high glucose (HG)-induced injury of rat renal tubular epithelial cells and its related regulatory mechanisms were investigated. In this study, NRK-52E cells were cultured in a normal glucose concentration (5.5 mM glucose) or HG concentration medium (30 mM glucose), and treated with the concentration gradient of *H. diffusa* extract (50, 100, 200 µg/ml). The results suggested that *H. diffusa* extract has a protective effect on the cell viability of NRK-52E cells and inhibited the HG-induced oxidative stress through the detection of malondialdehyde content and specific activities of superoxide dismutase and glutathione peroxidase. The extract also inhibited the HG-induced apoptosis. In addition, the epithelial-to-mesenchymal transition (EMT) indicators including alpha-smooth muscle actin (α-SMA), E-cadherin, N-cadherin and vimentin were measured, it was found that the HG-induced EMT of NRK-52E cells was inhibited by the extract. Moreover, *H. diffusa* extract exerted an inhibitory effect on the PI3K/AKT signaling activation induced by HG. Hence, *H. diffusa* extract could protect rat renal tubular epithelial cells from HG-induced injury by inhibiting PI3K/AKT signaling pathway.

KEYWORDS: *Hedyotis diffusa* Willd, renal tubular epithelial cells, oxidative stress, apoptosis, PI3K/AKT pathway

INTRODUCTION

Diabetes mellitus (DM) is an endocrine and metabolic disease that seriously threatens human health. DM causes many complications, including diabetic retinopathy, diabetic cardiovascular disease and diabetic nephropathy (DN) [1, 2]. Among them, DN is the most common and most serious complication of DM, and DN has become the main cause of chronic renal failure [3]. Alleviating oxidative stress and reducing inflammation are closely related to DN treatment [4]. In addition, regulating the inflammatory level of cells has an important effect on the onset and treatment of DN. Not only that, fibrosis caused by epidermal-mesenchymal transition (EMT) is also an important reason for the progress of DN [5]. However, so far, traditional treatment methods only focus on regulating blood glucose levels which cannot control the occurrence of complications. Therefore, the existing treatments for DN are limited, and new treatments need to be proposed to reduce the necessity of kidney transplantation and reduce the burden of this disease.

Studies have reported that many medicinal plants can be used to treat diabetes. The preventive and therapeutic effects of medicinal plants with strong antioxidant effects and anti-inflammatory effects on these diabetic complications including DN have attracted widespread attention [6]. It has been reported that celastrol could reduce kidney damage in DN rats by inhibiting PI3K/AKT pathway, inhibit glomerular

basement membrane thickening, and achieve podocyte homeostasis [7]. Acteoside improved diabetic kidney disease by regulating the β-catenin pathway [8]. *H. diffusa* Willd is an herb of the Rubiaceae family. It is widely distributed in southern China and other Asian countries. Pharmacological studies have shown that varieties of phytochemicals compounds were identified from *H. diffusa* Willd, including iridoids, flavonoids, anthraquinones and phenolics [9]. Moreover, these plant extracts exhibited significant anti-cancer, anti-inflammatory, antioxidant, and neuroprotective effects [10, 11]. *H. diffusa* Willd was shown to have a protective effect on HG-induced kidney inflammation in mice [12]. However, there are few studies on the effects of this type of herb on HG-induced injury of rat renal tubular epithelial cells and its related regulatory mechanisms. Therefore, this study explored the role of *H. diffusa* Willd extract in HG-induced NRK-52E cell injury and underlying mechanisms.

MATERIALS AND METHODS

Cell culture

Rat renal tubular epithelial cell line NRK-52E cells were purchased from the American Type Culture Collection (ATCC; VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C in a humidified atmosphere of 5% CO₂. For the high glucose induction, cells were cultured in normal glucose (5.5 mM glucose) or high

glucose (HG; 30 mM glucose) for 48 h [13].

Preparation of *H. diffusa* Willd extract

H. diffusa Willd extract was purchased from DASF Biotechnology Co., Ltd. (Nanjing, China) and dissolves in 40% dimethyl sulfoxide (DMSO) at a concentration of 200 mg/ml. NRK-52E cells were treated with different concentration of the herb extract (50, 100, 200 µg/ml) for 24 h.

