



Research Article

Piper nigrum L. and its main component piperine ameliorate hyperuricemia and hyperuricemia-related renal inflammation through suppressing the JAK2-STAT3-XDH/SOCS3/TNF- α signalling pathway

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Abstract: Hyperuricemia is a metabolic disease characterized by serum uric acid (UA) levels remaining above normal levels for a long period of time, which has become a huge challenge faced by society. The fruits of *P. nigrum* L. (*P. nigrum*) as traditional Indian medicine was used to treat various diseases. However, the anti-hyperuricemia effect and mechanism of the ethanol extract of *P. nigrum* (PE) and its primary component, piperine (PI) still need to be elaborated. In this study, we systematically evaluated the effects of PE and PI on hyperuricemia, as well as their underlying mechanisms in the hyperuricemic mouse and cell models. Our results showed that PE effectively prevented abnormal elevation of UA level, and restored renal function in hyperuricemic mouse model. Transcriptomic and western blot analysis revealed that PE respectively relieved hepatic synthesis of UA and enhanced renal excretion of UA through suppressing the expressions of xanthine dehydrogenase (XDH) and glucose transporter member 9 (GLUT9) *in vitro* and *in vivo*. Additionally, PE ameliorated renal inflammation by reducing the expressions of suppressor of cytokine signalling 3 (SOCS3) and TNF- α . Furthermore, the experimental results for PI were consistent with those observed for PE. Mechanistically, the gene overexpression of Janus Kinase 2 (JAK2), luciferase reporter gene and molecular docking results demonstrated that the anti-hyperuricemia effect of PE was associated with the blocking of the JAK2/ signal transducers and activators of transcription 3 (STAT3) signalling transduction, and PI could specifically bound to JAK2. These data suggested that PE could ameliorate hyperuricemia and hyperuricemia-related renal inflammation through suppressing the JAK2-STAT3-XDH/SOCS3/TNF- α signalling pathway, and the beneficial effects of PE presumably were attributed to PI. In summary, our study suggests that PE may be an effective natural product for improving hyperuricemia by restoring the metabolic balance of UA and suppressing renal inflammation.

Keywords: *Piper nigrum* L.; piperine; hyperuricemia; XDH; renal inflammation; JAK2

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1 Introduction

Hyperuricemia is a metabolic disease characterized by serum uric acid (UA) level above 420 $\mu\text{mol/L}$ (in men) or 360 $\mu\text{mol/L}$ (in women) for a long time, which caused by abnormal purine metabolism^[1,2]. With the change of people's dietary structure and lifestyle, the incidence of hyperuricemia presents a sharp increase trend year by year. The recent epidemiological studies have shown that the prevalence of hyperuricemia is approximately 20% in the United States^[3]. In China, the prevalence of hyperuricemia is

about 14%, and the suffers gradually tends to be younger^[4]. More importantly, the growing clinical evidences suggest that hyperuricemia probably induce various complications, including gout, chronic kidney disease, hypertension and cardiovascular disease. Therefore, maintaining an appropriate serum UA level is essential for the human health.

Serum UA level is mainly determined by UA production and excretion rates^[5,6]. Physiologically, xanthine oxidase (XO) and xanthine dehydrogenase (XDH) as a rate-limiting enzyme, catalyzes the oxidation of hypoxanthine to xanthine, and further

xanthine to serum UA in the liver^[7]. On the other hand, in the kidney, the UA transport system composed of multiple UA transporters, including urate transporter 1 (URAT1), glucose transporter member 9 (GLUT9), ATP-binding cassette transporter subfamily G member 2 (ABCG2) and organic anion transporters (OATs) is responsible for the reabsorption and excretion of UA, and maintains a dynamic equilibrium of UA metabolism^[8-14]. Nevertheless, under hyperuricemia pathological conditions, an increase in production and reabsorption, or the decrease in excretion or both result in the excessive accumulation of UA in the blood and the occurrence of hyperuricemia^[15]. Notably, the higher UA level has been reported to be closely associated with kidney inflammation and injury^[16-18]. The growing evidence supports that the excessive accumulation of UA can form the urate crystal and initiate the Janus Kinase 2/signal transducers and activators of transcription (JAK2/STAT3) signalling pathway in kidneys, consequently promoting the expression of inflammatory cytokines, such as interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α and suppressor of cytokine signalling 3 (SOCS3)^[19,20]. The secretion of inflammatory cytokines further causes kidney inflammation and injury, ultimately promoting progression of hyperuricemia to chronic kidney disease^[21,22]. Therefore, inhibiting UA production and reabsorption, accelerating UA excretion, simultaneously alleviating the inflammation is a crucial approach for the prevention and treatment of hyperuricemia and hyperuricemia associated nephritis.

At present, a variety of clinical drugs, including XO inhibitors (allopurinol and febuxostat) and the UA excretion promoter (benzbromarone and probenecid) are used to treat hyperuricemia^[6]. However, these drugs often act on a single target and exert a series of side effects, such as UA stones, hypersensitivity, liver and kidney damage, diarrhea and higher risks of cardiovascular disorders, which limit their therapeutic effect and lead to intolerance in patients^[23-28]. Thusly, there is an urgent need to explore a new natural and nontoxic therapeutic strategies for hyperuricemia.

Natural products from medical plants, such as traditional Chinese medicine, Indian medicine and other ethnic medicine, are always an important source of leading compounds and direct therapeutic agents^[29,30]. Currently, a growing number of herbal extracts and natural products have been used to improve and treat human diseases^[31-35]. *Piper nigrum* L. (*P. nigrum*) is a perennial woody vine in the *Piperaceae* family, native to India and widely grown in tropical regions such as Vietnam, Brazil, and southern China^[36,37]. Traditionally, the fruits of *P. nigrum* as traditional Indian medicine was used to treat convulsions, gastrointestinal diseases and liver damage diseases^[38-40]. In recent years, *P. nigrum* fruits, have attracted more attention because it exhibits variety of novel therapeutic effects, such as anti-atherosclerosis, anti-cancer and anti-Alzheimer's disease^[41-44]. Studies have revealed that *P. nigrum* fruits primarily comprises alkaloids, flavonoids, amino acids, triterpenoids, organic acids, and other bioactive constituents^[45]. Among these components, the therapeutic effect of *P. nigrum* fruits is probably attributed to its alkaloids component^[46]. Piperine (PI) is a principle bioactive alkaloid derived from *P. nigrum* fruits, with a content of up to 2% to 9%^[47]. Previous study has reported that *P. nigrum* fruits extracts could alleviate acute inflammation of gout and kidney damage caused by high UA^[48]. In addition, a recent study showed that PI could suppress UA reabsorption and renal inflammation by reducing the expression levels of URAT1, GLUT9 and inflammatory factors, while ameliorating hyperuricemia and hyperuricemia

associated nephritis^[47]. These studies suggest that *P. nigrum* fruits extracts has a therapeutic effects against UA metabolism imbalance and hyperuricemia associated nephritis, and PI, as the key active component from *P. nigrum* fruits extracts, probably exerts a crucial effect in this process. However, whether *P. nigrum* fruits extracts can ameliorate hyperuricemia and hyperuricemia-related renal inflammation, and the effect of PI on UA production still remains unclear and requires further investigation. Meanwhile, the direct target and detailed mechanism of *P. nigrum* fruits extracts and PI also need to be elucidated.

In this study, we evaluated the anti-hyperuricemia effects of *P. nigrum* fruits extracts and its main active component, PI *in vivo* and *in vitro*. In addition, we also systematically investigated the direct target and mechanism through which *P. nigrum* fruits extracts and PI reduce UA level and alleviate hyperuricemia associated nephritis by molecular docking, enzyme-linked immunosorbent assay (ELISA) and gene overexpression experiments. The present study will provide a valuable reference to understand the potential therapeutic effects of *P. nigrum* fruits against hyperuricemia.

2 Materials and methods

2.1 Reagents

The fruits of *P. nigrum* were purchased from Tongrentang Chinese Medicine Co., Ltd. (Beijing, China), PI (P886278), potassium oxonate (PO) (P831461), xanthine sodium salt (XSS) (X923359), allopurinol (A800424) and benzbromarone (B838527) were purchased from Macklin Co., Ltd. (Shanghai, China). UA (U2625) was acquired from Sigma-Aldrich (MO, USA). Commercial kits for detecting the levels of UA (C012-2-1), XO (M001-1-1), creatinine (Cr) (C011-2-1), and blood urea nitrogen (BUN) (C013-2-1) were purchased from Nanjing Jiancheng Bioengineering Institute Co., Ltd. (Nanjing, China). The pSTAT3-TA-Luc vector (D4404) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The pcDNA-JAK2 (TP320503) were purchased from Origene Wuxi Biotechnology Co., Ltd. (Wuxi, China). The anti-DDK Tag (AE005), anti-JAK2 (A11497), anti-p-JAK2 (AP0373), anti- β -actin (AC038), anti-XDH (A9022) and anti-SOCS3 (A0694) were purchased from ABclonal Technology Co., Ltd. (Wuhan, China). The anti-GLUT9 (67530-1-Ig) was purchased from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, China). The anti-STAT3 (bs-55208R) and anti-p-STAT3 (bs-1658R) were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China).

2.2 Preparation of different extracts of *P. nigrum* fruits

The preparation of *P. nigrum* fruits different extracts were performed as described in a previous study with minor modifications (Fig. 1A)^[49]. The fruits of *P. nigrum* were collected and dried at air ambient, dry *P. nigrum* fruits (100 g) were crushed and refluxing extracted with petroleum ether for 6 h at 35 °C (1:10; g/mL), the solution was concentrated 50 $\times$ and dried with a vacuum freeze dryer to obtain the petroleum ether extract of *P. nigrum* fruits. After removing excess solvent from the residue of *P. nigrum* fruits, continue to extract with 80% ethanol reflux at 45 °C for 6 h (1:10; g/mL). Subsequently, the solution was concentrated 50 $\times$ and dried using a vacuum freeze dryer to obtain the *P. nigrum* fruits ethanol extract (PE). After continue removing ethanol from the residue of *P. nigrum* fruits, the residue was extracted with water reflux at 85 °C for 6 h (1:10; g/mL), and

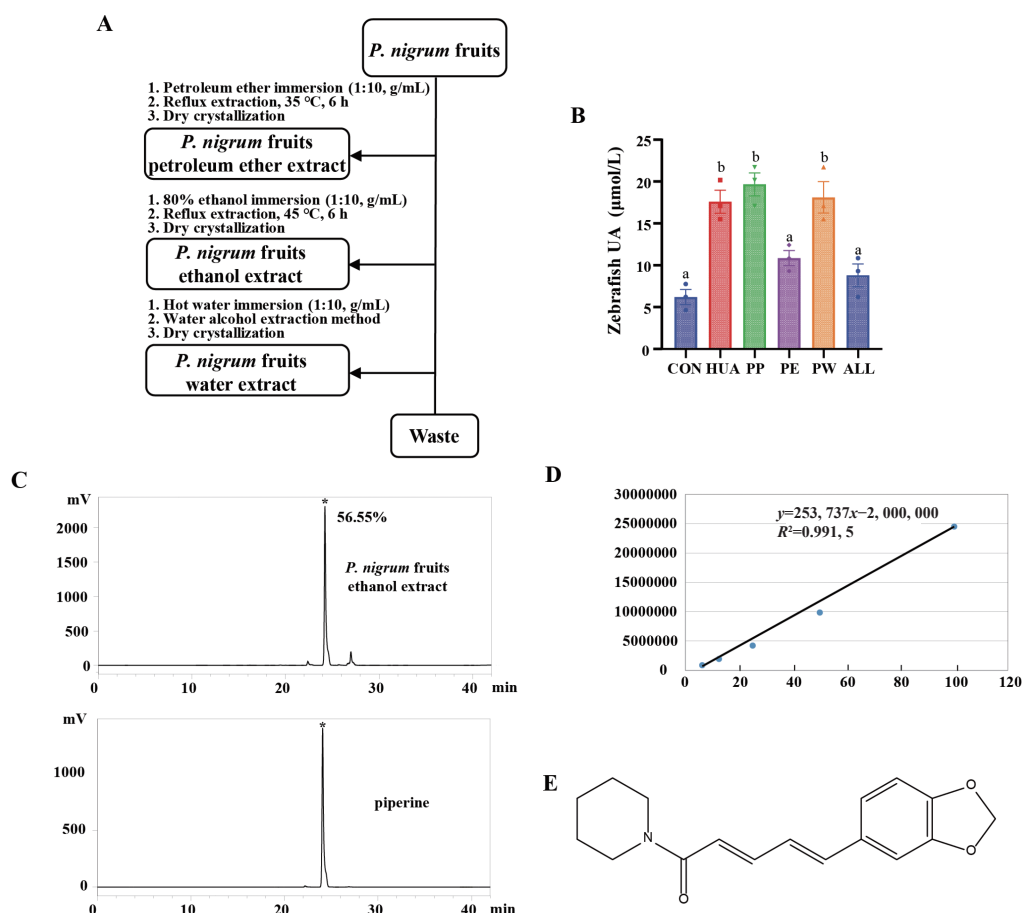


Figure 1 Extraction of *P. nigrum* extract. (A) Extraction method. (B) Zebrafish UA. (C) HPLC. (D) Standard curve. (E) Piperine structure. Data are presented as the means $\pm$ SEMs ($n=3$). W/V, weight (g)/volume (mL). CON, control group; HUA, hyperuricemia group; PP, *P. nigrum* fruits petroleum ether extract group; PE, *P. nigrum* fruits ethanol extract group; PW, *P. nigrum* fruits water extract group; ALL, Allopurinol group.

medicinal solution is used to remove protein through alcohol precipitation the water extract of *P. nigrum* fruits was obtained after drying.

