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Hepatoprotective effects of *Malus hupehensis* tea against isoniazid- and rifampicin-induced liver injury by regulating cytochrome P450 in mice

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ABSTRACT

Malus hupehensis (Pamp.) Rehd (MH) is a dual-purpose plant used as medicine and food, which possesses various biological activities including hepatoprotective effect. In this study, the hepatoprotective activity of extracts of MH (MHE) against RFP + INH-induced liver injury was investigated. Animals in model group were given saline and INH + RFP. Animals in MHE groups were given MHE and INH + RFP. Animals in normal control group were given equal volume of saline. Subsequently, blood and liver samples were collected and assessed. MHE significantly alleviated the liver injury, as evidenced by decreased activities of the ALT, AST, TBIL, MDA and GSH-ST, and increased levels of SOD and GSH. MHE also effectively reduced the pathological tissue damage. Moreover, the CYP450 levels, and expression of CYP3A4, CYP2C9 and NADPH4 protein were inhibited by MHE. MHE exerts a protective effect against RFP + INH-induced liver injury in mice, and could be developed as a liver protectant for treatment of DILI.

1. Introduction

Drug-induced liver injury (DILI) is a common adverse drug reaction (ADR), which can lead to liver failure and even death. In China, the leading cause of DILI were traditional Chinese medicines and dietary supplements (26.81%) and antituberculosis (anti-TB) medications (21.99%) (Shen et al., 2019), in which anti-TB drugs account for a large proportion of liver injury. Tuberculosis (TB) is a disease infected by *Mycobacterium tuberculosis* (MTB), which is the number one cause of death from infectious disease globally (Floyd et al., 2018). Isoniazid (INH) combined with rifampicin (RFP) are the first-line drugs widely used in anti-TB. The combination of them can synergize and reduce drug resistance. However, it can significantly increase liver toxicity and even lead to acute liver failure which may be life-threatening. Previous research results show that one of the reasons of hepatotoxicity is that the interaction of two drugs changes the activity of Cytochrome P450 (CYP450) and its subtypes, which leads to the increase of metabolites with hepatotoxicity (Chen et al., 2015). CYP450 in liver plays an important role in drug metabolism (Kerns and Di, 2008). Many commonly used drugs can induce or inhibit CYP450, therefore, drug interaction will occur when two or more drugs are combined. During treatment process of anti-TB, the hepatotoxicity is mainly mediated by metabolites including hydrazine and acetyl hydrazine of INH (Ramappa and Aithal, 2013). RFP is an inducer of P450 enzyme, which can accelerate the metabolism of INH, increase the amount of intermediate metabolites of hydrazine and acetyl hydrazine, and increase the hepatotoxicity (Tostmann et al., 2008).

Malus hupehensis (Pamp.) Rehd (MH) belongs to the family *Rosaceae Malus* Mill (Guo et al., 2020), which is a traditional medicine and food plant. Before its independent name, MH was named "linqin" and "Huahong". "Linqin" was first recorded in "Qianjin Shizhi". The leaves of "Huahong" for medicinal use was first recorded in "Diannan Materia Medica", and it used as medicine with the name of MH was first recorded in "Xinhua Compendium of Materia Medica". It was documented that it is sour taste and neutral in nature, and is mainly used to treat the stagnation of food retention, dyspepsia, dysentery and malnutrition

Abbreviations: MH, *Malus hupehensis* (Pamp.) Rehd; MHE, extracts of MH; RFP, rifampicin; INH, isoniazid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; MDA, malondialdehyde; GSH-ST, glutathione S-transferase; SOD, superoxide dismutase; GSH, glutathione; DILI, drug-induced liver injury; ADR, adverse drug reaction.

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(Editorial board of Chinese materia medica of State Administration of traditional Chinese Medicine, 1999). According to the liver and spleen theory recorded in "Jingui Yaolue", liver diseases could be treated by reinforcing spleen (Zhao et al., 2016). It has been used as tea drinking for more than four hundred years, especially in Hubei province in China (Liu et al., 2019). As an important class of tea, the leaves of MH are nicknamed "San-Pi-Guan" implying that a pot of tea can be prepared by using only three pieces of leaves (Liu et al., 2018; Liu et al., 2015). MH is reported to possess effects of clearing away heat, relieving toxicity, relieving pain, tonic, promoting urination and digestion, alleviating edema, activating blood, and relieving thirst (Fu et al., 2018).

