



Research Article

# Supplements extracted from *Lophatherum gracile* Brongn. ameliorates hyperuricemia by regulating nucleotide metabolic enzymes and urate transporters

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**Abstract:** Hyperuricemia (HUA) has gradually become the fourth most prevalent chronic disease and the incidence rate is getting younger. In this study, *Lophatherum gracile* Brongn. with uric acid (UA)-lowering effects were screened from medicine food homology (MFH) plants by UA overproduction and excessive absorption cell models. Further, a murine model of potassium oxonate and yeast-induced HUA was used to evaluate the *in vivo* anti-high UA and nephroprotective effects of *L. gracile*, which significantly reduced serum UA and increased urine UA levels in HUA mice. *L. gracile* effectively inhibits the overproduction of UA by decreasing the activities of xanthine oxidase and adenosine deaminase (ADA), promoting excretion by regulating the mRNA expression of glucose transporter 9. Moreover, active components of *L. gracile* were analyzed by high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry/mass spectrometry (HPLC-QTOF-MS/MS), network pharmacology was used to identify active ingredients further and validate targets. The results of network pharmacology showed that the components of the *L. gracile* acted on the targets xanthine dehydrogenase (XDH), adenosine deaminase (ADA), solute carrier family 2 member 9 (SLC2A9), and so on, which was consistent with the previous verification. The most likely active substances of *L. gracile* hydrojuglone glucoside. The effect of reducing UA is discovered for the first time in *L. gracile*. These findings suggested that *L. gracile* could be used as a potential confrontational strategy for the development of anti-HUA functional foods, providing the theoretical basis for the application of MFH plants and the development of related functional food products.

**Keywords:** uric acid; *Lophatherum gracile* Brongn.; hydrojuglone glucoside; nucleotide metabolic enzymes; urate transporters; hyperuricemic model

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## 1 Introduction

Uric acid (UA) is one of the human metabolites, it can result in hyperuricemia (HUA) once it accumulates too much in the body. With the globalization of the economy, people's diets have become higher in sugar and fat, which causes the increased incidence of HUA<sup>[1,2]</sup>. The overall prevalence of HUA adults is 14% only in China<sup>[3]</sup>. This trend is becoming more pronounced, and epidemiological surveys predict that it is expected to reach 1.42 million in 2030 in the world<sup>[4]</sup>. Not only that, the population of the disease is getting younger. Elevated morbidity has facilitated research into HUA. Studies have shown that HUA is closely related to metabolic syndrome, chronic kidney disease, cardiovascular disease and diabetes<sup>[5,6]</sup>. It has reported that cardiovascular disease mortality rate in persons with high UA was over double that in persons without high UA<sup>[7]</sup>. HUA occurs due

to increased levels of UA in the body. Urate homeostasis depends on the balance between its production and excretion<sup>[8]</sup>. UA is the end product of the purine metabolism in the human liver, adenosine deaminase (ADA) and xanthine oxidase (XO) are the key enzymes that catalyze the formation of UA<sup>[9]</sup>. Meanwhile, the synthesis of UA can be reversed by a remedial synthesis pathway with the help of adenine phosphoribosyltransferase (APRT). Clinically the main reason caused by HUA is insufficient kidney excretion<sup>[10]</sup>. As a negatively charged polar molecule, UA relies on transporters to maintain renal metabolic homeostasis<sup>[11]</sup>. The main transporters are UA transporter 1 (URAT1) and glucose transporter 9 (GLUT9), organic anion transporter 1/3 (OAT1/3) and ATP binding cassette transporter (ABCG2)<sup>[12]</sup>.

Current clinical HUA treatment agents include allopurinol, benzbromarone and febuxostat. But all of them have adverse effects such as kidney stones, and all-cause cardiac death<sup>[13,14]</sup>. For

the han Chinese ethnic group in particular, the risk of allopurinol hypersensitivity syndrome is higher with use (6%–8%) than for whites (2%)<sup>[15]</sup>. Patients with HUA often suffer from kidney disease<sup>[16]</sup>, which means the side effects of drug-induced liver and kidney damage can create a vicious cycle with the patient's symptoms. Based on the deficiencies of treatment, it is urgent to seek natural products with better efficacy and fewer side effects, which may be potential agents for HUA prevention and treatment.

Medicine food homology (MFH) refers to a category of food ingredients that possess medicinal value<sup>[17]</sup>. They have already been wild used as raw materials in functional foods and have shown some preventive effects on chronic diseases such as hypertension<sup>[18]</sup> and hyperlipidemia<sup>[19]</sup>. These physiological activities are often the result of the metabolite's multiple targets and multiple pathways working together<sup>[20]</sup>. However, there are few reports on their use in the treatment of HUA. Therefore, this study screened MFH for their UA-lowering effects and further identified those with proven efficacy in reducing UA levels and analyze potential active ingredients.

## 2 Materials and methods

### 2.1 Preparation of herb extract

Nine kinds of herbs belonging to MFH were selected based on literature review and Chinese medicine theory, which were used to be candidates for anti-HUA herbs. *Euryales Semen*, *Portulacae herba*, *Houttuyniae herba*, *Lophatheri herba*, *Imperatae Rhizoma*, *Phragmitis rhizoma*, *Rubi Fructus*, *Rosae Rugosae Flos*, *Prunellae Spica*. The samples were authenticated based on *China Pharmacopoeia* (volume I, 2020 edition) standard by Prof. B. Y. Yu from the School of Traditional Chinese Pharmacy, China Pharmaceutical University.

A total of 20 g of each herb was soaked to single distilled 400 mL water or 60% ethanol and extracted twice by using rotary evaporator, each for 40 min combine the filtrates, concentrate under reduced pressure, and freeze-dry for subsequent experiments.

### 2.2 Main chemicals and reagents

Adenosine was purchased from Sigma-Aldrich (Shanghai, China). XO was purchased from Shanghai yuanye Bio-technology Co., Ltd. (China). Allopurinol, benzbromarone and UA sodium were purchased from macklin (Shanghai, China). Hank's Balanced salt solution, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution (HEPES), phenylmethylsulfonyl fluoride (PMSF) were obtained from Jiangsu Keygen Biotech Co., Ltd. (Jiangsu, China). TongFengShuPian (TFSP, Z20090318, No. 20210904, PANLONG Pharmaceutical Co., Shanxi, China) was purchased from pharmacy. Potassium oxonate (PO) and yeast was purchased from Solarbio (Beijing, China). Adenosine, UA assay kit (C012-2-1), ADA assay kit (A048-2-1), XO activity assay kit (A002-1-1), Creatinine assay kit (C011-2-1) and Urea nitrogen assay kit (C013-2-1) were purchased from NJJC (NJJC Bio, Nanjing, China). HPLC grade acetonitrile, anhydrous ethanol (analytical grade) were purchased from Merck (Darmstadt, Germany).

