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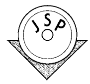

ORIGINAL PAPER

Eurycoma longifolia alkaloid components ameliorate hyperuricemic nephropathy via regulating serum uric acid level and relieving inflammatory reaction

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Abstract

Hyperuricemia is an independent risk factor for chronic kidney disease. We have previously showed the uric-acid-lowering effect of *Eurycoma longifolia* Jack, yet the renal protective effect and mechanism of *E. longifolia* remain obscure. The mouse model of hyperuricemic nephropathy was induced by adenine combined with potassium oxonate in male C57BL/6 J mice. *E. Longifolia* alkaloid components could reduce the level of serum uric acid by regulating the expression of hepatic phosphoribosyl pyrophosphate synthase (PRPS), hypoxanthine-guanine phosphoribosyl transferase (HPRT), and renal urate transporter organic anion transporter 1 (OAT1) and ATP-binding box subfamily G member 2 (ABCG2) in HN mice. Additionally, *E. Longifolia* alkaloid components alleviated renal injury and function caused by hyperuricemia, which was characterized by improving renal histopathology, reducing urea nitrogen and creatinine levels. *E. Longifolia* alkaloid components treatment could reduce the secretion of pro-inflammatory factors by inhibiting the activation of NF-κB and NLRP3 inflammatory signaling pathways, including tumor necrosis factor α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1β), and regulated activated normal T cell expression and secretion proteins (RANTES). Meanwhile, *E. longifolia* alkaloid components improved renal fibrosis, inhibited the transformation of calcium-dependent cell adhesion molecule E (E-cadherin) to α-smooth muscle actin (α-SMA) transformation, and decreased collagen 1 expression in HN mice.

Keywords Hyperuricemic nephropathy · *Eurycoma longifolia* alkaloid components · Uric acid · Renal fibrosis · Inflammation response

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Abbreviations

ABCG2	ATP-binding cassette super family G number 2
BUN	Blood urea nitrogen
cDNA	Complemental deoxyribonucleic acid
Cur	Clearance of uric acid
ELISA	Enzyme-linked immunosorbent assay
E-cadherin	Calcium-dependent cell adhesion molecule
EMT	Epithelial-to-mesenchymal transition
ECM	Extracellular matrix
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT9	Glucose transporter 9
GMP	Guanosine monophosphate
H&E	Hematoxylin–eosin staining
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
IL-1β	Interleukin-1β
Masson	Masson-trichrome staining
MCP-1	Monocyte chemoattractant protein-1
NF-κB	Nuclear factor-kappa B

NLRP3	Nod-like receptor pyrin domain-containing protein 3
OAT1	Organic anion transporter 1
PAS	Periodic acid Schiff reaction
PBS	Phosphate buffered saline
qRT-PCR	Real-time quantitative polymerase chain reaction
PRPS	Phosphoribosyl pyrophosphate synthetase
RANTES	Chemokine (C–C motif) ligand 5
TNF-α	Tumor necrosis factor-α
UPLC	Ultra-performance liquid chromatography
URAT1	Urate-anion transporter
α-SMA	1α-Smooth muscle actin

Introduction

Hyperuricemia (HUA) is an independent risk factor for chronic kidney disease (CKD) [1]. The risk of kidney disease in people with serum uric acid levels higher than 9 mg/dl is three times higher than that normal uric acid levels [2]. Patients with long-term HUA have a greater risk of CKD, which means that HUA may accelerate the progression of CKD [1]. In the population with estimated glomerular filtration rate (eGFR) $\geq$ 90 mL/min/1.73m^2, the prevalence of HUA is 11–13%, but the prevalence of HUA is about 6–7 times higher in people with eGFR between 15 and 29 mL/min/1.73m^2 [3].

Long-term HUA can cause excessive accumulation of uric acid in the kidneys, resulting in hyperuricemic nephropathy (HN). HUA was once thought to be monosodium urate (MSU) deposited in the collecting duct of the kidney, causing kidney disease in a manner similar to gouty arthropathy [4]. Recently, new mechanisms of HN have been reported, including endothelial dysfunction, activation of the renal angiotensin system, oxidative stress, and renal tubular epithelial interstitialization [5, 6]. In addition, uric acid can also induce vascular endothelial injury and inflammation by activating transcription factors (such as NF-κB) and the NLRP3 inflammasome signaling pathway, causing kidney damage [7]. Whether it is in the form of MSU or soluble urate, the mechanism of kidney damage caused by uric acid is related to the participation of inflammatory reactions [8, 9].

At present, the safety and efficacy of uric-acid-lowering drugs for the treatment of HN have yet to be verified. Allopurinol and benzbromarone are the first-line uric-acid-lowering drugs in clinical practice. Allopurinol may cause severe hypersensitivity, agranulocytosis, and aggravate nephrotoxicity by impairing pyrimidine metabolism [10]. In patients with kidney injury, although benzbromarone can effectively reduce the blood uric acid concentration, it cannot improve the renal function damage and can cause severe liver damage [11]. Therefore, it is necessary to find new compounds that has no toxic side effects and can treat HUA complicated with renal damage.

Eurycoma longifolia Jack (*E. longifolia*) is a widely popular herbal medicine in Southeast Asia. *E. longifolia* is a traditional medicine used to treat sexual dysfunctions and intermittent fever (malaria) [12]. *E. longifolia* is rich in various biologically active compounds, such as quassinoids, alkaloids, squalene derivatives, benzene lignans, hydantoins, and bioactive steroids [13]. *E. longifolia* have shown a wide dynamic range of pharmacological activities including aphrodisiac [14], antimalarial [15], anti-inflammatory [16], anti-diabetic [17], anti-hyperuricemia [18], and anticancer [19]. We have recently showed the uric-acid-lowering effect of *E. longifolia* [18], yet the renal protective effect and mechanism of *E. longifolia* on hyperuricemic mice remains obscure. To further study the anti-HN effects and mechanisms of *E. longifolia* alkaloid components, this study combined with in vivo and in vitro models to evaluate the effects of alkaloids from *E. longifolia* in reducing uric acid and improving HN kidney damage, and explore its potential mechanisms.

Materials and methods

Materials

The stems of *E. longifolia* were collected from Thailand. The stems of *E. longifolia* were identified by Dr. Wang Tao from Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine (TJUTCM). Voucher specimen was deposited at the Institute of Traditional Chinese Medicine of TJUTCM (Voucher Number: TJUTCM-18-0155).

E. longifolia alkaloid components are provided by Tianjin Key Laboratory of Chemistry and Analysis of Traditional Chinese Medicine, the contents of the target analytes in *E. longifolia* alkaloid components were 28.96 and 44.34 μg/g for canthin-6-one, and 9-methoxycanthin-6-one, respectively [20]. The compounds, Compound **1** (PubChem CID: 5,320,161), Compound **2** (PubChem CID: 10,446,368), Compound **3** (PubChem CID: 102,004,796), Compound **4**(PubChem CID:259,218), Compound **5** (PubChem CID: 97,176), and Compound **6** (PubChem CID: 9,881,423) were purchased from Yuanye Biotechnology Co., Ltd. Shanghai, China. Their purity was HPLC $\geq$ 95%.

Animals

This study was performed on mice of SPF-grade C57BL/6 J (8–10 weeks old, male). All the mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were fed on a standard diet, with an indoor temperature of 22 °C ± 2 °C and a fixed artificial light time

of 12 h. Before the formal experiment, the mice were adaptively fed for at least 7 days. The animal experiment of this study was approved by Science and Technological Committee and the Animal Use and Care Committee of TJUTCM (No. 202010006, 6 October 2020).

