Protective Effect of Trillium Tschonoskii Maxim Saponin on CCl4-induced Acute Liver Injury of Rats through Apoptosis Inhibition

Journal: Canadian Journal of Physiology and Pharmacology

Manuscript ID: cjpp-2016-0228.R1

Manuscript Type: Article

Date Submitted by the Author: 27-May-2016

Complete List of Authors: Wu, Hao; Hubei University for Nationalities, College of Science and Technology
Qiu, Yong; Hubei University for Nationalities, College of Medicine
Shu, Ziyang; Wuhan Sports University, College of Health Science
Zhang, Xu; Wuhan Sports University, College of Health Science
Li, Renpeng; Hubei University for Nationalities, College of Science and Technology
Liu, Su; Affiliated Hospital of Hubei University for Nationalities
Chen, Longquan; Hubei University for Nationalities, College of Medicine
Liu, Hong; Hubei University for Nationalities, College of Medicine
Chen, Ning; Wuhan Sports University, College of Health Science

Keyword: Trillium tschonoskii Maxim (TTM), liver injury, hepatomegaly, inflammatory factor, apoptosis
Manuscript type: Original paper

**Protective Effect of *Trillium Tschonoskii* Maxim Saponin on CCl₄-induced Acute Liver Injury of Rats through Apoptosis Inhibition**

Hao Wu¹,²,#, Yong Qiu²,#, Ziyang Shu³, Xu Zhang³, Renpeng Li¹, Su Liu⁴, Longquan Chen², Hong Liu²,* Ning Chen³,*

¹College of Science and Technology, Hubei University for Nationalities, Enshi 445000, China
²College of Medicine, Hubei University for Nationalities, Enshi 445000, China
³Hubei Key Laboratory of Sport Training and Monitoring, College of Health Science, Wuhan Sports University, Wuhan 430079, China
⁴Affiliated Hospital of Hubei University for Nationalities, Enshi 445000, China

**Running title:** TTM against liver injury

#These authors contributed equally to this project.

*Corresponding author:
Ning Chen, PhD, Professor of Biochemistry
College of Health Science, Wuhan Sports University, Wuhan 430079, China
Tel: 86-27-67846140
Fax: 86-27-67846140
E-mail: nchen510@gmail.com

Hong Liu, Professor of Pharmacology
School of Medicine, Hubei University for Nationalities, Enshi 445000, China
Tel: 86-718-8437479
Fax: 86-718-8437479
E-mail: 2548449651@qq.com

https://mc06.manuscriptcentral.com/cjpp-pubs
Abstract

In order to explore hepatoprotective role and underlying mechanisms of TTM, 36 rats were randomly divided into control, CCl₄-induced liver injury model, and DDB and low, moderate and high-dose TTM treatment groups. After CCl₄-induced model establishment, the rats from DDB and TTM groups were administrated with DDB at 0.2 g/kg·d and TTM at 0.1, 0.5 and 1.0 g/kg·d, while the rats from control and model groups were administrated with saline. Upon 5 days treatments, all rats were sacrificed for determining serum ALT and AST levels and liver index, examining histopathological changes in liver through HE and TUNEL staining, and evaluating TNF-α and IL-6 mRNA expression by RT-PCR, and Caspase-3, Bcl-2 and Bax expression by Western blot. Results indicated that CCl₄ could induce acute liver injury and abnormal liver function in rats with obvious hepatomegaly, increased liver index, high ALT and AST levels, up-regulated TNF-α and IL-6, and overexpressed Bax and Caspase-3. However, DDB and TTM could execute protective role in CCl₄-induced liver injury in rats through reducing ALT and AST levels, rescuing hepatomegaly, down-regulating inflammatory factors and inhibiting hepatocyte apoptosis in a dose-dependent manner. Therefore, TTM has obvious protective role in CCl₄-induced liver injury of rats through inhibiting hepatocyte apoptosis.

