Puerarin inhibits inflammation and lipid accumulation in alcoholic liver disease through regulating MMP8

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Citation: Ying HU, Shuxian WANG, Lan WU, Kai YANG, Fan YANG, Junfa YANG, Shuang HU, Yan YAO, Xun XIA, Yixin LIU, Li PENG, Jihong WAN, Chuanpu SHEN, Tao XU, Puerarin inhibits inflammation and lipid accumulation in alcoholic liver disease through regulating MMP8, *Chinese Journal of Natural Medicines*, 2023, 21(9), 670–681. doi: 10.1016/S1875-5364(23)60399-1.

View online: https://doi.org/10.1016/S1875-5364(23)60399-1

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Hypoglycemic activity of puerarin through modulation of oxidative stress and mitochondrial function via AMPK
Puerarin inhibits inflammation and lipid accumulation in alcoholic liver disease through regulating MMP8

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Available online 20 Sep., 2023

[ABSTRACT] Alcoholic liver disease (ALD) is a growing global health concern, and its early pathogenesis includes steatosis and steatohepatitis. Inhibiting lipid accumulation and inflammation is a crucial step in relieving ALD. Evidence shows that puerarin (Pue), an isoflavone isolated from Pueraria lobata, exerts cardio-protective, neuroprotective, anti-inflammatory, antioxidant activities. However, the therapeutic potential of Pue on ALD remains unknown. In the study, both the NIAAA model and ethanol (EtOH)-induced AML-12 cell were used to explore the protective effect of Pue on alcoholic liver injury in vivo and in vitro and related mechanism. The results showed that Pue (100 mg·kg⁻¹) attenuated EtOH-induced liver injury and inhibited the levels of SREBP-1c, TNF-α, IL-6 and IL-1β, compared with silymarin (Sil, 100 mg·kg⁻¹). In vitro results were consistent with in vivo results. Mechanistically, Pue might suppress liver lipid accumulation and inflammation by regulating MMP8. In conclusion, Pue might be a promising clinical candidate for ALD treatment.

[KEY WORDS] Puerarin; MMP8; Alcoholic liver disease; Inflammatory; Lipid accumulation


Introduction

Alcohol consumption is a critical risk factor leading to alcoholic liver disease (ALD), which may induce huge economic, social, and clinical consequences. In 2016, it caused nearly 3 million mortalities worldwide and accounted for 5.3% of global deaths, according to the Global Status Report on Alcohol and Health (from World Health Organization in 2018) [1]. As the major cause of chronic liver diseases, ALD consists of a wide range of disorders, such as steatosis or fatty liver (asymptomatic ALD at the early stage), steatohepatitis, alcoholic hepatitis, cirrhosis (advanced ALD), and hepatocellular carcinoma (HCC) [2, 3]. Medical treatment options for ALD involve the use of glucocorticoids or pentoxifylline alone or in combination, which have been reported to reduce short-term mortality. Unfortunately, not all patients respond to glucocorticoids, and the long-term benefit is uncertain [4, 5]. Lipid metabolism and inflammatory response are closely related to ALD. Hepatic steatosis, the asymptomatic early ALD, is a reversible pathology, and mainly featured by an accumulation of lipid droplets in liver cells [6, 7]. The progression of ALD includes twice hits. The first hit refers to the lack of hepatocyte viability and the accumulation of triglycerides in the liver caused by ethanol (EtOH). The second hit includes lipid peroxidation and oxidative stress. Furthermore, based on the optimal microenvironment provided by the first hit, the second hit will induce the activation and proliferation of hepatic stellate cells, damage mitochondrion function, and lead to the pathological changes from fatty liver to inflammation and even liver fibrosis [8, 9]. Therefore, it is crucial to determine potential targets which suppress inflammation and...
lipid accumulation to reduce ALD.