This study randomly divided mice into five groups: normal group (Sham), model group (HN), positive drug group (Ben-50), *E. longifolia* alkaloid components medium-dose group (T-200), and *E. longifolia* alkaloid components high-dose group (T-200), with 10 mice in each group.

Adenine- and potassium-oxonate-induced HN mice

HN mice model was established by oral administration of potassium oxonate and adenine (Sigma-Aldrich Co., MO, USA). Benzbromarone and *E. longifolia* alkaloid components were suspended in normal saline and orally administrated, with an interval of 1 h, the mice had intragastric administration of adenine (75 mg/kg/day) and potassium oxonate (200 mg/kg/day). The normal and HN groups were only administered orally the same volume of normal saline. The experiment lasted 21 days. The mice were anesthetized with isoflurane. The mice were then euthanized with carbon dioxide. The carbon dioxide filling rate is 10%–30% of the chamber volume.

Ultra-performance liquid chromatography (UPLC) was used to determine serum uric acid levels in mice [18]. The clearance of uric acid (Cur) and creatinine (Ccr) was then calculated [21].

Histopathology of renal tissues

Kidney tissue was fixed with 10% formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS) or Masson's trichrome (MASSON). The stained sections were observed with an optical microscope with a magnification of × 400. The histopathological changes were evaluated by epithelial cell necrosis and renal tubule dilatation. The positive staining area of renal interstitial fibrosis was quantitatively measured by Image J program (National Institutes of Health, Bethesda, Maryland, USA).

Immunohistochemistry

The kidney embedded wax block was cut into 4 μm slices. After deparaffinization, they were treated with citrate buffer and then treated with 0.3% H_2O_2. 3% BSA was used to block non-specific proteins. After blocking, each slide was incubated with CD68 primary antibody (Abcam plc. Cambridge, MA, USA) at 4 °C overnight. The next day, the tissue section was incubated with the secondary antibody. Images were observed and captured with an Axio Imager 2 (Zeiss, Oberkochen, Germany).

Western blot and qRT-PCR analysis

The methods for western blotting and qRT-PCR were consistent with our previous studies [22]. Anti-ABCG2, anti-OAT1, anti-PRPS, anti-IκBα, anti-phosphor-IκBα, anti-NLRP3, anti-Caspase 1, anti-NF- κB, anti-phosphor-NF-κB, anti-Collagen 1, anti-E-cadhrein, anti-α-SMA and β-actin antibodies are purchased from Abcam plc. Cambridge, MA, USA. Other antibodies used in this study included: anti-URAT1 (ProteinTech Group. Chicago, USA) and anti-GLUT9 (Millipore Co. Ltd. Bedford, MA, USA). qRT-PCR was performed by the SYBR Green QuantiTect RT-PCR Kit (Thermo Fisher Sci. Inc., St. Austin, TX, USA). The results were detected using the $2^{-\Delta\Delta CT}$ method. The PCR primer sequences were shown in Table 1.

Cell culture and processing

Screening of active compounds to inhibit the absorption of urate

To establish HEK293T cells with URAT1 gene overexpression, the cDNA of URAT1 was subcloned into A pHB-CMV-MCS-EF1-ZSgreen-puro (Hanheng Biotechnology Co., Ltd. Shanghai, China). HEK293T cells were then transfected using Lipofiter™. After 24 h, the transfection solution was removed and the successfully transfected cells were screened.hURAT1-HEK293T cells (HEK293T cells with URAT1 overexpression) were cultured in DMEM containing 10% fetal bovine serum and 1% diabody, and placed in a constant temperature incubator with a CO_2 concentration of 5% (temperature was set at 37 °C). The compound was administered at a concentration of 5 μmol/L. The concentration of uric acid model was 100 μmol/L. When

Table 1 Sequences of the primers

Gene	sequence (5'-3')
TNF-α	5'-CTCCTACTGCTTCCTCGTCT-3'
	5'-GGTTCTGTTCTTGGTCTCCG-3'
MCP-1	5'-TTCAGGTTATGTGGTTCAAGATG-3'
	5'-AGGAAGAGGATGGAAGGGTCAGT-3'
RANTES	5'-ATATGGCTCGGACACCACTC-3'
	5'-SCTTGGCGGTTCCTTCGAG-3'
IL-1β	5'-GTTGCTCCCCTACTGCTGATGGCTT-3'
	5'-AGATTCGGGTCGTCCTTGCTTGTCC-3'
GAPDH	5'-AACTTTGGCATTGTGGAAGG-3'
	5'-GGATGCAGGGATGATGTTCT-3'

the cells grew logarithmically, they were seeded in 6-well plates. After washing with chlorine-free HBSS buffer, HBSS buffer was added for incubation. After removing the HBSS buffer, the HBSS solution containing 100 μmol/L urate and the corresponding concentration of alkaloid monomer was incubated for 30 min. RDEA3170 acted as a urate-anion transporter 1 (URAT1) inhibitor. 0.1 mmol/L NaOH was added to each well to lyse the cells, and then the uric acid content of the sample was measured.

Compounds inhibit hyperuric-acid-induced inflammatory response in renal tubular epithelial cells

NRK-52E cells (Hanheng Biotechnology Co., Ltd, Shanghai, China) were cultured in DMEM medium containing 10% fetal bovine serum concentration and 1% diabody concentration, and placed in a constant temperature incubator with a CO_2 concentration of 5% (temperature was set to 37 °C). The final concentration was 15 mg/dL uric acid for 48 h to build the model. Select the logarithmic growth phase cells, inoculate them in a 6-well plate, when the cells were 70–80% of the bottom plate, starve for 11 h, and 5 μmol/L alkaloid monomer compound and inhibitor were pre-administered for 1 h. Uric acid solution was added to each well and cultured for 24 h. 5 μmol/L alkaloid monomer compound and inhibitor was added to each well to incubate for 24 h. The experiment was ended and subsequent experimental tests were carried out.

Enzyme-linked immunosorbent assay (ELISA)

For the determination of the expression levels of IL-1β, TNF-α, MCP-1 and RANTES in mouse kidneys, mouse kidneys were fully homogenized in 9 volumes of pre-chilled Tris–HCl buffer (50 mM, pH 7.4) containing 10 μg/mL indomethacin. Centrifuge at 5000 rpm for 10 min to remove insoluble proteins. All steps are carried out at 4 °C. Add antigen diluted to appropriate concentration with coating diluent in each well, 37 ℃ for 4 h. 5% calf serum was added into each well and sealed at 37 ℃ for 40 min. After washing for 3 times, add the sample to be tested. The well was washed three times after reacting at 37 ℃ for 40 min. Each well was added enzyme labeled antibody for 1 h, then added substrate solution away from light at 37 ℃ for 3–5 min. At last, each well was added termination solution, and the absorbance of the solution in each well was detected at 450 nm wavelength by a microplate reader (MuATiskanMK3, Thermo Fisher Sci. Inc., St. Austin, TX, USA).

Data analysis of network pharmacology

Use Pharmmapper online tool for compound target enrichment analysis, and then use Cytoscape software for network construction, analysis, and beautification.

Statistical analysis

The result data are shown as the mean ± S.E.M. The SPSS 20.0 statistical software was used for data analysis (version 20, SPSS; IBM, Armonk, NY, USA). The significant differences between the data were evaluated by one-way ANOVA, LSD and Dunnett's (Tukey) test were used for post hoc evaluations. $P < 0.05$ was considered statistically significant.

Results

The alkaloid component of *E. longifolia* could significantly reduce the level of serum uric acid, improve the renal dysfunction and renal histopathological damage caused by high uric acid in HN mice.