In previous study, total flavonoids of MH have an obviously inhibitory effect on the hepatic fibrosis induced by *Schistosoma japonicum* infection (Du et al., 2011). Total flavonoids of MH have an obvious inhibitory action on the hepatic fibrosis induced by carbon tetrachloride (CCl_4), which may be related to its antioxidant characteristic. In CCl_4-induced rats, antioxidant capacity of organizations was enhanced, the level of lipid peroxidation was reduced, the cell membrane was protected from damage, and the expression of transforming growth factor β1 (TGF-β1) was inhibited to reduce the degree of liver fibrosis (Feng et al., 2012). It was also found that phloridzin (PHL), the main component of MH, has hepatoprotective effect. PHL has the protective effect on CCl_4-induced acute liver injury mice, which may be related to its antioxidant characteristic. In CCl_4-induced mice, antioxidant capacity of organizations was enhanced, the level of lipid peroxidation was reduced, the cell membrane was protected from damage (Feng et al., 2010). These studies demonstrated that PHL exerted beneficially hepatoprotective effects on hepatic fibrosis induced by CCl_4, mainly by enhancing antioxidant capacity of liver organizations, reducing the level of lipid peroxidation and protecting hepatocyte membranes from damage to alleviate hepatic fibrosis (Deng et al., 2012). Based on these studies, it would be of great interest to estimate whether MH has beneficial effects on anti-TB drug-induced hepatoxicity and to explore its underlying mechanism. To test the hypothesis, RFP + INH-induced liver injury model was performed to study the hepatoprotective effects of MH in mice.

2. Materials and methods

2.1. Plant material and chemicals

The leaves of *Malus hupehensis* (Pamp.) Rehd (MH) were gathered from the Changyang Tujia Autonomous County in Yichang City of Hubei Province of China and were identified by Doc. Yu-bing Wang of the College of Biological and Pharmaceutical Sciences, China Three Gorges University. A voucher specimen (No. 201905MH) was deposited in the Hubei Key Laboratory of Natural Products Research and Development (China Three Gorges University, Hubei, China).

The leaves of MH (17.00 g) were dried and extracted under reflux two times with 75% ethanol (250 ml) at 80℃ for 3 h. The extracts were combined and concentrated under reduced pressure to obtain 3.997 g extract (yield 23.51%) (MHE). The MHE were quantitatively analyzed by HPLC to obtain quality control of the main components. The separation was performed on a Diamonsil Plus C_{18} column (250 × 4.6 mm i.d., 5 μm; Dikma, China).The mobile phase was a mixture of H_2O (A) and acetonitrile (ACN) (B). The gradient program of mobile phase was carried out as follows: 0–10 min, 10%-25% B; 10–30 min, 25%-50% B; 30–40 min, 50%-100% B. The detection wavelength was set at 289 nm. The flow rate was set at 1 ml/min.

INH was purchased from Tianjin Lisheng Pharmaceutical Co., Ltd (Tianjin, China, batch number: 1801004). RFP was purchased from Shenyang Hongqi Pharmaceutical Co., Ltd., (Shenyang, China, batch number: 1710161). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), glutathione S-transferase (GSH-ST) and protein quantitative assay kits were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). CYP450 ELISA kit was acquired from Jiangsu Meimian industrial Co., Ltd (Jiangsu, China). Cytochrome P450 3A4 (CYP3A4), Cytochrome P450 2C9 (CYP2C9) and NADPH oxidase 4 (NADPH4) antibodies were purchased from Abcam (Cambridge, MA, USA).

2.2. Animals

Kunming male mice, weighing 20 ± 2 g, SPF, were provided by the Experimental Animal Center of China Three Gorges University (Hubei, China). Studies were conducted according to protocols approved by the institutional ethical committee. The mice were maintained under controlled conditions (temperature: 25 ± 2 °C, relative humidity: 60 ± 10%, room air change: 12–18 times per hour, and 12/12-h light/dark cycle) and allowed ad libitum access to food and water.

2.3. Treatments

Mice were randomly divided into the following five groups (n = 10 per group) and treated for 28 consecutive days (4 weeks): normal control group, model group (INH + RFP group), low dose group of MHE (MHE-L), medium dose group of MHE (MHE-M) and high dose group of MHE (MHE-H). All mice were treated as follows by gavage: (i) normal control group: animals were given equal volume of saline. (ii) model group: after administration of equal volume of saline for 1 h, animals were given INH (100 mg/kg/day) + RFP (100 mg/kg/day). (iii) MHE-L group: after administration of MHE (30 mg/kg/day) for 1 h, animals were given INH (100 mg/kg/day) + RFP (100 mg/kg/day). (iv) MHE-M group: after administration of MHE (60 mg/kg/day) for 1 h, animals were given INH (100 mg/kg/day) + RFP (100 mg/kg/day). (v) MHE-M group: after administration of MHE (120 mg/kg/day) for 1 h, animals were given INH (100 mg/kg/day) + RFP (100 mg/kg/day). Mice in each group were fasted for 1 h before administration.

