Revista Brasileira de Farmacognosia 29 (2019) 637–643

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Protective effect of kinsenoside on acute alcohol-induced liver injury in mice

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ARTICLE INFO

Article history:
Received 23 February 2019
Accepted 18 June 2019

Keywords:
Kinsenoside
Alcoholic liver injury
CYP2E1
Oxidative stress

ABSTRACT

Anoectochilus roxburghii (Wall.) Lindl., Orchidaceae, is a Chinese medicinal plant which can be effective for some diseases such as hepatitis, nephritis, pneumonia. Its active ingredient is kinsenoside. The mechanisms of kinsenoside on the liver-protective effect have not been fully explored until today. The present study was aimed to investigate the protective effect and mechanism of kinsenoside on acute alcoholic liver injury. The protected activity of kinsenoside (10, 20 and 40 mg/kg) were investigated on acute alcoholic liver injury in mice. Male C57BL/6 J mice were fed with non-fat feed for 30 days and oral administrated 14 ml/kg bw of ethanol (50%) on the 31st day. The activities of serum aspartate aminotransferase, serum alanine aminotransferase, triacylglyceride and very low density lipoprotein were determined in serum. The hepatic levels of oxidative stress as glutathione, malondialdehyde were measured in liver homogenates. The levels of cytochrome P450 2E1 (CYP2E1) were measured by immunohistochemistry. Furthermore, histopathological observations were carried out on the separated livers of mice. It was suggested that the trends of acute hepatic injury and fatty degeneration induced by alcohol were reduced in the ethanol group after kinsenoside treatment. Compared to ethanol groups, triacylglyceride, malondialdehyde, very low density lipoprotein, reduced glutathione, serum alanine aminotransferase and serum aspartate aminotransferase levels of kinsenoside (20, 40 mg/kg) groups were decreased ($p < 0.05$). Meanwhile kinsenoside significantly decreased the level of protein CYP2E1. In conclusion, kinsenoside enhances antioxidant capacity of mice and antagonizes alcohol-induced lipid metabolism disorders. Besides, kinsenoside inhibits alcohol-caused hepatocyte apoptosis, reduces oxidative stress, and relieves hepatocyte death, which may be a mechanism of kinsenoside in the treatment of alcoholic liver.

Introduction

Alcohol products are widely consumed in the world, while alcohol abuse is one of the main causes of liver disease worldwide and has become a social problem. Acute drinking can cause physiological and psychological changes such as rapid glycogen consumption, hypoglycemia and acidosis. Acute alcohol liver injury(ALI)can result in related liver diseases and the generation of reactive oxygen species (ROS), which associated with the cytochrome P450 2E1 (CYP2E1) enzyme can influence lipid metabolism such as the index of triacylglyceride (TG), very low density lipoprotein (VLDL) and oxidative stress as glutathione (GSH), malondialdehyde (MDA) (Cao et al., 2015; Ding et al., 2012). Studies has shown that ALI has a relationship with the increase of alcohol-induced CYP2E1 activity (Chen et al., 2014). But there are no efficacious therapeutic modalities or drugs to pre-protect livers and slow down the progression of ALI (Wang et al., 2015). Herbal medicines and active monomers have attracted increasing attention because of their low level of toxicity, multi-target actions and other effects (Mathurin and Bataller, 2015). Kinsenoside (**1**) (KD) is a main component of *Anoectochilus koshunensis, A. roxburghii* (Wall.) Lindl., and *A. formosanus,* family Orchidaceae, and it has many protective effects on pleurodynia, diabetes, nephritis and hypertension (Shih et al., 2005). In this paper, the degree of hepatic tissue injury was observed by the determination of GSH, MDA, AST, ALT, TG, VLDL, and oil red O liver tissue slices in hepatic homogenization by using the model of acute alcoholic liver injury. Then the pharmacological mechanism was discussed using inflammatory results and CYP2E1 (a metabolic enzyme, related to alcohol-induced hepatocyte apoptosis and oxidative stress)

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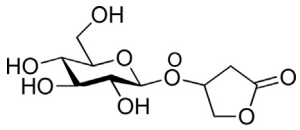
https://doi.org/10.1016/j.bjp.2019.06.006

expression analyzed by immune-histochemical detection and H&E staining method (Stewart et al., 2001; Chen et al., 2015; Ding et al., 2015).