Matrix metalloproteinases (MMPs) belong to zinc-containing endopeptidases that regulate cell-matrix composition and the processing of bioactive molecules. Under the pathological conditions, aberrant expression of MMPs were reported to induce chronic inflammation, cartilage degradation, and lipid disorder [10-12]. Emerging evidence has confirmed the pivotal activity of MMP8 in serum which acts as a marker in ALD progression [13-15]. MMP8 concentrations in alcohol-induced liver cirrhosis (stage C) patients based on the Child-Turcotte-Pugh criteria, were notably up-regulated compared with normal controls [14]. MMP8 is not only considered a regulator of lipid and glycerol metabolism through modulating related lipogenic genes [16], but also plays a vital part in inflammatory response [17]. In MMP-8-null mice, the influx of impaired leukocytes into the liver was clearly observed and LPS-induced and neutrophil-specific CXC chemokines were not released, resulting in better survival outcomes [18]. Therefore, the inhibitors of MMP8 may be an appropriate choice for inflammation and lipid cumulation in ALD.

Puerarin (Pue) is an isoflavone derivative with crown expanding effect and the most effective component from the roots of Pueraria thunbergii and Pueraria lobata (Willd) Ohwi [19], which were traditionally used in China for the treatment of diverse disorders related to alcohol consumption such as alcohol-induced osteonecrosis [20] as well as anxiogenic effect due to alcohol withdrawal [21]. Moreover, multiple biological effects of Pue have also been confirmed such as anti-lipid accumulation [22] and anti-inflammatory effects [23]. In this study, we found that abnormally elevated MMP8 enhanced inflammation and lipid deposition in ALD, which was alleviated by Pue as a potential MMP8 inhibitor.

Materials and Methods

Materials and reagents

Puerarin (purity > 98%) was from Chengdu Derick Biotechnology Co., Ltd. (Chengdu, China). Silymarin (Sil) was bought from Sigma Chemical Company (St. Louis, MO, USA). EtOH and controlled liquid diets were provided by the TROPHIC Animal Feed High-Tech Co., Ltd. (Nantong, China). 4% Paraformaldehyde (PFA) (BL539A) was purchased from Biosharp (Shanghai, China). Aspartate aminotransferase (AST) commercial kit and alanine aminotransferase (ALT) commercial kit were bought from Nanjing Ji-ancheng Institute of Bioengineering (Nanjing, China). MMP8 (AF0219) monoclonal antibody was purchased from Affinity (USA). Phospho-p65 (p-P65) (Ser536) antibody (AF2006) was provided by Affinity (USA). P65 (BS3157) monoclonal antibody was provided by Bioworld (Nanjing, China). IL-6 (66146-1-lg), IL-1β (66737-1-lg) and TNF-α (60291-1-lg), SREBP-1c (66875-1-lg), and PPAR-α (66826-1-lg) polyclonal antibodies were from Proteintech (Wuhan, China). Anti-β-actin (TA-09) polyclonal antibody was bought from ZSGB-BIO (Beijing, China). The primers of MMP8, IL-1β, IL-6, TNF-α, PPAR-α, SREBP-1 and GAPDH were from Sangon Biological and Technological Company (Shanghai, China). ECL-chemiluminescent kit (P2100) was bought from New Cell & Molecular Biotech Co., Ltd. (Suzhou, China). RIPA Lysis and Extraction Buffer, Primary Antibody Dilution Buffer, PMSF and Tween were bought from Beyotime (Hangzhou, China). F12 medium was bought from HyClone (Beijing, China). Trizol and Lipofectamine™ 2000 were bought from Invitrogen (Carlsbad, CA, USA). Opti-MEM and Fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). Triton X-100 (ST797), Hematoxylin Staining Solution (C0107), Citrate Antigen Retrieval Solution (P0081), BSA (Fatty Acid & IgG-Free, BioPremium), Liquid Blocker Super PAP Pen (FPPP06B), DAB Horseradish Peroxidase Color Development Kit (DBA)(P0202), and PVDF (0.2 μm) (FFP24) were from Beyotime (Shanghai, China). Tris-buffered saline (TBS) was bought from Boster (Boster, China). The rabbit two-step detection kit (PV9001) was from ZSGB-BIO (Beijing, China). Matrix metalloproteinase-8 inhibitor (MSI) (Apigenin-7-glucuronide, CAS: No.29741-09-1) was purchased from MedChemExpress (New Jersey, USA). The SYBR® Green Premix Pro Taq HS qPCR Kit was bought from Accurate Biology (Hunan, China).