At the end of the experiment, after fasting for 12 h, all mice in each group were weighed and sacrificed. The blood and liver samples were collected. The blood was immediately centrifuged at 3,000 rpm for 15 min to obtain serum, which was stored and later assessed for biological activities of hepatic biomarkers. The liver samples were immediately washed with cool saline at least three times, dried with filter paper, weighed and then separated into two parts. One part of the liver samples was immediately stored at −80℃ for future analysis. The other part was fixed in 4% paraformaldehyde solution for pathological examination.

2.4. Calculation of the liver index

The liver index was calculated as following: liver index (%) = (mouse liver weight / mouse weight) × 100%.

2.5. Analysis of hepatic biomarkers

The contents of ALT, AST and TBIL in serum were measured using commercially available kits according to the manufacturer's instructions, and the extent of liver injury was evaluated.

2.6. Estimation of antioxidant enzymes and lipid peroxidation products

The liver samples were homogenized, and the homogenates were immediately centrifuged at 2,600 rpm for 10 min. Levels of SOD, GSH, GSH-ST, and MDA were determined in supernatants using the corresponding assay kits according to the manufacturer's instructions.

2.7. Determination of CYP450

The concentrations of CYP450 in serum were determined in supernatants using the commercially available kits according to the manufacturer's instructions.

2.8. Pathological examination

The liver tissues of mice were fixed with 4% paraformaldehyde for 24 h, and then transferred to 75% ethanol. After conventional dehydration, paraffin embedding, the tissues were sliced into 5-μm thick sections and stained with HE. The pathological changes were observed with the microscope, and damage of hepatocytes in different groups was compared.

2.9. Western blot analysis

The protein expression levels of CYP3A4, CYP2C9 and NADPH4 in the livers of mice were measured by western blotting. Briefly, 80–100 mg of liver tissue of each group was weighed, then 1 ml NP-40 SURFACT-AMPS DETERGENT (Thermo, USA) lysate homogenate containing protease inhibitor was added, after centrifuged at 12,000 rpm in 4℃ for 10 min, protein quantification was performed using the BCA protein assay kit (Nanjing Jiancheng bioengineering Institute, China). An equivalent amount of protein was loaded and separated using 10% SDS-PAGE (Beyotime Institute of Biotechnology, China). All proteins were transferred to the PVDF (biosharp, China) membrane, and sealed with 5% skimmed milk powder for 2 h. Target proteins were incubated overnight at 4 °C with the following primary antibodies: anti-CYP3A4 antibody (1:2,000, Abcam, USA), anti-CYP2C9 antibody (1:1,000, Abcam, USA), anti-NADPH4 antibody (1:1,000, Abcam, USA), anti-β-Actin antibody (1:5,000, Abcam, USA), After incubation with primary antibody, the membranes were washed with TBST for three times, and incubated with secondary antibody goat anti-rabbit IgG (1:2,000, Abcam, USA) for 1 h at room temperature. Automatic luminescence analyzer (Tanon5200 type) was used for chemiluminescence color rendering, and Image J software was used to analyze the gray value of protein strip.

2.10. Statistical analysis

Statistical analysis was performed with SPSS.19.0 software, and one-way analysis of variance (ANOVA) was used to compare mean values among the groups, and Tukey's test was used in the post hoc multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Quality control analysis of MHE

MHE were analyzed by HPLC. PHL, the main component of MHE was quantified (0.88%), and the chromatogram was shown in Fig. 1.

3.2. Effects of MH on liver index in mice

Compared with the normal control group, liver index in model group increased significantly after treatment with INH + RFP ($P < 0.01$), indicating that INH + RFP induced hypertrophy of liver tissue. Liver index of MHE groups were lower than model group, and high dose MHE group was significantly reduced compared with that in model group ($P < 0.05$) (Fig. 2).

3.3. Effects of MH on serum ALT and AST

The contents of ALT and AST in serum of mice were determined to evaluate the extent of liver injury. Compared with normal control group, serum ALT and AST contents in model groups were significantly increased ($P < 0.01$). Compared with model group, the contents of AST in MHE groups were significantly decreased ($P < 0.01$). The contents of ALT in low, medium and high dose MHE groups were lower than in model group ($P < 0.05$ or $P < 0.01$) (Fig. 3).