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Materials and methods

Reagents

Kinsenoside (**1**) was extracted from *Anoectochilus roxburghii* (Wall.) Lindl., Orchidaceae, by 80% ethanol solution three times. After silicone column chromatography and ODS purification, the extract was identified by thin layer TLC with HPLC. HPLC-ELSD analysis detects the quality of kinsenoside from the dry extract of *A. roxburghii*. HPLC-ELSD was proceed by an Ultimate AQ-C18 at 30 °C and using 100% distilled water as the mobile phase. The evaporative light-scattering detector (ELSD) was performed at 2.5 l/min gas flow and 55 °C (Fig. 1). The glucosides component, Kinsenoside, was quantified by the standard substance which was obtained by repeated silica gel column chromatography. Finally, after HPLC-ELSD, and NMR identification, it is indicated that the purity of Kinsenoside was over 95%. Silymarin was supplied by Sigma (USA). Commercial kits used for determining the activities of triacylglyceride (TG), very low density lipoprotein (VLDL, ELISA method), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), malondialdehyde (MDA) and reduced glutathione (GSH) were provided by Nanjing Jiancheng Biological Engineering (Nanjing, China). The botanical origin of *A. roxburghii* from Fujian Yongan Huangnijia Co., Ltd. (Fujian, China) was authenticated carefully by Prof. Wang Yingming from Wuhan Institute of Botany, Chinese Academy of Sciences. The voucher specimen was deposited at Institute of Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, Wuhan, China.

Animals and drug treatment

The animal treatments for the study were approved by the institutional animal care committee of Tongji Medical College, Huazhong University of Science and Technology (IACUC Number: 856). We conducted the experiments in strict accordance with the relevant regulations on animal ethics (IACUC Number: 856). In total, 72 male C57BL/6 J mice (15–18 g, SPF) were supplied from Beijing Huafukang Biotechnology Co, Ltd (Beijing, China). All mice were arranged in specific pathogen fee (SPF) laboratory with a normal temperature (21.5 ± 0.5 °), humidity (52 ± 5%) and controlled room with a 12 h light-dark cycle for one week before experimentation. 72 C57BL/6 J mice were divided into normal control group, model group (ethanol group), silymarin group, low dose kinsenoside group (KDL group, 10 mg/kg bw), medium dose kinsenoside group (KDM group, 20 mg/kg bw), high dose kinsenoside group (KDH group, 40 mg/kg, BW), with twelve mice in each group. All animals were fed with pelleted food for 30 days. Every day at about 2:00 pm, the normal group and the ethanol group were given the same amount of distilled water, while other groups were given silymarin or kinsenoside (0.1 ml/10 g) by intragastric administration. The animals were weighed once a week and the dosages of the samples were adjusted for their weight. On the 31st day, after final treatment for 2 h, the hepatic injury model was caused by 50% ethanol (14 ml/kg, bw) via intragastric administration and the normal group was given the same amount of water distilled by 0.5% CMC-Na. All mice were fasted for 16 h and anesthetized with 10% chloral hydrate. Then the blood was collected, all serum samples were separated using a conventional centrifugation (4 °C, 1500×*g*, 10 min). Then, the animals were sacrificed to obtain the liver for histopathological and biochemical assays. The samples were placed in −80 °C refrigerator.

Measurement of VLDL and TG

The TG and VLDL levels of serum samples were detected by Bio-Tek synergy2 Multiscan Spectrum (Botten Instruments Co, Lctd, USA).

Liver function analysis

Uniformly, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities of serum samples were determined by Bio-Tek synergy2 Multiscan Spectrum (Botten Instruments Co, Ltd, USA).

Antioxidant and lipid peroxidation analyses

The liver tissue homogenates were prepared in ice-cold saline by using ultrasonic cell disruptor. After once centrifugation (624×*g*, 10 min, 4 °C), the hepatic levels of active substances (MDA, GSH) in the super natant were assayed by commercial detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturers' instructions. The antioxidant activity of kinsenoside was quantified as the amount of GSH using kits. The photometric value was detected at 405 nm. Levels of MDA and GSH were expressed as nmol/mg live protein weight.

Histopathological evaluation

The hepatic histology study was analyzed by Oil Red O staining. Portions of liver were fixed for histopathological evaluation according to the standard operating procedure. Another portion of liver were stored at −80 °C. Hepatic steatosis was determined by staining of 10 mm thick frozen sections with Oil Red O. The stained section was inspected at 400× magnification.

Hematoxylin-eosin Stain (H&E Stain) was used to demonstrate the normal components and general morphological structures of various tissues for comprehensive observation. The liver tissues were fixed with 4% paraformaldehyde and paraffin embedded sections were stained with hematoxylin and eosin, then they were observed using light microscopy at 200× magnification.

