Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Anti-fibrotic effects of marein-enriched *Coreopsis tinctoria* flavonoids on CCl_4-induced hepatic fibrosis in rats *via* blocking the TGF-β1/Smad signaling pathway

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ARTICLE INFO

Article history:
Received 2 July 2024
Received in revised form 18 July 2024
Accepted 7 February 2025

Keywords:
Coreopsis tinctoria
Marein
Flavanomarein
Hepatic fibrosis
TGF-β/ERK/Smad pathway
Antioxidant
Anti-inflammation
Flavonoids

ABSTRACT

Coreopsis tinctoria Nutt., an edible flowering plant, belongs to the Chrysanthemum family and is mainly grown at high altitudes in Northwestern China. It is rich in polyphenolic compounds, particularly marein and flavomarein, and possesses multiple health-promoting properties, such as antioxidant, hypoglycemic and vasorelaxant effects. Previous bioactivity investigations majorly focused on *C. tinctoria* and its crude extract. The aim of the present study was to prepare marein-dominant *C. tinctoria* flavonoids (CF), further investigate the CF protective effects of liver fibrosis induced by carbon tetrachloride and elucidate the associated molecular mechanisms. Results have demonstrated that CF effectively attenuated hepatofibrogenesis by increasing the activity of glutathione (GSH) and superoxide dismutase (SOD); suppressing the hepatic stellate cell (HSC) activation, inhibiting transforming growth factor β (TGF-β) activation and the production of α-smooth muscle actin (α-SMA), alleviating the phosphorylation of extracellular signal-regulated kinases1/2 (ERK1/2) and small mothers against decapentaplegic1/2 (Smad1/2), thus maintaining the collagen metabolic homeostasis in the liver. Our study revealed that CF possesses an efficacious protective effect against chronic hepatic fibrosis due to their strong inhibitory effects of oxidative stress and chronic inflammation.

1. Introduction

Coreopsis tinctoria Nutt., an edible plant and a member of the Chrysanthemum family and grown vastly in mountainous areas with high altitudes of about 3 000 meters above sea level, such as Xinjiang area of China, is rich in polyphenolic compounds majorly marein, flavomarein and caffeyolquinic acids[1-3] (Fig. 1), and possess multiple health-promoting properties such as antioxidant[4-5], antiageing[6-7], vasorelaxant effect and lowering high blood pressure[5,8], hypoglycemic[9-10] and protective effects against diabetic nephropathy among others[11-13]. In particular, *C. tinctoria* ameliorated hepatosteatosis in rats induced with a high fat diet and improved insulin homeostatis *via* the inhibition of the expression of glucose-6-phosphotase and phosphoenolpyruvate carboxykinase (PEPCK) and other mechanisms[14-15].

C. tinctoria and its extracts have also demonstrated to possess hepatoprotective effects against liver injury and other pathological conditions. In a model of acute liver injury induced by

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Peer review under responsibility of Beijing Academy of Food Sciences.

