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# Puerarin, isolated from *Pueraria lobata* (Willd.), protects against hepatotoxicity via specific inhibition of the TGF-β1/Smad signaling pathway, thereby leading to anti-fibrotic effect

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ABSTRACT

Recently, the TGF-β1/Smad signaling pathway has been investigated in the pathogenesis of hepatofibrosis, and pharmacological treatment of liver fibrosis targeted this pathway to determine its contribution to the inhibition of fibrotic development. Importantly, ethnopharmacology-derived *Pueraria lobata* has been reported to effectively reverse the fibrotic process in the liver. In the present study, we performed dimethylnitrosamine (DMN)-induced liver fibrosis in rats to assess the benefits of puerarin (PR), which was isolated from *Pueraria lobata* (Willd.), on ECM-derived hepatocytes associated with the TGF-β1/Smad pathway. Our results showed that the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), laminin (LN), type III precollagen (PCIII) and type IV collagen (CIV) were significantly reduced by PR treatment, while hepatic homogenates showed decreased levels of hydroxyproline (Hyp) and collagen I (Col I). Masson's trichrome staining indicated that the DMN-induced liver fibrosis was alleviated. In addition, the protein expression levels of transforming growth factor-β l (TGF-β l), smad2, smad3, α-SMA and TIMP-1 were downregulated specifically by PR treatment, whereas the protein expression levels of smad7 and MMP-1 were upregulated. Furthermore, we evaluated the PR-mediated inhibitory effect on TGF-β1-treated proliferation and activation in a rat liver stellate cell line (HSC-T6). These data resulted in inhibition of the cell growth of HSC-T6 in a dose-dependent manner and a reduction in TβRI, smad2 and smad3 expressed proteins in the presence of PR on TGF-β1-treated HSC-T6 cells, while smad7 levels were downregulated. Taken together, these findings identify a unique effect for PR-regulation of the TGF-β1/Smad pathway in blocking fibrotic development and provide a promising strategy for hepatofibrosis treatment.



## Introduction

Hepatic fibrosis is a pathophysiological progression associated with excessive extracellular matrix (ECM) deposition in response to chronically damaged liver tissue (Bataller and Brenner 2005; Lee and Friedman 2011). The formation of hepatic fibrosis in correlative fibrous connective tissue is a reparative or reactive process (Anty and Lemoine 2011). Hepatic stellate cells (HSCs), the major cell type involved in liver fibrosis, play a pivotal role in ECM remodeling and hepatic fibrosis development. When the liver is lesioned, HSCs are activated and play subsequent roles in proliferation, contractility and chemotaxis. Activated HSCs can also secrete collagen constituted scar tissue, which can result in cirrhosis (Sarem et al. 2006; Li et al. 2013a,b). Although the precise pathogenic mechanisms remain unclear, several medications have been used to treat clinical liver fibrosis. Silymarin therapy with the effective dosage can mitigate the symptoms related to hepatopathy. Silymarin has also been reported to protect against cytotoxicity in hepatocytes (Saller et al. 2001; Fehér and Lengyel 2008). In addition, multiple drug therapies have been used to treat hepatic fibrosis with some success, including toxicity reduction and efficacy reinforcement (Sun and Li 2000; Lian et al. 2005; Zhang 2012). Phytomedicine refers to traditional medicine in which the herbs used are scientifically validated and are categorized as natural medicines. In

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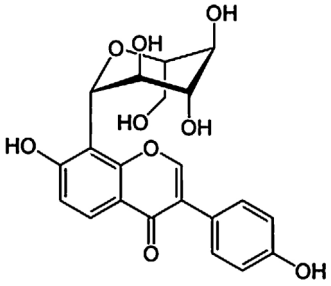


**Fig. 1.** The structure of puerarin (PR).

traditional Chinese medicine (TCM), *Pueraria lobata* has traditionally been used as a remedy for alcoholism and hangovers in China, where it is thought to detoxify the liver and alleviate its subsequent symptoms (McGregor 2007; Penetar et al. 2011). *Pueraria lobata* or its extracts have been promoted as a supplement or therapy for a diversity of symptoms, including headaches, alcoholism, intestinal obstruction, diarrhea, angina pectoris and hypertension (Lukas et al. 2005; Wong et al. 2011; Yang et al. 2010). In addition, our previous studies found that puerarin (PR) resulted in attenuation of hepatofibrosis via a reduction in ECM deposition in rats with $CCl_4$-induced hepatic fibrosis (Li et al. 2013a,b). However, PR-targeted TGF-β1/Smad signaling pathway involved in hepatofibrosis progression has remained largely unexplored.

Liver fibrosis is also tightly associated with the TGF-β1/Smad signaling pathway at multiple levels. Thus, we proposed that PR might effectively exert resistance to collagenation by mediating this signaling pathway. In this study, we examined the expression of collagenic components using *in vivo* and *in vitro* studies involved in the PR-mediated regulation of the TGF-β1/Smad signaling pathway, and demonstrated the anti-fibrotic effect of PR and its underlying mechanism.