MTT assay

MTT assays were performed to evaluate cell viability of NRK-52E cells with different treatments. NRK-52E cells were plated onto 96-well plates at a density of 5×10^3 cells per well. After treatment, 10 µl of MTT (5 mg/ml) was added to each well. Then the cells were cultured for another 4 h. Subsequently, the MTT formazan was resuspended in 100 µl DMSO and absorbance was measured at 490 nm. All the experimental sets were performed in triplicate.

Measurement of SOD, GSH-Px and MDA

The levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were all measured using the commercial kits purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Three kits including total superoxide dismutase assay kit with WST-8 (S0101S), cellular glutathione peroxidase assay kit with NADPH (S0056) and lipid peroxidation MDA assay kit (S0131S) were used and all the assays were performed as the users' instructions.

Flow cytometry

Flow cytometry was used to detect the apoptosis of NRK-52E cells. NRK-52E cells were cultured in normal glucose (5.5 mM glucose) or HG (30 mM glucose) and treated with different concentration of *H. diffusa* Willd extract (50, 100, 200 µg/ml). Then cells were harvested and the percentage of apoptotic cells was examined using a commercial Annexin V-FITC/PI Apoptosis Detection Kit (Sigma-Aldrich, MO, USA) according to the users' instructions.

Western blotting

Western blotting was performed as previously described [14]. Primary antibodies used in this study including Bax antibody (ab32503), Bcl-2 antibody (ab182858), cleaved caspase-3 antibody (ab32042) alpha-smooth muscle actin (α-SMA; ab7817), E-cadherin (ab231303), N-cadherin (ab245117) and Vimentin (ab92547) were purchased from Abcam (MA, USA). In addition, antibodies including phosphorylated PI3K (#17366), total PI3K (#4257), phosphorylated AKT (#4060), total AKT (#9272) and GAPDH (#5174) were obtained from Cell Signaling Technology (MA, USA). The antibody was diluted to a ratio of 1:1000.

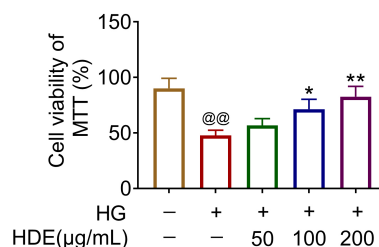


Fig. 1 *H. diffusa* Willd extract promotes the viability of NRK-52E cells. Cell viability was examined by MTT assays NRK-52E cells. NRK-52E cells were treated with high glucose (30 mM glucose) and *H. diffusa* extract (50, 100, 200 µg/ml). High glucose, HG; *H. diffusa* extract, HDE. indicates a group versus vehicle group; * indicates a group versus the group treated with HG. @@ $p < 0.01$, * $p < 0.05$ and ** $p < 0.01$.

Statistical analysis

The results in this study were presented as mean±standard deviation (SD). Data was analyzed using the independent Student's t-test through the GraphPad Prism 8.0 software.

RESULTS

H. diffusa Willd extract promotes the cell viability of NRK-52E cells

NRK-52E cells were treated with HG and cell viability was examined by MTT assay. It was found that cell viability was significantly decreased as a result of HG treatments. Moreover, the viability of HG-cultured cells was significantly rescued by treatments with *H. diffusa* Willd extract in a dose-dependent manner (50, 100, 200 µg/ml; Fig. 1). The data suggested that *H. diffusa* Willd extract could promote the viability of NRK-52E cells.

H. diffusa Willd extract attenuates HG-induced oxidative stress of NRK-52E cells

The levels of SOD, GSH-Px and MDA were then detected to determine the oxidative stress status. HG treatments significantly decreased the levels of SOD and GSH-Px in NRK-52E cells, but *H. diffusa* Willd extract increased the two enzyme specific activities (Fig. 2A,B). On the contrary, MDA levels were significantly increased after HG treatments but decreased upon treated with the extract (Fig. 2C). These results suggested that *H. diffusa* Willd extract could attenuate HG-induced oxidative stress of NRK-52E cells.