Immunohistochemical analysis

The tissue samples of liver of mice, preserved in 2.5% glutaraldehyde-polyoxymethylene solution, were dehydrated and embedded in paraffin following routine methods. After incubated with blocking buffer (normal goat serum) at room temperature for 20 min and dropped into the primary antibody for 2 h, the samples were rinsed in PBS-T. After incubated with secondary antibody (Peroxidase/DAB+, Rabbit/Mouse, DAKO) for 30 min, the paraffin sections were again rinsed in PBS-T (3 × 5 min). After operating according manufacturer's specification, six fields (400× magnification) from each sample have been selected randomly. The measurement of the integral optical density (IOD) value was used to evaluate the influence between alcohol and CYP2E1. Throughout this process, the consequences were shown as IOD/area by Image-Pro Plus 6.0 (IPP) software. The result was demonstrated by IOD/area in the form of histogram.

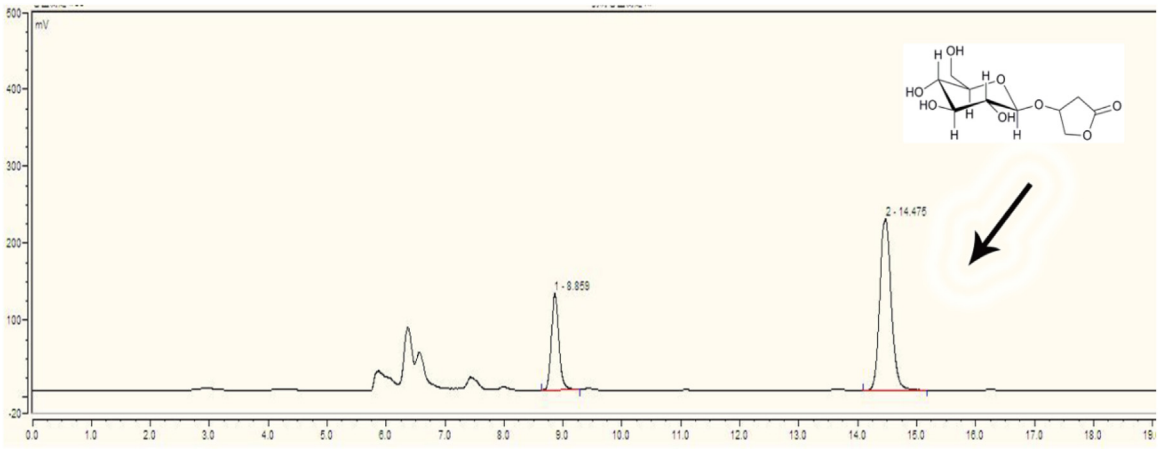


Fig. 1. Quantitative HPLC-ELSD analysis of *Anoectochilus roxburghii* extract and chemical structure of Kinsenoside HPLC was performed using an Agilent Zorbax AQ-C18 ($250 \times 4.6\,mm^2$ *i.e.*, 5 μm) at 30 °C and using 100% distilled water as the mobile phase. Flow rate was maintained at 1.0 ml/min and the sample injection volume was 10 μl.

