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Effect of flavonoid compounds extracted from *Iris* species in prevention of carbon tetrachloride-induced liver fibrosis in rats

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ABSTRACT. We investigated the effect of flavonoid compounds extracted from species of genus *Iris* L. on carbon tetrachloride (CCl_4)-induced rat liver fibrosis. Thirty Sprague-Dawley rats were randomly divided into normal control group, liver fibrosis model group, and drug treatment group (N = 10 each). Next, 0.2 mL/100 g CCl_4 was subcutaneously injected for 6 weeks in both model and treatment rats to generate the liver fibrosis model. In the control group, an equal volume of castor oil was injected subcutaneously. Rats in the treatment group also received 100 $mg \cdot kg^{-1} \cdot day^{-1}$ flavonoid compounds via gastric tubes. After 6 weeks, rats were sacrificed, and their liver tissues were examined for pathological changes, including alanine aminotransferase, aspartate aminotransferase, total bilirubin, hyaluronic acid, laminin, and procollagen type-3. Liver tissues from control rats showed no significant pathological changes, while model animals showed significant liver fibrosis. In the treatment group, liver fibrosis significantly decreased compared to the model group ($P < 0.05$). Liver fibrotic indices, including hyaluronic acid, laminin, and procollagen type-3, in treatment rats were all significantly lower than those in the

model group ($P < 0.05$), but not significantly different compared to the normal group ($P > 0.05$). Other liver function indices, including alanine aminotransferase, aspartate aminotransferase, and total bilirubin, in treatment rats were also significantly lower than those in model rats ($P < 0.01$) but higher than those in control animals ($P < 0.05$). Flavonoid compounds extracted from *Iris* plants showed significant inhibitory effects on CCl_4-induced rat liver fibrosis.

Key words: CCl_4; Flavonoid compounds; Liver fibrosis

INTRODUCTION

Liver fibrosis is a repair mechanism after chronic liver damage and represents an intermediate stage during the progression of chronic liver disease. Without effective management, liver fibrosis often evolves into liver sclerosis or even liver failure (Cengiz et al., 2014; Li et al., 2014a). With timely and effective prevention and treatment, liver fibrosis can be slowed to impede the occurrence and development of liver sclerosis and its complications, thereby reducing pain in patients with chronic liver disease. The pathogenesis of liver fibrosis is complicated, since multiple inducing factors are involved, including the oxidation response, cytokines from different cells, and several growth factors, all of which can induce complicated pathophysiological changes (Kessoku et al., 2014). Although dozens of pathogenic factors exist, the pathological alternations of liver fibrosis are similar: hepatic stellate cell activation is a key step underlying the occurrence of liver fibrosis. The proliferation of hepatic stellate cells can elevate the synthesis and secretion of the extracellular matrix while inhibiting its degradation, causing deposition of extracellular matrix within liver tissues, abnormal hyperplasia of fibrous connective tissues, changes in the structure of hepatic lobules, and finally liver sclerosis. Therefore, the prevention and treatment of liver fibrosis is critical for reversing or inhibiting liver sclerosis. However, the development of effective drugs for reversing liver fibrosis is a major clinical challenge. Recently, promising results in the treatment of liver fibrosis have been reported using Chinese medicine (Li et al., 2014b; Zhang et al., 2014a), which often contain flavonoid compounds (Fu et al., 2014). Current studies have revealed multiple pharmacological roles of flavonoid compounds, including the treatment of tumors, cardiovascular disease, and diabetes (Chavez-Santoscoy et al., 2014; Daddam et al., 2014; Sankari et al., 2014). To further study the effect of flavonoid compounds extracted from *Iris* plants on liver fibrosis, we developed liver fibrosis model rats using carbon tetrachloride (CCl_4) and measured indices of liver function, liver fibrosis, and tissue pathological changes to evaluate their effects on CCl_4-induced rat liver fibrosis.