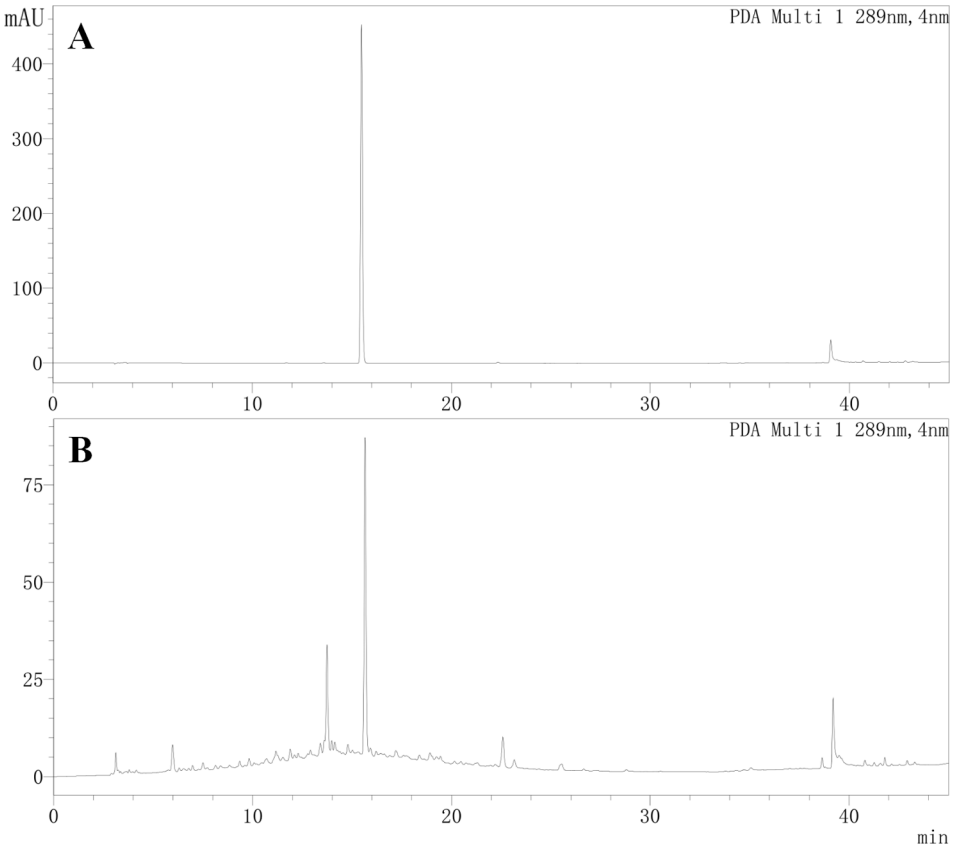

Fig. 1. HPLC chromatogram of the PHL(A) and MHE(B).

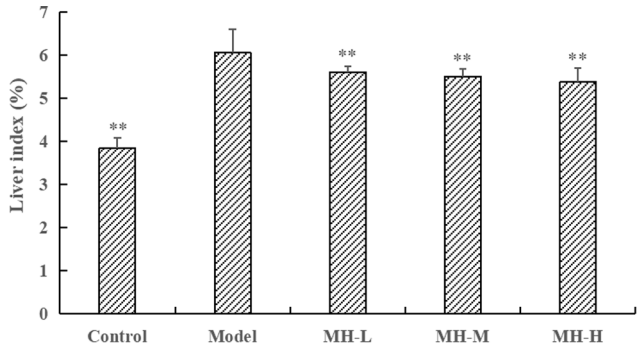

Fig. 2. Effect of MHE on liver index in IHN- and RFP- induced hepatic injury in mice. Results are presented as mean ± SD (n = 10). *P < 0.05 and **P < 0.01 compared with INH + RFP model group.

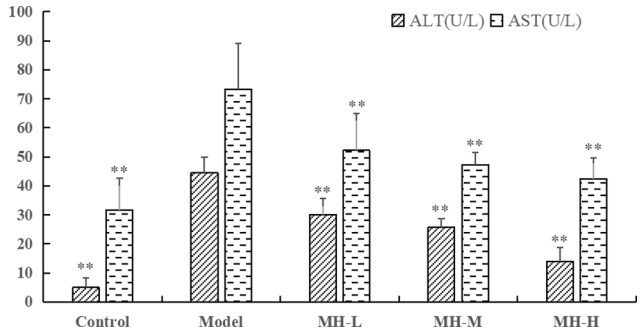

Fig. 3. Effect of MHE on serum ALT and AST in IHN- and RFP- induced hepatic injury in mice. Results are presented as mean ± SD (n = 10). *P < 0.05 and **P < 0.01 compared with INH + RFP model group.