## Materials and methods

### *Materials*

PR (purity > 99.0%; the structural formula is shown in Fig. 1) was provided by the Department of Pharmaceutical Chemistry, Guangxi Medical University. Silymarin (purity > 95.0%) was purchased from the Xi'an Rongsheng Biotechnology Co., Ltd. (Xi'an, China). Analytical grade dimethylnitrosamine was purchased from the Chengdu Kelong Chemical Reagent Factory (Chengdu, China). Other chemicals and reagents were labeled as described below.

### *Acute toxicity testing*

Ten Sprague Dawley (SD) rats were fasted for 16 h and the animals' body weight was recorded pre-treatment. A specific dose of PR (final concentration: 500 mg/ml; 6000 mg $kg^{-1}$ body weight) was orally administered to each rat and changes in the behaviors, vital signs and mortality were observed.

### *Animals and drug intervention*

Six-week-old healthy male SD rats weighing approximately 200 ± 20 g were purchased from the Medical Laboratory Animal Center of Guangxi Medical University, China (Certificate No. SCXK-Gui-2009-0002). The study was conducted according to protocols approved by the Institutional Ethical Committee of Guilin Medical University. All of the rats were housed under controlled conditions with a temperature of 25 ± 2 °C, relative humidity of 60 ± 10%, room air changes 12–18 times/h, and a 12-h light/dark cycle. The study was conducted in accordance with the U.S. guidelines (NIH publication #85-23, revised in 1985) for laboratory animal use and care.

To screen the fibrotic process, the rats were randomly divided into two groups: control group and hepatic fibrosis model group. Hepatic fibrosis animals intraperitoneally received 0.6 ml $kg^{-1}$ (for a period of 1 week) and 1.2 ml $kg^{-1}$ (for a period of 5 week) 0.5% DMN (dissolved in distilled water) twice a week for 6 consecutive weeks. The control animals received an equivalent volume of saline twice a week. Two rats from model group and one rat from control group were randomly chosen, and a pathological examination was performed to monitor the formation of hepatofibrosis at week 2, 4 and 6, respectively.

Rats were randomly assigned into six groups: Group I ($n$ = 10): normal rats were administered normal saline and used as normal controls; Group II ($n$ = 20): fibrotic rats were administered normal saline and used as model controls; Group III ($n$ = 20): fibrotic rats were administered Silymarin (SM, 400 mg $kg^{-1}$ body weight) by oral gavage once a day for 4 weeks; Group IV ($n$ = 20): fibrotic rats were administered PR (200 mg $kg^{-1}$ body weight) by oral gavage once a day for 4 weeks; Group V ($n$ = 20): fibrotic rats were administered PR (400 mg $kg^{-1}$ body weight) by oral gavage once a day for 4 weeks; Group VI ($n$ = 20): fibrotic rats were administered PR (800 mg $kg^{-1}$ body weight) by oral gavage once a day for 4 weeks.

### *Analysis of functional liver enzymes*

The serum levels of alanine transaminase (ALT), aspartate transaminase (AST), hyaluronic acid (HA), laminin (LN), type III precollagen (PCIII) and type IV collagen (CIV) were measured using commercially available kits (Wuhan Boster Bio-Engineering Limited Company, Wuhan, China) and performed according to the manufacturer's protocol.

### *Tissue sampling*

After 4 weeks of PR administration, all of the rats were anesthetized with 20% urethane, and the serum samples were collected and stored in tubes containing heparin. Liver specimens were dissected and washed immediately with ice-cold saline to remove excessive blood. In addition, some of the specimens were properly conserved for subsequent tests. Other specimens were fixed in 10% formalin solution for subsequent staining procedures.

### *Measurement of liver index*

Liver index = (Liver weight/Body weight) × 100%,
Thymus index = Thymus weight/Body weight,
Spleen index = Spleen weight/Body weight.

### *Pathological examination*

Liver tissue samples ($n$ = 10, each group) were sectioned, and Masson's trichrome staining procedure was performed. The pathological changes were monitored and imaged under a light microscope (Olympus, CX41, Japan). In addition, the morphological changes of the liver lesion were evaluated using the following criteria: score 0, absent fibrosis; score 1, presence of fibrosis; score 2, mild fibrosis; score 3, moderate fibrosis; and score 4, severe fibrosis (Li et al. 2013a,b).

### *Estimation of liver collagenic parameters*

Hepatic tissues were washed with normal saline to remove excess blood and clots. The tissues were then homogenized on ice with Tris–HCl (6 mmol/l) containing 3 mmol/l EDTA (pH 7.2). Homogenates were centrifuged at 1500 × $g$ for 10 min at 4 °C.