### 2.3 Screening from MFH on cell model

#### 2.3.1 UA over-production cell model

The cell model of UA over-production is referenced in the

report of Hou's<sup>[21]</sup> and some modifications are made. L0-2 (Human hepatic cell line) cells were cultured in 96-well plates ( $4 \times 10^5$  cells/well) for 48 h, the control and model groups were placed in DMEM medium with 10% FBS and 1% Penicillin-Streptomycin (PS) solution, while the experimental groups and the positive drug group were pre-incubated for 24 h with 500 µg/mL extracts of MFH herbs and 100 µmol/L allopurinol, respectively. Subsequently, each well was washed and 2.5 mmol/L ADA in serum-free medium was added to groups, while the control group was maintained in fresh medium without ADA. After 30 h incubation, 0.005 U/mg XO was added to each well. The culture supernatant was collected 8 h after treatment, and the UA level was measured by the assay kit.

#### 2.3.2 UA absorption cell model

This model is referenced by previous report<sup>[22]</sup> and enrichment process was improved. Human kidney-2 (HK-2) cells were cultured in 100 mm cell culture dishes ( $9 \times 10^5$  cells/dish), and UA uptake assay was performed when the density reached nearly 100%. After washed, all the groups were placed in HHBS (hanks buffer with 25 mmol/L HEPES) for 20 min while experimental groups were pre-incubated with 500 µg/mL extracts and 50 µmol/L Benzbromarone, respectively. Then each well was placed in HHBS with UA (1 mmol/L) except control group. After 60 min incubation, each well was washed three times. The cells were digested and transferred into EP tube, freeze-thaw several times, PMSF (100 µmol/L) were added, then vortex and centrifugation (4 °C, 12,000 r/min, 10 min). The supernatant was dried by nitrogen blowing and dissolved in 150 µL of water, vortex and centrifugation again then is used to determine UA levels by the assay kit.

### 2.4 Animals

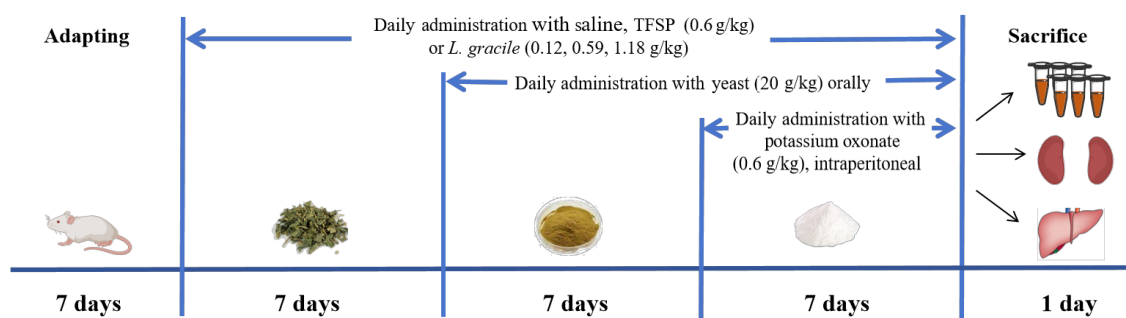
Adult male Kunming mice, 6–7 weeks old, 20–22 g, were purchased from SPF (Beijing)Biotechnology Co., Ltd. (animal license No.: SCXK(Jing)2019-0010). The mice were placed in a temperature-controlled environment ((25 ± 1) °C) for a 12/12 h light/dark cycle. All animal care protocols were approved by the Animal Care and Use Committee of China Pharmaceutical University (protocol number: 2023-02-016).

### 2.5 Establishment of HUA mice

Mice were randomly divided into 6 groups ( $n = 8$ ): control, model, positive drug 600 mg/kg (TFSP), *Lophatherum gracile* groups: low dose (L), medium dose (M) and high dose (H) group (0.12, 0.59, and 1.18 g/kg). PO and yeast were administered as a suspension formulated by 0.5% sodium carboxymethyl cellulose (CMC-Na). After adapting, *L. gracile* were given for 3 weeks. Yeast (20 g/(kg. day)) was oral gavage for 2 weeks and PO (600 mg/(kg. day)) was intraperitoneally injected for last week. The *L. gracile* were given 1 h earlier before HUA modelling. On the 21<sup>th</sup> day, all mice were given 0.3% pentobarbital sodium 1.5 h after modelling. The blood was obtained then centrifuge (3,000 r/min, 10 min, 4 °C) to collect serum. The weight of the kidneys was measured, then one kidney was fixed for renal histological. All samples were stored at –80 °C for further assays. The flow chart of animal experiments is shown in Fig. 1.

### 2.6 Biochemical assay

On the 20<sup>th</sup> day, urine was collected by pressing the bladder 1.5 h after modeling. The liver specimens were homogenized with



**Figure 1** Experimental schedule of PO and yeast induced HUA in mice.

0.9% ice saline for 30 s, centrifuged at 12,000 *g* at 4 °C for 15 min, and the supernatant was taken. Serum uric acid (SUA), urine uric acid (UUA), adenosine deaminase (ADA), blood urea nitrogen (BUN) and creatinine (CR) levels in urine were analyzed using commercially available kits according to the manufacturer's instructions.

## 2.7 Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from kidney and liver using RNA extraction kit and cDNA synthesis using a HiScript II Q RT SuperMix kit (Nanjing Vazyme Biotech Co., Ltd., China). Corresponding primers were obtained from Qinko Biotechnology Co., Ltd. (Wuhan, China), refer to Table S1. Real-time PCR was performed using ChamQ SYBR qPCR Master Mix (Nanjing Vazyme Biotech Co., Ltd., China) and QuantStudio® Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA). The calculation of the relative expression of target genes was performed using the  $2^{-\Delta\Delta Ct}$  method with  $\beta$ -actin as the internal reference.

## 2.8 Renal histological examination

The kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5  $\mu$ m thickness. After deparaffinization and rehydration, the kidney sections were stained with Hematoxylin and Eosin (H&E) for morphological analysis. Tissue damage was assessed using a tubular damage score, as previously described<sup>[23]</sup>. Injury was scored in a blinded manner according to the percentage of damage including loss of the brush border, tubular dilation, inflammatory cell infiltration and intratubular hemorrhage: 0, no damage; 1, <25%; 2, 25%–50%; 3, 50%–75%; 4, >75%.

## 2.9 Statistical analysis

All data were presented as mean  $\pm$  SEM and the analysis was performed using GraphPad Prism 8 (version 8.2.1) software. The data were analyzed using the ANOVA test to compare the data between the individual experimental groups, and  $P < 0.05$  was considered as significant difference.

## 2.10 HPLC-QTOF-MS/MS analysis

### 2.10.1 Sample preparation

*L. gracile* powder were extracted with 60% ethanol for 30 min under ultrasonication. The concentration is equal in amount of 0.1g/mL crude herb amount. After cooling, add weight. The supernatant was filtered before injection into the HPLC-QTOF-MS/MS system.