Animals and treatment

C57BL/6J male mice (6–8 weeks, weighing 20–22 g) were bought from the Experimental Animal Center at Anhui Medical University. The mice were fed with standard diet and drinking water in the laboratory, and maintained in a controlled environment (50% ± 5% relative humidity, 20–25 °C, and a dark/light cycle of 12/12) for seven days before experiments. The NIAAA model [24] was established within 16 days, which requires acclimatization to liquid diet for 5 days, a modeling period for 10 days, and a single gastric gavage of alcohol (20%) and sample collection for 1 day. Briefly, 48 mice were randomly divided into six groups (n = 8). The Control (Con) group: the mice were fed with controlled liquid diet in the modeling period and gavaged with a single gavage of 0.9% normal saline (5 g kg⁻¹) on the last day. The EtOH group: the mice were fed with liquid diet containing EtOH (5% V/V) in the modeling period and given a single binge treatment with 20% EtOH (5 g kg⁻¹) on the last day. The low-dose Pue group: in additional to the same modeling method in the EtOH group, Pue (25 mg kg⁻¹) was administered by gavage. The medium-dose Pue group: in additional to the same modeling method in the EtOH group, Pue (50 mg kg⁻¹) was administered by gavage. The high-dose Pue group: in additional to the same modeling method in the EtOH group, Pue (100 mg kg⁻¹) was administered by gavage. The Sil group: in additional to the same modeling method in the EtOH group, Sil (100 mg kg⁻¹) [20] was administered by gavage. Then, 9 h after the last EtOH gavage, the mice were euthanized and liver tissues and blood samples were harvested. The animal experiments were conducted following the ethical code for use of the laboratory animals at Anhui Medical University. The present study was in accordance with the ethical standards of the Helsinki Declaration and approved by
the Health Medical Research Ethics Committee in Anhui Medical University (20190246).

**Serum aminotransferase activity**

After centrifugation at 5000 r min⁻¹ for 10 min, the activities of serum AST and ALT were evaluated by commercial kits.

**Cell culture**

The murine non-transformed hepatocyte cell line AML-12 was preserved in our laboratory (School of Pharmacy, Anhui Medical University). The cells were cultured with F12 supplemented with 10% FBS and 1% penicillin and streptomycin in an incubator (37 °C and 5% CO₂). To establish a cell model of ALD, the cells were stimulated with EtOH for 24 h. Furthermore, the cells were exposed to various concentrations of Pue, according to the experimental protocols.

**Screening of MMP8 inhibitor concentrations**

AML-12 cells (2 × 10⁵ cells per well in a 6-well plate) were treated with M8I (MMP8 inhibitor) before EtOH stimulation for 1 h, and total protein was extracted for Western blot analysis to detect the expression of MMP8.

**Western blot**

The total protein extracted from frozen liver samples and AML-12 cells were separated by SDS-PAGE and blotted onto PVDF membrane. After incubation with TBST containing skim milk (5%) at room temperature for 2 h, the PVDF membrane was incubated at 4 °C with primary antibodies diluted in primary antibody dilution buffer at an appropriate concentration overnight. Of note, the primary antibodies dilution ratios were β-actin (1 : 1000), SREBP-1c (1 : 1000), PPAR-α (1 : 1000), p65 (1 : 1000), P65 (1 : 1000), TNF-α (1 : 1000), IL-6 (1 : 1000) and IL-6 (1 : 1000). Then, the membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies) at 1 : 10 000 dilution in TBST at room temperature for 1 h, before washing in Tween 20 + TBST (0.075%) for three times (10 min). Finally, the proteins were observed with the ECL-chemiluminescent kit and quantified by Image J software.