3.4. Effects of MH on serum TBIL

The content of TBIL in serum of mice was determined to evaluate the DILI. Compared with normal control group, the serum TBIL content in model group were significantly increased (P < 0.01). Animals treated with all dosages of MHE exhibited a significant decrease in the contents of TBIL compared with those in model group (P < 0.05 or P < 0.01) (Fig. 4).

3.5. Effects of MH on antioxidant enzyme and lipid peroxidation

The GSH levels were significantly higher in normal control group, MHE-M, and MHE-H groups than in model group (P < 0.05 or P < 0.01, Fig. 5**A**). The GSH-ST activities were significantly lower in normal control group, MHE-L, MHE-M, and MHE-H groups than in model group (P < 0.01, Fig. 5**B**). The MDA levels were lower in normal control group, MHE-L, MHE-M, and MHE-H groups than in model group (P < 0.05 or P < 0.01) (Fig. 5**C**). Furthermore, compared with that in model group, the SOD activities were higher in normal control group and MHE groups, however, the SOD activity were not significantly different in normal control group, MHE-L and MHE-M groups (P > 0.05), and the enzyme activity was only significantly higher in MHE-H group (P < 0.05, Fig. 5**D**).

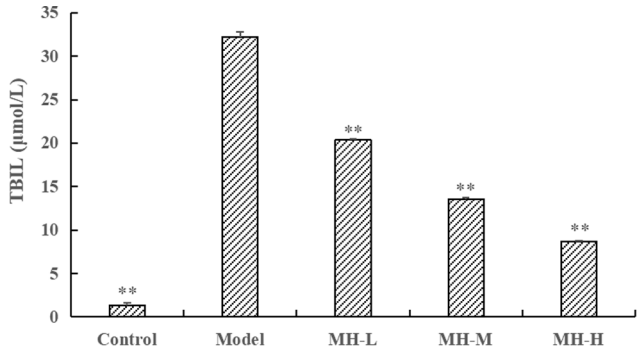

Fig. 4. Effect of MHE on serum TBIL in IHN- and RFP- induced hepatic injury in mice. Results are presented as mean ± SD (n = 10). *P < 0.05 and **P < 0.01 compared with INH + RFP model group.

3.6. Effects of MH on CYP450

Compared with normal control group, the CYP450 levels in model group increased significantly after treatment with INH + RFP (P < 0.01), indicating that INH + RFP affected the CYP450 activities in liver injury mice. The CYP450 levels of MHE groups were significantly lower than model group (P < 0.05) (Fig. 6).

3.7. Histopathological examination

In normal control group (Fig. 7**A**), the structure of hepatic lobules was intact, the hepatic cord was clear and arranged radially around the central vein, the hepatic sinusoids and the morphology of hepatocytes were normal. In the IHN- and RFP- induced model group (Fig. 7**B**), there were ballooning degeneration, swelling, expansion of portal area, and infiltration of inflammatory cells. The normal structure of hepatic cells was destroyed, the arrangement of hepatocytes was irregular, the hepatic lobules were not clear, the hepatic sinusoids were squeezed, and a small amount of hemorrhagic foci. Compared with model group, the extent of liver injury in the MHE groups was reduced (Fig. 7**C, 7D and 7E**).

3.8. Effects of MH on the protein expression of CYP3A4, CYP2C9 and NADPH4 in mice with INH + RFP-induced liver injury.

The protein expression levels of CYP3A4, CYP2C9 and NADPH4 in livers of mice were evaluated by western blotting.

Compared with normal control group, the protein expression levels of CYP3A4, CYP2C9 and NADPH4 in model group were significantly increased (P < 0.01). Compared with model group, the protein expression of CYP3A4, CYP2C9 and NADPH4 in MHE groups were decreased (P < 0.01 or P < 0.05) (Fig. 8**A, 8B, 8C and 8D**).

4. Discussion

RFP combined with INH is the preferred alternative in the treatment of TB. However, hepatotoxicity caused by these drugs is a major concern during the long-term therapy. Therefore, the discovery of the effective agents that can reverse the liver injury induced by these anti-TB drugs is of great importance (Wang et al., 2018). MH is a dual-purpose plant used as medicine and food, which is a traditional Chinese herb whose hepatoprotective effect was confirmed in a previous study. In this study, the comprehensive analysis of its detoxification activity in RFP and INH-induced mice was conducted for further development of a novel drug with high efficacy and low toxicity for treatment of anti-TB drug-induced hepatoxicity.