### 2.10.2 HPLC-QTOF-MS/MS analysis of *L. gracile*

Separation using Zorbax SB-C<sub>18</sub> (4.6 mm  $\times$  150 mm, 5  $\mu$ m, Agilent Corporation, Santa Clara, CA, USA) on Agilent 1260 Infinity II HPLC-DAD system (190–400 nm) (California, USA). The eluents A (water-formic acid, 1,000:1, V/V) and B (acetonitrile) were used for separation at a flow rate of 1.0 mL/min. The column temperature was set at 40 °C. The injection volume was set to 15  $\mu$ L with detection set to 330 nm. The gradient elution program was set as flows: 0–10 min, 5% B; 10–20 min, 5%–10% B; 20–30 min, 10%–13% B; 30–40 min, 13%–15% B; 40–60 min, 15%–16% B. Agilent 6530 quadrupole time-of-flight (Q-TOF) mass spectrometer (California, USA) was used for MS detection, the electrospray ionization mass spectrometry (ESI-MS) spectra were obtained in negative electrospray ionization mode at the mass ranges of  $m/z$  100–2,000. ESI ion source, capillary voltage, 3,500 V. Drying gas flow, 9.0 L/min. Atomization pressure is 35 psi, fragmentor voltage is 120 V, collision voltage is 10–50 V. Capillary temperature is 435 °C. Agilent Mass Hunter Qualitative Analysis B.07.00 software was used for data acquisition and processing.

## 2.11 Network pharmacology

### 2.11.1 Screening potential bio-active targets and disease targets of HUA

The main active compounds of *L. gracile* were collected from HPLC-QTOF-MS/MS results for research. The corresponding potential targets were identified by the SwissTargetPrediction (<http://www.swisstargetprediction.ch/>). All targets were restricted to human origins. Applied the keyword of HUA to retrieve in the following databases: Gene Cards databases (<http://www.genecards.org/>) and DisGeNET (<https://www.disgenet.org/>). The overlapping targets between HUA and active compounds were visualized by the Venny (<https://bioinfo.cnb.csic.es/tools/venny/>) online tools. The herb-component-target (HCT) network was established based on the active ingredients and overlapping genes.

### 2.11.2 Network construction

These overlapping genes were imported into the String database version 11.0 (<https://string-db.org/>) with *Homo sapiens* as the organism and a high confidence interaction score of >0.4. The interaction results were imported into Cytoscape software 3.7.1 for building a protein-protein interaction (PPI) network and network analysis.

### 2.11.3 GO and KEGG pathway enrichment

Gene Ontology (GO) terms and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses were

assessed using the DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>). The results were visualized as bubble charts using the RS tudio software.

### 3 Results

#### 3.1 *L. gracile* inhibited the overproduction of UA on LO-2 cell

The cell viability of all extracts at a concentration of 500 µg/mL exceeds 90%, indicating that the extracts do not have any significant impact on normal cell growth (Fig. S1). After administration with same concentration respectively, as Figs. 2A, 2B showed, compared to the control group, supernatant UA level in the model group was increased 3.3 times. Compared to the model group, supernatant UA level in the positive group, allopurinol, were significantly decreased. The ethanol extract of *Phragmites communis* Trin. (mean = 3.099), *Rubus chingii* Hu. (mean = 3.224) and the aqueous extract of *Houttuynia cordata* Thunb. (mean = 3.168) showed the tendency of decreasing UA level. The ethanol extract of *L. gracile* and the aqueous extract of *Imperata cylindrica* Beauv. var. major (Nees) C.E.Hubb., *P. communis* Trin. and *Rosa rugosa* Thunb. were significantly decreased UA level. Among them, ethanol extract of *L. gracile* was the most effective.

#### 3.2 *L. gracile* has the trend of suppressing the UA excessive absorption on HK-2 cell

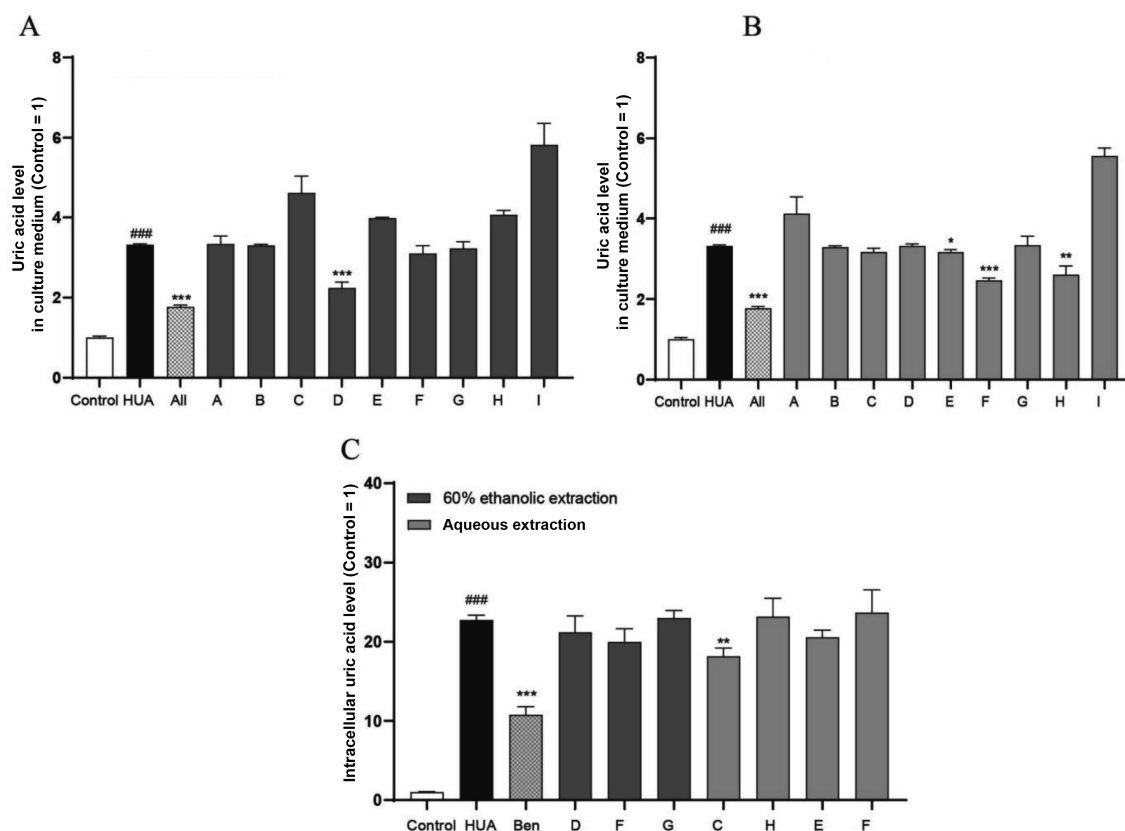
Four extracts with an effect and three extracts with a tendency

were screened out to be detected by the UA excessive absorption cell model. After being administrated with extracts at a concentration of 500 µg/mL respectively, as shown in Fig. 2C, compared to the control group, the intracellular UA level in the model group was increased about 22 times. Compared to the model group, benzbromarone significantly decreased intracellular UA level. The water extract of *H. cordata* Thunb. decreased intracellular UA level significantly. The ethanol extract of *L. gracile* showed the tendency of decrease intracellular UA level.