Key words: *Trillium tschonoskii* Maxim (TTM), liver injury, hepatomegaly, inflammatory factor, apoptosis.
Introduction

Liver plays an important role in metabolism and detoxification in human body. It is also one of the organs sensitive to a series of stimuli such as trauma, viral infection, autoimmune disease and other exogenous materials including drugs, alcohol and toxins, thus resulting in its acute or chronic damage (Shi et al. 2014; Wang et al. 2007). Severe acute liver injury could result in life-threatening clinical problems such as jaundice, serious blood coagulation disorders and high death rate (Bernal et al. 2010). At the same time, the long-term accumulated liver injury also can lead to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC), so it is very important for the prevention of liver injury (Dong et al. 2016). Although there is an increasing drug demand for the protection of the liver, modern medicine is still lack of reliable liver-protective drugs (Lu et al. 2016), and severe acute liver injury is still a great challenge in the field of clinical medicine (Auzinger and Wendon 2008).

During long application history in China, Chinese herbs or natural products have obvious treatment efficacy for a series of diseases with little side effects and low cost (Kou and Chen 2012; Kou et al. 2013; Kou et al. 2015; Zhang et al. 2014). Therefore, more and more people are exploring a novel strategy for the prevention and treatment of liver diseases through Chinese herbs (Akhter et al. 2015; Yan et al. 2015).

Trillium tschonoskii Maxim (TTM), a pharmacologically important medicinal plant containing steroidal saponins and alkaloids from Liliaceae, is widely distributed in central and western China (Li et al. 2005; Zhang et al. 2013). Its dried rhizome and root have medicinal roles in promoting blood circulation and stanching bleeding, detoxification, tranquilizing and allaying excitement, so that it is usually used for the treatment of
hypertension, neurasthenia, dizziness, headache, traumatic injury and bleeding as well as a series of liver disorders (Fu 1992). TTM is honored as one of four magical herbs from Tujia nationality due to its obvious curative effects for a series of diseases. However, as it’s a kind of medicinal plant from the minority in China, its clinical application is limited in the regions of Tujia and Miao nationalities without extension all over the China.

According to previous reports, saponins from several herbs are known to induce apoptosis in cancer cells and are proposed to be promising modulators of drug resistance. Paris saponin VII extracted from *T. tschonoskii* can reverse multidrug resistance of adriamycin-resistant MCF-7/ADR cells via P-glycoprotein inhibition and apoptosis augmentation (Li et al. 2014), inhibit metastasis by modulating matrix metalloproteinases in colorectal cancer cells (Fan et al. 2015b) and reduce migration and invasion in human A549 lung cancer cells (Fan et al. 2015a). The saponin TTB2 isolated from *T. tschonoskii* Maxim can exert anticancer effects and may be a potential candidate for the development of anticancer drugs (Huang and Zou 2015). The true incidence of phytotherapy-related hepatotoxicity and its pathogenic mechanisms are largely unknown (Tarantino et al. 2009). It is important to increase the awareness of both clinicians and patients about the potential dangers of herbal remedies. Meanwhile, the role of TTM in liver disorders and underlying mechanisms are also not fully understood. In order to further confirm its treatment efficacy and extend its clinical application, we established CCl₄-induced acute liver injury model in rats to explore its protective roles and underlying mechanisms for diseases associated with acute liver injury.
Materials and methods

The preparation of TTM saponin

TTM provided by Specimen Center of Hubei University for Nationalities was smashed after dried in 60 °C constant temperature oven, then sequentially immersed in 75% alcohol for 2 hours and completed ethanol reflux extraction for three times. The alcohol extract after filtration was concentrated to 1 g/mL for future use. The saponin in TTM extract was analyzed by high-performance liquid chromatography to reveal total saponin of 18.54%.

