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Hepatoprotective Effects of Yintian Granule on Experimental Liver Injury in Mice

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ABSTRACT

Objective Yintian Granule (YTG), as a type of local preparation and applied for Chinese patent, is mainly composed of several traditional Chinese herbs used as both drug and food such as *Lonicera macranthoides*, *Gardenia jasminoides*, and *Asparagus cochinchinenis*, and has been reported to demonstrate the beneficial effects on human health in other researches. In this paper, the protective effects of YTG against experimental acute liver injury of mice were investigated to assess the value of this innovative Chinese herbal compound. **Methods** Carbon tetrachloride (CCl_4) and 50% ethanol were used respectively to induce the acute liver injury model in mice pre-administered with YTG. Lai's method was used to detect the level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum, Coomassie brilliant blue method was used for the determination of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content, and hematoxylin-eosin (HE) staining was used for the observation of liver histomorphometry. **Results** YTG significantly lowered the elevated ALT and AST levels, increased the SOD activity, decreased the MDA content, and inhibited the deterioration of liver. **Conclusion** YTG exerts protective effected against hepatocyte damage in mice induced by CCl_4 and 50% ethanol, respectively.

Key words

Hepatoprotection; liver injury; Yintian Granule

1. Introduction

Liver is a vital organ in human body and easy to be attacked by various factors, such as poisoning, metabolic disturbance, and circulatory disturbance. Liver injury is a process that involves variation in multiple kinases, free radical injury, oxidative stress, and lipid peroxidation (Sanjoy et al, 2013; Carmen et al, 2013). Recently, the usage of Chinese materia medica (CMM) on the treatment of liver disease is getting more and more attention worldwide. The idea of

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"preventive care" focusing on health has been recently brought in this field. Yintian Granule (YTG) is a prescription of Chinese potent medicine firstly produced by our team (Zhang hui et al, 2012). Application for national technology invention patent has been filed. YTG was made from *Lonicera macranthoides* Hand.-Mazz*, Gardenia jasminoides* Ellis, and *Asparagus cochinchinensis* (Lour.) Merr., all of which were Chinese herbal medicine, fitting for medicinal and edible use for thousands of years. YTG contains many major active ingredients, such as chlorogenic acid (CGA), geniposide, *A. cochinchinensis* glycoside, polysaccharides, etc. CGA exhibits potent anti-oxidant and anti-inflammatory activities, reduces blood pressure and liver injury, and improves the oxidation of low density lipoprotein (LDL) (Jennifer et al, 2007; Yun et al, 2012; Joao, 1994). Geniposide is from the fruit of *G. jasminoides* and is useful against hyperlipidemia and fatty liver. It can significantly inhibit liver fibrosis and possess anti- inflammatory and anti-angiogenic activities as well (Kojima et al, 2011; Ma et al, 2011; Koo et al, 2006). *A. cochinchinensis* glycoside was extracted from the roots of *A. cochinchinensis*. Recent studies have suggested that *A. cochinchinensis* glycoside could show anti-oxidant activity and protect mice liver against oxidative damage by inhibiting the hepatic microsomal lipid peroxidation (Chun et al, 2011; Jian et al, 2013; Xiong et al, 2011). The main objective of this study was to investigate the protective effects of YTG against liver injury. Carbon tetrachloride (CCl_4) and 50% ethanol were used to induce acute liver injury model (Handa and Sharma 1990; Tsukamoto et al, 1995) in mice pre- administered with YTG. The results suggested that YTG could be used in designing new anti-hepatic disease agents.

2. Materials and methods

2.1 Preparation of YTG

Dried *Lonicera macranthoides* Hand.-Mazz*, Gardenia jasminoides* Ellis, and *Asparagus cochinchinensis* (Lour.) Merr. were obtained from Southwest Hosptal (Chongqing, China) and the voucher specimens were deposited in Natural Medicinal Chemistry Research Center of this Institute. Water extract was obtained at 70 ^{o}C (1 h × 3 times). The extracts were filtered through Whatman No. 1 filter paper and the filtrate was concentrated under vacuum using a rotary evaporator (RE−2000A, Shanghai, China). The concentrated extracts were dried at 80 ^{o}C. TLC chromatogram was used for identification of the extracts, and the active constituents were tested by HPLC (Agilent−1200 series, USA).