The alkaloid component of *E. longifolia* lowered uricemia and attenuated renal injury in HN mice

At the end of the study, the HN mice showed higher kidney coefficients and lower body weight levels (Fig. 1a–c), as well as significantly increased levels of uric acid (Fig. 1d), and serum creatinine (Fig. 1e), blood urea nitrogen (BUN) (Fig. 1f), and reduced uric acid clearance (Fig. 1g) in comparison to those of control. The alkaloid component of *E. longifolia* reduced uricemia and restored renal function parameters in a dose-dependent manner (Fig. 1c–f). Histopathological results further confirmed this point, among which *E. longifolia* alkaloid components improved the pathological changes of HN mice kidney.

The alkaloid component of *E. longifolia* reduced the synthesis of uric acid and promoted the excretion of uric acid in HN mice

Hyperuricemia is related to increased uric acid production and decreased excretion. The positive control group of benzbromarone is known to promote uric acid excretion, which is consistent with our results, that is, the level of uric acidemia in the mice fed with benzbromarone is even lower than that of the control group (Fig. 1d). *E.*

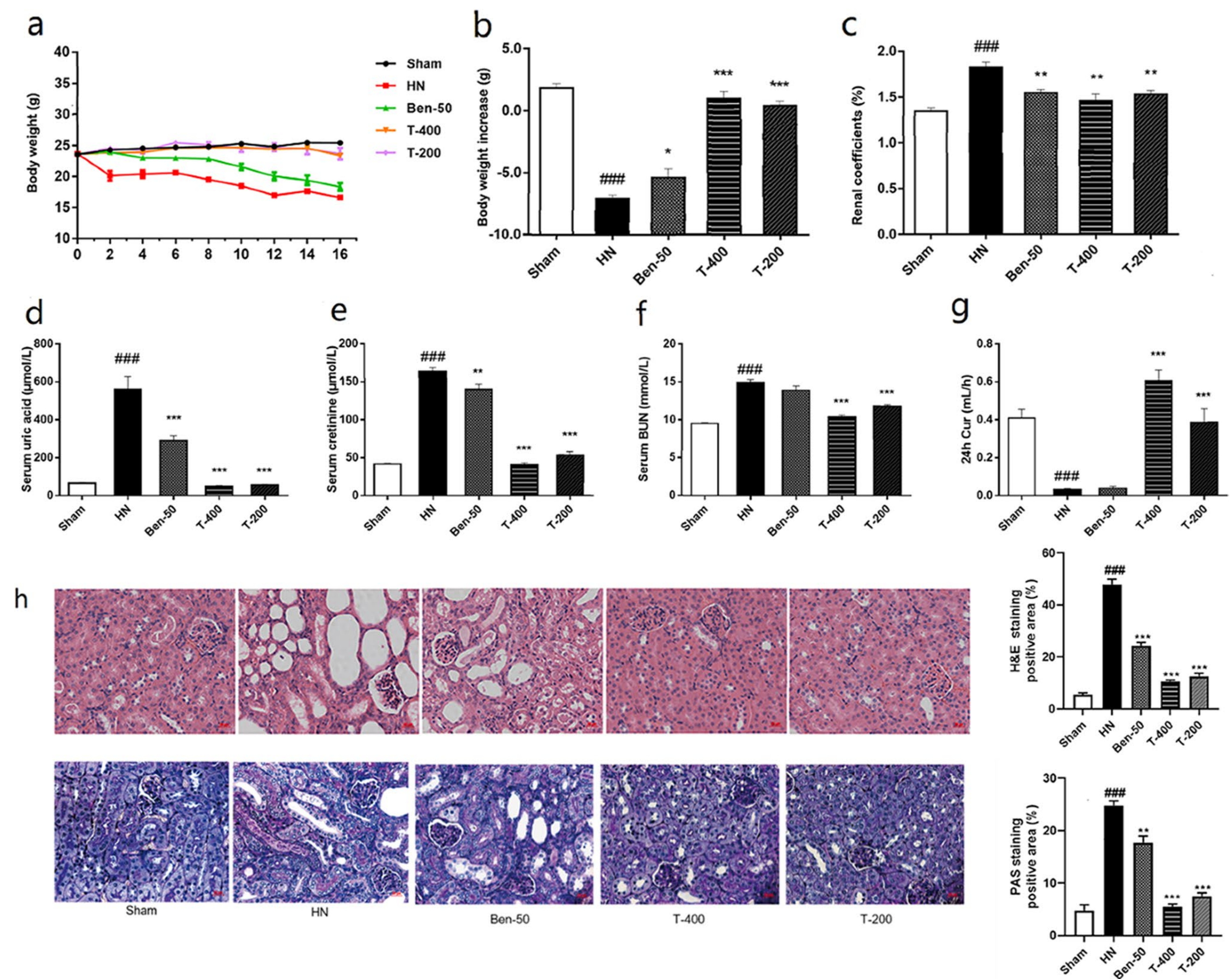

Fig. 1 Effects of *E. longifolia* alkaloid components on plasma uric acid, creatinine, urea nitrogen and kidney injury in HN mice. **A** Body weight; **B** body weight increase; **C** kidney coefficient; **D** serum uric acid; **E** serum creatinine; **F** blood urea nitrogen, **G** uric acid clearance; **H** representative photomicrographs (×400) of H&E and PAS staining in mice kidneys. Sham: blank control group; *HN* hyperuricemia nephropathy group, *Ben-50*: benzbromarone 50 mg/kg; *T-200 E. longifolia* alkaloid components, low-dose group 200 mg/kg; *T-400 E. longifolia* alkaloid components, high-dose group 400 mg/kg. Data are expressed as mean ± SEM ($n = 10$). Compared with Sham group, $^{###}P < 0.001$; compared with HN group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

longifolia alkaloid components reduced uric acidemia to near normal levels. The liver is the main site for uric acid synthesis, phosphoribosyl pyrophosphate synthetase (PRPS) and hypoxanthine-guanine phosphoribosyl transferase (HPRT) are the rate-limiting enzymes for uric acid synthesis. We found through western blot analysis that the alkaloid component of *E. longifolia* significantly reduced the expression of PRPS and HPRT in the liver of HN mice (Fig. 2a–c). Uric acid transporter in the kidney mediates renal uric acid excretion. We found through western blot analysis that *E. longifolia* alkaloid components significantly restored the abnormal expression of organic anion transporter 1 (OAT1) and ATP-binding box subfamily G member 2 (ABCG2) in the kidney of HN mice (Fig. 2d–f). Therefore, it can be concluded that the regulation of uric acid transporter and key enzymes for uric acid synthesis may be part of the mechanism of reducing uric acid by the alkaloid component of *E. longifolia*.