INH is metabolized into two metabolites, hydrazine and acetyl hydrazine, which cause hepatotoxicity by inducing oxidative stress (Roy et al., 2008; Chowdhury et al., 2006). RFP is a potent inducer of several metabolic enzyme pathways in particular CYP450 system, which leads to increased metabolism of INH yielding toxic metabolites (Ramappa and Aithal, 2013). In this study, compared with normal control group, the concentration of CYP450 in model group was significantly increased, indicating the induction effect of RFP and INH on CYP450 enzyme. The concentrations in MHE-treated groups were decreased into normal level, which declared the moderation effect of MHE on CYP450 system in RFP- and INH- induced liver injury.

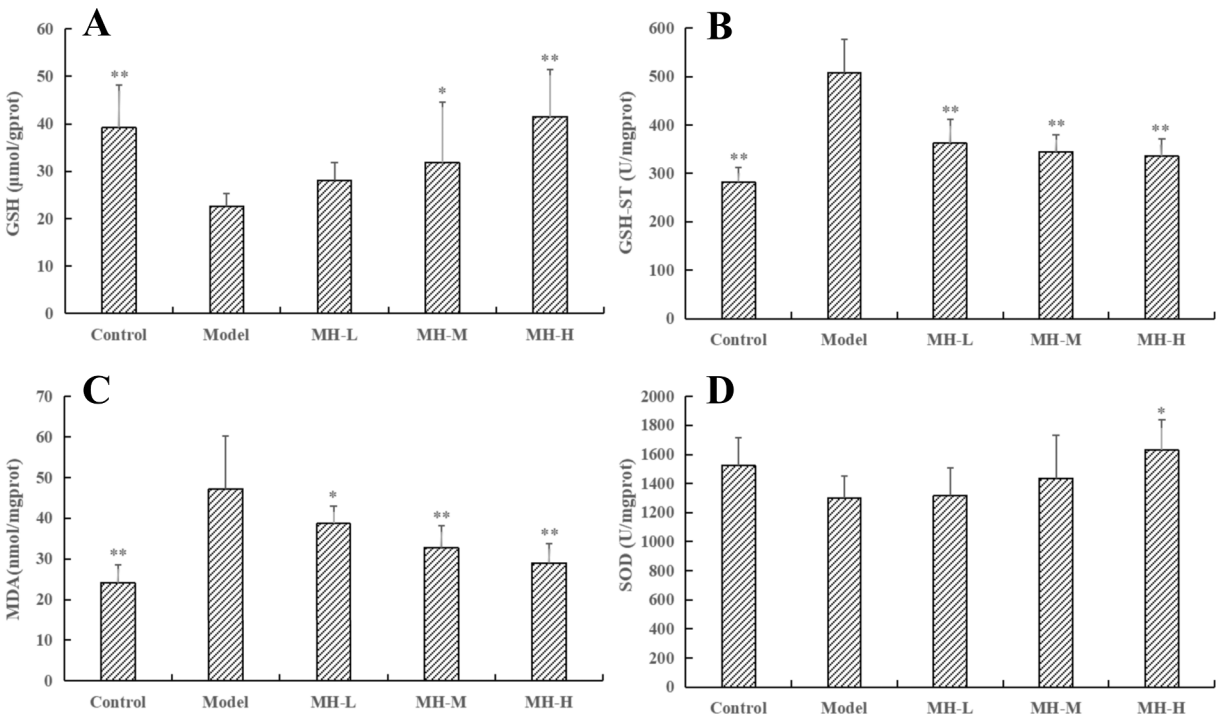

Fig. 5. Effect of MHE on GSH (A), GSH-ST (B), MDA (C), and SOD (D) in IHN- and RFP- induced hepatic injury in mice. Results are presented as mean ± SD (n = 10). *P < 0.05 and **P < 0.01 compared with INH + RFP model group.

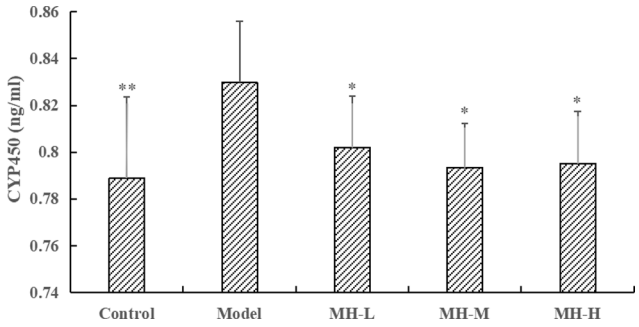

Fig. 6. Effect of MHE on CYP450 in IHN- and RFP- induced hepatic injury in mice. Results are presented as mean ± SD (n = 10). *P < 0.05 and **P < 0.01 compared with INH + RFP model group.