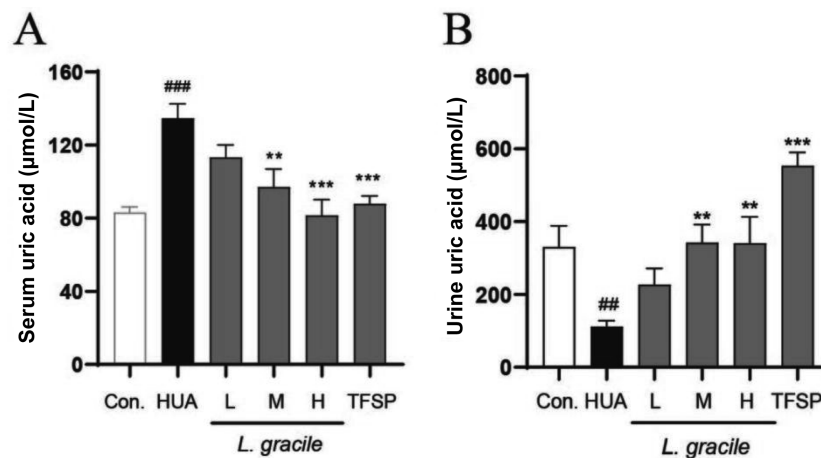
Combining the results of the two models, the ethanol extract of *L. gracile* and the water extract of *H. cordata* Thunb. had the best effect in UA production and excretion model, respectively. However, the performance of both in the other model was barely satisfactory, *H. cordata* Thunb. even increased the UA level in production model, yet *L. gracile* had a slight tendency to reduce UA production. Therefore, *L. gracile* was chosen for next study.

#### 3.3 *L. gracile* lowered SUA levels and increased UUA levels

Anti-HUA activity of *L. gracile* was determined by measuring SUA and UUA levels in HUA mice. As shown in Fig. 3A, the SUA level in HUA mice was about 1.6 times that in the control group ( $P < 0.001$ ), indicating the abnormal elevation of SUA in mice. Compared to the model group, positive drug, TFSP groups demonstrated significant decreasing SUA levels ( $P < 0.001$ ). The SUA concentration in the *L. gracile* groups ( $0.12, 0.59, \text{ and } 1.18 \text{ g/kg}$ ) were ( $113.1 \pm 9.8$ ), ( $97.05 \pm 8.70$ ), and ( $81.5 \pm 6.8$ ) µmol/L respectively, indicating that *L. gracile* induced a concentration dependent reduction in SUA levels. As shown in



**Figure 2** Effect of MFH extracts on cell model. (A) Nine kinds ethanol extracts of MFH on UA overproduction model. (B) Nine kinds of aqueous extracts of MFH on UA overproduction model. (C) The effect of 7 extracts on excessive absorption model. All: allopurinol; Ben: Benzbromarone; A: *Euryale ferox* Salisb.; B: *Portulaca oleracea* L.; C: *H. cordata* Thunb.; D: *L. gracile* Brongn.; E: *I. cylindrica* Beauv. var. major (Nees) C.E.Hubb.; F: *P. communis* Trin.; G: *R. chingii* Hu.; H: *R. rugosa* Thunb.; I: *Prunella vulgaris* L. All data are shown as the mean  $\pm$  SEM. ### $P < 0.001$ , vs control group; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs HUA model group.



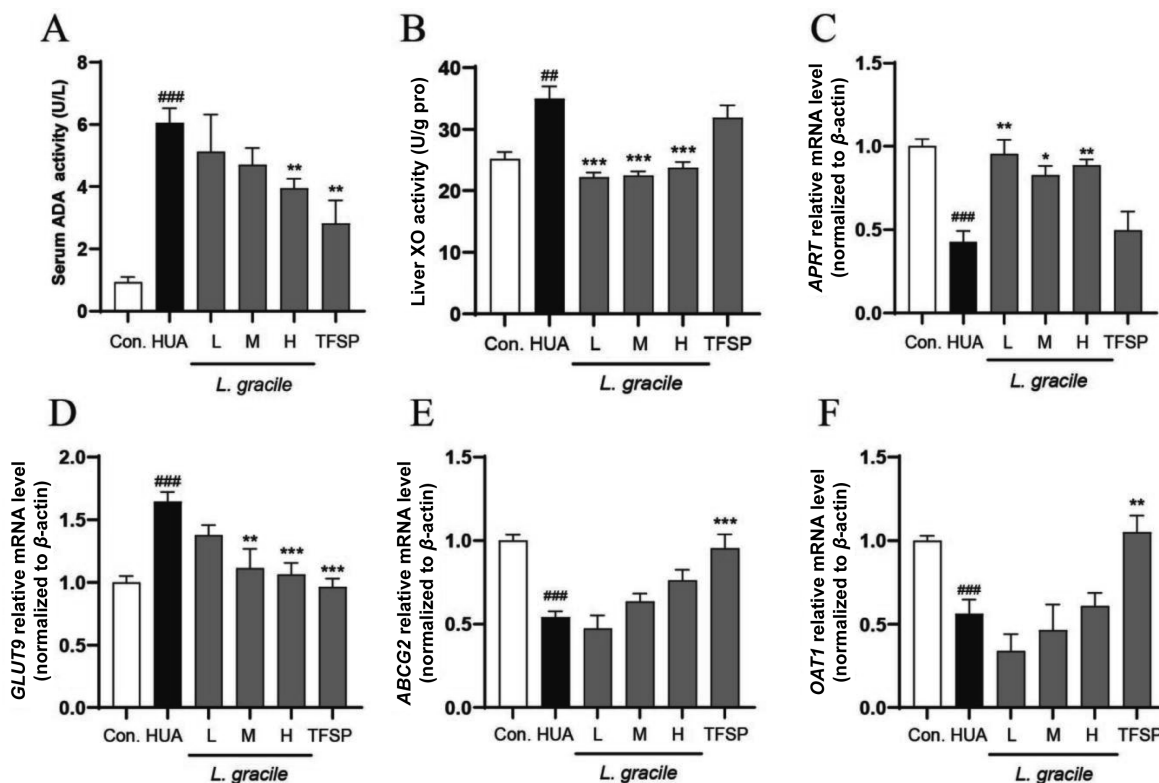
**Figure 3** SUA and UUA in HUA model. (A) SUA level of *L. gracile*. (B) UUA level of *L. gracile*. All data are shown as the mean  $\pm$  SEM.  $^{##}P < 0.01$ ,  $^{***}P < 0.001$ , vs control group;  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs HUA model group.

Fig. 3B the UUA level significantly decreased compared with those in the control group ( $P < 0.01$ ). Compared to the model group, UUA levels in TFSP group increased significantly ( $P < 0.001$ ). The UUA concentration in the *L. gracile* groups (0.59, and 1.18 g/kg) were  $(342.1 \pm 49.4)$ ,  $(340.7 \pm 72.4)$   $\mu\text{mol/L}$  respectively, significantly increased the UUA levels ( $P < 0.001$ ), indicating that *L. gracile* induced a concentration-dependent addition in UUA levels.