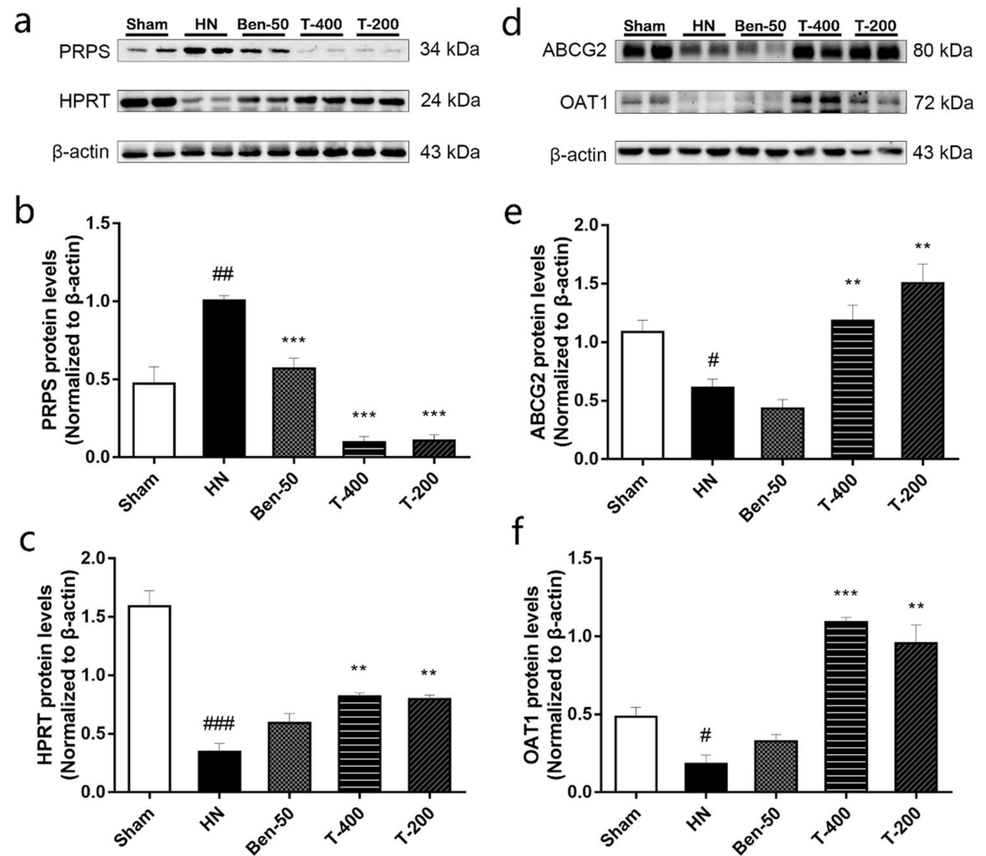

Fig. 2 Effects of *E. longifolia* alkaloid components on uric acid synthesis and transport in HN mice. **A** *E. longifolia* alkaloid components reduced the expression of PRPS, and increased the expression level of HPRT; **B**, **C** the ratio of PRPS and HPRT to β-actin was calculated; **D** *E. longifolia* alkaloid components increased the expression level of ABCG2 and OAT1; **E** the ratio of ABCG2 and OAT1 to β-actin was calculated. Sham: blank control group; HN: hyperuricemia nephropathy group; Ben-50: benzbromarone 50 mg/kg; T-200: *E. longifolia* alkaloid components, low-dose group 200 mg/kg; T-400: *E. longifolia* alkaloid components, high-dose group 400 mg/kg. Data are shown as mean ± SEM ($n = 10$). Compared with Sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared with HN group, $^{**}P < 0.01$, $^{***}P < 0.001$

The alkaloid component of *E. longifolia* improved renal tubulointerstitial fibrosis via relieving inflammatory reaction in HN mice

The alkaloid component of *E. longifolia* reduced pro-inflammatory production in the kidneys of HN mice

Excessive pro-inflammatory production plays a crucial role in the development of HUA-related kidney injury. Higher concentrations of tumor necrosis factor α (TNF-α), interleukin-1 β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), and regulated activated normal T cell expression and secretion proteins (RANTES) were detected in the kidneys of HN mice compared to the control group (Fig. 3). Specifically, *E. longifolia* alkaloid components significantly reduced mRNA levels and protein levels of these pro-inflammatory mediators (Fig. 3), validating that *E. longifolia* alkaloid components attenuated inflammation in the kidneys of HN mice.

The alkaloid component of *E. longifolia* inhibited the activation of NF-κB and NLRP3 inflammasome signaling pathway in the kidney of HN mice

Previous studies have shown that inflammation is closely related to HN, and NF-κB is expressed as an important inflammatory mediator in the context. The activation of NF-κB is the core step of NF-κB signal transduction. Through western blotting, we found that the increased phosphorylation of NF-κB and NF-κB inhibitory protein (IκBα) in the kidneys of HN mice was repressed by *E. longifolia* alkaloid components treatment (Fig. 4a–c). It has been confirmed that the activation of the NLRP3 inflammasome signaling pathway is a key driving factor for renal fibrosis. Therefore, the expression of NLRP3 and Caspase 1 in the kidney of HN mice was detected by immunoblotting. In Fig. 4F–H, increased expression of NLRP3 and Caspase 1 was detected in the kidney of HN mice, which was inhibited by *E. longifolia* alkaloid components treatment. Similarly, benzbromarone failed to inhibit the expression of NLRP3 and Caspase 1.

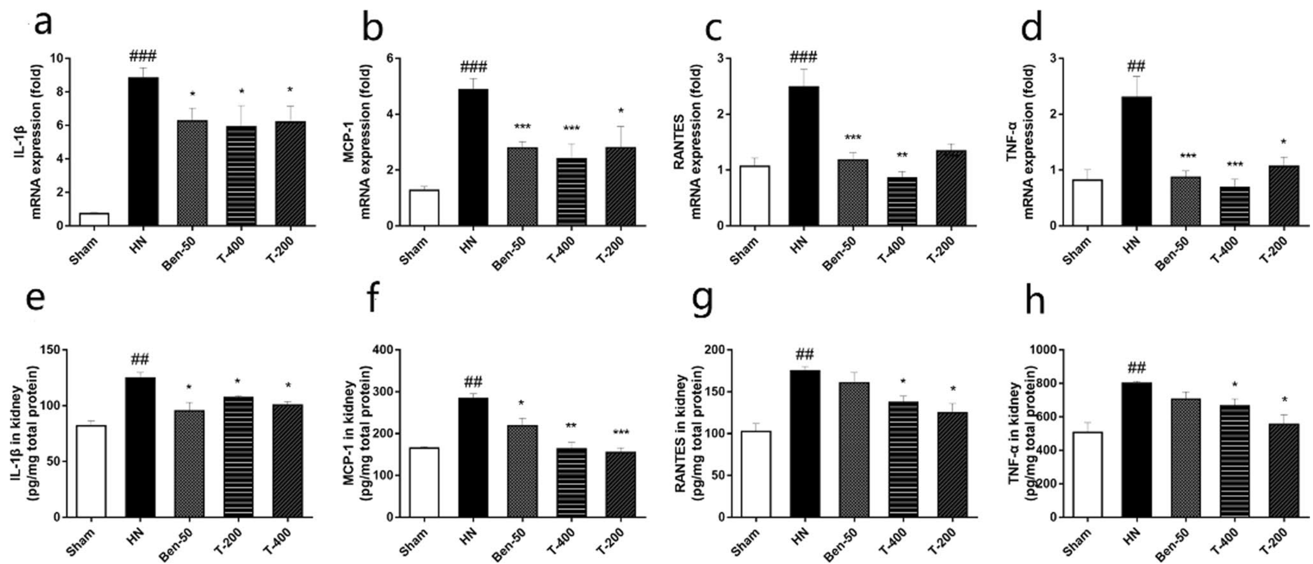

Fig. 3 Effects of *E. longifolia* alkaloid components on pro-inflammatory production in HN mice. **A–D** The mRNA expression levels of IL-1β, MCP-1, RANTES, and TNF-α were determined; **E–F** the concentration of IL-1β, MCP-1, RANTES, and TNF-α in HN mice kidney was detected by ELISA. Sham: blank control group; HN: hyperuricemia nephropathy group; Ben-50: benzbromarone 50 mg/kg; T-200: *E. longifolia* alkaloid components, low-dose group 200 mg/kg; T-400: *E. longifolia* alkaloid components, high-dose group 400 mg/kg. Data are shown as mean ± SEM ($n = 10$). Compared with Sham group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared with HN group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