H. diffusa Willd extract inhibits HG-induced apoptosis of NRK-52E cells

Furthermore, the effect of *H. diffusa* Willd extract on cell apoptosis were also determined by flow cytometry. The results indicated that HG significantly induced the apoptosis of NRK-52E cells, whereas this effect was abolished by treated with the extract (50,

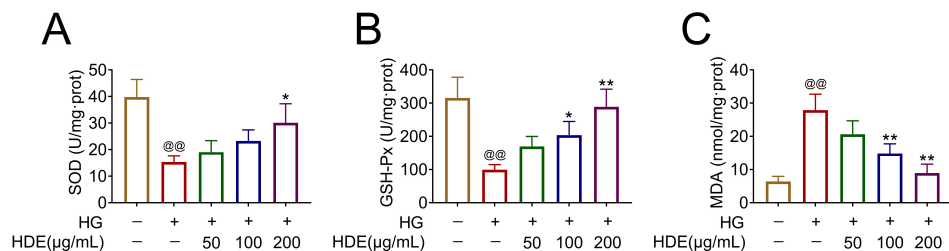


Fig. 2 *H. diffusa* Willd extract attenuates HG-induced oxidative stress of NRK-52E cells. The levels of (A) SOD, (B) GSH-Px and (C) MDA were detected to determine the oxidative stress status. NRK-52E cells were treated with HG and *H. diffusa* extract as mentioned in Fig. 1. All abbreviations and statistics are as indicated in Fig. 1.

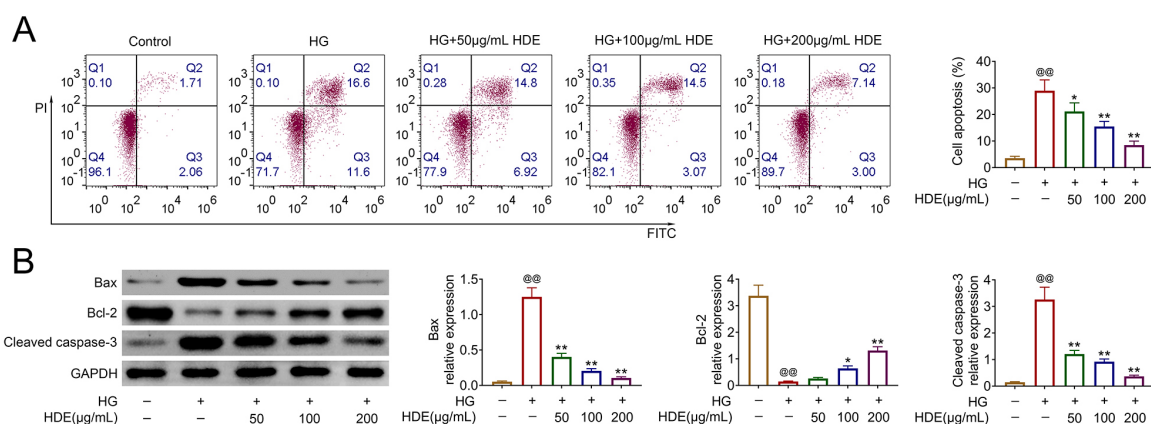


Fig. 3 *H. diffusa* Willd extract inhibits HG-induced apoptosis of NRK-52E cells. (A) The effect of *H. diffusa* extract on cell apoptosis were determined by flow cytometry. (B) The protein expression of Bax, Bcl-2 and cleaved caspase-3 were determined by Western blotting. NRK-52E cells were treated with HG and *H. diffusa* extract as mentioned in Fig. 1. All abbreviations and statistics are as indicated in Fig. 1. GAPDH was used as loading control.

100, 200 $\mu\text{g/mL}$; Fig. 3A). In addition, the apoptosis-related proteins were examined and the results demonstrated that the amounts of Bax and cleaved caspase-3 were significantly increased in HG-cultured NRK-52E cells, whereas the extract significantly decreased the amounts of both proteins. Moreover, this plant extract significantly increased the protein expression of Bcl-2 inhibited by HG (Fig. 3B). These results suggested *H. diffusa* Willd extract could inhibit HG-induced apoptosis of NRK-52E cells.

H. diffusa Willd extract inhibits HG-induced epithelial-to-mesenchymal transition (EMT) of NRK-52E cells

HG-induced EMT promotes the fibrosis in DN. The EMT indicators including α -smooth muscle actin (α -SMA), E-cadherin, N-cadherin and vimentin were measured. The results suggested that the protein expression of α -SMA, N-cadherin and Vimentin were significantly increased in HG-cultured NRK-52E cells. However, HG-induced upregulation of these proteins were reversed by this plant extract. In addition, E-cadherin was significantly decreased in HG-cultured

NRK-52E cells and the extract abolished this effect (Fig. 4). The results indicated that *H. diffusa* Willd extract could inhibit HG-induced EMT of NRK-52E cells.