Animal grouping and drug administration

The healthy male rats with body weights of 180-220 g were ordered from Hubei Research Center of Laboratory Animals (License number: 2015-0018). All rats were randomly divided into 6 groups including control group, CCl₄-induced acute liver injury model group, biphenyl dimethyl dicarboxylate (DDB) group at the dose of 0.2 g/kg·d, and TTM groups at low, moderate and high dosages (0.1, 0.5 and 1.0 g/kg·d), respectively. Six rats were in each group. All rats were subjected to adaptive feeding with regular feeds for 1 week. The rats were subjected to diet restriction for 12 h prior to model establishment. The rats from the control group were subjected to subcutaneous injection of peanut oil at the dose of 5 mL/kg, while the rats from other groups were subjected to the administration of peanut oil mixed with 25% CCl₄ at the dose of 5 mL/kg (equal to 2 g/kg for CCl₄) for the establishment of acute liver injury model. After model establishment, the rats from treatment groups were administrated with DDB and TTM at the designed dosages through gavage twice a day with an interval of 12 h for 5 successive days. The diets of the rats were restricted except water after the last drug administration. Twenty-four hours later, the rats were
anesthetized with 10% chloralhydrate, and blood and liver tissue samples were harvested for analysis.

**Liver index determination**

The body weight of each rat was measured prior to being sacrificed. Similarly, the wet weight of rat liver was measured after sacrificed. The liver index = liver weight/rat body weight × 100%.

**Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level measurement**

Blood samples of the rats after last drug administration for 12 h were harvested in a common anticoagulant tube, and then subjected to the centrifugation at the speed of 1000 ×g for 5 min. The supernatant was collected, and then serum ALT and AST levels were determined by immunity transmission turbidity method using commercially available assay kits (Mindray Bio-medical Electronics Co., Ltd, Shenzhen, China) in an automatic biochemical analyzer (BS-800M, Mindray Bio-medical Electronics Co., Ltd, Shenzhen, China).

**Real-time polymerase chain reaction (RT-PCR) detection**

Approximately 0.1 g liver tissue was subjected to complete homogenate. Total RNA was extracted using Trizon reagent (Invitrogen, USA) following manufacturer’s instructions and then reversely transcribed into cDNA. The mRNA expression levels of IL-6 and TNF-α were determined through fluorescence quantitative RT-PCR. The forward and reversed primers for IL-6 were 5’-GCTCTCGCAAGAGACTTCC-3’ and 5’-GGTCTGTGTTGAGTTGTTAC-3’, respectively. The forward and reversed primers for
TNF-α were 5'GTTGATTGGTCCCAACAE3' and 5'GTCTTTGAGATCCATGCCE3', respectively. The forward and reversed primers for β-actin as the internal control were 5’-CAGATGGAGGGGCCGACTCATC-3’ and 5’-TAAAGACCTCTATGCCAACACGT-3’, respectively. The relative expression level of IL-6 or TNF-α was calculated by the ratio of IL-6 or TNF-α to β-actin.

**Histology and immunohistochemistry**

The small slice of liver tissue from each rat was subjected to the fixing by 4% paraformaldehyde, regular embedding by paraffin, sectioning, hematoxylin-eosin (HE) staining and Ki67 staining (Abcam, Cambridge, USA), and the slides were observed for conventional morphological evaluation under a light microscope (Nikon Eclipse TE2000-U, NIKON, Japan) and photographed at 100× magnification.

**Terminal-deoxynucleoitidyl transferase-mediated nick end labeling (TUNEL) staining**

The sections of liver tissues were analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the in situ cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. TUNEL-positive cells were captured under a light microscope (Nikon Eclipse TE2000-U, NIKON, Japan) and analyzed through Image pro-plus 6.0 software (Media Cybernetics Inc., MD, USA).

**Apoptosis-related protein expression evaluated by Western blot**

Approximately 0.1 g of liver tissue after homogenizing was lysed with RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of PMSF (Beyotime Institute of Biotechnology, Jiangsu, China) at the speed of 4000 ×g at 4 °C for 20 min. The collected
supernatant was added with loading buffer and boiled at 95 °C water batch for 5 min. Then, 20 µg total protein was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membrane. The membrane was blocked with 5% skim milk at room temperature for 3 h. The targeted protein on the membrane was probed with primary antibody such as Caspase-3, Bax or Bcl-2 (Cell Signaling Technology, USA, 1:1000) at 4 °C overnight. The membrane was then washed with TBS-T buffer for three times with 15 min in each time. Sequentially, the membrane was incubated with secondary antibody, HRP-labeled anti-rabbit IgG (Cell Signaling Technology, USA, 1:30000) for 2 h, and washed with TBS-T buffer for three times. Then, the chemiluminescence agent was incubated with the membrane in dark room for 2 min and the protein probed by primary antibody in the membrane was imaged. The β-actin was used as an internal reference. The data were analyzed by the software Quantity one 4.6.1 (Bio-Rad Software Inc., California, USA).