**Total RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Frozen liver samples and cultured cells were homogenized using TRIzol reagent (Invitrogen). After reverse transcription, quantitative mRNA expression was performed using the SYBR Green Master Mix, according to the manufacturer’s instructions. Fold changes in the mRNA levels of target genes were correlated with constant control GAPDH. The PCR primer sequences were designed as follows (Table 1).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP8</td>
<td>F: 5'-TCAACCTGTTTCTCGTGCTGC-3' R: 5'-TCAACCTGTTTCTCGTGCTGC-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5'-TGACAAGCTCTAGCCACGTCCAGCAG-3' R: 5'-TTGCCTTTGAGATCATGAGCGAGG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: 5'-GAAGATGCCACTCACCAGACAG-3' R: 5'-AAGTGCAATCATCGTGTGACCA-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: 5'-GGTTAATTGACAGCTGGTGGTGGG-3' R: 5'-GTGCAATTGAGTTGCACTGGT-3'</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>F: 5'-GTCAATCAGCAGACACCTTTCCTCC-3' R: 5'-CTGCCCCACATATTCGACACCTC-3'</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>F: 5'-GGAGCCATGGATTGCACATT-3' R: 5'-GGCCCGGGAAGTCACTGTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-GTCGTGAGTATGTAGTCTGGAGATCT-3' R: 5'-ACAGTCTTCTGAGTGGCAGTGA-3'</td>
</tr>
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**Immunofluorescence (IF)**

The nuclear localization of p65 was detected by IF. After fixed with 4% paraformaldehyde for 30 min, AML-12 cells were permeablized with 0.3% Triton X-100 and then blocked with 10% BSA. Then, the cells were incubated with primary antibodies (p65, 1 : 200) at 4 °C overnight. Then, after incubation with secondary antibodies for 2 h, the cell nucleus was stained with DAPI. Images were captured with a fluorescence microscope.

**Cell proliferation assay**

The proliferation of AML-12 cells was tested by Cell Counting Kit-8 (CCK-8) assay. AML-12 cells were seeded in a 96-well plate, while sterile PBS was added in the outer wells. One day after cell adhesion, the cells were stimulated with Pue (0, 6.25, 12.5, 25, 50, and 100 μmol·L⁻¹) for 24 h. Then, 10 μL CCK-8 solution was added to each well. After incubation another 1 h, the absorbance was recorded at a wavelength of 450 nm.

**Molecular docking**

The potential drug targets of Pue were predicted and screened using the DS 2017 software. The structure of Pue was drawn using the Chemdraw 2017 software and imported into the DS software. After the ligand was prepared and the energy was minimized, the potential targets of Pue were virtually screened through the Ligand Profiler program in the DS software. The results were analyzed according to the fit value of the binding between the ligand and each protein (the higher the fit value was, the better Pue matched the corresponding protein). The target corresponding to the protein was the likely target of the compound. Then, Pue and MMP8 protein were combined through CDOCKER molecular docking for further analysis.

**Statistical analysis**

The above experiments were repeated at least three times. All data are expressed as the mean ± SEM. The differences between groups were compared by the one-way ANOVA.
GraphPad Prism version 9.0 was used to analyze statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001, indicated significant difference.

**Results**

**Pue suppressed the progression of ALD in vivo**

To explore the effect of Pue on C57BL/6J mice fed with EtOH liquid diet ad libitum, ALD model mice were established before intraperitoneal injections of Pue for 10 days (Fig. 1A). The detailed food intake and body weight changes of the mice within 16 days are shown in Figs. 1B and 1C. The liver tissues were collected for histopathological observation by H&E staining. The results showed that intercellular space dilatation, hepatic cords, and fat vacuoles were progressively increasing in ALD mice with several inflammatory cell infiltration (Fig. 1D). Furthermore, Pue treatment (100 mg·kg⁻¹)
reduced the liver weight to body weight ratio and the levels of serum ALT and AST in a dose-dependent manner (Figs. 1E–1G). In sum, these findings demonstrated that Pue exerted effective hepatoprotective effect against alcoholic liver injury.