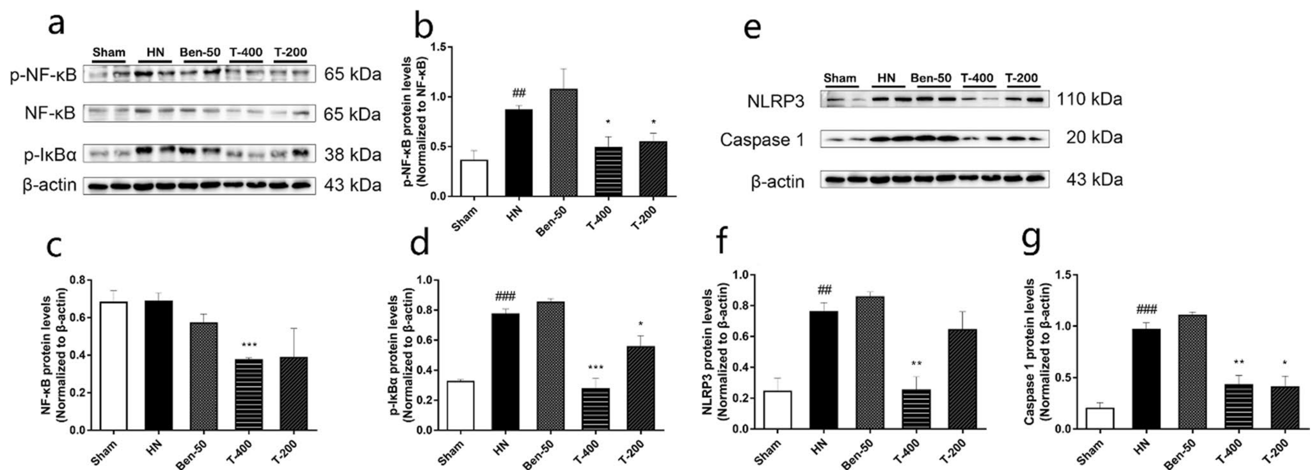

Fig. 4 Effects of *E. longifolia* alkaloid components on NF-κB and NLRP3 inflammasome signaling pathway in HN mice. **A** The protein expression levels of p-NK-κB, NK-κB, and p-IκBα were determined. **B–D** The ratio of p-NK-κB, NK-κB, and p-IκBα to β-actin was calculated. **E** The protein expression levels of NLRP3 and Caspase 1 were determined. **F–G** The ratio of NLRP3 and Caspase 1 to β-actin was calculated. Sham: blank control group; HN: hyperuricemia nephropathy group; Ben-50: benzbromarone 50 mg/kg; T-200: *E. longifolia* alkaloid components, low-dose group 200 mg/kg; T-400: *E. longifolia* alkaloid components, high-dose group 400 mg/kg. Data are shown as mean ± SEM ($n = 10$). Compared with Sham group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared with HN group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The alkaloid component of *E. longifolia* improved renal tubulointerstitial fibrosis in HN mice

Tubular interstitial fibrosis has been confirmed in the pathogenesis of HN and is characterized by abnormal or excessive extracellular matrix (ECM) deposition. According to Fig. 5a, b, HN mice showed increased Masson tricolor positive areas in the damaged renal tubular interstitial compartment, while *E. longifolia* alkaloid components improved the morphological

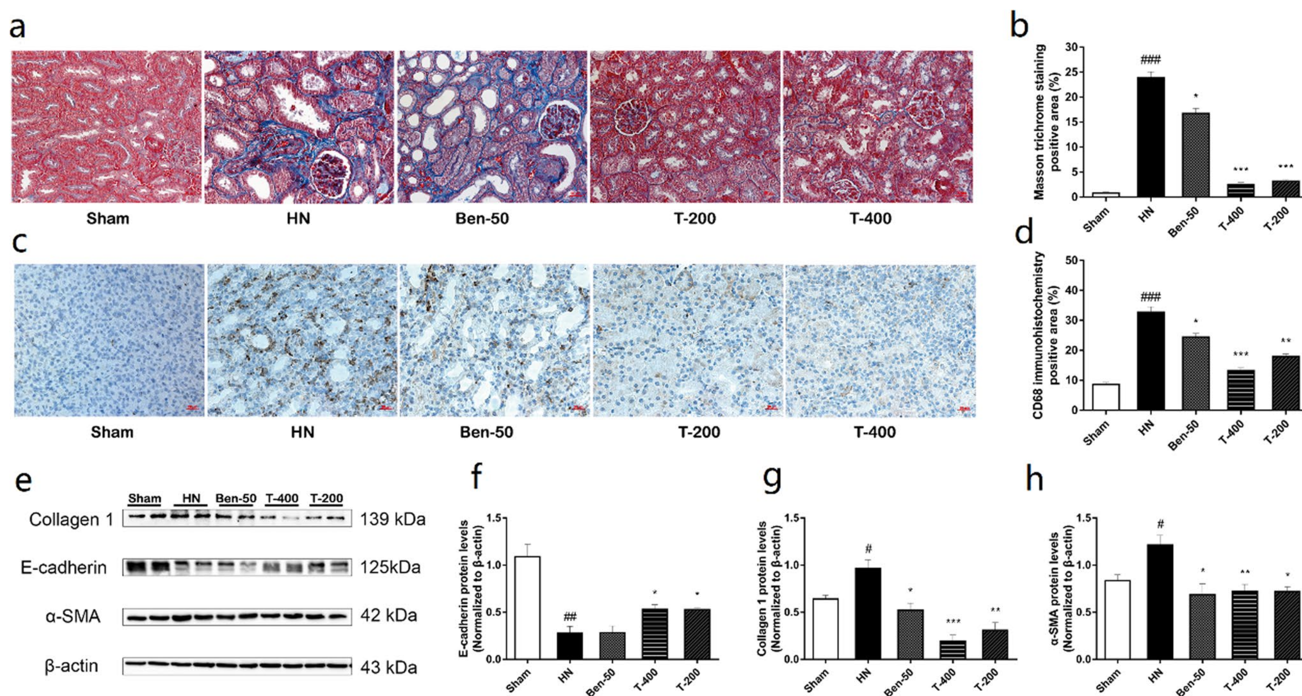

Fig. 5 Effects of *E. longifolia* alkaloid components on renal tubulointerstitial fibrosis in HN mice. **A** The photomicrograph of mice kidneys stained with MASSON (×400). **B** Fibrotic score. **C** Representative photomicrographs (×400) of CD68 immunohistochemistry of mice kidneys. **D** Score of kidneys in CD68 immunohistochemistry. **E** The protein expression levels of E-cadherin, Collagen 1, and α-SMA were determined by western blot. **F–H** The ratio of E-cadherin, Collagen 1, and α-SMA to β-actin was calculated. Sham: blank control group; HN: hyperuricemia nephropathy group; Ben-50: benzbromarone 50 mg/kg; T-200: *E. longifolia* alkaloid components, low-dose group 200 mg/kg; T-400: *E. longifolia* alkaloid components, high-dose group 400 mg/kg. The data are shown as mean ± S.E.M ($n = 10$). Compared with Sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared with HN group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

lesions of fibrosis. Moreover, the positive cells of HN mice increased significantly, suggesting a large number of macrophage infiltration in the kidney of HN mice, the positive expression of *E. longifolia* alkaloid components groups decreased significantly, and the macrophage infiltration was significantly improved (Fig. 5c, d).

