Toxicology Letters 217 (2013) 102–110

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

# Protective effect of genistein isolated from *Hydrocotyle sibthorpioides* on hepatic injury and fibrosis induced by chronic alcohol in rats

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## HIGHLIGHTS

- Genistein significantly decrease oxidative stress and production of inflammatory.
- Genistein effectively inhibit fibrogenic mediators and activation of HSCs.
- Genistein could be a possible new therapeutic approach for liver fibrosis.

## ARTICLE INFO

*Article history:*
Received 27 October 2012
Received in revised form
16 December 2012
Accepted 17 December 2012
Available online 26 December 2012

*Keywords:*
Genistein
*Hydrocotyle sibthorpioides*
Alcohol
Liver fibrosis

## ABSTRACT

This study examined the effect of genistein isolated from *Hydrocotyle sibthorpioides* on chronic alcohol-induced hepatic injury and fibrosis. Rats underwent intragastric administration of alcohol (5.0–9.5 g/kg) once a day for 24 weeks. A subset of rats were also intragastrically treated with genistein (0.5, 1 or 2 mg/kg) once a day. Genistein significantly decreased the plasma alcohol concentration, inhibited the activities of alanine and aspartate aminotransferases and decreased levels of inflammatory mediators, including interleukin 6, tumor necrosis factor-α and myeloperoxidase, *via* down-regulation of nuclear factor-κB. Moreover, genistein effectively inhibited collagen deposition and reduced pathological tissue damage as determined by hepatic fibrosis biomarkers, such as total hyaluronic acid, laminin, and type III collagen. Mechanistically, studies showed that genistein markedly reduced lipid peroxidation, recruited the anti-oxidative defense system, inhibited CYP2El activity, promoted extracellular matrix degradation by modulating the levels of tissue inhibitor of matrix metalloproteinase-1 and matrix metalloproteinase-2, induced HSC apoptosis by down-regulating B-cell lymphoma 2 mRNA, and inhibited the expression of α-smooth muscle actin and transforming growth factor $\beta_1$ proteins. In conclusion, genistein exerts a preventative effect to ameliorate developing liver injury and even liver fibrosis induced by chronic alcohol administration in rats.



## 1. Introduction

Alcohol-related liver disease is a major cause of morbidity and mortality worldwide, and the clinical syndrome of alcoholic liver disease (ALD) carries a particularly poor prognosis, such as liver cirrhosis1 or hepatocellular carcinoma (Kawaratani et al., 2011). Chronic alcohol ingestion is known to be associated with defective gut motility that indirectly results in an elevated level of endotoxin in the liver (Szabo and Bala, 2010). Furthermore, the major metabolic product of alcohol, acetaldehyde activates hepatic stellate cells (HSCs), triggering inflammatory and fibrogenic signals, such as tumor necrosis factor-α (TNF-α), interleukins (IL-6) and transforming growth factor-β1 (TGF-β1) (Mello et al., 2008). Numerous strategies have been employed to develop anti-fibrotic therapies, including inhibition of hepatic stellate cells (HSCs) (Weng et al., 2007), interference in the secretion of extracellular matrix (ECM) and cytokines (Iimuro and Brenner, 2008; Seki and Brenner, 2008), and prevention of oxidation through use of antioxidants (Comporti et al., 2008). Despite these efforts, controversy and uncertainty remain with respect to the ideal treatment option for liver fibrosis.

Herbal medicines have long been used as therapy of liver fibrosis. And many are now being collected and examined in an attempt to identify possible sources of anti-liver fibrosis (Zou et al., 2008). Natural compounds, because of their structural diversity, provide a good opportunity for screening for anti-liver fibrosis agents.