The CGA standard (0.0108 g) was taken into 100 mL volumetric flask, added with 50% methanol, and shaked. The CGA standard solution (108 μg/mL) was obtained. Geniposide (0.0110 g) was accurately weighed and added with 50% methanol into 100 mL volumetric flask, mixed and then the geniposide standard solution (110 μg/mL) was obtained.

Dried YTG (0.3 g) was taken into 25 mL volumetric flask, added with 20 mL of 50% methanol, and the sample was treated by ultrasonic for 30 min and filtered through a 0.45 μm membrane filter, the filtrate was detected by HPLC.

A Platisil-ODS column (250 mm × 4.6 mm, 5 μm) was installed and the column temperature was kept at 25 ^{o}C. The mobile phase composed of water containing acetonitrile and 0.2% phosphoric acid, and the split ratio to HPLC was 2:8. The flow rate was 1.0 mL/min, injection volume was 20 μL, and the detection wavelength was 326 nm. The retention time of CGA which was identified in *Lonicera macranthoides* Hand.-Mazz by HPLC was 5.5 min (Figure 1A).

An Agilent Zorbax SB-C18column (250 mm × 4.6 mm, 5 μm). The mobile phase composed of water containing acetonitrile and deionized water, the split ratio to the HPLC was 1:9 and the column temperature was kept at 25 ^{o}C. A flow rate was 1.0 mL/min, injection volume was 20 μL and detection wavelength was 238 nm. The retention time of geniposide was 19.2 min (Figure 1B).

2.2 Animals and experiment protocol

Male ICR mice with body weight of 20.0 ± 2.0g were obtained from the Experimental Animal Center, Chongqing Medical University (China) and housed under controlled environment [(22 ± 2) ℃, 12 h light/dark cycle, diet and water *ad libitum*] in the Experimental Animal Center, College of Pharmaceutical Sciences & College of Chinese Medicine, Southwest University (Chongqing, China). All animal protocols were performed in accordance with international guidelines for care and use of laboratory animals.

2.3 Protective effects of YTG on experimental acute liver injury induced by CCl_4

Sixty ICR mice were randomly divided into normal control, model, positive control [0.01 g/kg bifendatatum (DDB)], low-, mid-, and high-dose (6.6, 13.2, and 26.4 g/kg, ig administration) YTG groups (n = 10). The mice in nomal control and model groups were ig administered equal volume of normal saline. All animals were ig administered for 14 d. After 2 h of the last treatment, all animals except those in the normal control group got acute experimental hepatic injury induced by 0.10% CCl_4 solution (20 mL/kg), fasting but water *ad libitum* for 16 h, and then all mice were sacrificed and the whole blood was collected from the suborbital vein under chloral hydrate anesthesia. The liver, kidney, and spleen were removed and weighed.

2.4 Protective effects of YTG on experimental acute liver injury induced by 50% ethanol

Sixty ICR mice were grouped using the method consistent with liver injury induced by CCl_4. All animals except tose in the normal control group were ig administered for 14 d. The mice in normal control group were ig administered with 50% ethanol solution in doses of 14 mL/kg body weight at intervals of 2 h. At the last treatment, the mice in all groups stopped feeding for 12 h, water *ad libitum*. All animals were sacrificed and the whole blood was collected