In addition, the expression of two key interstitial matrix components, Collagen 1 was significantly increased and calcium-dependent cell adhesion molecule E (E-cadherin) was significantly decreased in the kidneys of HN mice, and *E. longifolia* alkaloid components successfully inhibited its expression (Fig. 5e, g). The contractile protein α-smooth muscle actin (α-SMA) expressed by mesenchymal fibroblasts has been regarded as a key fibrotic factor and plays a vital role in the progression of renal fibrosis. In our research, the enhanced renal expression of α-SMA was found in HN mice, and its expression was inhibited after being treated with *E. longifolia* alkaloid components (Fig. 5h). Therefore, *E. longifolia* alkaloid components can alleviate tubular interstitial fibrosis in HN mice.

E. Longifolia alkaloid monomer could reduce cellular uric acid uptake, inhibit NF- κB and NLRP3 / Caspase 1 signaling pathway activation

Inhibition of *E. longifolia* alkaloid monomer on uric acid uptake in hURAT1-HEK293T cells

As shown in Fig. 6a, we identified 6 major alkaloids from *E. longifolia*. By comparing its chemical and spectral data with the data reported in the literature, it is determined that its structure is 4-methoxy-5-hydroxycanthin-6-one (1), 9,10-dimethoxycanthin-6-one (2), 4,9-dimethoxycanthin-6-one (3), 5-methoxy-canthin-6-one (4), canthin-6-one (5), and 9-methoxycanthin-6-one (6). Due to the crucial role of URAT1 in renal urate reabsorption, hURAT1-HEK293T cells were used to determine the effect of *E. longifolia* alkaloid components on urate reabsorption. Role in urate reabsorption in kidney, hURAT1-HEK293T cells were

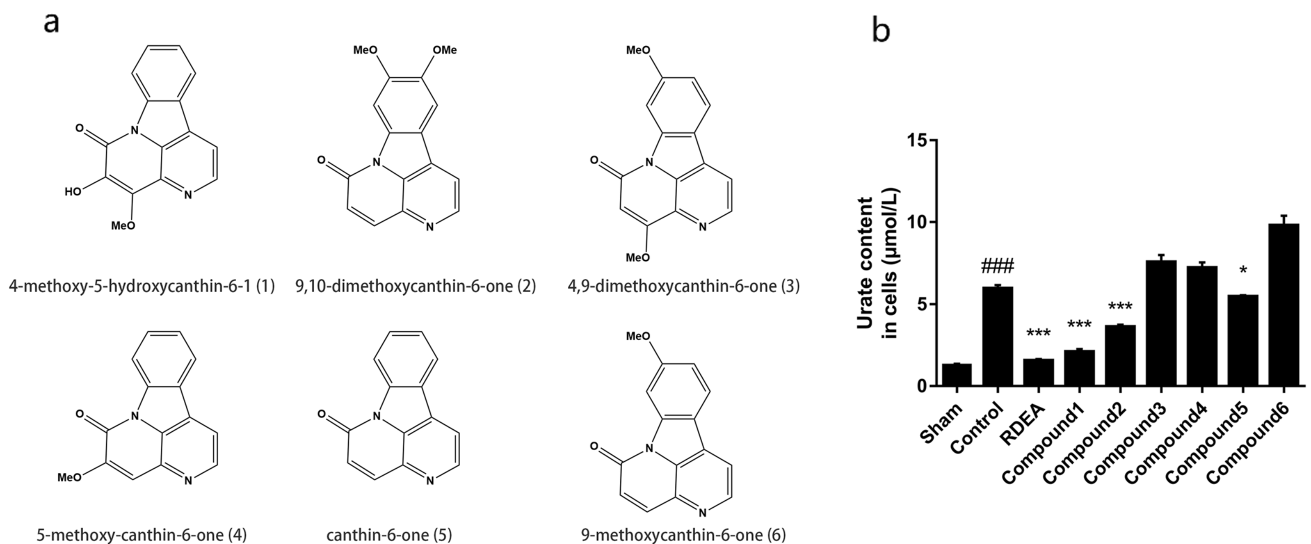

Fig. 6 Effects of *E. longifolia alkaloid* monomer on uric acid uptake in hURAT1-HEK293T cells. **A** Chemical structure of *E. longifolia alkaloid* monomer. **B** Uric acid concentration in hURAT1-HEK293T cells. Sham: blank control group; control: 100 mol/L urate group; RDEA: 1 μmol/L RDEA; Compounds **1–6**: 5 μmol/L Compound **1-6**. The data are shown as mean ± S.E.M ($n=5$). Compared with Sham group, ###$P<0.001$; compared with HN group, *$P<0.05$, ***$P<0.001$

used to determine the effects of urate uptake regulation role of alkaloid components from *E. longifolia*. As shown in Fig. 6b, uric acid absorption increased significantly in cells expressing hURAT1. 1 μmol/L RDEA3170 and 5 μmol/L Compounds **1**, **2,** and **5** could reduce the uric acid uptake of hURAT1-expressing cells, while Compounds **3**, **4,** and **6** showed relatively low activity, indicating that the alkaloids in *E. longifolia* could inhibit URAT1.

Analysis of compound-target-pathway network of *E. longifolia*

To explain the potential pharmacological effects of *E. longifolia* in the treatment of HN, *E. longifolia* alkaloids were tested using network pharmacology. The results showed that Compounds **2**, **5,** and **6** targeted the inflammatory signal pathway, among which Compounds **2** and **6** TNF and IL-17 targeted signal pathway, and Compound **5** targeted the IL-17 signal pathway (Fig. 7). It has been confirmed by us that Compounds **2** and **5** have a significant inhibitory effect on uric acid absorption. Based on this result, we subsequently conducted follow-up in vitro experiments on Compounds **2** and **5** to explore the inhibitory effect and related mechanisms of Compounds **2** and **5** on hyperuric-acid-induced renal tubular epithelial cells NRK52E verification.

Compounds 2 and 5 alleviate pro-inflammatory effects by inhibiting the activation of NF-κB and NLRP3/Caspase 1 signaling pathways in uric-acid-stimulated NRK52E cells

We explored the effect of Compounds **2** and **5** in uric-acid-treated rat proximal tubule epithelial NRK52E cells. Further western blotting results demonstrated that Compounds **2** and **5** can inhibit the pro-inflammatory production induced by uric acid in a dose-dependent manner (Fig. 8a–d), which were consistent with *E. longifolia* in vivo. We further verified the mechanism by which Compounds **2** and **5** alleviate HN in NRK52E cells stimulated by uric acid. It was found that Compounds **2** and **5** inhibited the expression of NLRP3 and Caspase 1 induced by uric acid (Fig. 8e–g). In addition, the expression of α-SMA and NF-κB, the phosphorylation of NF-κB induced by uric acid was largely suppressed by compound **5** (Fig. 8h–k).