ALT and AST in liver are mainly located in hepatocyte plasma and mitochondria (Zhang et al., 2019). Due to various hepatic cell damages, the cytosol leakage will cause increment of ALT and AST in the serum (Guo et al., 2017; Dong et al., 2014). Currently, changes of serum ALT, AST and TBIL are the main laboratory indexes and markers of liver injury and the severity of DILI (Yu et al., 2017; You et al., 2017). In present study, the hepatoprotective activity of MHE was assessed by determined the levels of ALT, AST and TBIL. Comparing model group to normal control group, these serum indicators were increased remarkably, that means combination RFP with INH induced significantly damage in liver of mice. Comparing MHE groups with model group, the results showed that treatments with MHE reduced these biochemical alterations induced by RFP + INH, indicating that MH possesses potential activity to protect liver damage.

GSH is a low molecular weight thiol antioxidant with detoxification. It has been reported that the covalent binding of most toxicants to hepatic protein occurs only after depletion of GSH, and the severity of hepatic necrosis is related to the degree of covalent binding (Li et al., 2015). Compared with normal control group, the concentration of GSH in model group was significantly decreased, indicating significant depletion of GSH. The concentrations in MHE-treated groups were significantly increased, which declared the detoxification of MHE in liver injury. GSH-ST, with a high concentration in liver cytosol, plays an important role in the detoxification of liver by catalyzing the conjugation of GSH with reactive metabolites (Guo et al., 2017; Zhang et al., 2017). GSH-ST released into the blood when the liver cells were damaged, therefore, increased GSH-ST can be used as a sensitive indicator of liver damage. Compared with normal control group, GSH-ST activity was significantly increased, indicating hepatocyte damage. Different doses of MHE can decrease GSH-ST activity, suggesting that MHE can reduce hepatocyte damage.

SOD is an important antioxidant enzyme in vivo, which can scavenge superoxide radical and decompose them into low-activity H_2O_2 and protect liver against spontaneous oxygen toxicity and lipid peroxidation (Zhang et al., 2019; Xue et al., 2012). As a final produce of lipid peroxidation, MDA content is an index of intensified peroxidation process and closely related to the severity of oxidative stress damage (Zhang et al., 2019; Xue et al., 2012). In this study, the activities of SOD in liver tissue of MHE groups were higher than that of control group, However, the contents of MDA in liver tissue of MHE groups were significantly lower than that of control group, which indicated the certain antioxidant capacity and ability to reduce lipid peroxidation of MHE.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) (Liang et al., 2016), a multi-subunit transmembrane enzyme complex composed of seven members: NOX1, NOX2, NOX3, NOX4, NOX5, Duox1 and Doux2 (Wan et al., 2020), is a key source of reactive oxygen species (ROS) (Eun et al., 2019), which play crucial roles in the progression of liver diseases, including cell death of hepatocytes induced by oxidative damage. In this study, the up regulated expression of NADPH4 in model group was inhibited by MHE, and the oxidative damage was further regulated.

CYP3A4 and CYP2C9 were two subtypes of CYP450. The results of our present study showed that the expression of CYP3A4 and CYP2C9 protein was up regulated, especially the 3A4, which is the main subtype and approximately 28% of the total CYP enzymes present in human liver and plays a critical role in drug metabolism (Chen et al., 2018). In MHE