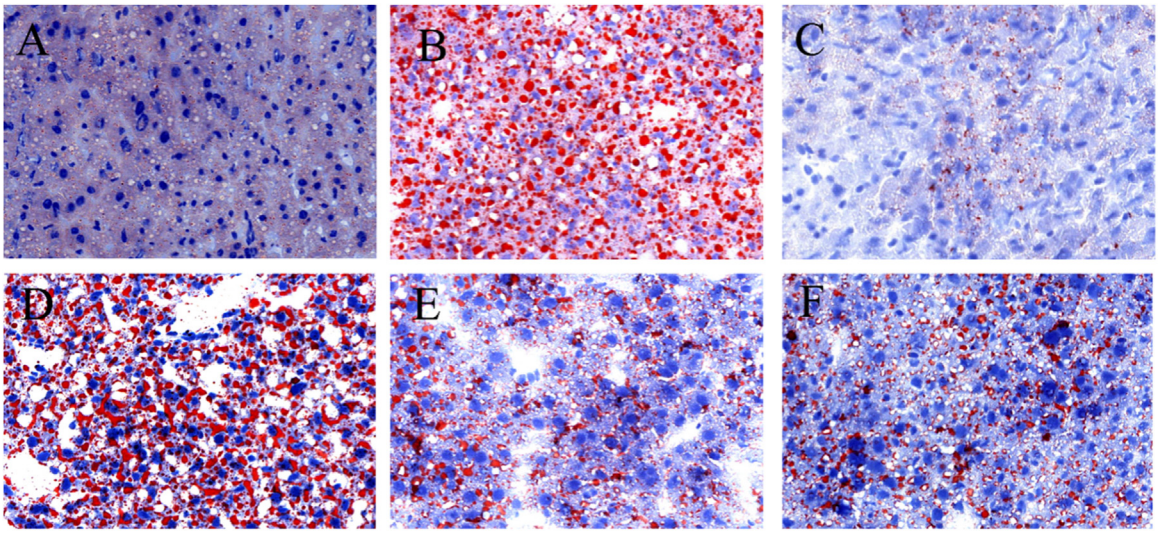


Fig. 2. Effect of kinsenoside on liver histopathology determined by Oil Red O staining (magnification, ×400). A. Normal group, normal liver; B. In the ethanol group, fatty degeneration was found in the space of liver tissues; C. the silymarin group (50 mg/kg); D. the KDL group (10 mg/kg); E. the KDM group (20 mg/kg); F. the KDH group (40 mg/kg). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

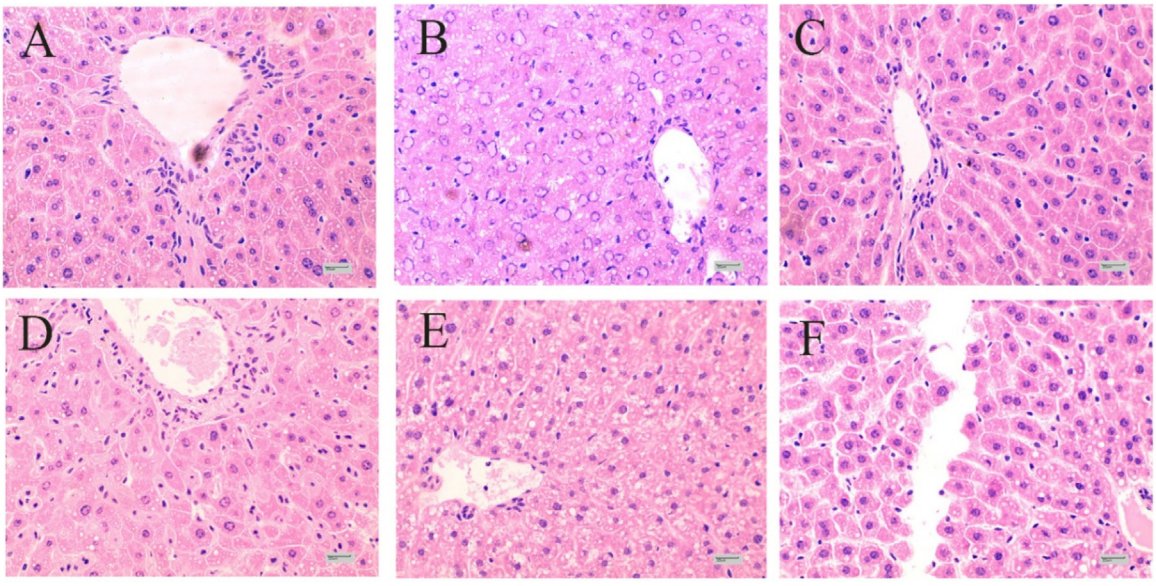


Fig. 3. Effect of kinsenoside on liver histopathology determined by H&E staining (magnification, ×200). A. Normal group, normal liver; B. the ethanol group, significant necrosis and inflammatory cells were found in the space of liver tissues; C. the silymarin group (50 mg/kg); D. the KDL group (10 mg/kg); E. the KDM group (20 mg/kg); F. the KDH group (40 mg/kg). Protection of kinsenoside (20 mg/kg and 40 mg/kg) reduced the liver injury and inflammation significantly.

Table 1
Effect of kinsenoside on pathological grading of liver injury induced by alcohol in mice.

Group	Kinsenoside (mg/kg)	N (Survival number)	The value of pathologic grading
Normal	–	12	0.3 ± 0.41
Model	–	9	3.3 ± 0.43[a]
Silymarin	50	11	1.4 ± 0.65[c]
KDL	10	10	2.8 ± 0.55
KDM	20	10	2.4 ± 0.71[c]
KDH	40	12	2.3 ± 0.77a[c]

[b] $p < 0.05$.
[a] $p < 0.01$ vs. the normal group.
[c] $p < 0.01$ vs. the ethanol group.

Statistical analysis

The experimental results were used to establish a database using Excel, and IBM SPSS Statistics 19 software was used for statistics analysis. One-way ANOVA and the LSD test were presented as the mean ± SD. A value of $p < 0.05$ was considered statistically significant. Through the analysis, the differences among the qualitative data were made by the variance analysis.