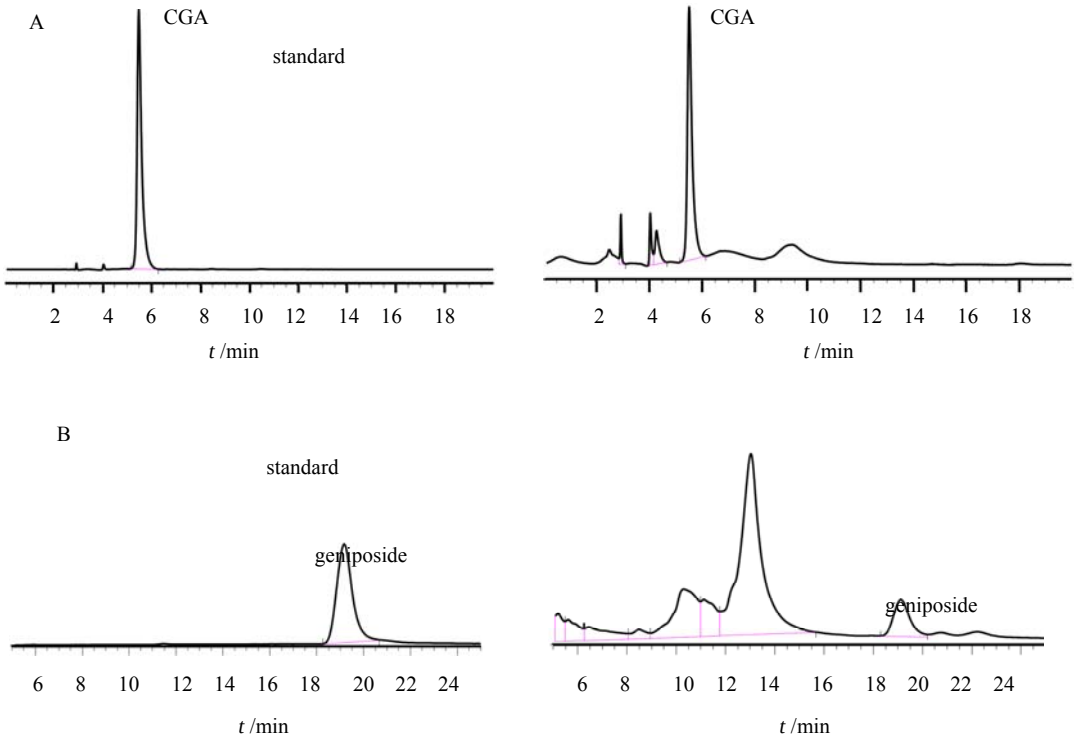


Figure 1 HPLC of CGA (A) and geniposide (B) from YGA

from the orbit under chloral hydrate anesthesia. The liver, kidney, and spleen were removed and weighed.

2.5 Serum biochemical analysis

Whole blood was kept at 4 °C and centrifuged at 1485 × *g* for 10 min to obtain serum. Enzyme Linked Immunosorbent Assay kit (Jiancheng Inst. Biotechnology, Nanjing, China) was used for the determination of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

2.6 Analysis on SOD activity and MDA content

Thin slices (300 mg) of the right lobe of the liver was taken and homogenized in cold normal saline to obtain 10% homogenate. Coomassie brilliant blue kits (Jiancheng Inst. Biotechnology, Nanjing, China) was used for the determination of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content.

2.7 Histological examination

The left lobe of the liver was excised, fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer system (PBS, pH 7.2) for 3 d at room temperature. Thin slices (0.3 cm × 0.5 cm) were taken and fixed in 10% formalin. The tissue samples were then dehydrated in a vacuum desiccator through a graded ethanol series, then defatted in xylene and embedded in paraffin, and sectioned in 5 μm-thickness slides. With the method of paraffin-embedded and stained with hematoxylin-eosin (HE), the slides were assessed for tissue damage and inflammation using inverted microscope (Ziss IX71, Germany).

2.8 Statistical analysis

All data were analyzed using SPSS version 18.0. Results were expressed as $\bar{x} \pm s$. The analysis of *t*-test was used for comparison among the groups. Results with $P < 0.05$ were considered as statistically significant.

3. Results

3.1 Effect of YTG on serum biochemical levels of ALT and AST in mice with liver injury induced by CCl_4

The serum levels of ALT and AST were significantly increased in the model group compared with those in the control group ($P < 0.01$). Compared with the model group, the serum levels of ALT and AST were significantly decreased in the three treatment groups ($P < 0.01$, Figure 2A).