H. diffusa Willd extract exerts an inhibitory effect on the activation of PI3K/AKT signaling induced by HG

To further explore the underlying mechanisms of *H. diffusa* Willd extract's protective effect on the HG induced injury of NRK-52E cells. The status of PI3K/AKT signaling was determined. The data indicated that phosphorylated PI3K (p-PI3K) and phosphorylated AKT (p-AKT) were significantly upregulated after treated with HG and this plant extract could significantly reversed the effect at a concentration of 50 $\mu\text{g/mL}$. The results indicated that *H. diffusa* Willd extract was able to exert an inhibitory effect on the PI3K/AKT signaling activation induced by HG.

DISCUSSION

HG stimulation was reported to induce a variety of abnormal physiological functions of renal tubular cells,

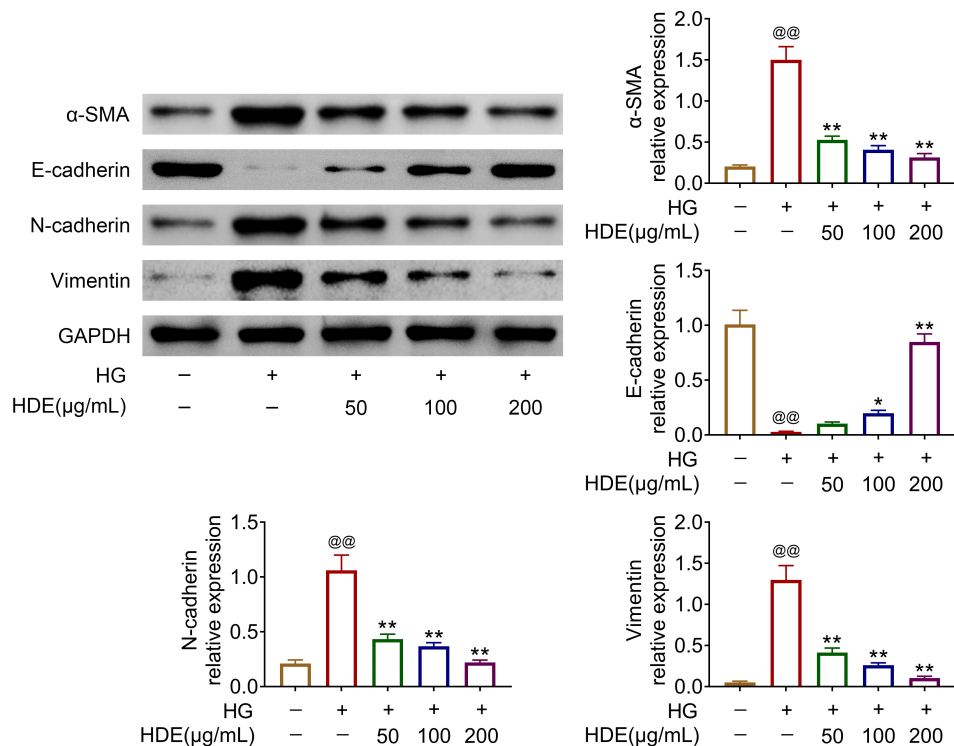


Fig. 4 *H. diffusa* Willd extract inhibits HG-induced epithelial-to-mesenchymal transition of NRK-52E cells. The EMT indicators including α -SMA, E-cadherin, N-cadherin and vimentin were determined by Western blotting. NRK-52E cells were treated with HG and *H. diffusa* extract as mentioned in Fig. 1. All abbreviations and statistics are as indicated in Fig. 1. GAPDH was used as loading control.