Discussion

HUA is an independent risk factor for chronic kidney disease (CKD), hypertension, cardiovascular and cerebrovascular diseases, diabetes, metabolic syndrome, atherosclerosis [21, 23]. With the increase of the prevalence of HUA,

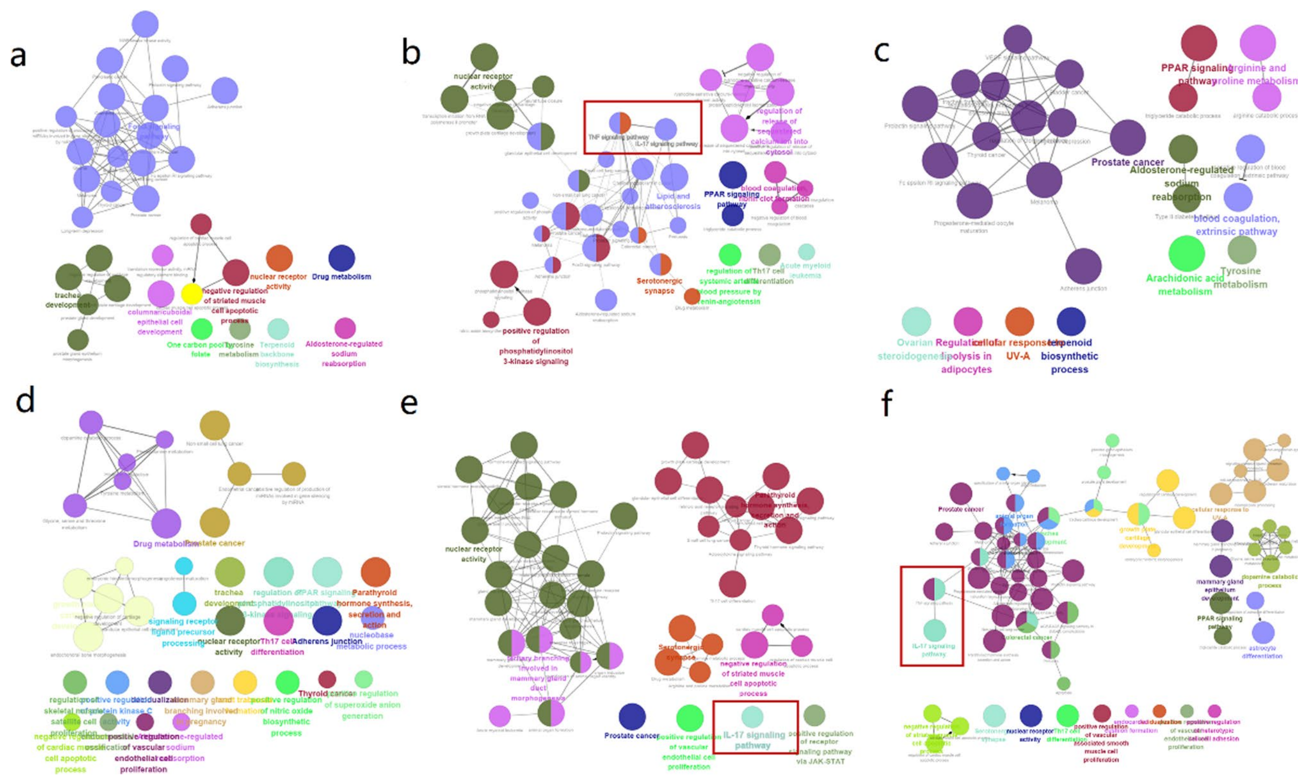

Fig. 7 Compound-target-pathway network of *E. longifolia*. **A**–**F** Compound (**1–6**)-target-pathway network of *E. longifolia*. The red box shows the targeted TNF and IL-17 signal pathway

the incidence of related complications has also increased, seriously endangering human health [21]. Epidemiological studies show that the incidence of renal disease in Chinese patients with HUA is as high as 15.1%, while the incidence of HN in people with normal blood uric acid is 2.9% [23]. The traditional uric-acid-lowering drugs, allopurinol and benzbromarone, could not reduce the damage of visceral function in HN [11]. Therefore, it is necessary to develop new treatments to prevent renal inflammation and fibrosis caused by HUA to improve the prognosis of HN.

Traditional natural medicine has the advantages of multi-target and low toxicity. Traditional natural medicine has been widely used in the treatment of patients with HN. *E. longifolia* has a variety of pharmacological properties, such as reducing blood sugar, blood pressure, and serum uric acid levels [17, 18]. In addition, as the main active component of *E. longifolia*, alkaloids have been proved to be effective inhibitors of NF-κB in vitro, but the renal protective effect and mechanism of *E. longifolia* on HUA remain unknown [24]. The purpose of this study was to investigate the effect and potential mechanism of *E. longifolia* alkaloid components in HN induced by adenine and potassium oxonate in mice. Our data suggested that oral *E. longifolia* alkaloid components could reduce the level of serum uric acid, alleviate kidney inflammation and renal fibrosis, thereby effectively relieving renal insufficiency caused by HUA.

Uric-acid-lowering treatment may help to prevent or alleviate the progression of CKD [25]. Consistent with this, in our study, the reduction of high uric acid by *E. longifolia* alkaloid components was accompanied by the improvement of renal function. Similarly, we found that *E. longifolia* alkaloid components potentially reduce uric acid by promoting the excretion of uric acid in the kidney and restoring the expression of uric acid transporter. In addition, *E. longifolia* alkaloid components inhibited the expression of PRPS and promoted the expression of HPRT in liver, thereby reducing uric acid production. Previous studies have shown that uric acid excretion disorder is initially caused by abnormal expression of urate transporters in proximal tubules. Our study confirmed that the expression of OAT1 and ABCG2 was maladjusted in HN mice, and the alkaloid component of *E. longifolia* significantly increased the expression of these proteins.

It was found that uric acid not only caused renal injury by crystal-dependent inflammatory reaction, but also caused indirect renal injury by amorphous effect [26, 27]. The level of serum uric acid in patients with HN increased significantly, accompanied by obvious renal pathological changes,