### 3.4 *L. gracile* suppress UA biosynthesis in HUA mice

ADA and XO are two key enzymes by tandem catalysis to affect the amount of UA were selected for further investigation. The effect of *L. gracile* on ADA and XO activity were assessed by spectrophotometrically. Compared to the model group (Fig. 4A),

serum ADA activity was reduced by *L. gracile*, high dose of *L. gracile* (1.18 g/kg) significantly inhibited serum ADA activity. As shown in Fig. 4B, the activity of liver XO in the model group was markedly higher than that in the control group, while the activity of liver XO in the *L. gracile* treatment group was significantly lower than that in the model group ( $P < 0.001$ ). The high activity of APRT enzyme can promote of the remedial synthesis pathway of nucleotides, which can reduce of purine metabolic end products. In Fig. 4C, the relative transcription of APRT in the model group were significantly decreased compared with the control group ( $P < 0.001$ ). Each dose of *L. gracile* showed a different addition on APRT relative transcription. As results have been noted, the results showed that *L. gracile* could inhibit the activities of ADA and XO and reduce APRT mRNA levels.



**Figure 4** *L. gracile* inhibit overproduction of HUA mice and regulate the UA transports proteins. (A) Serum ADA of *L. gracile*. (B) Liver XO of *L. gracile*. (C) APRT relative mRNA level of *L. gracile*. (D) GLUT9 relative mRNA level of *L. gracile*. (E) ABCG2 relative mRNA level of *L. gracile*. (F) OAT1 relative mRNA level of *L. gracile*. All data are shown as the mean  $\pm$  SEM.  $^{##}P < 0.01$ ,  $^{***}P < 0.001$ , vs control group;  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs HUA model group.

*L. gracile* can reduce the production of UA by inhibiting the enzyme activity at multiple sites in the purine metabolism pathway. The results are also consistent with the results of *in vitro* experiments.

### 3.5 *L. gracile* promote UA excretion in HUA mice

As described above in Fig. 3B, the UUA levels were increased in the treatment of *L. gracile*. UA is mainly excreted by the kidneys from the body, which involves many UA transporters and regulates its excretion. After HUA induction, the *GLUT9* mRNA expression were up-regulated significantly ( $P < 0.001$ ). Compared to the model group, the mRNA expression of *GLUT9* was significantly decreased with the treatment of TFSP ( $P < 0.001$ ). *L. gracile* reduced the *GLUT9* mRNA expression in a dose-dependent manner (Fig. 4D). On the other hand, the *OAT1* and *ABCG2* mRNA expression were reduced significantly ( $P < 0.001$ ) in model group, while results didn't show the significant differences in the presence of *L. gracile* (Figs. 4E, 4F), but there is an ameliorate trend to enhance the *OAT1* and *ABCG2* mRNA expression levels.

### 3.6 *L. gracile* alleviated injury caused by HUA in mice

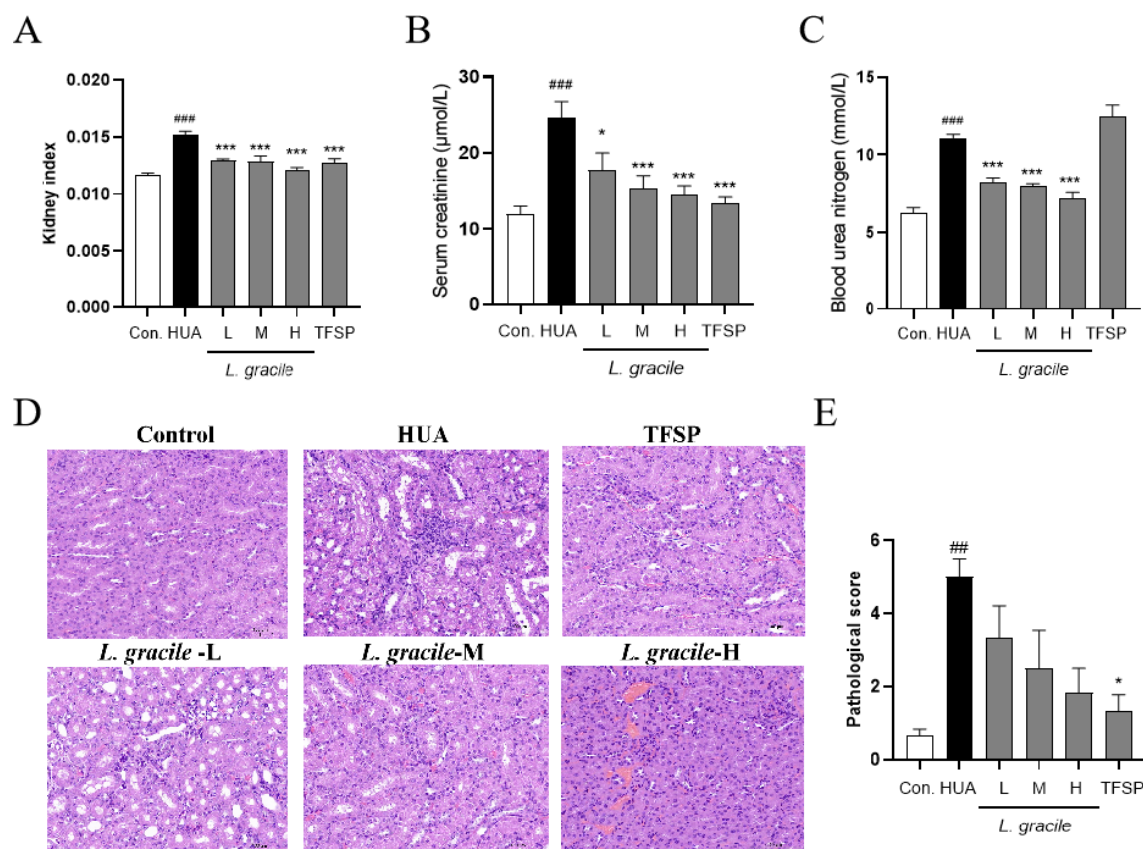
The accumulation of UA will form precipitation, with the first to bear the brunt is the kidneys. Compared to the control group, the kidney (Fig. 5A) and liver (Fig. S2) index was increased in the model group, which were restored to normal in the *L. gracile* groups. These suggest that preliminary indication of the protective effect of the extracts against kidney injury. To estimate renal functions, serum level of BUN and CRE were examined.

Compared to normal group, the CRE and BUN concentrations in the model group were substantially increased, while *L. gracile* substantially reduced the serum contents of CRE (Fig. 5B) and BUN (Fig. 5C). Sections of the mice kidney are shown in Fig. 5D, PO-yeast induced HUA mice exhibited severe renal tubular atrophy and inflammatory cell infiltration in renal interstitial, which were reversed by *L. gracile*. As shown by the pathological score (Fig. 5E), *L. gracile* showed amendment in recovering renal injury.