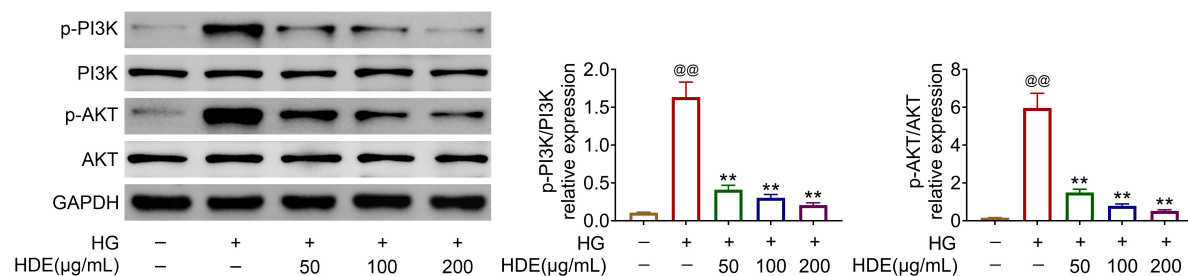


Fig. 5 *H. diffusa* Willd extract exerts an inhibitory effect on the activation of PI3K/AKT signaling induced by HG. The p-PI3K, total PI3K, p-AKT and total AKT were measured Western blotting. NRK-52E cells were treated with HG and *H. diffusa* extract as mentioned in Fig. 1. All abbreviations and statistics are as indicated in Fig. 1. GAPDH was used as loading control. ^{@@} $p < 0.01$ and ^{**} $p < 0.01$.

including imbalance of renal tubular reabsorption, senescence-like phenotypes of epithelial cells, apoptosis and tubular interstitial fibrosis, which could lead to chronic kidney disease and even eventually progress to end-stage renal disease [15]. In the present work, *H. diffusa* Willd extract showed a protective effect on the cell viability of NRK-52E cells and inhibited the HG-induced oxidative stress, apoptosis and EMT of NRK-52E cells. Moreover, this plant extract exerted an

inhibitory effect on the PI3K/AKT signaling activation induced by HG.

Oxidative stress is associated with chronic complications of DM diseases, such as DN, diabetic macrovascular disease, diabetic peripheral neuropathy, and diabetic cataract [16]. Under normal physiological conditions, the body's antioxidant system can quickly remove excess reactive oxygen species (ROS) and maintain a dynamic balance between oxidation and

antioxidant. Under pathological conditions such as hyperglycemia, stress, etc., dysfunction of antioxidant system leads to excessive accumulation of ROS or RNS which results in an imbalance between oxidation and antioxidant. The ROS and RNS damage DNA, proteins, and lipids, and induce gene mutations. Protein denaturation and lipid peroxidation are the cause of cell apoptosis or cell death [17]. In the current study, *H. diffusa* Willd extract exerted an inhibitory effect on the HG-induced oxidative stress and apoptosis of NRK-52E cells, which demonstrated that the extract may exhibit an excellent protective effect on the DN. In addition, previous studies showed that 2-hydroxymethyl anthraquinone from *H. diffusa* Willd exhibited significant antioxidative activity in acute lung injury [18]. Amentoflavone and total flavonoids from this type of plant inhibited the ROS accumulation in H₂O₂-induced HL-O2 cells [19]. These studies all demonstrated that *H. diffusa* Willd had excellent antioxidant properties.

Renal fibrosis is an important pathological process in many chronic kidney diseases including DN, and its main pathological sign is the accumulation of extracellular matrix [20]. In the process of renal fibrosis, the proximal tubule epithelial cells of the kidney produced extracellular matrix by expressing and releasing adhesion molecules such as cytokines and chemokines [21]. Prolonged hyperglycemia could induce the formation and accumulation of extracellular matrix [21]. In recent years, renal tubular EMT has been shown to participate in renal fibrosis [22]. Further research on the role and mechanism of tubular EMT in DN is of great significance for preventing and treating DN, preventing renal fibrosis, and protecting renal function [22]. In this study, *H. diffusa* Willd extract was shown to inhibit HG-induced EMT of NRK-52E cells. The findings suggested that this plant extract may be used in the prevention of renal fibrosis treatment of DN.

PI3K/AKT signaling pathway is the classical pathway of insulin signal transduction, which can increase glucose uptake, cell proliferation and apoptosis after activation [23]. PI3K/AKT/mTOR pathway was shown to be significantly activated in human renal cell carcinoma (RCC), and this signaling pathway was also significantly up-regulated in DN [24]. In addition, mangiferin could reduce inflammation and oxidative stress levels in DN, and inhibited renal interstitial fibrosis through downregulating the PTEN/PI3K/AKT signaling [25]. The findings of this study disclosed that *H. diffusa* Willd extract could exert an inhibitory effect on the PI3K/AKT signaling activation induced by HG. Collectively, the results of this study demonstrated that *H. diffusa* Willd extract could protect rat renal tubular epithelial cells from HG-induced injury by inhibiting PI3K/AKT signaling pathway. This finding suggested that this plant extract may be used to treat DN and alleviate the progression of the disease.

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