3.2 Effect of YTG on serum biochemical levels of ALT and AST in mice with liver injury induced by 50% ethanol

Treatment with 50% ethanol resulted the increased serum biochemical levels of ALT and AST, respectively compared with the control group. On the basis of these results, mice which were treated with YTG had decreased serum ALT and AST levels compared with model group, especially those in high-dose (26.4 g/kg) YTG group were significantly decreased compared with the model group ($P < 0.01$, Figure 2B).

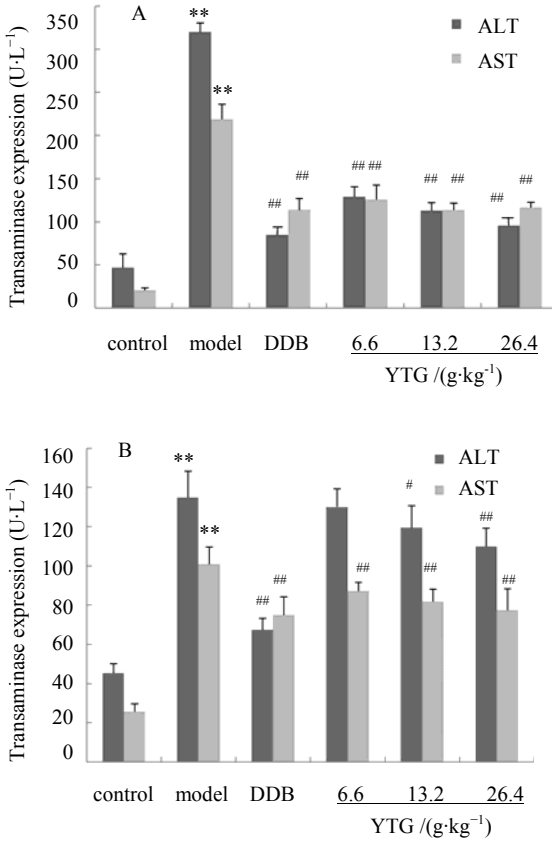


Figure 2 Effects of YTG on serum levels of ALT and AST in mice with liver injury induced by CCl_4 (A) and 50% ethanol (B) ($\bar{x} \pm s$, $n = 10$)

$^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ *vs* model group

3.3 Determination of SOD activity and MDA content in mice with liver injury induced by CCl_4

SOD activity was markedly decreased in the model group compared with that in control group ($P < 0.01$), and SOD activity was significantly increased in the treatment groups compared with the model group ($P < 0.01$, Figure 3A). Then, the content of MDA was increased in the model group compared with that in the control group. However, compared with the model group, the content of MDA was decreased in the treatment group (Figure 3B).

3.4 Determination of SOD activity and MDA content in mice with liver injury induced by 50% ethanol

SOD activity in liver of mice in model group significantly decreased ($P < 0.01$) compared with that in the control group. Administration of YTG could promote the SOD activity compared with the model group ($P < 0.01$, Figure 3C). Then, MDA content in liver markedly increased ($P < 0.01$) in the model group compared with the control group. Compared with the model group, the MDA content was significantly decreased in the treatment groups ($P < 0.01$). The results indicated that YTG could relieve the liver damage induced by 50% ethanol (Figure 3D).