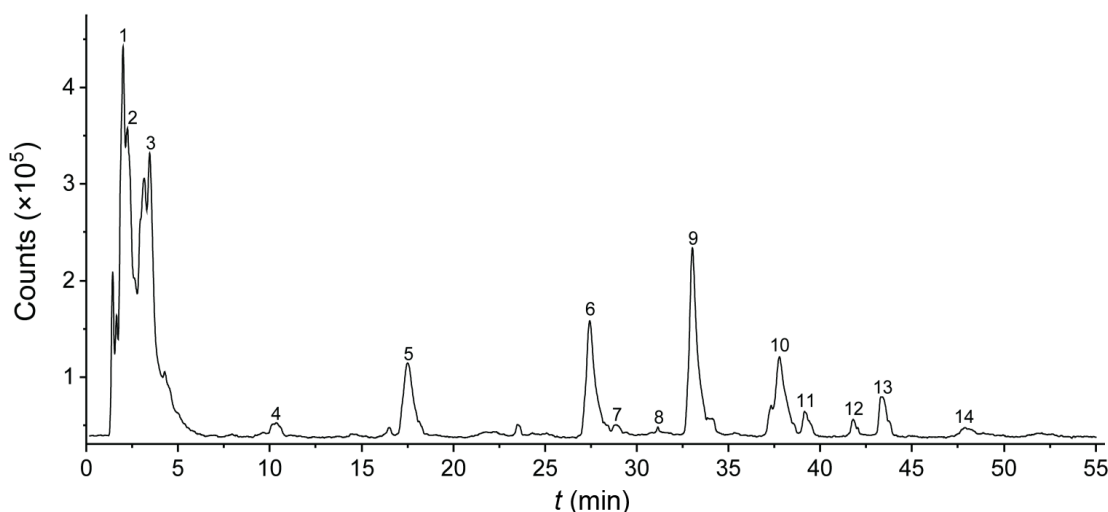
### 3.7 Active ingredients of *L. gracile*

The chromatographic characteristic profile of the *L. gracile* aqueous extract is illustrated in Fig. S3. The base peak chromatogram that resulted from the *L. gracile* ethanol extract is depicted in Fig. 6, where the peaks are numbered according to their elution order. Main ingredients present in *L. gracile* was identified separately by HPLC-QTOF-MS/MS combined with the data provided in literature. A total of 14 major constituents were detected in *L. gracile* as shown in Table 1. More detailed structures can be found in Fig. S4. The results showed that the main components of the ethanol extract of *L. gracile* were phenolic acids and flavonoids. Among them, the contents of hydrojuglone glucoside, isoorientin 3'-O-glucuronide, isoorientin and chrysoeriol 7-O-(6"-malonyl-glucoside) are much higher. They can be identified as its main components.

Isoorientin is a natural flavonoid compound, the UA lowering effect was once reported by An *et al.*<sup>[24]</sup>, the results showed the XOD activity and UA level were inhibited after the treatment of isoorientin *in vitro* and *in vivo*. Apart from this, there are no reports on the other ingredients lowering UA effects. However, it



**Figure 5** Nephroprotective effect of *L. gracile* in HUA mice. (A) Kidney index in *L. gracile*. (B) Serum SCR level in *L. gracile*. (C) Serum BUN level in *L. gracile*. (D) Representative H&E staining of the kidney tissue (magnification of  $\times 400$ ; scale bar = 100  $\mu\text{m}$ ). (E) Quantification of the tubular injury score in *L. gracile*. All data are shown as the mean  $\pm$  SEM. ## $P < 0.01$ , ### $P < 0.001$ , vs control group; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs HUA model group.



**Figure 6** Total ion chromatograms in negative ion mode in *L. gracile*. 1–14 are the peak numbers. Detailed information is given in Table 1.

**Table 1** Identification of the chemical constituents in *L. gracile* ethanol extract by UPLC-Q-TOF-MS analysis

| No. | Retention time (min) | Compound                                             | Molecular formula                               | Measurement ( <i>m/z</i> ) | Error (ppm) | MS fragment ion ( <i>m/z</i> ) |
|-----|----------------------|------------------------------------------------------|-------------------------------------------------|----------------------------|-------------|--------------------------------|
| 1   | 2.233                | Malic acid                                           | C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>    | 133.014,3                  | −1.62       | 71.014,4                       |
| 2   | 2.601                | Citric acid                                          | C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>    | 191.019,7                  | −0.2        | 111.008,4                      |
| 3   | 2.815                | <i>cis</i> -Aconitic acid                            | C <sub>6</sub> H <sub>6</sub> O <sub>6</sub>    | 173.009,2                  | −0.94       | 85.029,8                       |
| 4   | 10.432               | Caffeoyl C1-glucuronide                              | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | 355.067                    | 0.06        | 85.029,7, 191.019,5            |
| 5   | 17.439               | 5- <i>O</i> -Hydrojuglone glucoside                  | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | 337.092,8                  | 0.45        | 119.049,9, 163.039,7           |
| 6   | 27.411               | 3- <i>O</i> -Hydrojuglone glucoside                  | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | 337.093,1                  | −0.35       | 93.034,7, 173.045,3            |
| 7   | 28.837               | 2-Hydroxycinnamic acid                               | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>    | 163.039,8                  | 1.5         | 119.050,2                      |
| 8   | 31.146               | 3- <i>O</i> -Caffeoyl-4- <i>O</i> -methylquinic acid | C <sub>17</sub> H <sub>20</sub> O <sub>9</sub>  | 367.103,3                  | −0.35       | 112.985,6                      |
| 9   | 33.003               | Isoorientin 3'- <i>O</i> -glucuronide                | C <sub>27</sub> H <sub>28</sub> O <sub>17</sub> | 623.125                    | 0.81        | 327.050,3                      |
| 10  | 37.814               | Isoorientin                                          | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | 447.093,2                  | 0.51        | 112.985,6                      |
| 11  | 39.288               | Swertiajaponin                                       | C <sub>22</sub> H <sub>22</sub> O <sub>11</sub> | 461.103,5                  | 0.86        | 44.998,5                       |
| 12  | 41.787               | Kaempferide 5-glucoside-7-glucuronide                | C <sub>28</sub> H <sub>30</sub> O <sub>17</sub> | 637.141,2                  | −0.26       | 341.066,1                      |
| 13  | 43.306               | Chrysoeriol 7- <i>O</i> -(6"-malonyl-glucoside)      | C <sub>25</sub> H <sub>24</sub> O <sub>14</sub> | 593.114,6                  | 0.59        | 327.050,8                      |
| 14  | 47.777               | Azelaic acid                                         | C <sub>9</sub> H <sub>16</sub> O <sub>4</sub>   | 187.097,3                  | 1.54        | 125.097,1                      |

is too early to draw a conclusion on the active ingredients. Therefore, network pharmacology was added on.