2.3 High-performance liquid chromatography (HPLC)

A total of 3.65 g of PE was obtained from 100 g *P. nigrum* fruits in extraction experiment, resulting in a calculated PE production yield of 3.65%. The active ingredients content in PE was carried out by HPLC-diode array detection analysis with an Agilent 1260 Infinity II system (Agilent Technology, Santa Clara, CA) with Eclipse Plus C18 column (Agilent Technologies, USA, 4.6 $\times$ 250 mm, 5 μ m), and the mobile phases water (A) and methanol (B) were utilized in a gradient mode (0–5 min: 50% A, 50% B; 5–30 min: 0% A, 100% B; 30–35 min: 0% A, 100% B; 35–45 min: 50% A, 50% B) at ambient temperature. Flow rate: 1.0 mL/min; injection volume: 10 μ L; detection wavelength: 343 nm; column temperature: 25 °C. The PE and standard PI were precisely weighed and dissolved in methanol. Standard PI was used to generate a standard curve under the same condition (standard curve range 6.25–100 μ g/mL), and PI content in PE was calculated based on the standard curve for HPLC peak area versus PI content.

2.4 Zebrafish experiment

Healthy AB strain wild-type zebrafish aged 3 months were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. All the experimental water was prepared by reverse osmosis water purification mechanism, the water temperature was

kept at (28 ± 0.5) °C, the pH value was kept at 7.3 ± 0.3 , the light was alternated day and night for 12/12 h. the zebrafish were fed with the ground red worms. After zebrafish reproduction, 5 days post fertilization (5 DPF) larvae with normal development were selected and randomly divided into 6 groups (three replicates per group, with 33 juvenile fish per replicate): The control group (CON group), hyperuricemia group (HUA group), *P. nigrum* fruits petroleum ether extract group (PP group, 20 μ g/mL *P. nigrum* fruits petroleum ether extract were administrated to zebrafish using the water-soluble method), *P. nigrum* fruits ethanol extract group (PE group, 20 μ g/mL PE were administrated to zebrafish using the water-soluble method), *P. nigrum* fruits water extract group (PW group, 20 μ g/mL *P. nigrum* fruits water extract were administrated to zebrafish using the water-soluble method), and allopurinol group (ALL group, 20 nmol/L allopurinol were administrated to zebrafish using the water-soluble method). Apart from CON group, all zebrafish were administered with 200 μ mol/L PO combination with 10 μ mol/L XSS using the water-soluble method for 24 h to induce hyperuricemia, while drug supplementation undergoing drug treatment. The zebrafish were collected in 1.5 mL EP tubes after using MS-222 fish stabilizer, and homogenized for determination of UA levels.

2.5 Mouse experiment

Eight-week-old male Kunming mice (20–25 g) were purchased from the Shandong Laboratory Animal Center (permission number SCXK 2014-0007, Jinan, China). The mice were housed

under standardized laboratory conditions (22–26 °C, 50% relative humidity, 12 h light/dark cycle), had free access to sterilized food, and adaptively fed for 7 days. The mouse experiments were divided into two batches. The mice were given 5% fructose water (free drinking) combination with 250 mg/kg PO (intraperitoneal injection) to induce hyperuricemia. The control mice were treated with the same volume of 0.5% carboxymethylcellulose sodium. In batch 1, 40 mice were randomly divided into 5 groups ($n=8$): The control group 1 (CON1 group, the mice with normal UA levels), the hyperuricemia group 1 (HUA1 group), the low-dose PE group (PE-L group, hyperuricemia mice were given with 10 mg/kg PE), the high-dose *P. nigrum* ethanol extract group (PE-H group, hyperuricemia mice were given with 50 mg/kg PE), and benzbromarone group 1 (BEN1 group, hyperuricemia mice were given with 20 mg/kg benzbromarone). In batch 2, 40 mice were randomly divided into 5 groups ($n=8$): The control group 2 (CON2 group), the hyperuricemia group 2 (HUA2 group), the low-dose PI group (PI-L group, hyperuricemia mice were given with 10 mg/kg PI), the high-dose PI group (PI-H group, hyperuricemia mice were given with 50 mg/kg PI), the benzbromarone group 2 (BEN2 group, hyperuricemia mice were given with 20 mg/kg benzbromarone). The administrations were performed by intragastric gavage (i.g.) once daily and lasted for 4 consecutive weeks. At the end of the experiment, urine as well as feces were collected in metabolic cages, and blood was collected. Then, the mice were euthanized and dissected to separate liver and kidney tissues, which were stored at -80 °C for analysis. All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals of Shandong University of Technology and approved by the Animal Ethics Committee of Shandong University of Technology (Y LX20210906, Y LX20211103, Y LX20220407).

2.6 Biochemical measurement

The levels of UA, Cr, BUN, XO and TNF- α were detected by using commercial detection kits according to the manufacturer's instructions. The estimate glomerular filtration rate (eGFR) was analysed as previously described^[50].

2.7 Histological study

The kidney tissues were rinsed carefully with ice-cold phosphate-buffered saline (PBS). The kidney tissues from mice were fixed in 4% paraformaldehyde, then embedded in paraffin and cut into tissue sections (3 μ m). Thereafter, hematoxylin and eosin (HE) and sirius red staining were performed for histological evaluation as previous research^[50]. As previously reported, histopathological changes in the renal glomeruli and tubules were scored on a scale of 0–4, indicating varying degrees of damage (0 = no injury; 1 = mild vacuolization; 2 = moderate tubular/glomerular damage; 3 = severe injury; 4 = extensive structural destruction)^[50].

2.8 Transcriptome analysis

Total RNA extraction, cDNA library construction and transcriptome sequencing were accomplished at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Briefly, according to the manufacturer's instructions, total RNA of kidney tissue from CON1 group, HUA1 group, PE-L group and PE-H group mice were extracted by using total RNA extraction kit. Then RNA quality was determined by 5300 Bioanalyser (Agilent) and quantified by using the ND-2000 (NanoDrop Technologies).

Enrichment of mRNA was carried out by using Oligo (dT)-based magnetic methods. Short fragments were then obtained *via* mRNA cleavage in a fragmentation buffer. First-strand cDNA was synthesized using random primers for reverse transcription, followed by the synthesis of second-strand cDNA. The resulting cDNA underwent end-repair and subsequent 3' adenylation. Adapters were then ligated to the ends of these 3' adenylated cDNA fragments. After amplification of the fragments by PCR, a cDNA library was constructed for sequencing on an NovaSeq X Plus sequencing platform. Heatmap and volcano plots were generated using the online platform of Majorbio Cloud Platform (www.majorbio.com). As shown in Fig. 3D, the gene expression differences among various treatment groups were statistically compared to identify specific genes associated with the disease, allowing for a further analysis of the biological significance of these particular genes.

2.9 Western blot analysis

The 10 mg tissue (or 1×10^6 cells) was lysed using cold RIPA lysis buffer (containing 1 mmol/L PMSF), lysate was centrifuged and protein concentration was measured using a BCA protein concentration assay kit. Subsequently, the protein samples (80 μ g per hole) were separated by 10% SDS-PAGE and then transferred to 0.45 μ m PVDF membranes. The PVDF membranes were blocked with 5% nonfat milk for 1 hour, followed by overnight incubation with primary antibody at 4 °C and then incubation with the HRP-conjugated secondary antibodies 2 h. The membrane was washed with TBST three times for 8 min each after each step. The chemiluminescence signals of the target proteins were photographed using a Tanon densitometric instrument (Tanon-5200, China) and perform normalization processing by β -actin.

2.10 Cell culture and treatment

The HepG2 cells or HK-2 cells were cultured in DMEM (Gibco) containing 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 10% (v/v) fetal bovine serum at 37 °C in a 5% CO₂ atmosphere. For the stimulation experiments, the cells were seeded at 1×10^6 cells per well in 6-well plates. After culturing overnight, the cells were stimulated with 0.1% dimethyl sulfoxide (DMSO), UA (100 mg/L) alone, UA (100 mg/L) plus PE (0, 2.5 or 5 mg/L) or UA (100 mg/L) plus PI (0, 2.5 or 5 μ M) in a new medium for 24 h. In all groups, the final concentration of DMSO was 0.1%. After stimulation, the culture supernatant was collected to determine TNF- α secretion levels by commercial detection kits according to the manufacturer's protocols, and the cells were harvested and subsequently lysed by ice-cold RIPA lysis buffer for western blot analysis.

2.11 Luciferase reporter assay

The HepG2 cells or HK-2 cells were seeded at 5×10^5 cells per well in 12-well plates. After culture for 24 h, the pSTAT3-TA-Luc plasmid (1.5 μ g) and the control Renilla vector pRL-TK (0.5 μ g) were transiently cotransfected into cells using Lipofectamine 2000 (4 μ L) according to the manufacturer's protocol. For JAK2 overexpression experiments, the cells were transiently cotransfected with pSTAT3-TA-Luc plasmid (0.9 μ g), the control Renilla vector pRL-TK (0.25 μ g) and/or the overexpression vector pcDNA-JAK2 (0.9 μ g) using Lipofectamine 2000 (4 μ L). Twenty-four hours post-transfection, the cells were washed and then stimulated with DMSO, UA or/and PE (or PI) at the indicated

concentrations in the figures for 24 h. Finally, the firefly and Renilla luciferase activities were detected by Dual-Luciferase Reporter Gene Assay Kit (Beyotime) according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity in each sample and is expressed as the mean fold relative to that of the DMSO group.

2.12 Overexpression of JAK2 in HepG2 cells or HK-2 cells

The HepG2 cells or HK-2 cells were seeded at 1×10^6 cells per well in 6-well plates. After culture for 24 h, the cells were transiently transfected with the overexpression vector pcDNA-JAK2 (4 μ g) using Lipofectamine 2000 (10 μ L) according to the manufacturer's protocol. Forty-eight hours after transfection, the protein overexpression level of JAK2 was analysed by western blotting. For stimulation experiments, the cells were transiently transfected as described above. At 24 h post-transfection, the cells were stimulated as indicated in the figures for 24 h. Finally, the protein expression levels were analysed using western blotting.

2.13 Molecular docking

The binding activities and sites of JAK2 to PI or Fedratinib were analyzed using AutoDock 4.2.6. The crystal structures of JAK2-JH1 domain (PDB ID: 6vne) and JAK2-JH2 domain (PDB ID: 5ut0) were download from the Protein Data Bank^[51]. The 3D structures of PI and Fedratinib were drawn using ChemBio3D Ultra 12.0 softwares. Subsequently, the protein and ligand structures were prepared, and the docking tests were performed using AutoDock 4.2.6 package as described in our prior study^[52]. After docking, the best binding model as judged by docking energy was visualized and analyzed by PyMOL 1.7.6 software.

2.14 ELISA

To analyse and compare the binding activities of PI and Fedratinib to JAK2, PI and Fedratinib were respectively coated on the polystyrene plates as indicated concentrations in figure (50 μ L/well) at 4 °C overnight. Subsequently the plates were blocked with 1% gelatine at 37 °C for 1 h. Following washing with PBS, the plates were incubated with recombinant JAK2 protein (50 μ g/mL, 50 μ L/well) for 1 h. Next, the binding affinity of the PI and Fedratinib to JAK2, respectively, was tested by incubation with an anti-DDK-Tag antibody (1:1000) and subsequent incubation with an HRP-labelled goat anti-mouse IgG antibody (1:1000). Finally, the plates were visualized and quantified by incubation with H_2O_2 and ophenylenediamine. The reaction was terminated by adding 2 N H_2SO_4 , and the absorbance was detected at 492 nm.

2.15 Data analysis

All data are displayed as Means $\pm$ SEM, and the data processing and statistical software is SPSS Statistics V22.0. The differences between the experimental groups were analyzed using one-way ANOVA, and post hoc tests were conducted using Dunnett.

3 Results

3.1 PE reduced UA levels in hyperuricemia zebrafish

Preparation of different extracts of *P. nigrum* fruits was conducted as shown in Fig. 1A. Subsequently, to efficiently screen and determine the UA-lowering effects of various extracts from

P. nigrum seeds, we established a hyperuricemia zebrafish model by PO and XSS treatment. As allopurinol, PE effectively mitigated the abnormal elevation of UA levels induced by PO and XSS in zebrafish. In contrast, other extracts did not show a significant UA lowering effect (Fig. 1B). The results shown that *P. nigrum* can significantly alleviate hyperuricemia, and its benefits may come from a certain component of PE.