Pue attenuated inflammation in the liver of ALD mice

In order to further evaluate the effect of Pue on EtOH-induced ALD, the expression of inflammation cytokines was measured. According to the results of Western blot and qRT-PCR analysis, compared with the Con group, inflammation cytokines such as TNF-α, IL-6 and IL-1β were induced by EtOH, but then reversed by Pue treatment (Figs. 2A and 2B). Thus, Pue exhibited anti-inflammatory effect on ALD.

Pue attenuated lipid accumulation in the liver of ALD mice

To further evaluate the anti-lipid accumulation effect of Pue on EtOH-fed mice, Oil red O staining, Western blot and qRT-PCR were used. The results of Oil Red O staining revealed that administration of Pue at 50 and 100 mg·kg<sup>−1</sup> reduced the generation of lipid droplets compared with the EtOH group, and the protective effect of Pue (100 mg·kg<sup>−1</sup>) was comparable to Sil (100 mg·kg<sup>−1</sup>) (Fig. 3A). The same results were confirmed by biochemical detection of TG and TC in the serum and liver homogenate (Figs. 3B and 3D). In addition, compared with EtOH treatment, Pue significantly reduced the protein and mRNA levels of adipogenesis-related protein SREBP-1c, but elevated the expression of lipid metabolism-related protein PPAR-α (Figs. 3C and 3E). These results indicated that Pue attenuated lipid accumulation and protected against EtOH-induced liver injury.

Pue attenuated EtOH-induced inflammation and lipid accumulation in AML-12 cells

To establish an in vitro ALD model, AML-12 cells were stimulated by EtOH to detect the effect of Pue on EtOH-induced inflammation and lipid accumulation. First, Western blot results indicated that the protein levels of SREBP-1c, IL-1β, TNF-α and IL-6 were remarkably up-regulated in AML-12 cells treated with EtOH (25, 50, 100, 200, and 400 mmol·L<sup>−1</sup>) and peaked at 100 mmol·L<sup>−1</sup> EtOH (Figs. 4A and 4B). Then, the effect of Pue on EtOH-induced cell injury was further confirmed by CCK8 assay. As shown in Fig. 4C, treatment with less than 100 μmol·L<sup>−1</sup> of Pue for 24 h did not obviously affect the viability of AML-12 cells. Furthermore, Western blot results showed that the levels of lipid accumulation and inflammation indexes significantly increased by EtOH (100 mmol·L<sup>−1</sup>), which however were restored by Pue (6.25, 12.5, 25, and 50 μmol·L<sup>−1</sup>) in a concentration-dependent manner, and the optimal effect was seen at 50 μmol·L<sup>−1</sup> (Fig. 4D). Based on these results, EtOH (100 mmol·L<sup>−1</sup>) and Pue (50 μmol·L<sup>−1</sup>) were selected for subsequent experiments. According to the results of Oil Red O staining and intracellular TG levels, Pue treatment (50 μmol·L<sup>−1</sup>) resulted in a great quantity of lipid droplets and high levels of TG in EtOH-induced AML-12 cells (Figs. 4E and 4F). Similarly, qRT-PCR analysis also confirmed that the mRNA levels of IL-6, TNF-α, IL-1β, and IL-6 were remarkably up-regulated in AML-12 cells treated with EtOH (25, 50, 100, 200, and 400 mmol·L<sup>−1</sup>) and peaked at 100 mmol·L<sup>−1</sup> EtOH (Figs. 4A and 4B). Thus, Pue exhibited anti-inflammatory effect on ALD.