### 3.8 Network pharmacology analysis of the constituents in *L. gracile*

Network pharmacology was used to identify possible active ingredients of *L. gracile* in HUA. A total of 14 compounds revealed a total of 37 overlapping genes identified by comparison with disease-associated genes (Fig. 7A). To intuitively visualize the interaction correlation between the core bio-active components and the common genes, the HCT network was established as shown in Fig. 7B, the 37 gene targets include *XDH*, *ADA*, *ABCG2*, *SLC2A9*, and *SLC22A6*. These correspond to the results of the previous results, showing that *L. gracile* does indeed exhibit inhibition of *XOD* and *ADA* and regulation of *UA* transport proteins. In the HCT network, 13 components of *L. gracile* are more or less related to HUA gene targets, with the strongest correlation being with, which is also the main component of *L. gracile*, hydrojuglone glucoside.

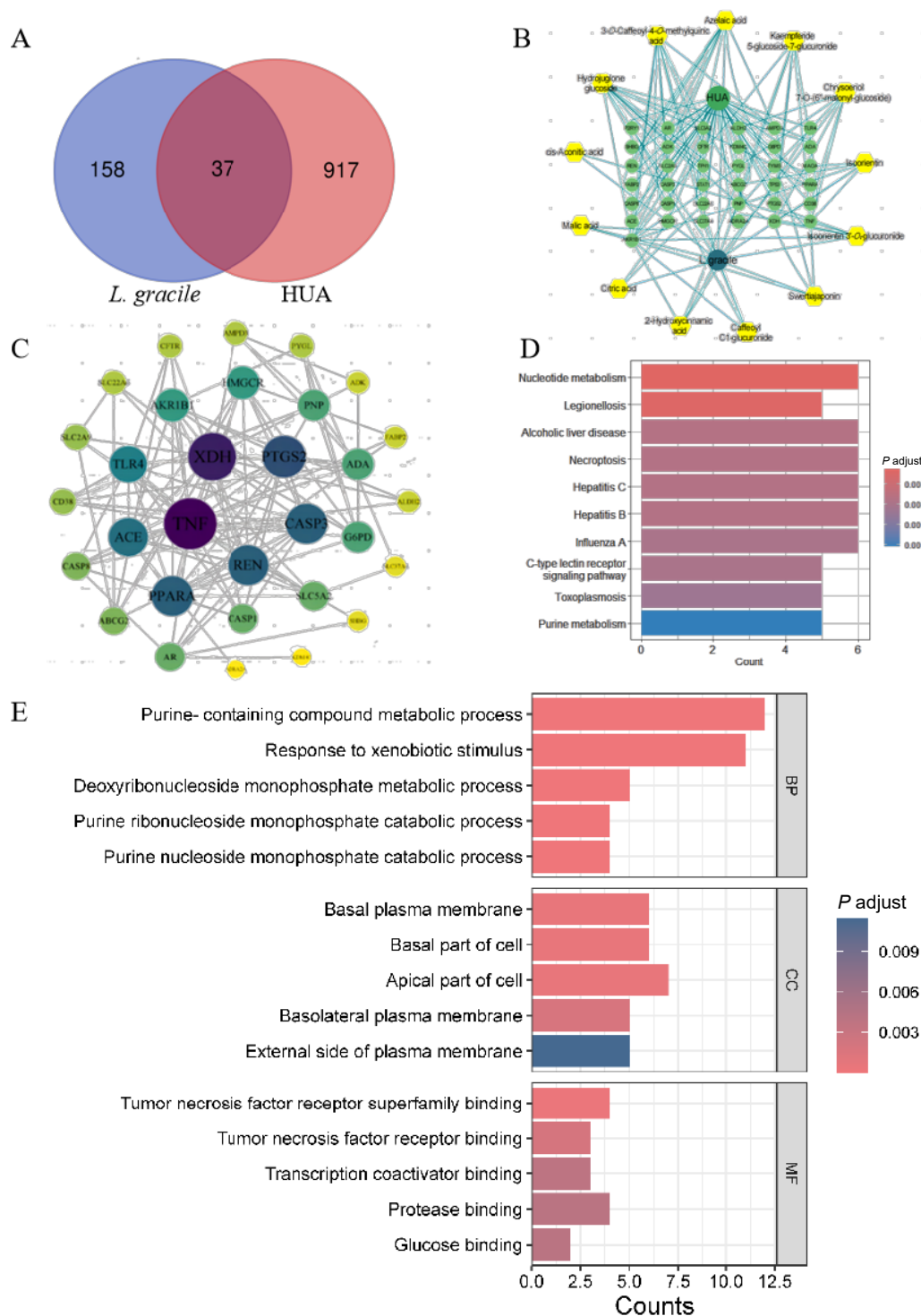
### 3.9 PPI network construction and analysis

The intersectional targets of ingredients in *L. gracile* and HUA

were imported into STRING database and were screened with the median of degree value as threshold to obtain the PPI network (Fig. 7D). According to the degree values of the targets, the main intersectional targets consisted of tumor necrosis factor (TNF), xanthine dehydrogenase (XDH), prostaglandin-endoperoxide synthase 2 (PTGS2), caspase-3 (CASP3), peroxisome proliferator-activated receptor alpha (PPAR-α), renin (REN), and so on.

### 3.10 GO and KEGG enrichment analysis

The above 234 key nodes were imported into Metascape platform for GO enrichment analysis, which included BP, CC, and MF (Fig. 7E). Among them, the BP was mainly involved in the response to purine-containing compound metabolic process, response to xenobiotic stimulus signal pathway. The CC were concentrated in plasma membrane, extracellular exosome and macromolecular complex. The MF mainly focused on the identical protein binding and enzyme binding. Meanwhile, the above 48 key nodes were imported into Metascape platform for KEGG pathway analysis (Fig. 7C). The results indicated that it was mainly involved in the metabolic pathway, nucleotide metabolism, purine metabolism, which may be the core targets in the treatment of HUA.



**Figure 7** Results of KEGG and GO enrichment analysis and pathway network. (A)Venn diagram showing the targets of the *L. gracile* and HUA. (B) The HCT network. Yellow circles, green circles represent active compounds of *L. gracile* and targets, respectively. (C) PPI enrichment analysis. (D) KEGG of common targets. (E) GO enrichment analysis.

## 4 Discussion

The early stage of HUA is typically referred to as asymptomatic HUA<sup>[25]</sup>. During this stage, UA levels in the blood are elevated, but the patient does not exhibit any noticeable symptoms. However, long-term asymptomatic HUA still poses a risk. For HUA without any symptoms at an early age, prevention is important. But there were very few numbers of herbs currently used to prevent it. The

initial thoughts of this study were to find new herbs that can regulate UA levels and to evaluate the effects. In this study, *L. gracile* was screened out from MFH, and the UA-lowering efficacy was confirmed for the first time by *in vitro* and *in vivo* experiments. Composition analysis and network pharmacology showed that the main active ingredient of UA-lowering effect is hydrojuglone glucoside in *L. gracile*.