3.2 PI is the main components of PE

In the HPLC fingerprint, the different components were eluted at different time -points, and HPLC analysis indicates that the primary component of PE is PI (Fig. 1C). The regression equation for the standard PI curve was $y = 253,737x - 2 \times 10^6$ ($R^2 = 0.991,5$). According to the standard curve, PI accounts for 56.55% of the composition of PE (Fig. 1D). Furthermore, the molecular structure of PI is illustrated in Fig. 1E.

3.3 PE reduced UA levels in hyperuricemia mice

Next, to further validate the efficacy of PE in ameliorating hyperuricemia, a hyperuricemia mouse model was employed. The experimental procedure is illustrated in Fig. 2A. Throughout the 4-week administration period, the body weight of each group was monitored (Fig. 2B). Unfortunately, administration of both 10 mg/kg and 50 mg/kg PE did not reverse the weight changes induced by PO and fructose water in hyperuricemia mice (Fig. 2B). Notably, PE administration significantly mitigated the abnormal elevation of serum UA levels; specifically, compared to mice receiving 10 mg/kg PE, those treated with either 50 mg/kg PE or allopurinol had lower serum UA levels (Fig. 2C). In terms of urinary UA and fecal UA excretion, PE administration effectively reversed the abnormal increases observed in these parameters due to hyperuricemia (Figs. 2D-E). These findings suggest that PE can substantially alleviate hyperuricemia in mice.

3.4 PE restored the BUN and Cr levels in hyperuricemia mice

There was no significant difference in serum BUN levels among all mice (Fig. 2F). Furthermore, administration with PE at doses of 10 mg/kg or 50 mg/kg significantly inhibited the increase in urine BUN levels (Fig. 2G) and serum Cr levels (Fig. 2H) induced by fructose water and PO. Meanwhile PE increased the urine Cr levels (Fig. 2I). This effect is comparable to that observed with benzbromarone. Importantly, PE increased the estimated glomerular filtration rate (eGFR) (Fig. 2J), a critical indicator of renal function, suggesting that PE can ameliorate glomerular dysfunction associated with hyperuricemia.

3.5 PE reversed kidney damage in hyperuricemia mice

To demonstrate the renal protective effects of PE, histological studies were performed. HE staining revealed renal pathological alterations. PE administration significantly ameliorated abnormal pathological conditions in hyperuricemia mice, which exhibited vacuolization of the glomerular epithelium, thickening of the glomerular basement membrane, tubular dilation, and vacuolization within the tubules (Fig. 2K). Sirius red staining indicated that renal fibrosis and inflammation. Renal impairments, which characterized by tubular atrophy, severe vacuolization and inflammation, were performed in hyperuricemia mice compared to untreated controls, while PE administration markedly reduced the degree of these impairments (Figs. 2L-M). Furthermore, although benzbromarone significantly

decreased serum UA levels, renal tissues from mice in the BEN1 group still displayed extensive renal impairments. These histological findings suggest that PE showed a renal protection.

3.6 Transcriptome analysis suggests key genes *XDH*, *SLC2A9* and *SOCS3*

In order to further investigate the UA lowering mechanism of PE in hyperuricemia mice, transcriptome sequencing and analysis

of the kidneys from CON1 group, HUA1 group, PE-L group and PE-H group mice were performed. The heatmap indicated significant differences among the four groups (Fig. 3A). Specifically, the volcano plots showed 772 differential genes (571 up-regulated and 201 down regulated) in the comparison between the CON1 group and the HUA1 group, 47 differential genes (40 up-regulated and 7 down regulated) in the HUA1 group and the PE-L group, and 538 differential genes (96 up-regulated and 442 down regulated) in the HUA1 group and the PE-H group

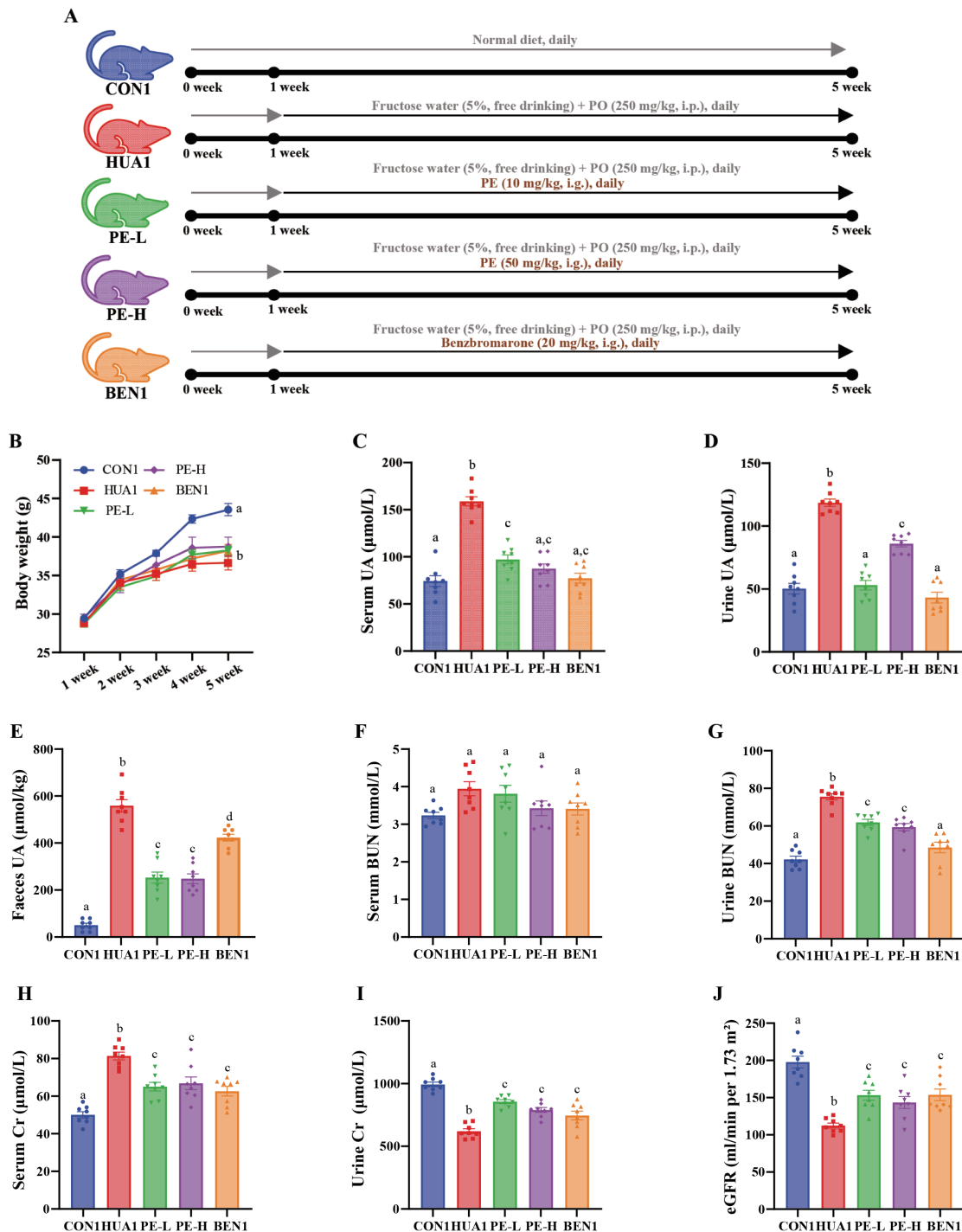


Figure 2 Effects of PE in hyperuricemia mice. (A) Animal experiment process. (B) Body weight. (C) Serum UA. (D) Urine UA. (E) Faeces UA. (F) Serum BUN. (G) Urine BUN. (H) Serum Cr. (I) Urine Cr. (J) eGFR. (K) HE staining. (L) Sirius red staining. (M) Kidney injury score. PO, potassium oxonate; PE, *P. nigrum* fruits ethanol extract; UA, uric acid; BUN, blood urea nitrogen; Cr, creatinine. eGFR, estimate glomerular filtration rate. HE, hematoxylin and eosin. CON1, control group 1; HUA1, hyperuricemia group 1; PE-L, low-dose *P. nigrum* fruits ethanol extract group; PE-H, high-dose *P. nigrum* fruits ethanol extract group; BEN1, benzbromarone group 1.

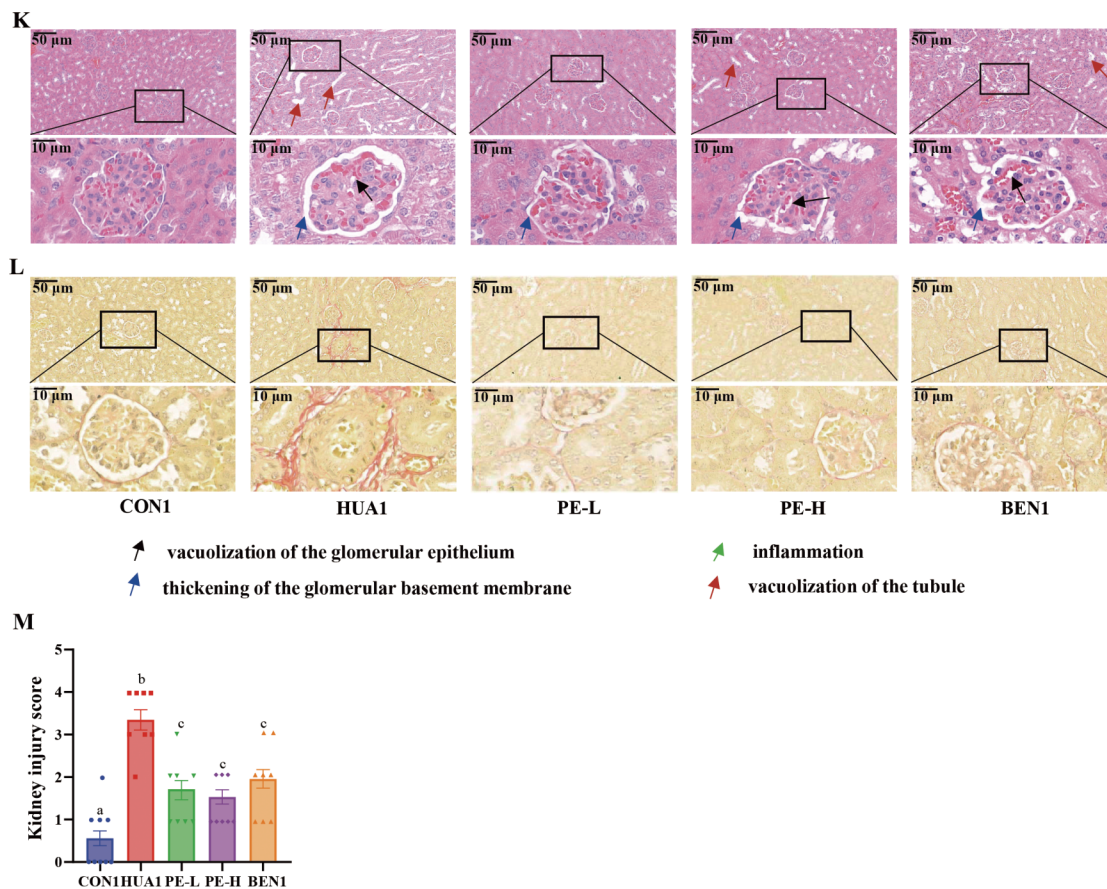


Figure 2 (Continued).

(Fig. 3B). In addition, UA metabolism (Fig. 3C) and differential gene analysis (Fig. 3D) shown that PE treatment significantly reversed the abnormal expression of *XDH*, *GLUT9*, and *SOCS3* genes caused by PO combination with fructose water (Table 1). *XDH* protein encoded by *XDH* gene can catalyze the production of UA^[53]. In our study, compared with control mice, the expression level of *XDH* in hyperuricemia mice increased, while PE reduced the expression levels of *XDH* gene (Fig. 3E). *GLUT9* protein encoded by *SLC2A9* gene can increase the reabsorption of UA in the kidneys^[54]. Compared with mice with hyperuricemia, PE significantly inhibited the transcription level of the renal *GLUT9* gene (Fig. 3F). Moreover, the inflammatory *SOCS3* protein encoded by *SOCS3* gene can promote the development of hyperuricemia associated nephritis^[55]. The results indicate that PE can prevent the high expression of *SOCS3* gene caused by hyperuricemia (Fig. 3G). In a word, our transcriptome analysis results further corroborate that the mechanism of PE in combating hyperuricemia is not only related to the regulation of UA synthesis and UA transporters, as well as the subsequent promotion of UA excretion, but also to renal inflammation.