Statistical analysis

All data were expressed as mean ± standard deviation (M ± SD). All statistical analyses were conducted using SPSS19.0 software (SPSS, Inc., Chicago, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, USA) through ANOVA with post-hoc tests. The significant difference and very significant difference were considered at $P < 0.05$ and $P < 0.01$, respectively.

Results

TTM rescues CCl$_4$-induced acute liver injury
Serum ALT and AST levels, as the golden biomarkers for liver damage, are well-accepted indicators to evaluate the severity of liver injury (Shi et al. 2014). In order to validate the treatment efficacy of TTM on acute liver injury, we established the rat model with CCl₄-induced acute liver injury to explore the treatment efficacy of TTM. Compared with the control group, the levels of serum ALT and AST in the model group revealed an obvious increase ($P < 0.01$ and $P < 0.05$, respectively), indicating that the establishment of CCl₄-induced acute liver injury model was successful. In contrast, after treatment with DDB and TTM, the serum ALT levels of the rats treated with DDB and TTM at low, moderate and high dosages revealed a very significant reduction ($P < 0.01$); the serum AST levels of the rats treated with DDB and high-dose TTM exhibited a significant decrease ($P < 0.05$), indicating that TTM treatment can decrease serum ALT and AST levels in a dose-dependent manner (Figures 1A and 1B).

**TTM inhibits CCl₄-induced hepatomegaly and liver index**

In order to explore the effect of TTM on CCl₄-induced histopathological change of liver tissues in rats, we compared fresh liver tissues of the rats from various treatments. As shown in Figure 2A, the rats from CCl₄-induced model group revealed obvious hepatomegaly when compared with the rats from control group and treatment groups. Subjected to the treatments with DDB and TTM, the hepatomegaly of the rats exhibited an obvious inhibition when compared with that of the CCl₄-induced model rats, suggesting that both DDB and TTM have obvious protective roles in CCl₄-induced hepatomegaly.

Liver index reflects liver pathological changes and nutritional status in the body (Chen 1993). Based on the results of liver index, its significant difference between model group and
control group was observed \((P < 0.01)\), suggesting that \(\text{CCl}_4\) could result in hepatomegaly, while the treatment with BBD and TTM at various dosages could inhibit \(\text{CCl}_4\)-induced hepatomegaly in rats (Figure 2B).

In addition, the liver tissues were examined with HE staining. As shown in Figure 2C, normal liver lobule structure and liver cells in rats from normal control group were observed. In contrast, in the \(\text{CCl}_4\)-induced model group, diffused edema, necrosis and inflammatory cell infiltration in liver tissues were observed. However, the treatments with DDB and TTM at various dosages could rescue the liver damage with the significant reduction of diffused edema, necrosis and inflammatory cell infiltration in liver tissues when compared with the model group. Moreover, the protection of TTM on liver tissues revealed an obvious dose-dependent manner.

**TTM reduces inflammatory factors for \(\text{CCl}_4\)-induced acute liver injury**

\(\text{TNF}-\alpha\) and IL-6 are two important pro-inflammatory factors causing cell inflammation and death (Diehl 2000; Schmich et al. 2011; Tilg 2001). Then, we examined the mRNA expression of both factors through RT-PCR. Results indicated that acute liver injury induced by \(\text{CCl}_4\) could result in an obvious increase of \(\text{TNF}-\alpha\) and IL-6 \((P < 0.05, \text{ and } P < 0.01, \text{ respectively})\) (Figures 3A and 3B). On the other hand, after treatment with DDB and TTM at various dosages, the expression of \(\text{TNF}-\alpha\) and IL-6 in DDB and TTM groups revealed an obvious reduction. Therefore, TTM can alleviate inflammatory response of liver cells to execute the protective function for liver.