MATERIAL AND METHODS

Research animals

Rats were used for all experiments, and all procedures were approved by The First Affiliated Hospital of Shantou University Medical College (Guangdong, China).

Thirty healthy male Sprague-Dawley rats aged 2 months (specific pathogen-free grade, body weight 250 ± 20 g) were purchased from the Research Animal Center of Medical Faculty in Shantou University Medical College and were kept in a specific pathogen-free-grade animal center.

Drugs and reagents

CCl_4 was purchased from Shanghai Chemical Reagent Factory (Shanghai, China) and diluted in castor oil to prepare a 20% (v/v) solution. Flavonoid compounds from *Iris* plants were extracted in the chemical pharmacy laboratory of our university. Major steps in the extraction process included microwave extraction with 70% ethanol, column filtration using D-101 macroporous resin and polyamide, elution using 70% ethanol, and vacuum drying. The final product was determined to contain flavonoid compounds by using an HCl-Mg qualitative assay. Hematoxylin and eosin were purchased from Boshide Bio. Corp. (Wuhan, China). The radioimmunoassay kits for laminin (LN), hyaluronic acid (HA), and procollagen type-3 (PCIII) were from Kemei Bio. Tech. Corp. (Beijing, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) assay reagents were purchased from Fuxing Changzheng Med. Sci. Corp. (Shanghai, China).

Animal treatment

Sprague-Dawley rats were randomly divided into 3 groups: normal control group, liver fibrosis model group, and treatment group (N = 10 in each group). Liver fibrosis rats were prepared as previously described (Zhang et al., 2014b). CCl_4-castor oil solution was subcutaneously injected daily into rats at 0.2 mL/100 g for 6 weeks. Treatment rats also received flavonoid compounds at 100 $mg \cdot kg^{-1} \cdot day^{-1}$ with daily gastric application for 6 weeks in addition to CCl_4 treatment in the model rats. In the normal control group, an equal volume of saline was applied daily via gastric tubes for 6 weeks. Food and water were given *ad libitum*.

Histopathology assays

After 6 weeks of treatment, all rats were anesthetized using 2% pentobarbital sodium. Serum was separated from whole blood collected from the abdominal aorta and was stored at -20°C. Rats were then sacrificed, and their liver tissues were fixed in 10% neutral-buffered formaldehyde and stained using hematoxylin and eosin. Liver fibrosis scores were based on the standard recommendation of the Chinese Society of Hepatology (2002) and used to evaluate the severity of liver fibrosis in experimental animals. Briefly, a score ranging from 0-29 was assigned based on the severity of liver fibrosis, with a higher score representing more severe conditions (Qin et al., 2013).

Assays of liver fibrosis and liver function indices

Serum levels of ALT, AST, TBIL, HA, LN, and PCIII were measured using an automatic biochemical analyzer and radioimmunoassay analyzer.

Statistical analysis

The SPSS ver. 12.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze all collected data, which are reported as means ± SE, unless otherwise specified. Liver fibrosis scores between groups were compared using the rank sum test. Between-group comparisons were conducted using analysis of variance. Statistical significance was defined as $P < 0.05$.

RESULTS

Histopathology observations of rat liver tissues

Liver tissues from all rats were sectioned, stained using hematoxylin and eosin, and observed under a light-field microscope to examine histopathology changes. The results showed no fibrosis in the liver tissues of control rats indicated by: 1) no significant change in liver and hepatic lobular structure and 2) no infiltration of inflammatory cells in liver tissues. The model rats, however, displayed liver fibrosis, including: 1) near the portal area, there were large amounts of collagen fibers, which separated and destructed the hepatic lobular structure; 2) significant alternations in liver tissue and hepatic lobular structure; and 3) numerous infiltrative inflammatory cells developed in the liver tissue, which displayed necrotic hepatocytes and fatty degenerated hepatocytes. After flavonoid treatment, the severity of fibrosis in rats significantly reduced, with fewer fatty degenerated and infiltrative inflammatory hepatocytes. The change in liver fibrosis following treatment was statistically significant compared to model rats ($P < 0.05$; Figure 1 and Table 1), suggesting a significant role of flavonoid compounds extracted from *Iris* plants in the prevention and treatment of liver fibrosis.