3.7 PE abated the protein expressions of GLUT9, XDH, SOCS3 and TNF- α in hyperuricemia mice

To further validate the transcriptome analysis results, we performed corresponding western blot measurements. As expected, compared to hyperuricemia mice, PE treatment significantly reduced the levels of GLUT9 in kidney (Fig. 3H), suggests that PE may promote the excretion of UA with urine. What's more, compared to mice with hyperuricemia, PE treatment not only significantly reduced XDH expression (Fig. 3I) in kidneys, but also inhibited kidney XO activity (Fig. 3J), indicates

that PE may inhibit the production of UA in the renal region. In terms of renal inflammation, compared with the HUA1 group, PE treatment significantly downregulated the protein expression of *SOCS3* (Fig. 3K) in mouse kidneys, and ELISA results also showed a significant decrease in TNF- α levels (Fig. 3L), denotes that PE has a certain protective effect on renal inflammation. Considering that more UA is produced in the liver, we conducted a similar study on mouse liver^[56]. Western blot showed that the trend of *XDH* and *XO* in mouse liver was consistent with that in kidney (Figs. 3M-N). The above results indicate that PE may improve hyperuricemia by reducing the expression of GLUT9 and XDH, as well as inhibiting XO activity. In addition, PE can alleviate hyperuricemia associated nephritis by reducing the expression of *SOCS3* and TNF- α .

3.8 PE retarded the JAK2/STAT3 signalling pathway in hyperuricemia mice

As shown in Figs. 3O-P, hyperuricemia significantly increased the phosphorylation levels of STAT3 and JAK2 in the kidneys of mice. In contrast, PE administration reversed this increase. Meanwhile, we observed a similar trend in the mouse liver (Figs. 3Q-R). The data suggests that PE can retard the activation of JAK2/STAT3 signalling pathway in mice caused by hyperuricemia. Further research is needed to determine whether inhibiting the JAK2/STAT3 signalling pathway is associated with improving hyperuricemia and hyperuricemia associated nephritis.

3.9 PE inhibited the JAK2/STAT3 signalling pathway in HK-2 and HepG2 cells

To further validate the findings of mice experiments, the

hyperuricemia cell models were established in the human kidney proximal tubelial cell (HK-2 cell) and the human liver

cell (HepG2 cell) by inducing with UA. At first, we evaluated the effect of PE on JAK2/STAT3 signal transduction in HK-2 cells and

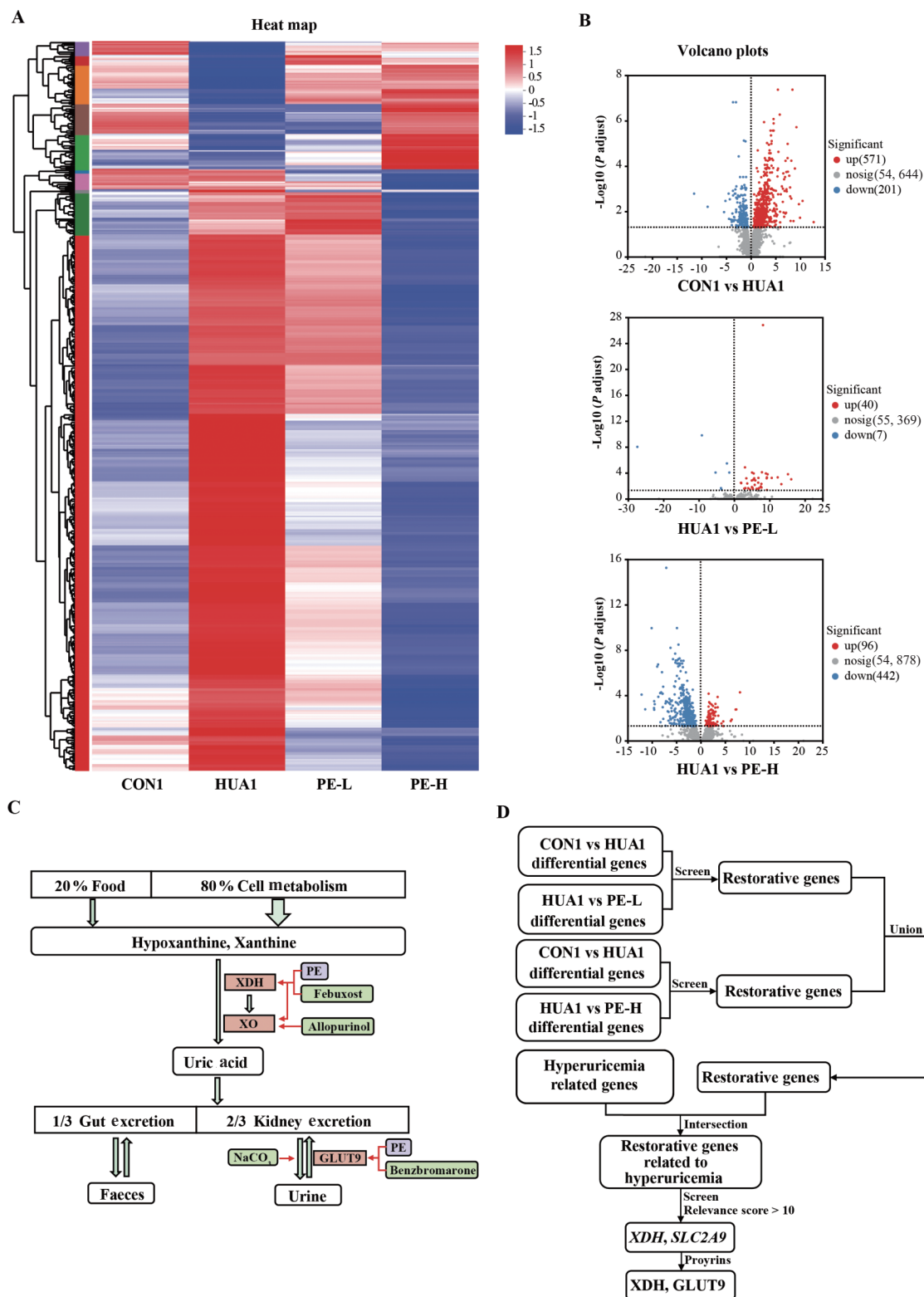


Figure 3 Transcriptome of PE in hyperuricemia mice. (A) Heatmap. (B) Volcano plots. (C) UA metabolism. (D) Transcriptome analysis process. (E) *XDH* gene. (F) *SLC2A9* gene. (G) *SOCS3* gene. Unfilled bars indicate data points excluded from analysis due to excessively discrete outlier values. (H) GLUT9 level in kidney. (I) *XDH* level in kidney. (J) Kidney XO activity. (K) *SOCS3* level in kidney. (L) *TNF- α* lever in kidney. (M) *XDH* level in liver. (N) Liver XO activity. (O) *STAT3* phosphorylation level in kidney. (P) *JAK2* phosphorylation level in kidney. (Q) *STAT3* phosphorylation level in liver. (R) *JAK2* phosphorylation level in liver. GLUT9, glucose transporter member 9; *JAK2*, janus kinase 2; PE, *P. nigrum* fruits ethanol extract; *SOCS3*, suppressor of cytokine signaling 3; *STAT3*, signal transducer and activator of transcription 3; UA, uric acid; *XDH*, xanthine dehydrogenase; XO, xanthine oxidase. CON1, control group 1; HUA1, hyperuricemia group 1; PE-L, low-dose *P. nigrum* fruits ethanol extract group; PE-H, high-dose *P. nigrum* fruits ethanol extract group.

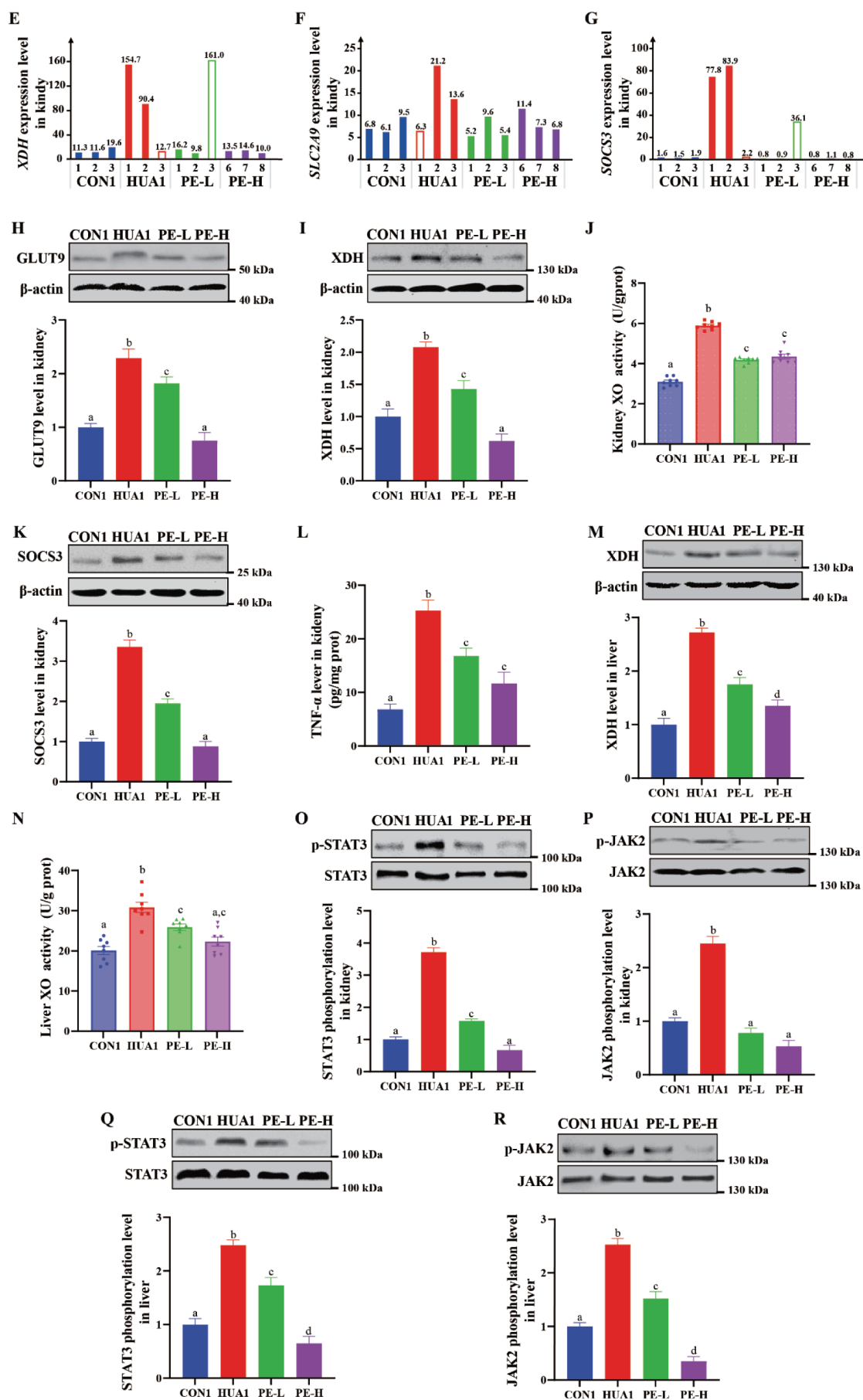


Figure 3 (Continued).

Table 1 Relevance scoring of transcriptomic DEGs associated with hyperuricemia

Gene name	CON vs HUA	HUA vs PE-L	HUA vs PE-H	Relevance score
<i>Slc2a9</i>	up	down	down	13.72
<i>Xdh</i>	up	down	down	10.81
<i>Nlrp3</i>	up	down	down	4.75
<i>Myc</i>	up	down	down	2.98
<i>Tnf</i>	up	down	down	2.48
<i>Fga</i>	up	down	down	2.34
<i>Slc37a1</i>	up	down	down	1.64
<i>Il1b</i>	up	down	down	1.52
<i>Serpine1</i>	up	down	down	1.39
<i>Fasn</i>	down	up	up	1.34
<i>Fth1</i>	up	down	down	1.34
<i>Havcr1</i>	up	down	down	1.34
<i>Lcn2</i>	up	down	down	1.34
<i>Slc22a7</i>	down	up	up	1.33
<i>Edn1</i>	up	down	down	1.28
<i>B2m</i>	up	down	down	1.1

HepG2 cells. As shown in Figs. 4A-B, PE dose-dependently reduced the phosphorylation levels of STAT3 and JAK2, and inhibited transcriptional activation of STAT3 caused by UA in HK-2 cells (Fig. 4C). Similar effects were observed in HepG2 cells (Figs. 4D-F). These results showed that the PE could suppress JAK2/STAT3 signalling pathway in HK-2 cells and HepG2 cells.

3.10 PE reduced the protein expressions of GLUT9, XDH, SOCS3 and TNF- α in HK-2 and HepG2 cells

As shown in Figs. 4G-J, UA (100 mg/L) treatment prominently increased the expressions of GLUT9, XDH, SOCS3 and TNF- α in HK-2 cells compared to untreated cells. Meanwhile, UA stimulation also significantly upregulated XDH expression level in HepG2 cells (Fig. 4K). However, PE could dose-dependently reverse the increased expression of these proteins caused by UA in HK-2 cells and HepG2 cells. The above data confirmed mice experiment results and suggested that PE ameliorate hyperuricemia and hyperuricemia associated nephritis by suppressing the expressions of GLUT9, XDH, SOCS3 and TNF- α .