**TTM inhibits apoptosis of liver cells induced by \(\text{CCl}_4\)**
In order to further understand the protective mechanism of TTM for acute liver injury, the cell apoptosis in liver tissues from the rats subjected to CCl₄ induction and DDB and TTM treatments at various dosages were examined through TUNEL staining. Compared with normal control group, a large number of cell apoptosis in liver tissues due to the CCl₄ induction in the model group was observed (Figure 4A); in contrast, subjected to the treatments with DDB and TMM at moderate and high dosages, the apoptotic cell number revealed a significantly decrease and the reduced apoptosis of liver cells exhibited as a dose-dependent manner for TTM (Figure 4B). Similarly, compared with the normal control group, CCl₄-induced liver tissues revealed an obviously increased Caspase-3 and Bax and reduced Bcl-2; on the other hand, the treatments with DDB and TTM at moderate and high dosages could result in the down-regulation of Caspase-3 and the up-regulation of Bcl-2 when compared with those in CCl₄-induced liver injury model group (Figure 4C). Therefore, the protective role of TTM in acute liver injury should be correlated with the inhibition of liver cell apoptosis.

Discussion

The mechanisms of the diseases with acute liver injury are very complex. Current reports are mainly focused on oxidative stress, inflammation and apoptosis (Abouzied et al. 2016; Chan et al. 2014; Schmich et al. 2011). The CCl₄-induced acute liver injury rat model is well accepted, which can result in substantial liver cell damage and liver dysfunction (Masuda 2006; Weber et al. 2003). Once CCl₄ is absorbed by the body, it can activate liver cytochrome (P450) to generate trichloromethyl free radicals (CC₃•) and peroxide
trichloromethyl free radicals (CCl₃O₂•), thus attacking phospholipid molecules on cell membrane of liver, increasing cell membrane permeability, and finally leading to liver cell swelling, necrosis, and symptoms of acute liver injury (LeSage et al. 1999; Weber et al. 2003).

In the present study, we utilize CCl₄-induced rat model with acute liver injury to clarify the treatment efficacy of TTM and underlying mechanisms. The administration of TTM at various dosages for model rats with acute liver injury by lavage for five days can reduce the levels of serum ALT and AST and liver index, and down-regulate the expression of TNF-α and IL-6. HE staining revealed that TTM treatment could alleviate inflammatory necrosis of liver tissues, and present an obvious protective effect on liver damage.

It is well known that the liver has strong capacity of regeneration and recovery (Kim and Kim 2013). Therefore, we suspect TTM protection against acute liver injury may be due to the promotion from liver cell regeneration. In our study, compared with CCl₄ model group, the expression of Ki67 revealed an obvious increase after the treatment with DDB; in contrast, no obvious expression of Ki67 was observed in liver tissues from the rats treated with TTM at various dosages (Figure 5), suggesting that the protective effect of TTM on acute liver injury may be not correlated with the regeneration of liver cells.