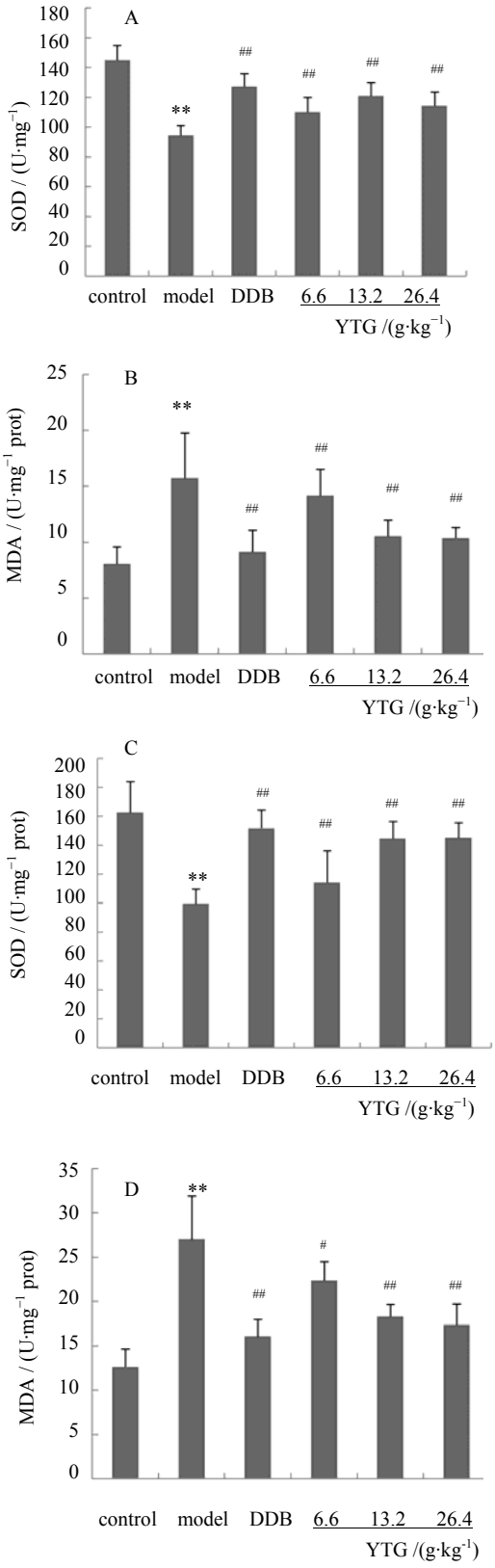


Figure 3 SOD activity (A) and MDA content (B) in mice with liver injury induced by CCl_4 and SOD activity (C) and MDA content (D) in mice with liver injury induced by 50% ethanol ($\bar{x} \pm s$, $n = 10$)

$^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ *vs* model group

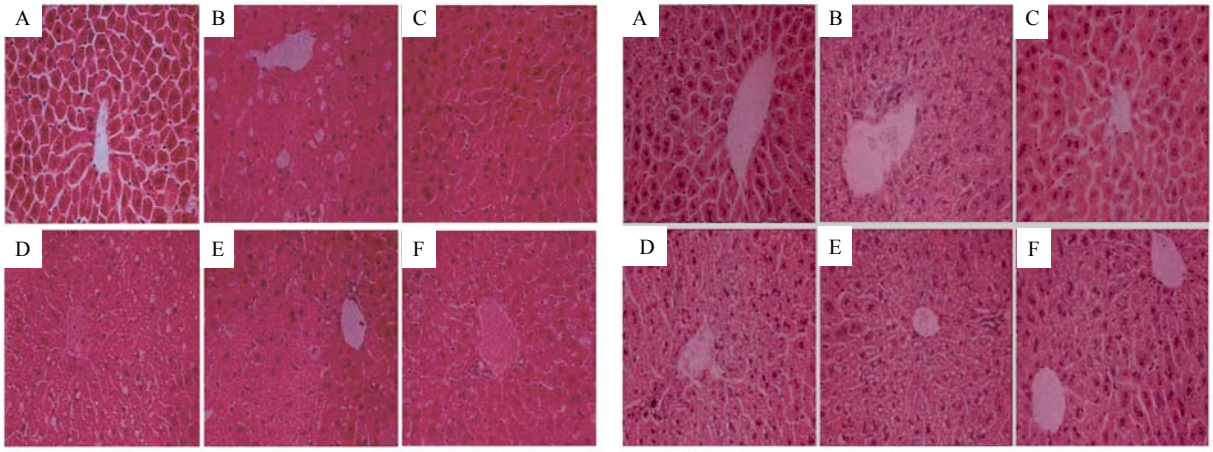
3.5 Effect of YTG on hepatic histopathological changes