Unlike other cellular models for HUA, which mainly focus on a

single target of XO. Both models used in this study maximally simulate the real situation in the human body. They also reflect the real situation of the action of traditional Chinese medicines more appropriately, which are usually multi-targeted and synergistic. In the section on cellular screening of drugs, noticeable, the ethanol extract of *L. gracile* significantly reduced the production of UA ( $P < 0.001$ ). However, the aqueous extract didn't show the effect. This suggests that it is highly likely to have an inhibitory effect on UA production and active substance is concentrated in the less polarized fraction.

It has been now widely accepted that UA level is the representative hallmark of HUA<sup>[26]</sup>. CRE and BUN levels are the most important biochemical indexes to detect abnormal renal function<sup>[27]</sup> and pathological sections make abnormal changes visually. The present study revealed that *L. gracile* reduced SUA, CRE and BUN levels in serum, elevated UA in urine, alleviated renal injury by pathology scores, indicating that it could alleviate yeast-induced HUA, which might be a therapeutic strategy in renal injury. HUA occurs when UA is overproduced or under excreted due to disorders of the modulating system of UA<sup>[28,29]</sup>. UA production is the process of purine metabolism. Multiple enzymes, including ADA, APRT and XO, participate in UA biosynthesis. These enzymes play important roles in the synthesis of UA through a series of tandem catalysis reactions. In the liver, ADA hydrolyzes adenosine to inosine, which is further degraded to hypoxanthine and it eventually forms UA by XO<sup>[30,31]</sup>. APRT can catalysis adenine to AMP through salvage pathway, which can reduce purine metabolism. In this study, *L. gracile* treatment significantly decreased XO and ADA levels as well as the mRNA levels of APRT in HUA mice, suggesting that *L. gracile* inhibit UA production by affecting multiple targets.

Genome-wide association studies were consistent with the fact that about 90% of patients suffer from the under excretion type in HUA<sup>[32]</sup>. The UA transporters play an important role in UA excretion<sup>[33]</sup>. Recent studies identified GLUT9, which is a kind of urate absorptive transporters, as a major determinant of plasma UA levels and of gout development<sup>[34]</sup>. Transporter OAT1 and ABCG2 are the main transporters regulating renal urate handling<sup>[35]</sup>, while ABCG2 also regulate intestinal transport<sup>[36]</sup>. Our results confirm that *L. gracile* reduce the gene expression of GLUT9. At the same time, it also has the trend to promote UA excretion transporter OAT1 and ABCG2 mRNA expression level. These demonstrate that *L. gracile* can increase urinary UA levels by modulating the UA transporter.

According to Chinese medicine theory, HUA is often classified as one type of disease with gout, and medicines with the effect to dispel dampness, remove paralysis and reduce inflammation are commonly used to treat with them<sup>[37]</sup>. *L. gracile* has the effect of clear heat, purge fire, diuretic, quench thirst and diuretic<sup>[38]</sup>. This is also consistent with the results presented in above, part of the UA-lowering effect of *L. gracile* is mediated by increased excretion of UA *in vitro*. As previously described many phytochemicals derived from natural products have been investigated for prevention and treatment of HUA, including flavonoids and phenols. Flavonoids can bind to XO sites to inhibit XO activity and reduce UA, such as quercetin, galangin, baicalin<sup>[39-42]</sup> and apigenin<sup>[43]</sup>. It also has been reported that kaempferol inhibited XO activity reversibly in a competitive manner<sup>[44]</sup>. We detected glycosylated derivatives with similar structures in *L. gracile*. The UA-lowering effect of isoorientin has been clearly confirmed<sup>[24]</sup>, and the XOD inhibitory activity of the adlay bran free phenolic extract was shown to be related to hydroxycinnamic acids<sup>[45]</sup>,

which were also detected in the *L. gracile*. In this light, anti-HUA effects by uricosuric or inhibition of UA production way and nephroprotective effect presented by the *L. gracile* extracts can be partially attributed to flavonoids. But there are also reports shown that multiple active component combinations of MFH could amplify the therapeutic efficacy of each agent<sup>[46]</sup>. Luteolin exhibited a stronger synergistic effect with kaempferol than did morin at the lower concentration<sup>[28]</sup>. After all, multiple components and multiple targets work together, which is also one of the characteristics of traditional Chinese medicine. Therefore, hydrojuglone glucoside is the most likely active ingredient as we mentioned, However, this does not contradict the possibility that the UA-lowering effect of *L. gracile* is also synergistic with other detected components.

Network pharmacology analysis indicated that 13 components derived from *L. gracile* may play important role in the treatment of HUA by acting on 37 HUA related targets. PPI results revealed the main intersectional targets consisted of TNF, ADH, PTGS2, PPAR- $\alpha$ , REN, CASP3. This matches the results verified in the previous part of this article. Animal and cell experiments have proved that *L. gracile* can directly reduce XOD activity. TNF and PTGS2 play a key role in HUA. TNF aggravates joint and tissue damage by promoting inflammatory responses, while PTGS2 further induces inflammation and pain by increasing the synthesis of prostaglandins, aggravating HUA-related symptoms. The inflammatory pathways in which both participate have a significant impact on the pathogenesis of HUA, making them potential therapeutic targets. Similarly, PPAR- $\alpha$  also affects UA levels by regulating lipid metabolism and anti-inflammatory responses. Histopathological analysis showed that the groups treated with *L. gracile* had the effect of alleviating HUA-induced inflammation. CASP3 is associated with apoptosis in HUA. The deposition of urate crystals activates CASP3, thereby inducing apoptosis and inflammatory responses. Activation of CASP3 leads to damage to joint and kidney tissues, further aggravating the symptoms of HUA. GO analysis showed that HUA-relieving effect of *L. gracile* mostly cellular components are located on the cell membrane. The KEGG analysis further indicated that the HUA-relieving mechanism of *L. gracile* was possibly associated with the metabolic pathway.

*L. gracile* has been widely used as raw materials for food and beverages in southeast Asia surrounding areas. Especially, *L. gracile* is frequently used as an ingredient in the formulation of various herbal teas in Guangdong province of China<sup>[47]</sup>. Commonly, the dried leaves are traditionally used for herbal tea or medicine, whereas the fresh leaves are used as a raw vegetable or flavoring herb. Due to the good foundation of food development and good taste, we believe *L. gracile* has a good prospect as an ingredient for the development of UA-lowering functional food and easy accepted by people.

## 5 Conclusions

To summarize, our findings confirmed that *L. gracile* could have alleviated HUA and ameliorated renal damage in mice with yeast-induced HUA for the first time. Screening of UA-lowering drugs from MFH, *L. gracile* demonstrated its excellent UA-lowering effect in cellular models. *L. gracile* not only inhibited the overproduction of UA better but also showed an effect of increasing UA excretion *in vivo*. Furthermore, LC-MS analysis combined with network pharmacology indicates that the most likely UA-lowering effective component of *L. gracile* is

hydrojuglone glucoside. Thus, *L. gracile* might have been considered as a new potential drug or supplement in protecting against HUA and HUA-caused renal injury.

## Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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## Electronic Supplementary Material (ESM)

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