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0378-4274/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2012.12.014

An example of a traditional herb that is often used in popular folk medicine in China is Apiaceae *Hydrocotyle sibthorpioides* Lam., which is often used for treating immune disorders and liver diseases. Genistein isolated from *H. sibthorpioides* has several health benefits and thus has attracted the medical and research professionals. It was argued in previous studies that the beneficial effects of genistein were associated with its antioxidant effect (Hsieh et al., 2010; Ji et al., 2011; Valsecchi et al., 2011). Treatment with genistein reduced acute liver damage induced by carbon tetrachloride ($CCl_4$) (Kuzu et al., 2007). Furthermore, genistein inhibited the growth of HepG2 cells and induced apoptosis (Chodon et al., 2007), and it improved liver function and attenuated non-alcoholic fatty liver disease in a rat model of insulin resistance (Mohamed Salih et al., 2009). Based on these reports, it would be of great interest to determine the effects of genistein on hepatic fibrosis. In the present study, we evaluated the therapeutic effects of genistein on alcohol-induced liver fibrosis in rats. In addition, we investigated the underlying mechanism using histopathological analysis, measurement of serum enzymes and identification of cytokines.

## 2. Materials and methods

### 2.1. Chemicals

*H. sibthorpioides* was purchased from Nanning Qianjinzi Chinese Pharmaceutical Co. Ltd. (Nanning, China). Voucher specimen (HSL2011062639) was identified by Q.F. Huang in the First Affiliated Hospital of Guangxi Traditional Chinese Medicine University and deposited in the herbarium of Department of Pharmacology of Guangxi Medical University.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) and catalase kits were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Total hyaluronic acid (HA), laminin (LN), and type III collagen terminal peptide (PC-IIINP) kits were obtained from Beijing Furui Bioengineering Research Company (Beijing, China). Interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) kits were purchased from Wuhan Boster Bio-engineering Co. Ltd. (Wuhan, China).

### 2.2. Preparation of genistein

The powder of dried plant of *H. sibthorpioides* (8 kg) was extracted with 80 L 95% ethanol by filtration. The solvent was evaporated under a vacuum to obtain 127.6 g crude extract, which was extracted with petroleum ether at 60–90 °C. The petroleum ether (62.4 g) was then subjected to chromatography on a silica gel column (200–300 mesh, Yantai, PR China; Ø10 cm × 300 cm) eluting with a gradient mixture of $CHCl_3$ and ethyl acetate (0–100% ethyl acetate, 1500 ml each fraction). The second fraction yielded a yellow powder after concentration, which was purified by preparative HPLC to produce compound (23.5 mg). Its structure was elucidated on the basis of physicochemical properties and spectral data: mp. 295.7–296.3 °C; IR (KBr, $\nu$/cm$^{-1}$) $\delta$: 3411, 1652, 1614, 1570, 1309, 1043, 789; ESI-MS $m/z$: 269[M−H]$^+$; $^1$H-NMR(300 MHz, DMSO-$d_6$) $\delta$: 12.95 (1H, s, $C_5$-OH), 10.89 (1H, s, $C_7$-OH), 9.60 (1H, s, $C_{4'}$-OH), 8.34(1H, s, $C_2$-H), 7.37 (2H, *d*, *J* = 8.4 Hz, $C_{2'}$-H, $C_{6'}$-H), 6.82 (2H, *d*, *J* = 8.4 Hz, $C_{3'}$-H, $C_{5'}$-H), 6.40 (1H, *d*, *J* = 1.9 Hz, $C_8$-H), 6.22 (1H, *d*, *J* = 1.8 Hz, $C_6$-H). The results showed that the compound is genistein, with its molecular formula being $C_{15}H_{10}O_5$. The compound was normally stored at 4 °C. It was dissolved in distilled water and diluted with physiologic saline for test in animal.

### 2.3. Animals and treatments

Male SPF-Wistar rats, weighing 180 ± 10 g, were provided by the Experimental Animal Center of Guangxi Medical University (Guangxi, China). The research was conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University (approval no.: 2011080618). All 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.

After a period of one week, the animals were divided into six groups of consisting of 15 rats per group. Rats in Group I served as the normal control and were given saline only; and Group II served as the genistein control, and the rats were orally administered 2.0 mg/kg genistein. Animals in Groups III–VI were given intragastric alcohol infusions to induce liver fibrosis. The doses of alcohol was increased gradually according to the method of Zhang et al. (2010): 5.0 g/kg/day from 1 to 4 weeks, 7.0 g/kg/day from 5 to 8 weeks, 9.0 g/kg/day from 9 to 12 weeks, and 9.5 g/kg/day from 13 to 24 weeks. Group III served as the alcohol-induced liver fibrosis model. In addition to alcohol, rats in groups IV -VI were also orally administrated genistein daily. The details of the treatments are as follows:

Group I: received the same volume of saline.
Group II: received 2.0 mg/kg genistein.
Group III: received alcohol.
Group IV: received alcohol + 0.5 mg/kg genistein.
Group V: received alcohol + 1.0 mg/kg genistein.
Group VI: received alcohol + 2.0 mg/kg genistein.