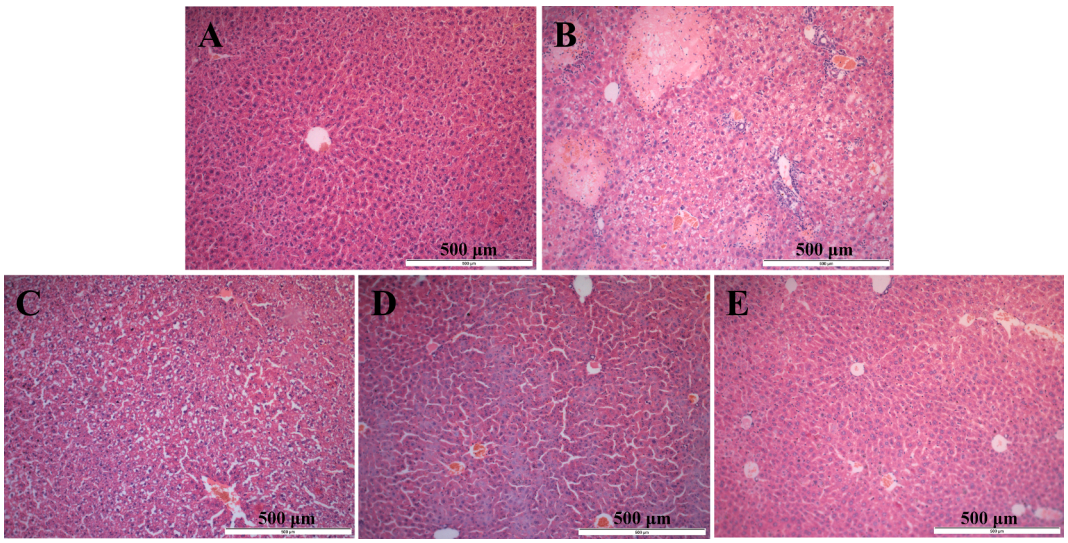

Fig. 7. Histological results of HE staining in INH + RFP-induced liver injury mice (100×). (A) the normal control group, (B) INH + RFP-treated model group, (C) the MHE-L group, (D) the MHE-M group, (E) the MHE-H group.

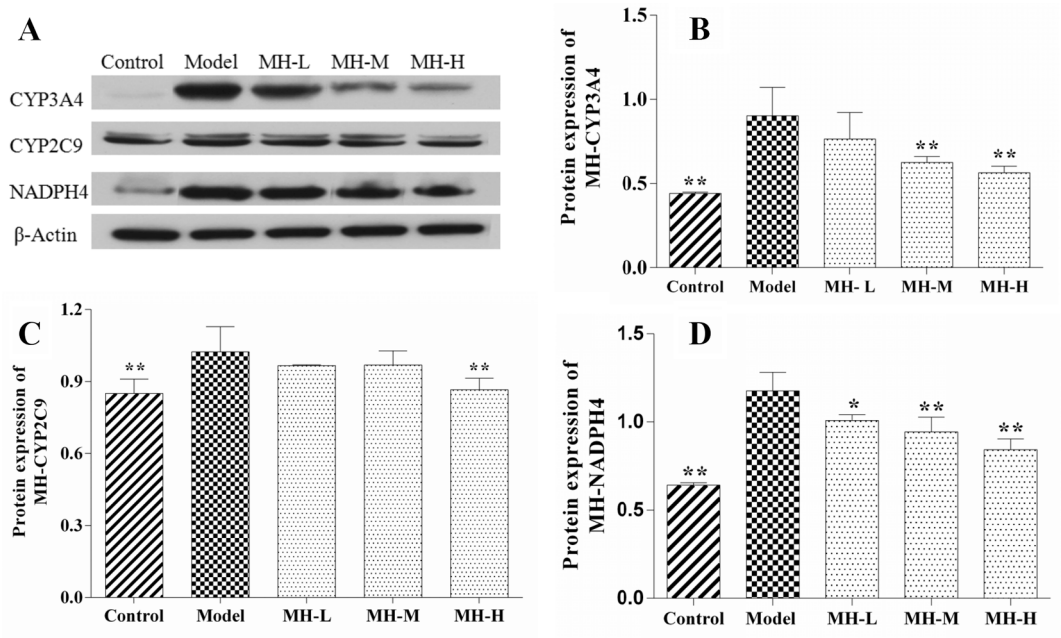

Fig. 8. Effects of MH on protein expression of CYP3A4, CYP2C9 and NADPH4 in mice with INH + RFP-induced liver injury. Results are presented as mean ± SD (n = 3). *P < 0.05 and **P < 0.01 compared with INH + RFP model group.

groups, these two subtypes of CYP450 were down regulated.

5. Conclusion

With the analysis of results discussed above, the conclusion may be drawn that RFP- and INH-induced hepatic injury was normalized by MHE via regulating CYP450 enzymes to reduce the production of toxic metabolites of INH in process, and resisting oxidative damage of toxic metabolites of INH at the end stage. MHE should be regarded as a novel and promising agent with a high potential in prevention and treatment of anti-TB drug-induced liver injury. Meanwhile, MH can be developed into functional food or drink to protect the liver in daily life.

Ethics statement

Animals were treated with humane care according to the guidelines of Animal Research Ethics Committee of the China Three Gorges University. Ethical clearance number: 2019010Y.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Authors contribution

Gaigai Deng conceived and designed the experiments. Guorong Li and Jiao Yang wrote the paper. Ying Yang and Guorong Li fed the animals and performed the experiments. Yujie Suo and Hailong Xu performed pathological examination. Ping Liu prepared the MHE. Junzhi Wang and Tianyan Feng reviewed the literature.

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