![Western blot analysis for the protein and mRNA levels of IL-6, IL-1β and TNF-α in AML-12 cells. (A) qRT-PCR analysis for the protein and mRNA levels of IL-6, IL-1β and TNF-α in AML-12 cells. Data are expressed as means ± SD (n = 3).](image_url)
α, IL-1β and SREBP-1c were induced by EtOH (100 mmol·L⁻¹) but suppressed by Pue (50 μmol·L⁻¹). The mRNA levels of PPAR-α decreased by EtOH, but increased by Pue (Fig. 4G). Therefore, the above results indicated that Pue exerted marked anti-inflammatory and anti-lipid accumulation effect in EtOH-induced AML-12 cells.

Pue attenuated lipid accumulation and inflammation of ALD by inhibiting MMP8

To explain the interaction between Pue and MMP8 at the molecular level, molecular docking was applied. M8I (MMP8 inhibitor), apigenin-7-glucuronide, was used as the positive control. The molecular docking results showed that Pue might be an ideal MMP8 inhibitor as M8I for it occupies the ATP binding pocket of MMP8 and thereby inhibits its activity (Figs. 5A and 5B), which was also confirmed by ELISA (Fig. 5C). Then, to explore whether MMP8 participates in regulating inflammation and lipid deposition in EtOH-induced AML-12 cells, the protein expression of related pro-
The results indicated that M8I suppressed the levels of SREBP-1c, TNF-α, IL-6 and IL-1β in EtOH-stimulated AML-12 cells in a dose-dependent manner (Fig. 5D), while the levels of PPAR-α markedly increased (10 μmol·L⁻¹). Moreover, the inhibitory effect of Pue on the above indexes was similar to that of M8I (Fig. 5E), while the effect of M8I was not further strengthened (Fig. 6). These results indicated that Pue attenuated lipid accumulation and inflammation of ALD by inhibiting MMP8 as the inhibitor of MMP8.

Pue suppressed the NF-κB signaling pathway through inhibiting MMP8

Previous studies proposed that MMP8 activated the NF-κB signaling pathway and thus acted as a proinflammatory mediator in microglia [27]. To explore the regulatory effect of MMP8 on inflammation response in ALD, the total expression of P65 in the ALD model liver was measured. Western blot results showed that Pue inhibited the phosphorylation of P65 in a dose dependent manner (Fig. 7A). Furthermore, the immunofluorescence results indicated that M8I significantly promoted the transfer of P65 into the nucleus, which was not reversed by Pue (Fig. 7B). Similarly, compared with the M8I group, the phosphorylation of P65 and the total expression of P65 protein did not increase or was further inhibited in the M8I + Pue group (Figs. 7C and 7D). Overall, these results suggested that Pue suppressed the NF-κB signaling pathway through inhibiting MMP8.

Discussion

ALD is currently one of the most prevalent liver dis-
orders around the world, and its death toll accounts for about 25% of cirrhosis. In 2017, approximately 123 000 000 individuals globally and 2 100 000 in the US suffered from alcohol-associated cirrhosis [28], and individuals with excessive alcohol use (> 3 per day for men and > 2 drinks per day for women) are at risk of ALD. ALD begins with hepatic steatosis from triglycerides accumulation in hepatocytes, and later alcoholic hepatitis and fibrosis [5]. Liver damage caused by alcohol, including two hits. First, alcohol increased the concentration of reactive oxides by increasing oxidative stress and inducing fat accumulation in the liver, acting as the first hit. Then, “the second hit” was completed by oxidative stress-related lipid peroxidation and inflammatory cytokines, which hit on the hepatocytes in the fatty liver, causing the following inflammation, necrosis, and fibrosis [29]. Although there are no satisfactory therapeutic approaches currently available for the treatment of ALD, natural products often play an adjuvant role in clinical therapy through inhibition of inflammatory and lipid deposition [30].