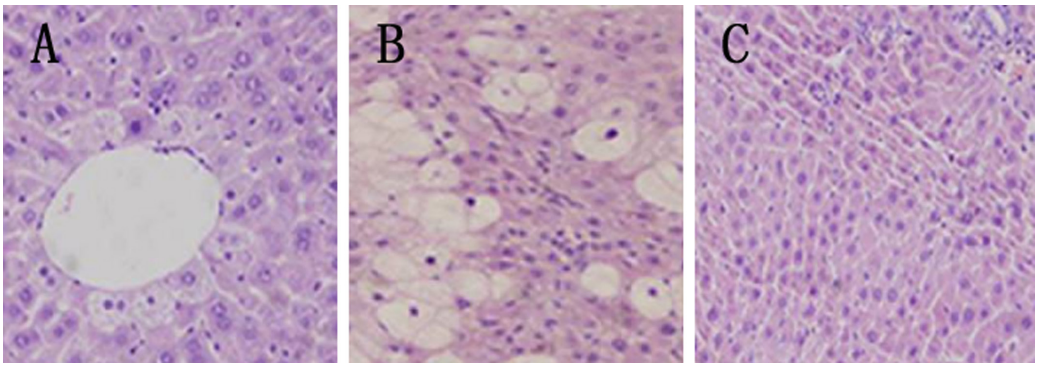

Figure 1. Histopathological changes in rat livers (hematoxylin and eosin staining, 200X). **A.** Normal control group; **B.** model group; **C.** treatment group.

Table 1. Histopathological changes in rat livers.

Group	N	Semi-quantitative score of liver fibrosis
Control	10	0.81 ± 0.09
Model	10	21.42 ± 3.73*
Treatment	10	6.71 ± 1.25*#

*$P < 0.05$ compared to control group; #$P < 0$ .05 compared to model group.

Indices of liver fibrosis in all groups

Blood samples were collected from the abdominal aorta in all rats. Serum was separated, and HA, LN, and PCIII levels were measured. The results showed significantly higher serum levels of HA, LN, and PCIII than those in control rats ($P < 0.05$; Table 2). After flavonoid treatment, these indices were found to be significantly lower than those in model group

rats ($P < 0.05$), but not statistically different compared to the normal group (Table 2), suggesting significant improvement of liver fibrosis following treatment with flavonoid compounds.

Table 2. Indexes of liver fibrosis in all groups.

Group	N	HA	LN	PCIII
Control	10	100.21 ± 11.03	5.23 ± 0.98	26.11 ± 2.34
Model	10	587.43 ± 32.66*	37.65 ± 4.15*	89.17 ± 7.36*
Treatment	10	106.69 ± 22.31#	8.17 ± 2.18#	38.24 ± 3.23#

*$P < 0.05$ compared to control group; #$P < 0.05$ compared to model group.

Liver function indices in all rats

Blood samples were collected from the abdominal aorta of rats in all groups. Serum was separated and tested for the levels of ALT, AST, and TBIL, which indicate hepatic functions. The result showed significantly elevated ALT, AST, and TBIL levels in model rats compared to control rats ($P < 0.05$; Table 3). After flavonoid treatment, these indices significantly decreased compared to the model group ($P < 0.05$), but remained significantly higher than those in control rats ($P < 0.05$; Table 3).

Table 3. Levels of ALT, AST, and TBIL in all groups.