Results

Kinsenoside of Anoectochilus roxburghii *extracts by HPLC-ELSD analysis*

The values of standard kinsenoside and tested samples showed an excellent linear relation, with $y = 1.4331 \times + 0.0269$ ($R^2 = 0.999$). Then, we figured out that the percentage of the kinsenoside content in *A. roxburghii* was around 15%.

Effect of kinsenoside on liver histopathology

Judging from the Oil Red O stain, the normal group (A) performed normal complete architecture, along with no deposition of liver collagen and ECM (Figs. 2A and 3A). But, ethanol group (B) hepatic fatty degeneration in mice was very obvious (Fig. 2B). Protection of KDM (20 mg/kg.d, bw), KDH (40 mg/kg.d, bw) and silymarin group reduced the degree of fatty degeneration obviously (Fig. 2C, E, F).

As shown in the photomicrograph in H&E Stain, the liver structure of alcoholic liver in model group was not complete and there were no whole hepatic cords and hepatic cell structure, with a small amount of inflammatory cell infiltration in central vein and portal area. In ethanol group, hepatocyte nucleus was marginalized, nuclear membrane structure was changed, suspected edema and hepatocyte had many fat vacuoles, with hepatocyte ballooning degeneration (Fig. 3A). Compared with ethanol group, the degree of hepatic pathological tissue injury in KDM and KDH was lighter and nuclear membrane structure of liver tissues remained intact (Fig. 3C, E, F). The effect was comparable to that of silymarin ($p < 0.01$) (Table 1). Nevertheless, the discrepancies were not significant in pathologic grading scores between the KDL (10 mg/kg) and the ethanol group ($p > 0.05$) (Table 1).

Effect of kinsenoside on liver function

Compared with the normal group, the serum ALT and AST levels of ethanol group were enhanced significantly. In Table 3, the index of liver function, serum ALT and AST activities of alcohol-induced mice were increased by 2.7 and 2.5 times ($p < 0.01$). Different from ethanol group, kinsenoside groups decreased the serum ALT and AST levels observably (10, 20, 40 mg/kg) ($p < 0.01$) (Table 2).

Table 2
Effect of kinsenoside on the activities of serum ALT and AST in mice induced by alcohol.

Group	Kinsenoside (mg/kg)	ALT(U/l)	AST(U/l)
Normal	–	79.01 ± 7.41	93.01 ± 9.32
Model	–	216.86 ± 12.52[a]	232.16 ± 12.31[a]
Silymarin	50	131.82 ± 11.8[c]	141.31 ± 15.66[c]
KDL	10	147.92 ± 15.2[c]	168.2 ± 13.28[c]
KDM	20	143.49 ± 13.6[c]	153.62 ± 10.32[c]
KDH	40	139.43 ± 10.4[c]	148.47 ± 13.13[c]

[b] $p < 0.05$.
[a] $p < 0.01$ vs. the normal group.
[c] $p < 0.01$ vs. the ethanol group.

Table 3
Effect of kinsenoside on the activities of serum TG and VLDL in mice induced by alcohol.

Group	Kinsenoside (mg/kg)	TG(μmol/ml)	VLDL (μg/ml)
Normal	–	1.336 ± 0.172	293.1 ± 30.4
Model	–	1.992 ± 0.437[a]	392.4 ± 37.2[a]
Silymarin	50	1.360 ± 0.163[c]	241.7 ± 32.4[c]
KDL	10	1.600 ± 0.236[c]	266.1 ± 22.6[c]
KDM	20	1.489 ± 0.310[c]	244.1 ± 33.7[c]
KDH	40	1.362 ± 0.301[c]	242.0 ± 49.7[c]

[b] $p < 0.05$.
[a] $p < 0.01$ vs. the normal group.
[c] $p < 0.01$ vs. the ethanol group.

Effect of kinsenoside on lipid metabolism

As shown in Table 3, the index of liver lipid metabolism, serum TG and VLDL activities of alcohol-induced mice were increased by 1.5 and 1.3 times ($p < 0.01$), respectively, when compared to those of normal control group. However, pre-treatment with KD significantly ($p < 0.05$) and dose-dependently lowered the liver index, TG and VLDL levels ($p < 0.01$) as compared to the model control group. Moreover, pre-treatment with KD alone did not reveal any significant changes in these indexes as compared to the normal control group. Lipid metabolism indexes were dose-dependent, indicating that KD could alleviate the abnormal lipid metabolism in acute alcoholic liver injury.