Mitochondrial apoptotic pathway is one of the major pathways of apoptosis. Bcl-2 protein family plays a vital role in mitochondrial apoptotic pathway (Tischner et al. 2010). Bax, Bcl-2, and Bcl-2 protein family can promote or inhibit the release of cytokines and other apoptotic factors through changing the mitochondrial outer membrane permeability (Rosse et al. 1998). Cytochrome C could mediate the activity of Caspase-3, and the activated Caspase-3
can induce cell apoptosis (Cusack et al. 2013). In the present study, CCl₄-induced liver cell apoptosis could be obviously alleviated in the presence of TTM treatment under the examination by TUNEL staining; similarly, based on the analysis of Western blot, TTM could reduce the expression of cleaved Caspase-3 and Bax, and improve the expression of Bcl-2, thus further verifying the inhibitory role of TTM in apoptosis of liver cells for the protection of acute liver injury. However, apoptotic cell death can be also associated with mitochondrial membrane depolarization and cytochrome C release. The exposure of hepatocytes to hepatotoxic substances may result in increased ROS production and mitochondrial damage, and is balanced by the presence of antioxidant substances. Therefore, circulating levels of cytochrome C, gamma-glutamyl transferase, triglycerides and unconjugated bilirubin, as well as Bcl-2 in overweight/obese patients is highly associated with non-alcoholic fatty liver diseases (Tarantino et al. 2011a; Tarantino et al. 2011b). Whether TTM can inhibit the release of cytochrome C, alleviate the production of ROS, reduce the damage of mitochondria, and be benefit for non-alcoholic fatty liver diseases needs to be further explored. As the crosstalk between apoptosis and autophagy for maintaining cellular hemostasis in a series of diseases (Chen and Karantza 2011; Chen and Karantza-Wadsworth 2009; Fan et al. 2016), whether TTM can execute the prevention and treatment of CCl₄-induced acute liver injury through the functional status of autophagy also needs to be further explored.

In conclusion, TTM has a protective effect against acute liver injury through reducing liver cell apoptosis rather than promoting liver cell proliferation. This study provides the
experimental basis for TTM application in acute liver injury to extend its application fields as a novel drug against acute liver injury.
Acknowledgements

This study was financially supported by grants from Hubei Province Scientific Research Foundation (D200729005), Chutian Scholar Program from Education Department of Hubei Province, Hubei Superior Discipline Groups of Physical Education and Health Promotion and Innovative Start-up Foundation from Wuhan Sports University to NC. The authors would like to express their gratitude to the School of Basic Medical Sciences from Wuhan University for providing a partial experimental platform.

Conflict of interest

All authors have declared no conflict of interest associated with this project.
References


Figure legends

Figure 1. TTM rescued CCl₄-induced acute liver damage based on examination of ALT (A) and AST (B) levels. All data were expressed as Mean ± SD (n = 6). #P < 0.05 and ##P < 0.01 compared with the control group; *P < 0.05 and **P < 0.01 compared with CCl₄-induced acute liver injury model group.

Figure 2. TTM inhibited CCl₄-induced hepatomegaly (A) and reduced liver index (B) as well as alleviated CCl₄-induced cell necrosis or cell death (C) through the evaluation by HE staining.

Figure 3. TTM reduced inflammatory responses from CCl₄-induced acute liver injury in rats through the determination of mRNA expression of TNF-α (A) and IL-6 (B) by RT-PCR. All data were expressed as Mean ± SD (n = 3) from three independent experiments. #P < 0.05 and ##P < 0.01 compared with the control group; *P < 0.05 and **P < 0.01 compared with the CCl₄-model group.

Figure 4. TTM executed hepatoprotection through inhibited apoptosis. TTM could obviously reduce liver cell apoptosis of rats induced by CCl₄ based on the TUNEL staining (A) and its statistical analysis (B). Similarly, TTM could result in down-regulation of Bax and Caspase-3 and up-regulation of Bcl-2 based on western blot analysis (C). ##P < 0.01 compared with the control group; *P < 0.05 and **P < 0.01 compared with the CCl₄-model group.
**Figure 5.** Effect of TTM on liver cell regeneration in the presence of CCl₄ induction. Compared with the CCl₄-induced model group, DDB could promote liver cell regeneration but TTM at various dosages did not exhibit the promotion role in liver cell regeneration, which was evaluated by Ki67 staining (A) and corresponding statistical analysis (B). **P < 0.01** compared with the control group; **P < 0.01** compared with the CCl₄-induced model group.
Figure 1

86x64mm (300 x 300 DPI)
Figure 2

85x63mm (300 x 300 DPI)
Figure 3

86x64mm (300 x 300 DPI)
Figure 4

86x64mm (300 x 300 DPI)
Figure 5

86x64mm (300 x 300 DPI)