3.5.1 Morphology of hepatic injury by CCl_4 in different groups

Compared with the control group (Figure 4a-A), the model group had ballooning degeneration necrosis, lobular cell disorded, and inflammatory cell infiltrated (Figure 4a-B); Hepatocytes in DDB group arranged in normal (Figure 4a-C); The tissue damage in low-dose YTG group (Figure 4a-D) reduced significantly, and hepatocytes arranged in better conditions than the model group; The cell necrosis in mid-dose YTG group (Figure 4a-E) was markedly reduced; The hepatocytes in high-dose YTG group arranged neatly and clearly, with cloudy swelling in few cells (Figure 4a-F).

3.5.2 Morphology of hepatic injury induced by 50% ethanol in different groups

Compared with the control group (Figure 4b-A), the mice in the model group had inflammatory cell infiltration and ballooning necrosis degenerated (Figure 4b-B). Liver tissue and hepatic lobules in DDB group were in normal (Figure 4b-C). Liver tissue damage reduced significantly in low-dose YTG group (Figure 4b-D). Hepatocytes in mid-dose YTG group (Figure 4b-E) arranged in better condition than the model group and cell necrosis were markedly reduced. Normal hepatic lobules in high-dose YTG group arranged neatly, with slightly cloudy swelling in few cells (Figure 4b-F).



(a) induced by CCl_4 (b) induced by 50% ethanol

Figure 4 Morphology of hepatic injury in control (A), model (B), DDB (C), low- (D), mid- (E), and high-dose (F) YTG groups (HE staining)

4. Discussion

Carbon tetrachloride (CCl_4) and 50% ethanol could induce the acute liver injury model in mice. Both of them could be metabolized by the microsomal cytochrome P450 metabolic epoxygenase. The metabolite chlorine radicals, trichloromethyl, and acetaldehyde could induce series lipid peroxidation, and then lead to cell structure such as cell membrane and cytoplasmic proteins disruption, followed by liver cell necrosis and fibrosis (Quan et al, 2002; Soren et al, 2005). ALT in the cytoplasm will be released into the blood when the cell membrane was broken down. AST will be further released from the liver mitochondrial when liver cell is under more severe damage. The liver injury could be detected by measuring the levels of transaminase in blood (Mosher et al, 2001). As the balance between pro-oxidant and anti-oxidant gets broken, it results in oxidative stress, cell injury, and liver disease (Mosher et al, 2001; Jose and Kaplowitz, 2005). The main active ingredients of YTG are chlorogenic acid (CGA), geniposide, and *A. cochinchinensis* glycoside. Many studies have indicated that CGA was the most abundant polyphenols in *L. macranthoides* and exerted the potent anti-oxidant and anti-inflammatory activities (Jennifer et al, 2007). CGA could markedly attenuate the elevated levels of ALT and MDA in liver tissues, and prevent the hepatic injury. In addition, it has been shown that CGA can scavenge superoxide radicals, hydroxyl radicals, and peroxynitrite *in vivo* (Graziani et al, 2004; Kono et al, 1997).

The liver protective effect of geniposide was associate with inhibiting P450 3A monooxygenase, increasing glutathione content (Ma et al, 2011), strengthening glutathione (GSH) activity, suppressing the expression of CYP2E (Kang et al, 1996), and increasing peroxisome proliferator- activated receptor-a (PPAR a) expression *in vivo* (Zhang et al, 2013). All of these benefits could be related to the increased SOD and decreased MDA in liver. ACE could reduce inflammatory cytokine production and decrease neutrophil-mediated myeloperoxidase (MPO) activity. Furthermore, ACE could evoke a significant inhibition of inflammatory reaction in mice which were induced by acetic acid (Lee et al, 2009).

In summary, this research showed that YTG has the protective effects on experimental acute liver injury and might be a good candidate for previntion of hepatic diseases.

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