Effect of kinsenoside on antioxidative defense

As shown in Fig. 4A–F, compared with the normal group, an obvious increase in MDA levels and a significant decrease in GSH activities was observed in liver homogenates from the ethanol group induced by alcohol ($p < 0.01$). Compared to normal control group, alcohol decreased the levels of GSH in model group by 23% and increased MDA level by 76.6% inversely. Compared with those values of model group, the values of the KDH group (40 mg/kg, $p < 0.01$) were expressed in dose-dependent manner significantly, whereas treatment of KDM (20 mg/kg, $p < 0.05$) suppressed partially the effects induced by alcohol. Nevertheless, treatment with KD alone did not show any marked alterations in all markers when compared to the normal control group.

Effect of kinsenoside on CYP2E1 level

To further elucidate the mechanism of KD preventive action on liver injury, the protein expressions of CYP2E1 in liver tissue were evaluated. CYP2E1 is an important enzyme resulting in oxidative stress in mice models induced by alcohol. As expected,

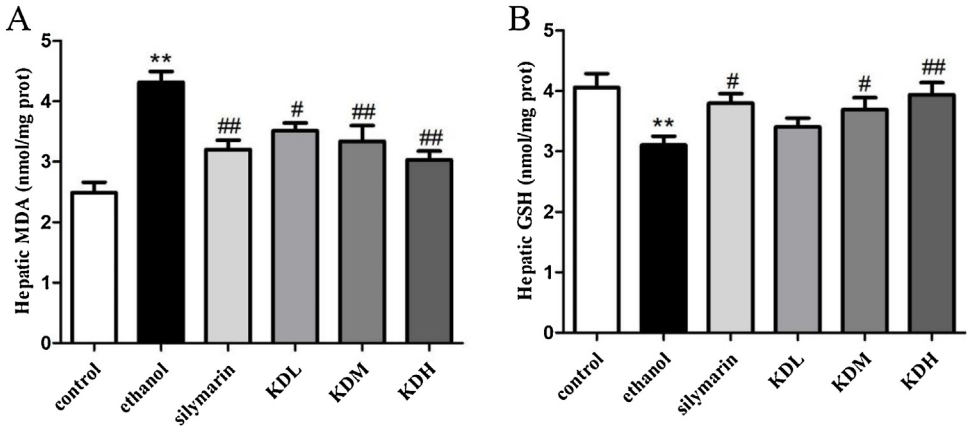
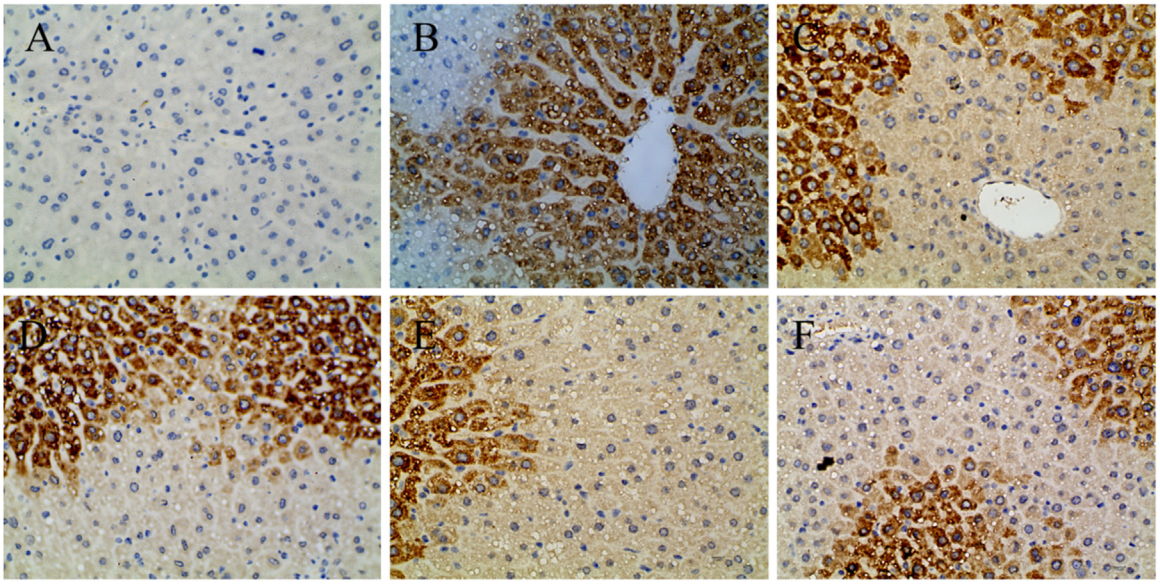


Fig. 4. Effect of kinsenoside on liver antioxidative defense. $^{**}p < 0.01$ vs. the normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the ethanol group.