3.11 PE inhibited UA metabolic disorders and renal inflammation via JAK2/STAT3 signalling pathway

To further determine whether JAK2/STAT3 signalling pathway is involved in PE suppressing the expressions of XDH, SOCS3 and TNF- α , we respectively established the overexpression cell model of JAK2 in HK-2 cells and HepG2 cells. As shown in Fig. 4L, after transfection with pcDNA-JAK2 overexpression vector, the JAK2 protein expression levels were significantly elevated in the HK-2 cells and HepG2 cells. Furthermore, JAK2 overexpression mediated by pcDNA-JAK2 vector in the HK-2 cells and HepG2 cells not only inhibited PE-induced reduction in the phosphorylation and transcriptional activation of STAT3, but also inhibited PE-induced reduction in the expressions of XDH, SOCS3 and TNF- α (Figs. 4M-V). Together with previous results, these data showed that PE can reduce UA reabsorption by reducing GLUT9, simultaneously PE inhibited JAK2 signal

transduction, leading to the attenuation of STAT3 activation and the reduction of STAT3 downstream protein expression (XDH, SOCS3 and TNF- α), thereby alleviating UA production and renal inflammation.

3.12 PI improved UA levels and hyperuricemia associated nephritis in hyperuricemia mice

Our previous component analysis studies showed that PI is the main component of PE. Therefore, we speculated that PE improved hyperuricemia and hyperuricemia associated nephritis through PI. To confirm our hypothesis, we analyzed the effects of PI on hyperuricemia and nephritis, the experimental procedures are described in Fig. 5A. After four weeks of administration, PI did not significantly recover the weight of hyperuricemia mice (Fig. 5B), but it could significantly reduce the UA level in blood of hyperuricemia mice (Fig. 5C), which was basically consistent with the efficacy of PE. And PI reversed the urine UA as well as faeces UA level of hyperuricemia mice (Figs. 5D-E). In addition, PI improved the levels of BUN and Cr induced by PO and fructose water (Figs. 5F-I), especially the level of serum BUN. As expected, the eGFR and section results showed that PI effectively alleviated the renal pathological damage and inflammation of hyperuricemia, and PI exerted renal protective effects comparable to benzbromarone (Figs. 5J-M). The results of animal experiments strongly support that PI is the main component of PE that exerts its UA lowering benefits.

3.13 PI suppressed the expression of GLUT9, XDH, SOCS3 proteins and inhibited the JAK2/STAT3 signalling pathway in hyperuricemia mice

Following the previous experimental steps, western blot was used to detect the expression of completely consistent trend with PE in kidneys and livers of mice. PI treatment inhibited the phosphorylation JAK2 and STAT3 in the kidneys and liver (Figs. 6A-D), reversed the expression levels of GLUT9, XDH (and XO activity), SOCS3, and TNF- α in the liver and kidneys (Figs. 6E-K). Overall, the exploration of the UA lowering mechanism of PI

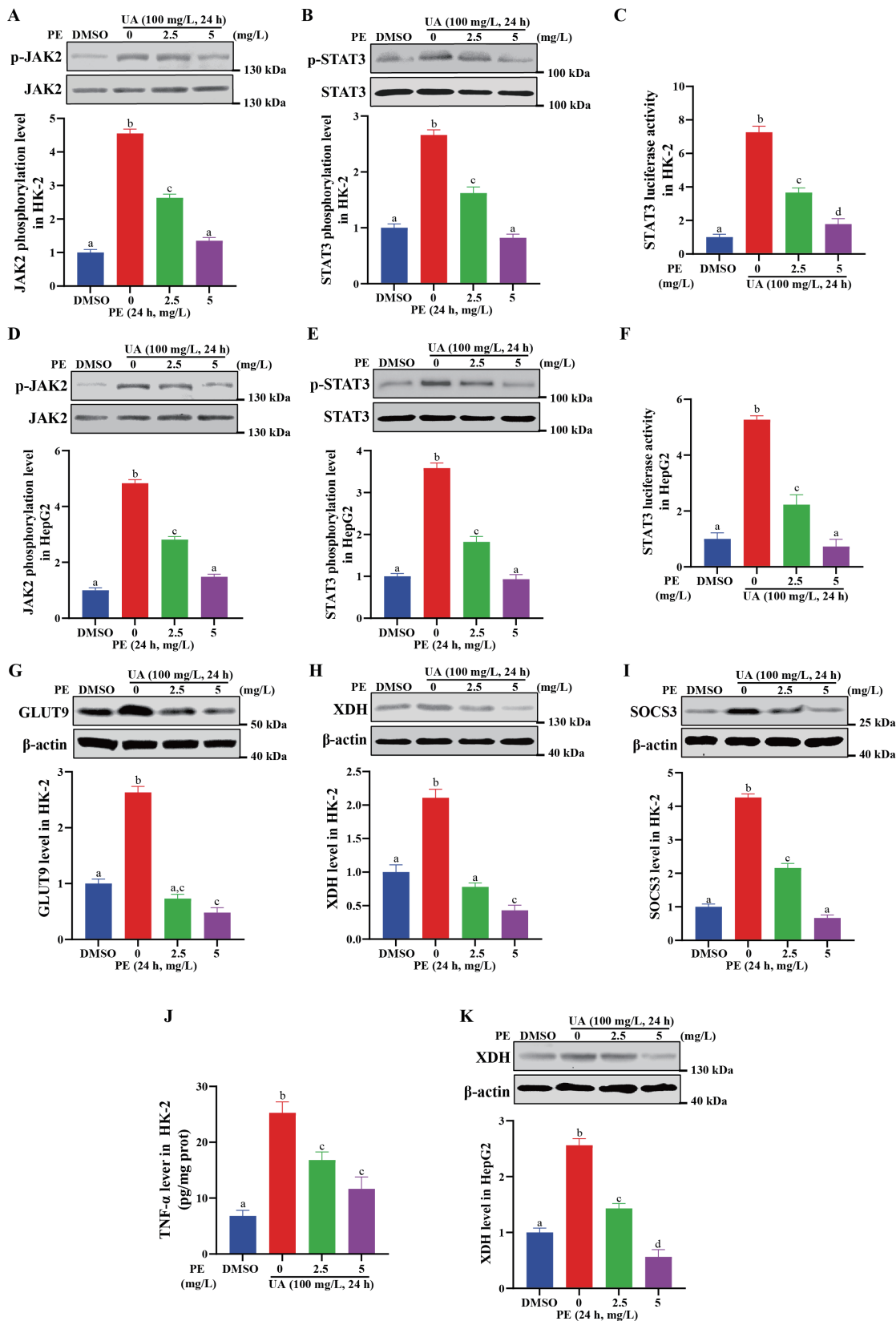


Figure 4 Effects of PE in HK-2 cells and HepG2 cells. (A) JAK2 phosphorylation level in HK-2 cells. (B) STAT3 phosphorylation level in HK-2 cells. (C) STAT3 luciferase activity in HK-2 cells. (D) JAK2 phosphorylation level in HepG2 cells. (E) STAT3 phosphorylation level in HepG2 cells. (F) STAT3 luciferase activity in HepG2 cells. (G) GLUT9 level in HK-2 cells. (H) XDH level in HK-2 cells. (I) SOCS3 level in HK-2 cells. (J) TNF-α lever in HK-2 cells. (K) XDH level in HepG2 cells. (L) JAK2 overexpression. (M) STAT3 luciferase activity in HK-2 cells. (N-Q) STAT3 phosphorylation level, XDH and SOCS3 level in HK-2 cells. (R) TNF-α level in HK-2 cells. (S) STAT3 luciferase activity in HepG2 cells. (T-V) STAT3 phosphorylation level and XDH level in HepG2 cells. DMSO, dimethyl sulfoxide; GLUT9, glucose transporter member 9; JAK2, janus kinase 2; PE, *P. nigrum* fruits ethanol extract; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; UA, uric acid; XDH, xanthine dehydrogenase.

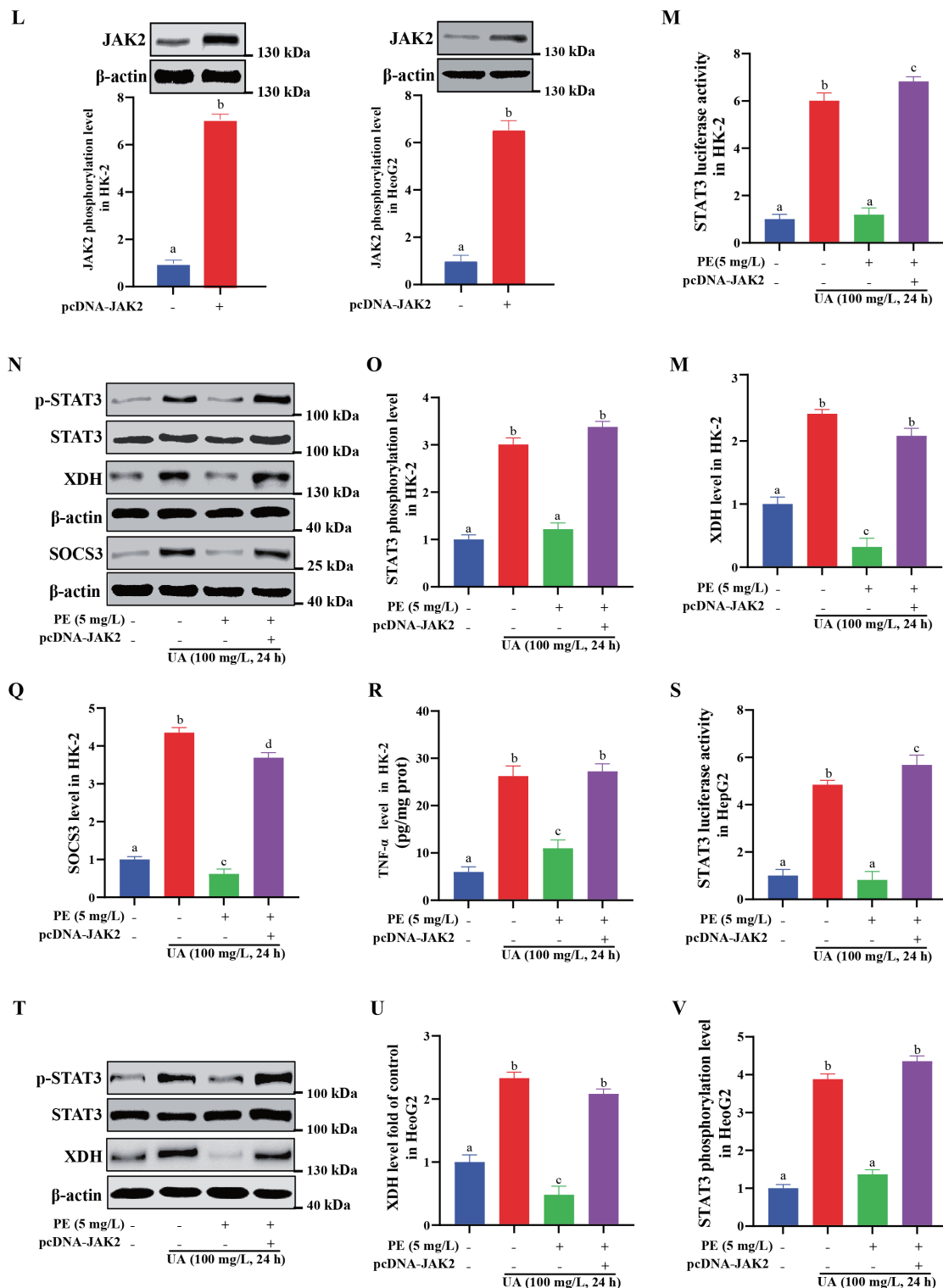


Figure 4 (Continued).

provides evidence that the benefits of PE in reducing UA come from PI.

3.14 PI suppressed the expression of GLUT9, XDH, SOCS3 proteins and inhibited the JAK2/STAT3 signalling pathway in HK-2 and HepG2 cells

To confirm the animal experiments results of PI, we performed western blot and luciferase reporter assays in HK-2 cells and

HepG2 cells. Consistent with animal experiments results, PI dose-dependently reduced the phosphorylation JAK2 and STAT3, inhibited the activation of STAT3 in HK-2 cells and HepG2 cells treated with UA (100 mg/L) (Figs. 6L-Q). Meanwhile, the expression levels of GLUT9, XDH, SOCS3 and TNF- α caused by UA (100 mg/L) also were suppressed by PI in a dose-dependent manner (Figs. 6R-V). Taken together, the above results confirmed that PI could accelerate UA excretion by reducing GLUT9 expression, meanwhile PI also ameliorate XDH-mediated UA

overproduction and SOCS3/TNF- α mediated renal inflammation by suppressing JAK2/STAT3 signalling pathway.