As a natural flavone glycoside isolated as a main constituent from the Chinese traditional herb Radix Pueraria, Pue is a well-known herbal remedy with hepatoprotective properties [31]. Previous studies demonstrated that Pue exerted beneficial activities on EtOH-induced liver diseases [32, 33]. For example, Wang et al found that disorders in lipid metabolism and oxidative injury in mice exposed to EtOH and microglia were reversed by Pue [34]. In addition, inflammatory responses and toxicity of acetaldehyde were also relieved by Pue in chronic EtOH-induced liver injury rats [35]. Although the protective effect of Pue on ALD has been individually investigated, the potential specific mechanism remains unclear. Based on the understanding of the possible hepatoprotective
Fig. 6  Pue attenuated lipid accumulation and inflammation of ALD by inhibiting MMP8. Western blot analysis for the levels of SREBP-1c, PPAR-α, TNF-α, IL-6 and IL-1β in EtOH-stimulated AML-12 cells with the presence of Pue and M8I. Data are expressed as the means ± SD (n = 3).

effect, this study used the chronic and binge ethanol feeding model to examine the efficacy of Pue on ALD. The results indicated that ALD mice presented disorganized hepatic cords, with an increased number of fat vacuoles and inflammatory cell infiltration, which could be reversed by Pue. Similarly, in vitro experiments also confirmed that Pue effectively inhibited the high expression of lipogenic-related protein SREBP-1c and inflammatory-related protein TNF-α, IL-1β and IL-6. These data suggested that Pue acts an effective role in preventing lipid deposition and inflammation in ALD.

To further explore the specific mechanism of Pue in ALD, the potential targets of Pue were virtually screened through the Ligand Profiler program in the DS software. The results demonstrated that the fitting value between MMP8 and Pue was the highest, and Pue was well matched MMP8 protein. MMP8, released from the polymorphonuclear neutrophil, was also called neutrophil collagenase-2 or collagenase. It has been proven that degrading HDL component apolipoprotein A-I and extracellular matrix components, and cleaving the triple-helical type I collagen, other ECM and non-ECM substrates, are essential in regulating inflammation [36, 37] and lipid metabolism [38]. Salminen et al. pointed out that MMP8 is expressed in various cell types in atherosclerotic lesions, and MMP8-deficient mice had significantly lower serum triglyceride levels and larger HDL particles [38]. In this study, we observed that the activity of MMP8 was activated by EtOH, but attenuated by Pue. Besides, M8I or Pue separately used decreased the levels of SREBP-1c, TNF-α, IL-1β and IL-6 in AML-12 cells induced by EtOH. However, the above protective effect could not be further strengthened by Pue + M8I. These observations indicated that liver protection mechanisms might be largely contributed to the anti-inflammatory and anti-lipid deposition effect of Pue, which related to the targeting of MMP8, leading to an improved understanding of molecular recognition.

In addition, MMP8 contributes to the regulation of the NF-κB signaling pathway. In lipoteichoic acid (LTA)-stimulated rat primary astrocytes, the activity of the NF-κB signaling pathway was significantly inhibited by M8I and yielded antioxidant and anti-inflammatory effects [37]. Generally, NF-κB is bound to a family of inhibitory proteins (IκBs) and sequesters in the cytoplasm. After activating, the NF-κB unit p65 was separated from IκBs and then translocated to the nucleus, whereas it modulated inflammatory gene expression [40]. In the ALD mice, Western blot results showed that Pue inhibited the phosphorylation of p65 in a dose dependent manner, and peaked at 100 mg·kg⁻¹. The results of IF in AML-12 cells induced by EtOH further indicated that M8I
significantly promoted the transfer of P65 into the nucleus, which could not be reversed by Pue. Similarly, compared with the M8I group, the phosphorylation of P65 and the total protein expression of P65 did not increase or was further inhibited in the M8I + Pue group. As shown in Fig. 8, our results indicated that Pue targeted and inhibited MMP8, and suppressed the NF-κB signaling pathway mediated by MMP8.

**Conclusion**

In summary, this study demonstrates that Pue exerts an effective liver protective effect in ALD mice. Consistent with the results in vivo, Pue inhibited the expression of pro-inflam-
Fig. 8  The specific mechanism by which Pue inhibits the inflammation and lipid accumulation in ALD by regulating MMP8.

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Cite this article as: HU Ying, et al. / Chin J Nat Med, 2023, 21(9): 670-681.