At the end of 24 weeks, animals were fasted overnight and then anesthetized with ketamine hydrochloride (30 mg/kg b.w., i.v.) prior to euthanasia. Blood samples were collected into heparinized tubes (50 U/ml). Liver samples were dissected out and washed immediately with an ice-cold saline to remove as much blood as possible. One part of the liver sample was immediately stored at −80 °C for future analysis. The other portion of the liver sample was fixed in a 10% formalin solution for histopathological analysis.

### 2.4. Determination of plasma alcohol concentration

The blood alcohol level was determined using a commercial kit (Changzhou SEO Biotechnologies Inc. Changzhou, China). This enzymatic test for alcohol utilizes the coenzyme nicotinamide adenine dinucleotide (NAD) and alcohol dehydrogenase (ADH). The formation of NADH can then be measured quantitatively by the increase in absorbance at 340 nm.

### 2.5. Estimation of hepatic alcohol metabolizing enzyme activities

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) were measured in liver homogenate according to the protocol established in previous studies (Kaur et al., 2012; Modig et al., 2002).

### 2.6. Assay of CYP2E1 enzyme activity

CYP2E1 enzyme activity was determined according to our previous studies (Lin et al., 2012). In brief, the livers were perfused with ice-cold 0.15 M KCl and homogenized in a homogenizer with 4 vol. (w/v) of 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 0.01 mM phenylmethylsulphonyl fluoride. Hepatic microsomal fractions were obtained by differential centrifugation. The microsomal fractions were used to determine CYP2E1-specific oxidative activities. Aniline hydroxylase was determined by measuring p-aminophenol formation activities, and the microsomal protein levels were determined using the Bradford method with bovine serum albumin as the standard (Banni et al., 2011). All of the assays were run in triplicate. CYP2E1 was detected immunochemically, as in a previous study (Lee et al., 2008).

### 2.7. Estimation of AST and ALT activities

Serum levels of ALT and AST were measured using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions.

### 2.8. Assays of plasma IL-6 and TNF-α and liver myeloperoxidase activity

Plasma IL-6 and TNF-α levels were determined using an ELISA according to the manufacturers's instructions. Myeloperoxidase (MPO) activity was measured according to the method of Yoshida et al. (2011). Tissue was homogenized in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammoniumbromide. The supernatant obtained after centrifugation was then mixed with 10 mM phosphate buffer (pH 6.0) and 1 ml of 1.5 mM *o*-dianisidine hydrochloride containing 0.2 mM $H_2O_2$. The change in absorbance at 450 nm was recorded for each sample. MPO activity was expressed as μmol of the oxidized product formed/min/mg protein using the extinction coefficient of 10,062 M$^{-1}$ cm$^{-1}$. The protein content was determined using bovine serum albumin as the standard.

### 2.9. Assay for nuclear factor-κB (NF-κB)

Nuclear factor-κBp65 DNA-binding activity in nuclear extracts of hepatic tissue samples was evaluated to measure the degree of NF-κB activation (Khan et al., 2011). The ELISA was performed following the manufacturer's protocol (NF-κBp65 Transcription Factor Assay Colorimetric, Invitrogen Corporation Carlsbad, CA, USA).

### 2.10. Estimation of antioxidant enzyme and lipid peroxidation

Liver tissue was washed with physiological saline to remove any blood and then homogenized on ice with Tris–HCl (5 mmol/L containing 2 mmol/L EDTA, pH7.4). Homogenates were centrifuged at 1000 × *g* for 15 min at 4 °C. The supernatants were used immediately for the SOD, GSH-Px, GSH-Rd and catalase assays. All of these enzymes were evaluated according to our previously established methods (Huang et al., 2011). Lipid peroxidation in the liver was determined by measuring the level of MDA, an end product of lipid peroxidation, using a thiobarbituric acid method (Janero, 1990). The level of hepatic MDA was expressed as μmol/g protein.