3.15 PI has good binding activity with JAK2

As our above results demonstrated that PI could suppress JAK2/STAT3 signalling pathway and reduce the expression of STAT3 downstream protein (XDH, SOCS3 and TNF- α), we speculated that JAK2 likely serves as a direct target for PI, and that PI bind to JAK2 to suppress the JAK2/STAT3 signal transduction. To assess this hypothesis, we performed molecular docking analysis. The previous studies have reported that both JH1 domain

and JH2 domain of JAK2 participate in regulating JAK2 protein kinase activity^[51]. Therefore, we respectively docked PI with JH1 domain and JH2 domain of JAK2 by using AutoDock 4.2.6. As shown in Figs. 7A-B, PI could respectively insert into the binding pockets of JH1 domain and JH2 domain of JAK2. Nevertheless, the binding of PI to JH1 domain has a significantly lower docking energy (-9.69 kcal/mol), compared with the binding of PI to JH2 domain (-2.66 kcal/mol), suggested that JH1 domain probably was the main binding region of PI for JAK2. Subsequently, we used the same parameters to dock JH1 domain of JAK2 with Fedratinib, which is the inhibitor for JAK2. As shown in Fig. 7C,

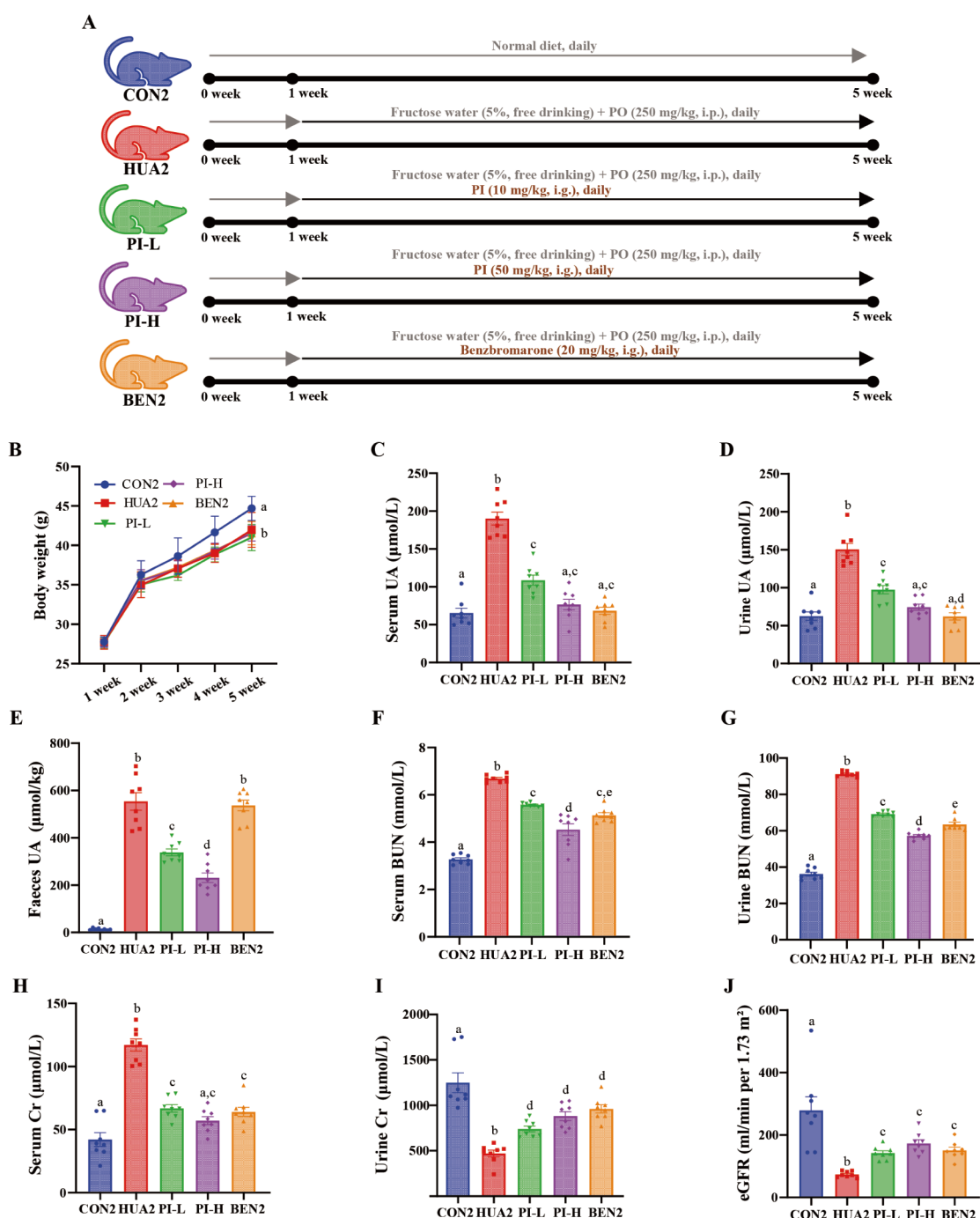


Figure 5 Effects of PI in hyperuricemia mice. (A) Animal experiment process. (B) Body weight. (C) Serum UA. (D) Urine UA. (E) Faeces UA. (F) Serum BUN. (G) Urine BUN. (H) Serum Cr. (I) Urine Cr. (J) eGFR. (K) HE staining. (L) Sirius red staining. (M) Kidney injury score. BUN, blood urea nitrogen; Cr, creatinine. eGFR, estimate glomerular filtration rate; HE, hematoxylin and eosin; PO, potassium oxonate; PI, piperine; UA, uric acid. CON2, control group 2; HUA2, hyperuricemia group 2; PI-L, low-dose piperine group; PI-H, high-dose piperine group; BEN2, benzbromarone group 2.

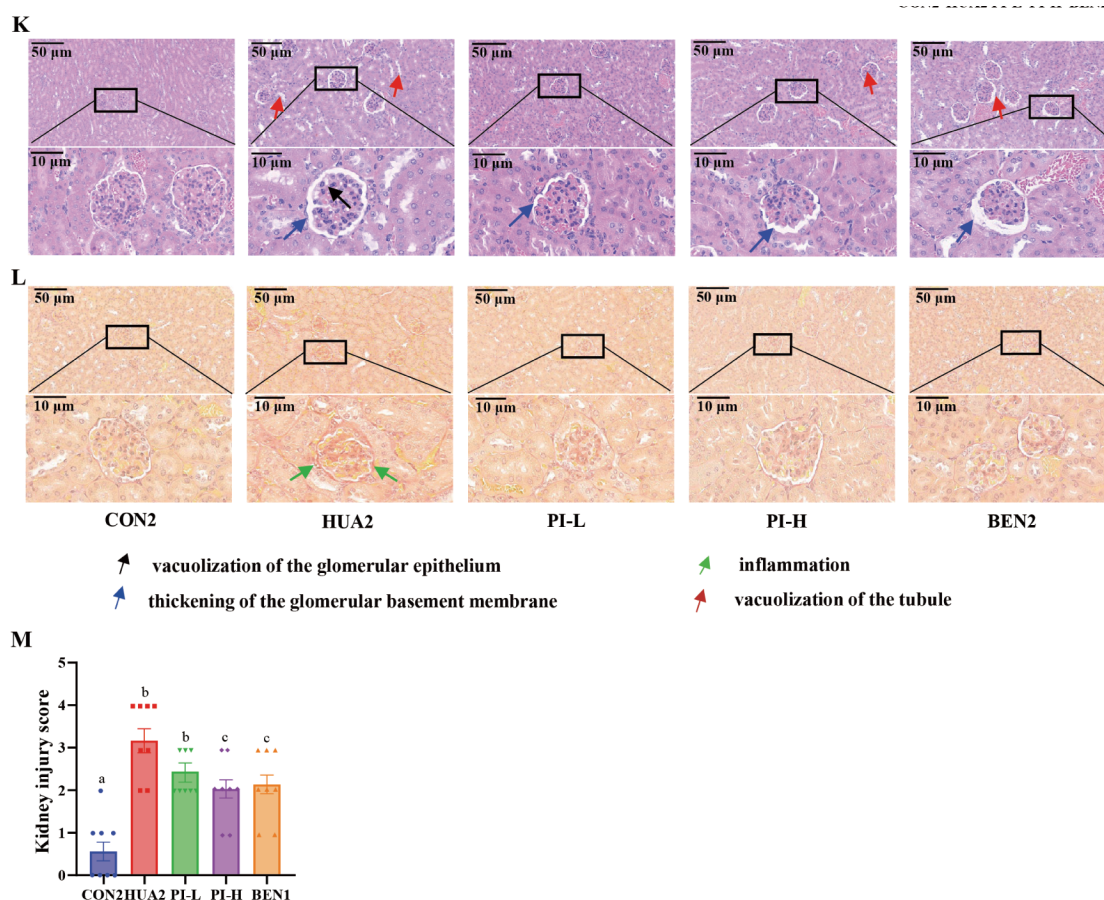


Figure 5 (Continued).

Fedratinib was buried inside the same binding pocket as PI, and has a similar docking energy (-9.19 kcal/mol). Detailed analysis showed that PI formed two hydrogen bonds with LEU932 and ARG980. Similar to PI, Fedratinib formed two hydrogen bonds with LEU932 (Fig. 7D). These data suggested that PI likely bound and inhibited JAK2. To further verify the aforementioned molecular docking results and confirm the binding interaction between PI and JAK2, we performed ELISA assays. As shown in Fig. 7E, PI (1, 2.5, and 5 μ mol/L) could dose-dependently bind JAK2, and exhibited a similar binding capable compared to JAK2 inhibitor Fedratinib. Taken together with *in vivo* and *in vitro* results, it is conceivable that PI, as the key active component from PE could target JAK2 to prevent JAK2/STAT3 signal transduction, leading to the reduction in the expressions of XDH, SOCS3 and TNF- α , thereby suppressing UA overproduction and subsequent renal inflammation.

4 Discussion

Prolonged high level of serum UA is a leading feature of hyperuricemia, and is also a risk factor for hyperuricemia associated nephritis, diabetes and atherosclerosis. Therefore, there is an urgent need to seek and develop safe and effective anti-hyperuricemia drugs. In this study, we systematically investigated the anti-hyperuricemia effect and the underlying mechanism of PE and its key active component PI in potassium oxonate (PO) combination with fructose induced hyperuricemia mice model and UA induced HK-2 cells and HepG2 cells. Our results indicated that PE and PI could inhibit UA overproduction by suppressing the JAK2/STAT3/XDH signalling pathway, and

simultaneously enhance UA excretion in renal by restraining GLUT9 expression, thereby reducing the excessive accumulation of UA in serum. Furthermore, PE and PI can also inhibit the expression and secretion of TNF- α and SOCS3 by suppressing the JAK2/STAT3 signalling pathway, thereby ameliorating hyperuricemia associated nephritis. These results showed that PE, as a natural plant active extract, can effectively improve and treat hyperuricemia and hyperuricemia associated nephritis. Meanwhile, PI, as a potential active ingredient, plays a key role in this process. Our research provides a new strategies and natural active ingredients for the treatment of hyperuricemia and hyperuricemia associated nephritis.

The fruits of *P. nigrum* in this study specifically refers to the dried immature fruit, also called black pepper, which retains the skin, flesh, and seeds. In fact, the promoting value of the effective components of *P. nigrum* fruits in the metabolism has been widely studied^[57]. The n-butanol extract of *P. nigrum* fruits (including gallic acid, chlorogenic acid, catechins, and rutin) plays a beneficial role in paracetamol-induced kidney damage in mice by inhibiting DNA damage in comet tail, glutathione S-transferase activity, lipid peroxidation, and overexpression of p53 protein^[58]. Furthermore, Duangjai *et al.* found that the PE and its main component PI can avoid significant lipid accumulation in a high-fat environment by downregulating the membrane levels of cholesterol transporters such as NPC1L1 and SR-BI proteins^[59]. The alkaloid components in *P. nigrum* fruits can effectively inhibit the upregulation of IL-1 β , TNF- α , IL-6 caused by lipolysis, relieve inflammatory reactions^[60]. We found, PE administration effectively prevented PO and XSS induced abnormal UA levels in zebrafish. This finding supports the notion that *P. nigrum* fruits contributes to UA reduction,

which is attributed to its ethanol extract component. Next, we identified PI is the main component of PE by HPLC analysis.