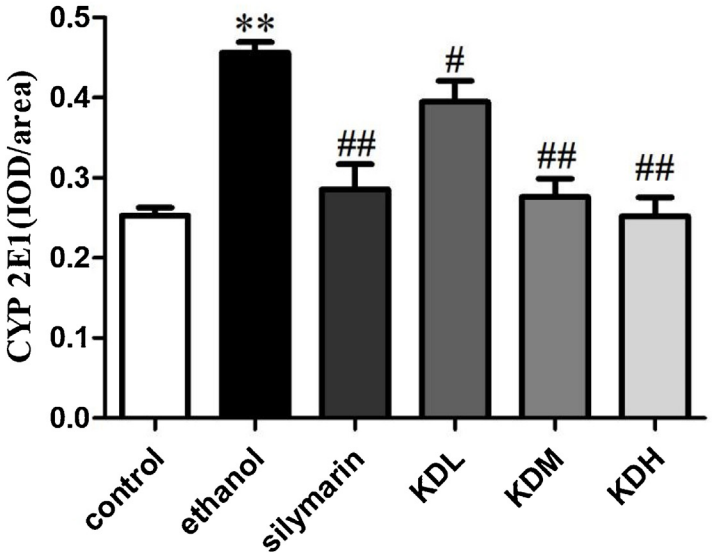


Fig. 5. Effect of kinsenoside on CYP2E1 expression. Histopathology sections of liver. A. Normal group, normal liver; B. the ethanol group, significant necrosis and inflammatory cells were found in the space of liver tissues; C. the silymarin group, 50 mg/kg; D. the KDL group, 10 mg/kg; E. the KDM group, 20 mg/kg; F. the KDH group, 40 mg/kg. Magnification 400× for each section. Protection of kinsenoside (20 mg/kg and 40 mg/kg) reduced the CYP 2E1 level significantly. $^{**}p < 0.01$ vs. the normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the ethanol group.

the CYP2E1 expression was apparently ($p < 0.01$) increased by 1.8 times in alcohol treated mice. But compared to model group, the CYP2E1 expression was inhibited in both KDM (20 mg/kg) and KDH (40 mg/kg) with decreased by 39.4% and 44.7% significantly ($p < 0.05$) (Fig.5F). However, silymarin group did not show any significant alterations in the protein expressions of CYP2E1 as compared to the normal control group.

Discussion

The main characteristics of the acute alcoholic liver injury model are the histological damage of mouse liver diseases, such as liver cell steatosis and necrosis, which are accompanied by the production of M̈allory bodyänd the destruction of cell structure, as well as the changes in the concentration and vitality of serum indicators related to liver function, such as TG, MDA and GSH. The serum activities of ALT and AST are very sensitive index employed in the diagnosis of liver diseases (Zeng et al., 2017). The current study shows that pretreatment of KD obviously attenuated the acute liver injury induced by alcohol. In our study, the index of ALT and AST were increased by alcohol. Elevated ALT and AST activity may be associated with liver cell membrane injury, as these enzymes are usually localized in the cytoplasm and released into the blood after cell injury happened (Gan et al., 2012). Nevertheless, the pretreatment of KD significantly decreased those damages, regulated liver cells, kept liver structure intact and relieved liver damage caused by alcohol.

From liver histopathology by the Oil Red O stain and H&E stain, we found that the liver structure was obviously destroyed by alcohol which caused fatty degeneration. In ethanol group, the liver structure of alcoholic liver was not complete, and there was no whole hepatic cords and hepatic cell structure, with a small amount of inflammatory cell infiltration in central vein or portal area and many fat vacuoles with hepatocyte ballooning degeneration (Fig. 3A). After KD pretreatment, the situation of liver was improved from fatty degeneration and liver structure.