Group	N	ALT (IU/L)	AST (IU/L)	TBIL (μM)
Control	10	37.35 ± 4.16	225.89 ± 41.62	1.26 ± 0.21
Model	10	438.43 ± 52.16*	667.37 ± 78.68*	4.27 ± 0.56*
Treatment	10	236.41 ± 67.18*#	395.13 ± 92.68*#	2.98 ± 0.67*#

*$P < 0.05$ compared to control group; #$P < 0.05$ compared to model group.

DISCUSSION

Because of environmental factors and changes in eating habits and lifestyles, the global occurrence rate of hepatic disease has increased in recent years. Liver sclerosis is a severe complication that impairs the quality of life of patients with hepatic disease. Chronic liver damage or liver sclerosis can be caused by multiple factors, including drug abuse, toxicants, viral infection, and ethanol toxicity, but in China, it often occurs after hepatitis is acquired (Mokdad et al., 2014; Xu et al., 2014). Recent studies have found that liver fibrosis occurs during the pathogenesis of liver sclerosis, which is chronic advancing hepatic damage. Therefore, the effective prevention and treatment of liver fibrosis is important for impeding or even reversing the development of liver sclerosis. However, few medications are available that target liver fibrosis, since the basic mechanism is not well understood. Pharmacological research of Chinese medicine has shown great advancements in recent years. These medicines have been shown to have an anti-fibrotic effect in animal studies and preliminary clinical trials (Chien et al., 2014; Wang et al., 2014).

The pharmacological functions of flavonoid compounds include multiple-treatment effects against various systematic diseases, including cardiovascular, neurological, immunological, endocrinology, and respiratory symptoms (Chavez-Santoscoy et al., 2014; Daddam et al., 2014; Sankari et al., 2014). These studies also revealed promising treatment effects

of flavonoid compounds against liver damage. In this study, we measured the function of flavonoid compounds extracted from *Iris* plants for preventing and treating liver fibrosis in a CCl_4-induced fibrosis rat model. The result showed significantly lower severity of fibrosis after flavonoid compound treatment, suggesting a role of flavonoid compounds from *Iris* plants in preventing and treating liver fibrosis. Further assays of indices of liver fibrosis showed that HA, LN, and PCIII levels after treatment significantly decreased, illustrating that flavonoid compounds from *Iris* plants can improve liver fibrosis. Furthermore, liver function indices, including ALT, AST, and TBIL, were improved after flavonoid treatment, but remained significantly high compared to control animals, suggesting that flavonoids can function as a replenishment method in ameliorating liver damage and significantly preventing liver fibrosis. Studies have shown that flavonoid compounds can protect the liver through multiple pathways, such as by preventing high liver iron levels, inhibiting lipid peroxidation and hepatic protein oxidation, and facilitating iron excretion (Feher and Lengyel, 2012; Sak, 2014). Additionally, flavonoid compounds have a stronger anti-oxidative activity than vitamin C and vitamin E (Mehta et al., 2013). In contrast, flavonoid compounds can exert their protective role by regulating cell apoptosis; these compounds can inhibit normal hepatic cell apoptosis while accelerating apoptosis of tumor cells and necrotic hepatic cells. Flavonoid compounds also protect liver against damage via modulation of cell mitosis and proliferation as well as the secretion of enzymes against platelets during coagulation and inflammation (Dong et al., 2013). Some research suggests that lipopolysaccharide can induce hepatic cells to produce tumor necrosis factor, which can further induce the inflammatory response to accelerate the development of liver sclerosis. Flavonoid compounds can inhibit tumor necrosis factor secretion to effectively impede liver damage (Park et al., 2013). The detailed cellular mechanism of flavonoid compounds extracted from *Iris* plants, however, should be further examined.

In summary, we demonstrated the potent anti-fibrotic role of flavonoid compounds extracted from *Iris* plants in animal experiments, providing a theoretical basis for its clinical application and revealing an alternative method for the clinical treatment of liver fibrosis.

Conflicts of interest

The authors declare no conflict of interest.

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