Combining with previous studies, both PE and PI have anti-gout effects, we further extended these findings to hyperuricemia

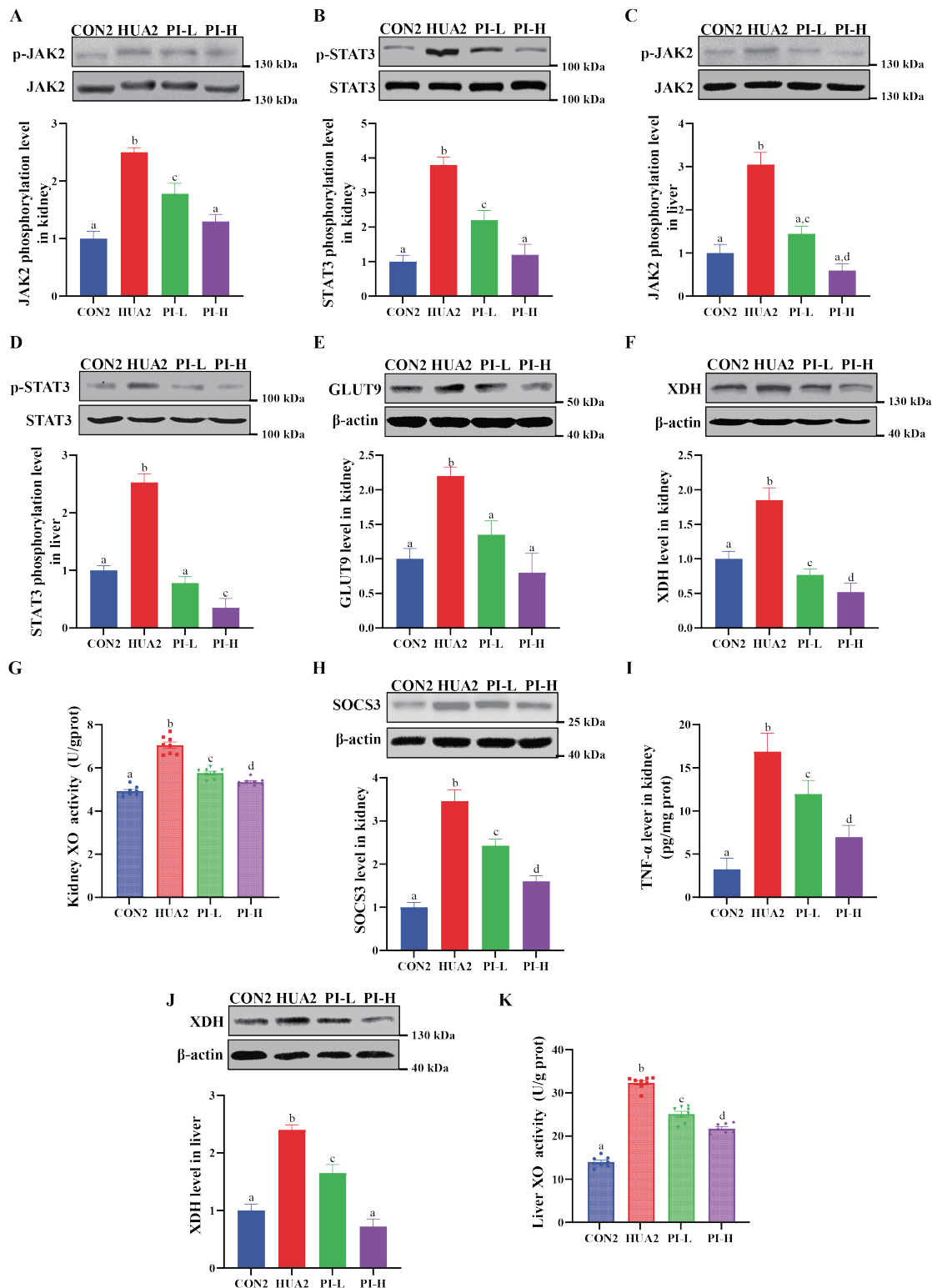


Figure 6 Effects of PI in hyperuricemia mice, HK-2 cells and HepG2 cells. (A) JAK2 phosphorylation level in kidney. (B) STAT3 phosphorylation level in kidney. (C) JAK2 phosphorylation level in liver. (D) STAT3 phosphorylation level in liver. (E) GLUT9 level in kidney. (F) XDH level in kidney. (G) Kidney XO activity. (H) SOCS3 level in kidney. (I) TNF-α level in kidney. (J) XDH level in liver. (K) Liver XO activity. (L) JAK2 phosphorylation level in HK-2 cells. (M) STAT3 phosphorylation level in HK-2 cells. (N) STAT3 luciferase activity in HK-2 cells. (O) JAK2 phosphorylation level in HepG2 cells. (P) STAT3 phosphorylation level in HepG2 cells. (Q) STAT3 luciferase activity in HepG2 cells. (R) GLUT9 level in HK-2 cells. (S) XDH level in HK-2 cells. (T) SOCS3 level in HK-2 cells. (U) TNF-α level in HK-2 cells. (V) XDH level in HepG2 cells. DMSO, dimethyl sulfoxide; GLUT9, glucose transporter member 9; JAK2, janus kinase 2; PI, piperine; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; UA, uric acid; XDH, xanthine dehydrogenase; XO, xanthine oxidase. CON2, control group 2; HUA2, hyperuricemia group 2; PI-L, low-dose piperine group; PI-H, high-dose piperine group.

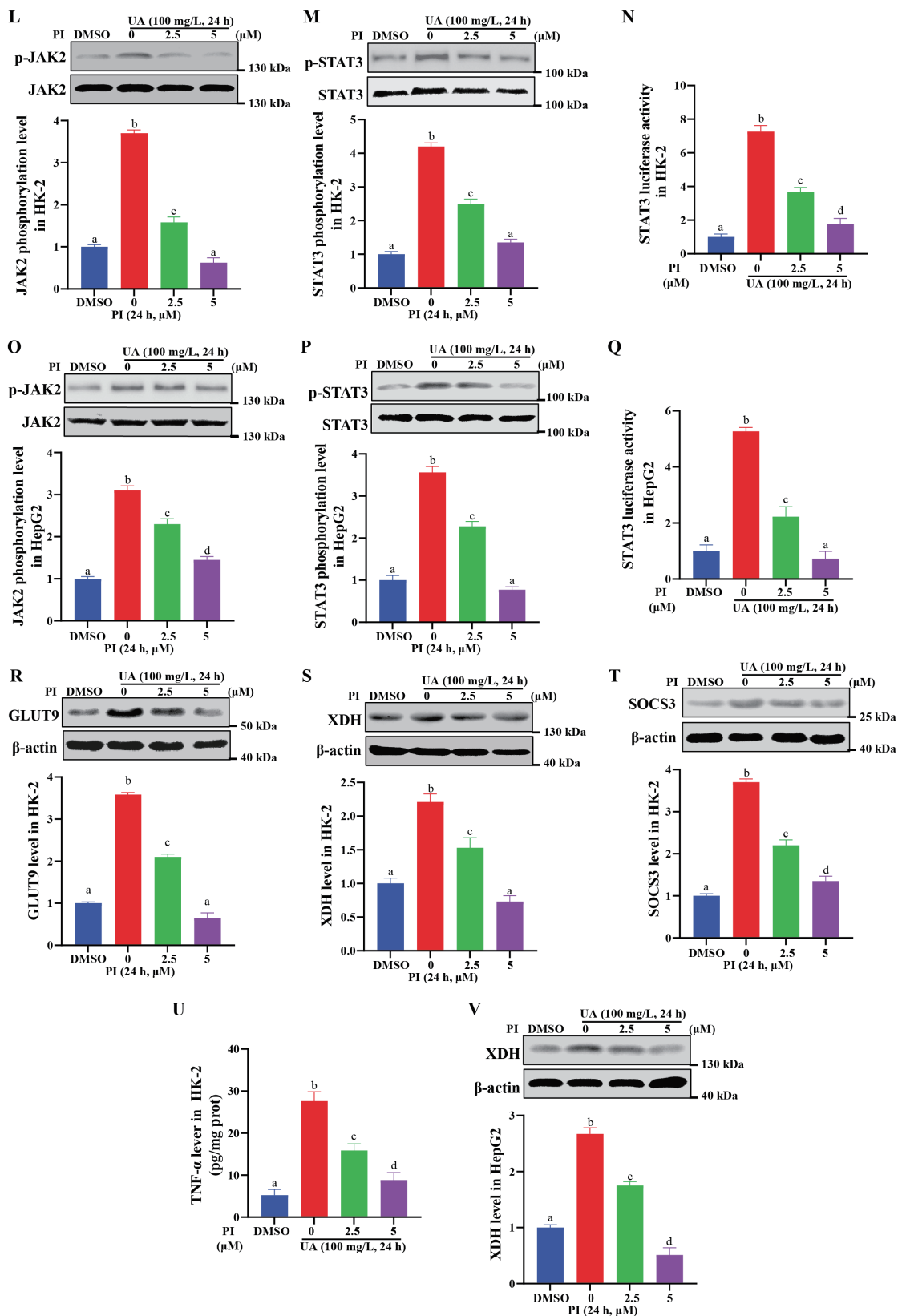


Figure 6 (Continued).

mice^[48,61]. The results showed that the PE and its main component PI both could significantly reduce the serum UA and urine UA levels induced by PO and fructose water in mice, effectively restore serum Cr levels and alleviated kidney inflammation caused by hyperuricemia. The more in-depth transcriptome analysis showed

that PE significantly downregulated the transcription of *SLC2A9*, *XDH*, and *SOCS3* gene in the kidneys. This means that unlike allopurinol (which can only inhibit UA production) and benzbromarone (which can only promote UA excretion), PE and its main component PI have dual effects of inhibiting UA

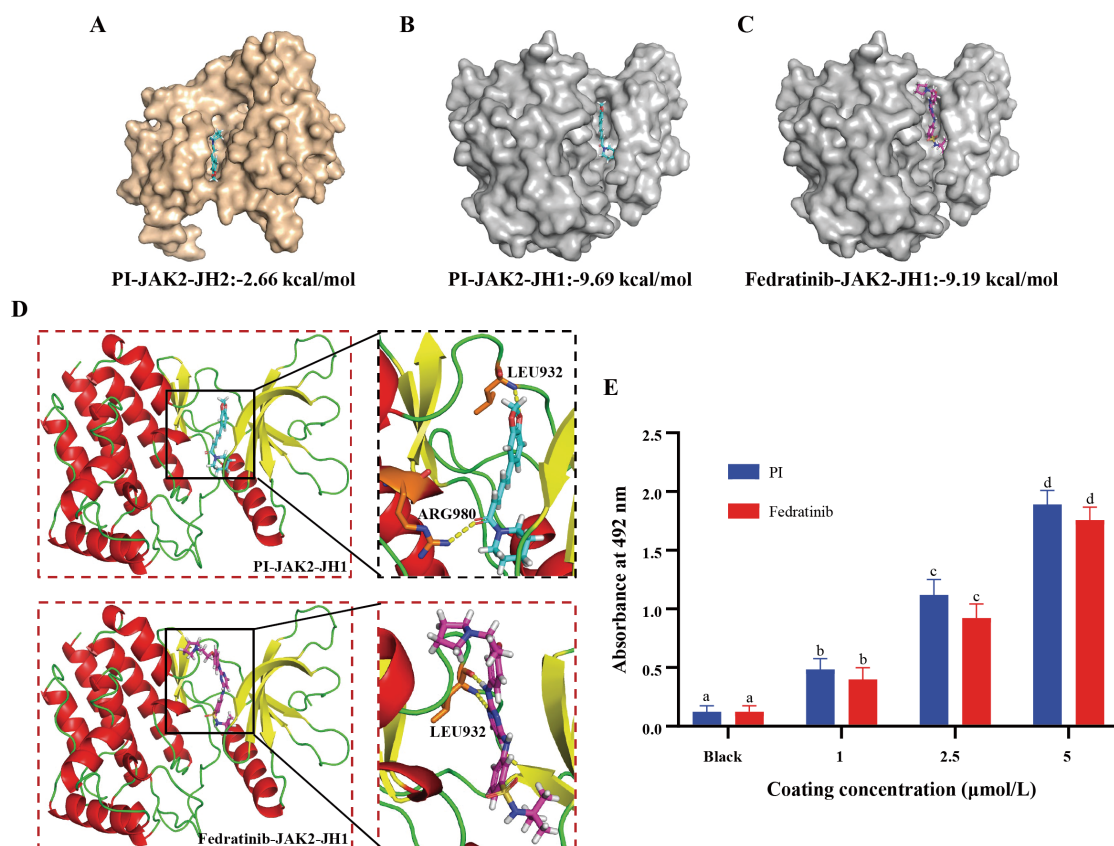


Figure 7 Molecular docking of PI and JAK2. (A) PI-JH2 domain. (B) PI-JH1 domain. (C) Fedratinib-JH1 domain. (D) Molecular docking JH1 domain. (E) Absorbance at 492nm. PI, piperine; JAK2, janus kinase 2.

production and promoting UA excretion. Moreover, compared to benzbromarone, PE and its main component PI have better renal protection. Undoubtedly, PE and its main component PI have more prospects in the treatment of hyperuricemia.

The renal excretion of UA is a key factor in combating hyperuricemia. A key factor for PE to exert its UA lowering effect is to promote renal excretion of UA. GLUT9 encoded by *SLC2A9* gene is mainly distributed in basolateral and apical membrane transporters in proximal tubular epithelial cells of the kidney, mediating the reabsorption of UA in the kidney^[1,62]. It has been reported that mice with renal GLUT9 deficiency are prone to hypouricemia^[54,63]. Similarly, functional loss mutations in human GLUT9 can lead to renal hypouricemia type 2 (RHUC-2), with significantly reduced serum UA levels and increased UA excretion fraction^[64]. Some patients may also experience exercise-induced acute kidney failure^[64]. For example, Matsuo *et al.* found multiple mutations in the GLUT9 gene associated with RHUC-2 in their study, which affect the normal function of GLUT9 and alter the metabolic balance of UA^[64]. In our experiment, transcriptome analysis of mouse kidneys indicated that the *SLC2A9* gene was downregulated after PE treatment. Moreover, reports showed that XO can act upstream of the PI3K/AKT signalling pathway and further affect GLUT9^[65]. But our western blot shows PE and its main component PI significantly inhibited the expression of GLUT9, both in the kidneys of hyperuricemia mice and in HK-2 cells stimulated by UA. These data confirm that PE and PI could increase UA excretion in renal by restraining GLUT9 expression, thereby suppressing the excessive accumulation of UA in serum.