Hepatocellular damage can be triggered by alcohol, through metabolism of CYP2E1, which forms free radical (Jiang et al., 2017). These free radicals react with proteins or lipids, or abstract hydrogen from polyunsaturated fatty acids, and result in lipid peroxidation such as MDA and GSH (Nada et al., 2010). Glutathione (GSH), an important intracellular thiol-based antioxidant, holds the post of an important line of defence against the oxidative stress that reduces H_2O_2, hydroperoxide (ROOH) and xenobiotic toxicity (Ma et al., 2012; Wu et al., 2010). MDA is an end product of lipid peroxidation that can be a marker of lipid peroxidation. In this study, from ethanol group, alcohol treatment led to obvious decrease in antioxidant enzymes activities such GSH level and increased in the level of lipid peroxidation marker MDA, with subsequent significant KD pretreatment, preventing these alters in a dose-dependent manner as KDH (40 mg/kg, $p < 0.01$) and KDM (20 mg/kg, $p < 0.05$). The results suggested that alcohol overwhelmed the antioxidant defense system and depleted the level of GSH, increased the formation of MDA in mouse liver and promoted lipid peroxidation, nevertheless the pretreatment of KD enhanced the antioxidant capacity and inhibited lipid peroxidation. These findings suggested that the hepatic protective effect of KD might be closely related to the reduction of oxidative stress.

However, the liver secretions of VLDL, as a product from liver, is also susceptible to other pathophysiological factors, including impaired liver function and inflammation (Browning et al., 2004; Loomba and Sanyal, 2013). It has been shown that hepatocellular oxidative stress mitochondrial dysfunction and endoplasmic reticulum stress, all related with inflammation are associated with impaired VLDL synthesis and lipid outflow (Brodsky and Fisher, 2008). In ethanol group, the levels of TG and VLDL were elevated by alcohol. Also, after KD treatment, these indexes were declined effectively.

CYP2E1, associated with strong ethanol induction, can metabolize and activate physiological toxicants, including ethanol and carbon tetrachloride. High expression of CYP2E1 is a risk factor for alcoholic liver disease (Cederbaum and Kessova, 2003; Lieber, 2004; Wang et al., 2009; Zeng et al., 2013). Studies have shown that CYP2E1 expression in rat and rabbit livers enhances NADPH oxidase activity because it appears to be weakly coupled with NADPH-CYP450 reductase (Yang and Cederbaum, 1997). The substance extracted from ethanol-treated rats, mainly CYP2E1, showed high content of superoxide and hydrogen peroxide (Das and Vasudevan, 2007; Nieto et al., 2002). In 2015, Huang et al. gave Wnt/β-catenin signaling pathway inhibitors to reduce the degree of hepatic steatosis and hepatocyte damage in rats. At heavy drinking, CYP2E1 is an important enzyme involved in alcohol metabolism in body (Guo et al., 2012; Huang et al., 2015; Groll et al., 2016). Many studies have shown that in the gastric ethanol feeding model, the obvious induction is the increase of CYP2E1 and the significant alcohol liver injury (Jiang et al., 2017). CYP2E1-deficient mice significantly reduced alcoholic oxidative stress and lipid accumulation in the liver. In addition, CYP2E1 expression was inhibited in rats with better tolerance to alcohol and less liver damage (Cao et al., 2015). CYP2E1 can also activate the JNK signaling pathway by generating a large number of reactive oxygen species (ROS) to affect autophagy and apoptosis (Wu and Cederbaum, 2013; Gerbal-Chaloin et al., 2014; Yang et al., 2014). From the perspective of immunohistochemistry, the expression of CYP2E1 increased after the mice were given a large amount of alcohol. After KD treatment, CYP2E1 expression decreased in a dose-dependent manner, reducing oxidative stress. In 2016, Liu et al reported that kinsenoside (**1**) induced a significant decrease in intracellular ROS generation, with an increase in NO production and inhibited the gene expression of NF-κB in AGEs-induced cells (Liu et al., 2016). Kinsenoside was capable of inhibiting toll-like receptor 2 (TLR2) and inflammasome NLR pyrin domain-containing 3 (NALP3), and attenuating MSU-induced activation of NF-κB/MAPK signaling *in vitro* (Qi et al., 2018).

In conclusion, this study suggests that KD has a potent hepatoprotective activity in acute alcohol-induced liver injury in mice. KD possess antioxidant activities, promotes the repair of liver cells, and ameliorate hepatic function of hepatic injury induced by alcohol, which may be the possible mechanism of KD in the treatment of alcoholic liver. Our investigation has provided convincing data supporting the potential clinical use and the quality preparation of KD.

Conflict of interest statement

The authors declare that there are no conflicts of financial interest.

Authors' contributions

SZ and JZ contributed to the conception of the study. SZ and YW contributed significantly to analysis, and manuscript preparation. QZ performed the data analyses and wrote the manuscript. LY helped perform the analysis with constructive discussions. YZ and JZ contributed to project administration and responses.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with

those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Acknowledgments

This work was financially supported by National Nature Science Fundation For Distinguished Young Scholars (No. 31700294).

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