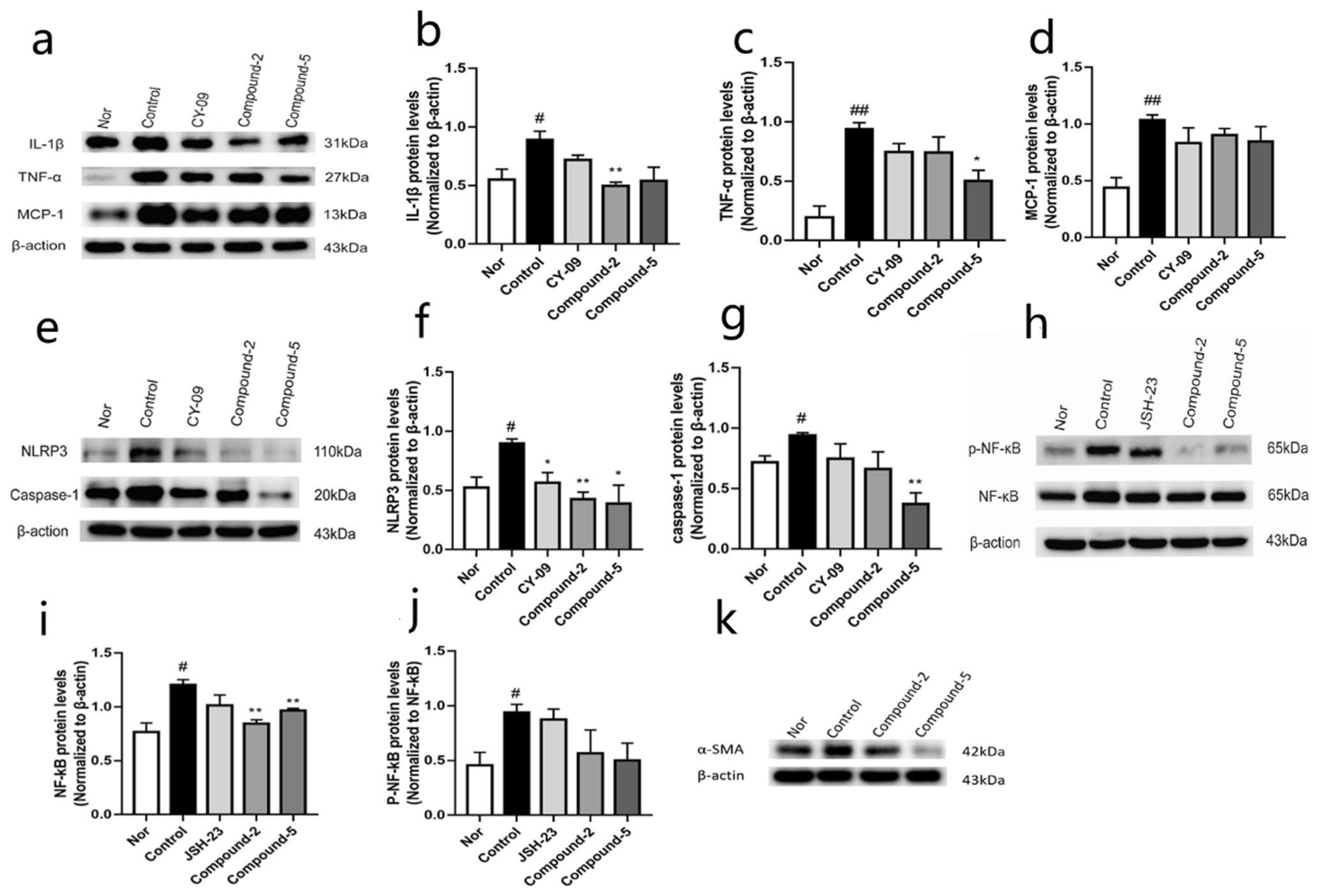

Fig. 8 Effects of Compounds 2 and 5 on the pro-inflammatory effect in uric-acid-stimulated NRK52E cells. **A** The protein expression levels of MCP-1, IL-1β, and TNF-α were determined. **B**–**D** The ratio of MCP-1, IL-1β, and TNF-α to β-actin was calculated. **E** The protein expression levels of Caspase 1 and NLRP3 were determined. **F**–**G** The ratio of Caspase 1 and NLRP3 to β-actin was calculated. **H** The protein expression levels of NK-κB and p-NK-κB were determined. **I**–**J** The ratio of NK-κB and p-NK-κB to β-actin was calculated. **K** The protein expression levels of α-SMA were determined. Nor: blank control group; control: 100 mol/L urate group; Compound **2** or **5**: 5 μmol/L Compound **2** or **5**; CY-09: an inhibitor of NLRP3; JSH-an inhibitor of NF-κB. The data are shown as mean ± S.E.M ($n = 5$). Compared with Nor group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared with control group, $^{*}P < 0.05$, $^{**}P < 0.01$.$^{***}P < 0.001$

mainly manifested in the infiltration of a large number of inflammatory cells and macrophages, interstitial inflammatory reaction and renal tubulointerstitial fibrosis. Whether in the form of MSU crystal or soluble urate, the mechanism of renal injury caused by uric acid is related to the participation of inflammatory response [8]. In addition, uric acid activates nuclear factor NF-κB in rat renal tubular epithelial cells (NRK-52E), promoting the expression of inflammation related cytokines such as MCP-1 and RANTES, induces renal inflammation [9]. Uric acid can induce renal interstitial inflammatory cell infiltration, and the infiltrated inflammatory cells can release pro-inflammatory chemokines and cytokines, promoting the occurrence and development of kidney disease [28]. We detected the level of renal inflammation in HN mice by CD68 immunohistochemistry, qRT-PCR, and ELISA. The results showed that the alkaloid component of *E. longifolia* significantly reduced the infiltration of inflammatory cells in the kidney and significantly reduced inflammatory factor and chemokine IL-1 β、TNF-α. The above results showed that the alkaloid component of *E. longifolia* reduced renal inflammation by downregulating the expression of inflammatory factors and chemokines. Uric-acid-induced renal inflammation was confirmed to be associated with transcription factors (such as NF-κB) and NLRP3 / IL-1β signal pathway [7]. Our results showed that the alkaloid component of *E. longifolia* could significantly downregulate phosphorylated NF-κB and its upstream IκBα. It can also downregulate the expression of NLRP3 and Caspase 1, thereby inhibiting activation of NF- κB and NLRP3/ IL-1β signal pathway.

Uric acid can also induce vascular wall thickening through amorphous effect, resulting in renal cavity occlusion and insufficient perfusion, leading to renal tubulointerstitial inflammation [29]. Studies have shown that uric acid can induce the transformation of renal tubular epithelial cells into interstitial cells, resulting in renal tubular interstitial

fibrosis [30]. Uric acid induces a large amount of accumulation of extracellular matrix (ECM), a key step of renal interstitial fibrosis, by activating the process of renal tubular epithelial mesenchymal transition (EMT), which eventually leads to renal tubular interstitial fibrosis [30]. Renal tubular interstitial fibrosis is an important pathological process in the progression of CKD to end-stage renal disease, and Collagen 1 is a key component of interstitial matrix. EMT is characterized by decreased expression of E-cadherin in epithelial cells, accompanied by increased expression of interstitial cell marker α-SMA. Uric-acid-lowering treatment has become a new treatment for renal injury in patients with HN. Studies have shown that in the CKD rat model, the damage of renal tubules caused the uric acid transporter to fail to function, and the increase in uric acid levels was mainly the result of reduced uric acid filtration in the glomerulus [24]. Therefore, when kidney injury occurs, the effect of uric-acid-lowering drugs that target renal transporters may be reduced [11]. In this study, western blot showed that the alkaloid component of *E. longifolia* could downregulated the expression of α-SMA and collagen 1, upregulated the expression of E-cadherin, which revealed that the alkaloid component of *E. longifolia* inhibited the process of epithelial interstitial, and then improved renal fibrosis induced by high uric acid.

Subsequently, in vitro experiments, we further verified the inhibitory effect of *E. longifolia* alkaloid monomer on uric acid reabsorption and uric-acid-induced inflammation of renal tubular epithelial cells. The results showed that Compounds **1, 2,** and **5** could inhibit the accumulation of uric acid and reduce the intracellular uric acid concentration in hURAT1-HEK293T cells. Combined with the results of network pharmacology, Compounds **2**, **5,** and **6** can target TNF and IL-17 inflammatory pathways. We used uric-acid-induced NRK-52E inflammatory model to detect the anti-inflammatory effect of alkaloid monomers Compounds **2** and **5**, and found that Compounds **2** and **5** can reduce inflammatory factor IL-1 β, MCP-1, and TNF-α by inhibiting the activation of NF-κB and NLRP3 inflammatory signaling pathways. Compounds **2** and **5** are Canthin-6-one alkaloids. Canthin-6-one alkaloids have been proved to be effective inhibitors of NF-κB. Therefore, we speculate that Canthin-6-one alkaloids may be the main uric-acid-lowering active components of *E. longifolia*.

Conclusion

In conclusion, the alkaloid component of *E. longifolia* had preventive and therapeutic effects on nephropathy caused by HUA. *E. longifolia* alkaloid components not only played a role in reducing uric acid by regulating hepatic uric acid synthase and renal uric acid transporter, but also reduced renal inflammation and fibrosis in HN mice through NF-κB and NLRP3 signaling pathway. Therefore, the alkaloid component of *E. longifolia* may be a promising natural drug for HN.

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Author contributions LL, DW, YZ, and TW completed the experimental design. KL, ML, QC, and HC were responsible for conducting experiments and analyzing data. DW and TW were responsible for writing the manuscript. ML, YZ, and TW checked the manuscript. TW and DW provided the funding.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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