With the transcriptome analysis of gene expression in mouse kidney, we also identified that PE significantly inhibited *XDH*

gene, which encodes the XDH protein. XDH is widely expressed in human tissues^[66]. In fact, XDH plays a crucial role in multiple physiological processes including purine metabolism, inflammatory response, and innate immunity^[65,67,68]. XDH and XO are encoded by the *XDH* gene, and XDH can be converted to XO through peptide bond cleavage or formation of disulfide bonds in enzymes to exert its function in mammalian purine metabolism^[69]. At present, XO is the most widely studied drug in the research of inhibiting purine metabolism to UA, but there is a lack of research on XDH^[70-76]. We supplemented the study that natural ingredients regulate XDH to inhibit uric acid production. On the one hand, XDH can be converted into XO, thereby metabolizing purines into UA under the condition of O₂ participation, while producing superoxide anions (O₂⁻) or hydrogen peroxide^[74,75]. On the other hand, XDH does not undergo conversion, but instead uses NAD⁺ or NADP⁺ as coenzymes to directly catalyze the oxidation of hypoxanthine to xanthine, which is then oxidized to UA^[76-78]. So inhibiting the expression and activity of XDH and XO in the liver and kidneys is crucial for reducing UA synthesis and inhibiting UA accumulation^[7]. In our experiment, the up-regulated *XDH* gene and its expression in mice and cells were significantly downregulated after administration of PE and its main component PI. This clearly suggests that PE and its main component PI treatment for hyperuricemia is related to the inhibition of UA synthesis.

In previous studies, XDH and XO has been found to be associated with the regulation of multiple signalling pathways, including PI3K/AKT signalling pathways, MAPK signalling pathways and NOX/FoxO3a/eNOS signalling pathways^[65,79,80].

Recent studies have found that STAT3 is key in regulating XDH in pulmonary microvascular endothelial cells and rat kidneys, but there have been no reports of liver and kidney related effects in mice^[81,82]. Our research has found that the JAK2/STAT3 signalling pathway may be the key to the role of PE and its main component PI. Indeed, the molecular components of the JAK/STAT signalling pathway were identified 30 years ago and have attracted much research attention as one of the core communication nodes in cellular function^[83]. As an important member of non-receptor tyrosine kinases, the JAK family consists of JAK1, JAK2, JAK3 and TYK2, which are downstream signal transduction components of receptor kinases on the cell membrane and play a key role in signal transduction^[84,85]. Cytokines derived from extracellular sources, such as interferons, interleukins, and growth factors, can act as activators of the JAK family^[86]. These cytokines bind to their receptors to form complexes that activate receptor-bound JAKs, which in turn catalyze the phosphorylation of receptor tyrosines and initiate a series of complex intracellular signalling processes^[87-89]. The four known members of the JAK family can each interact with specific cytokine receptors and recruit the corresponding STATs for different biological functions^[90]. The STAT family members include STAT1, STAT2, STAT3, STAT4, STAT5 α , STAT5 β , and STAT6, which act as transcription factors phosphorylated after JAK kinase activation and regulate gene expression in the nucleus^[91]. Among them, JAK2/STAT3 signalling pathway has been extensively studied and includes many biological processes such as inflammatory response, cancer, cell proliferation, immune escape and treatment resistance^[92-98]. In our experiment, after JAK2 overexpression in HK-2 cells as well as HepG2 cells, the regulatory effect of PE on XDH was reversed, validating that PE reduces XDH expression by suppressing JAK2/STAT3 signalling pathway, and consequently relieving UA synthesis. Although the present results verified the indispensable role of JAK2/STAT3 signalling pathway in the reduction of XDH expression induced by PE, whether the JAK2/STAT3 signalling pathway is involved in the inhibitory effect of PE on GLUT9 expression, and the precise mechanism still remains unclear and needs to be investigated in the future.

The other significant hazards of hyperuricemia lie in the complications it causes, among which hyperuricemia associated nephritis is the most prominent^[99-102]. In previous experiments, direct administration of UA stimulated JAK2 phosphorylation in the nucleus, followed by STAT3 phosphorylation^[19,20]. The phosphorylated STAT3 is then translocated to the nucleus, where it upregulates the expression of various cytokines, including SOCS3 and TNF- α ^[19,20,103,104]. These cytokines further activate the JAK2/STAT3 signalling pathway, establishing a positive feedback loop that enhances the inflammatory response^[105]. SOCS3, an important member of the SOCS family, also a protein factor that negatively regulates cytokine and growth-related factor signalling pathways, which can prevent excessive cellular responses and promote cell death/apoptosis, thereby exacerbating kidney injury^[106-110]. While TNF- α not only stimulates the release of neutrophils and monocytes, but also directly drives inflammatory cytokines (such as IL-6 and caspase-8), indirectly promoting cell death and participating in inflammatory immune responses and disease development^[111,112]. A multitude of natural plant compounds can target the JAK2/STAT3 signalling pathway to diminish the expression of inflammatory factors SOCS3 and TNF- α , and subsequently combat hyperuricemia associated nephritis, such as apigenin, quercetin, and rutin^[113]. Similar to these compounds, PE and its main component PI can alleviate

glomerular injury and inflammatory cell infiltration in mouse kidneys, and inhibit the JAK2/STAT3 signalling cascade in mouse kidneys and HK-2 cells, including its downstream regulatory factors such as SOCS3 and TNF- α . In this research, we found that PE and its main component PI, serve as novel JAK2 inhibitor, which can decrease the expression of SOCS3 in the kidneys of hyperuricemia mice and exert anti-inflammatory effects, rather than merely reducing UA.

Furthermore, the virtual molecular docking has gradually become a reliable method for assessing the safety of candidates. Zhang *et al.* validated the effectiveness of PI and β -lactase an enzyme through molecular docking^[114]. Molecular docking and ELISA studies confirmed the good binding activity between PI and JAK2, which may be the molecular basis for the inhibition of XDH, SOCS3, and TNF- α expression in *P. nigrum* to combat hyperuricemia. Notably, as the main ingredient of the most famous spice *P. nigrum* fruits, PI has been used as food for centuries, and there are few reports of PI-related toxicity in clinical practice, which further explains its safety^[57,115,116]. In addition to the beneficial effects mentioned in the introduction, reports have shown that PI can also serve as a bioavailability enhancer, maximizing the benefits of other medicinal compounds in treatment^[117,118]. Therefore, as with natural products with pharmacological diversity, we should be carefully assessed with consideration for many compounding factors affecting the clinical outcome of pharmacokinetic interactions (e.g., dose, dosing regimen, genetic variation and species). What more, plant extracts promote intestinal UA excretion, which includes regulating gut microbiota and promote UA transporters ABCG2 as well as GLUT9, is becoming an effective measure for the treatment of hyperuricemia^[119-123]. Such as stevia residue and dendrobium officinalis^[123,124]. In this study, PE administration significantly reduced renal GLUT9 levels and promoted renal excretion in mice with hyperuricemia. Although 2/3 of UA is excreted through the kidneys, 1/3 of UA is still excreted through the gut^[62]. We need to further consider the impact of the gut, including the gut barrier, gut microbiota, and gut UA transporters.

In conclusion, this study revealed that PE and its main component PI administration effectively improved hyperuricemia in mice by reducing UA synthesis and promoting UA excretion. In addition, PE and its main component PI can improve hyperuricemia associated nephritis by inhibiting the activation of inflammatory signalling pathway. PE and its main component PI might be less toxic and more efficient as a nature medicine for the treatment of hyperuricemia (Fig. 8).

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript. Additional data related to this paper may be requested from the corresponding author upon request.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work. Wen-Long Sun is an Editorial Board Member of Food & Medicine Homology, but was not involved in the processing, peer review or decision making of this article.

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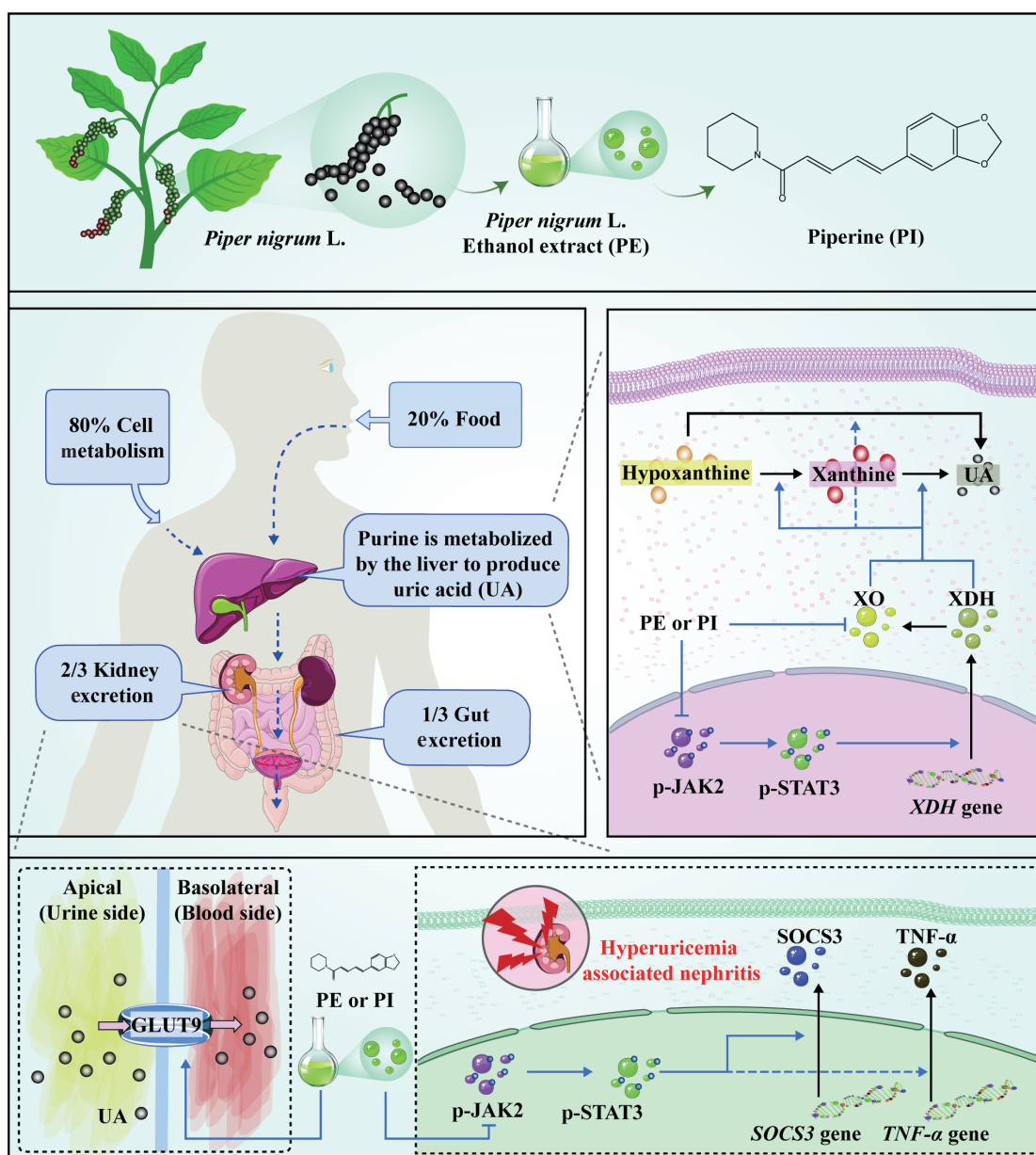


Figure 8 PE and PI suppress the JAK2-STAT3 signalling axis, resulting in the reduction of XDH expression and XO activity, thereby inhibiting hepatic and renal UA production. Meanwhile, PE and PI downregulate the expression of the renal urate transporter GLUT9, that facilitates UA excretion. Additionally, PE and PI can attenuate renal inflammation and preserve renal function by reducing SOCS3 and TNF- α expressions in the kidney.

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Author contribution statement

Nan-Chuan Li: Methodology, data curation. Mei-Ling Xin: Writing-original draft, data curation, methodology, visualization. Xin-Hua Song: Project administration, methodology. Hong-Yu Wang: Data curation, investigation. Fu-Yuan Yang: Resources, validation. Jing-Da Li: Writing-review & editing, supervision. Wen-Long Sun: Writing-review & editing, funding acquisition. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals of Shandong University of Technology and approved by the Animal Ethics Committee of Shandong University of Technology (YLY20210906, YLY20211103, YLY20220407).

Use of AI statement

None.

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