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2025.9250499

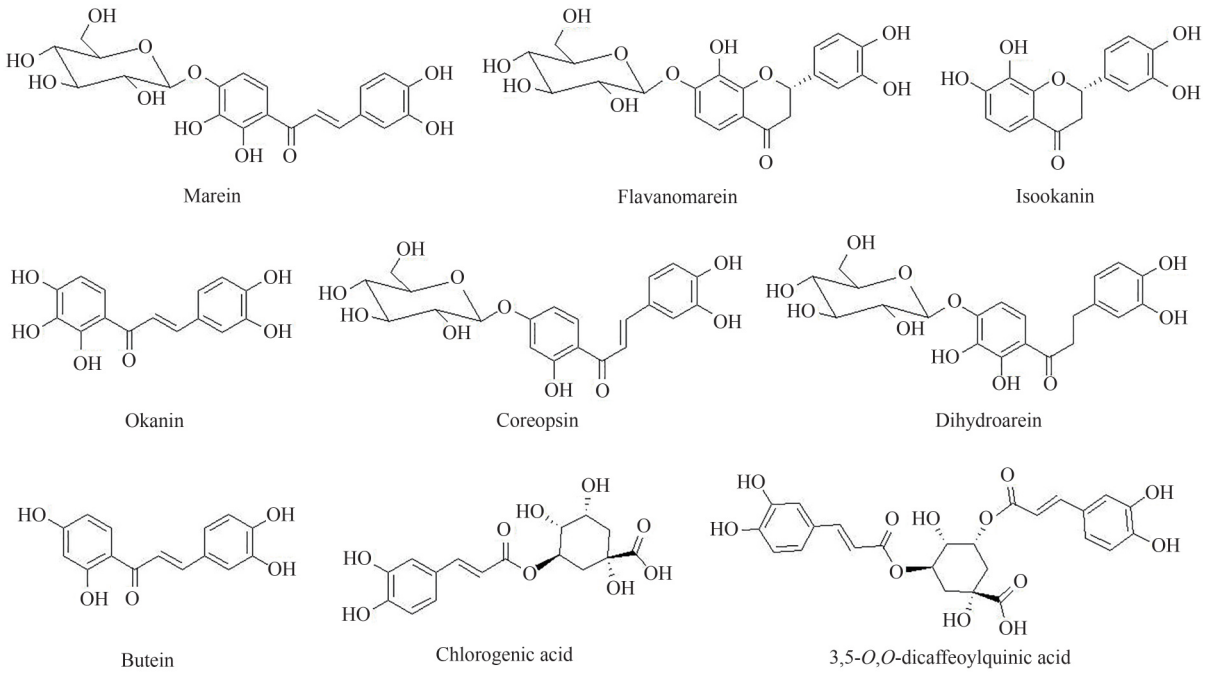


Fig. 1 Chemical structures of major flavonoids and polyphenolic acids in *C. tinctoria* Nutt.

D-galactosamine/lipopolysaccharide, the phenolic extract of *C. tinctoria* lowered the serum levels of aminotransferases-alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and malondialdehyde (MDA), and increased antioxidative enzyme glutathione (GSH), by an underlying mechanism of the up-regulation of nuclear factor-erythroid 2-related factor 2 (Nrf2), peroxisome proliferator-activated receptors alpha and gamma (PPARα and PPARγ), indicating phenolic compounds in *C. tinctoria* prevented acute liver injury[16-17]. In another acute liver injury model of mice induced by carbon tetrachloride (CCl_4), *C. tinctoria* ethanol extract demonstrated hepatoprotective effect against liver damage by reducing oxidative stress, increasing superoxide dismutase (SOD) and GSH reductase and inhibiting the expression of proinflammatory cytokines, namely, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6[18]. The mechanisms of hepatoprotective effect of *C. tinctoria* extract against acute liver damage demonstrated by the up-regulation of Nrf2, PPARα and PPARγ[16-17] and the inhibition of TNF-α, IL-1β and IL-6[18] provided evidence that the antioxidant and anti-inflammatory effects of *C. tinctoria* were the pathways for acute liver injury. However, the protective effect of *C. tinctoria* against chronic liver diseases such as heptatic fibrosis and the underlying mechanism remained unexplored. Furthermore, crude extracts of *C. tinctoria* with acetone[16-17] or ethanol[18] have been employed in previous studies against acute liver injury, for which the typical rich phytochemicals in *C. tinctoria* such as marein and/or flavanomarein were not examined. Therefore, the targeted evaluation of enriched flavonoid phytochemicals in *C. tinctoria* for their protective effects against chronic liver diseases and associated mechanisms is warranted.

Hepatic fibrosis, usually accompanied by continuous oxidative stress and chronic inflammation, is a preliminary chronic liver disease and mostly attributed to persistent hepatic injuries such as high fat consumption, alcohol abuse, toxins, genetic effects and other stimulating factors[19]. When left untreated, liver fibrosis is progressed to liver cirrhosis even liver cancer, which is the most common death of non-neoplastic disorder among hepatobiliary and digestive diseases globally. The management of liver fibrosis poses a significant burden on healthcare systems, with substantial annual expenditures and limited success from existing therapies[20]. Hence it is imperial to prevent liver fibrogenesis from developing into severe liver disease. Currently, there is no efficient treatment for liver fibrosis except the removal of inducing factors, underscoring the urgent need for therapeutic strategies that target fibrogenesis pathways to prevent the progression of liver fibrosis to more severe liver diseases. Therefore, the use of nutrients that target fibrogenesis pathways is a primary strategy and it is of great importance.

In this study, we have successfully prepared the marein-dominant *C. tinctoria* flavonoids (CF) shown in Fig. 2 and investigated its protective effects on liver fibrosis in a CCl_4-induced rat model and the associated underlying mechanism. Our results have revealed that CF has effectively reduced oxidative damage and attenuated pathological

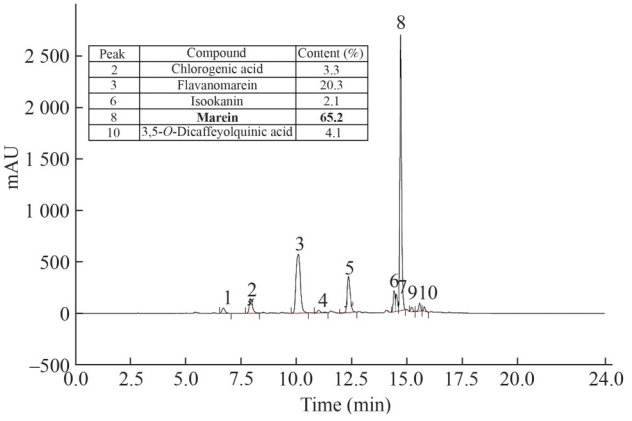


Fig. 2 HPLC profile and percentage content of prepared marein-dominant flavonoids (CF).

liver fibrosis by down-regulating the expression of transforming growth factor β (TGF-β), alleviating the activation of hepatic stellate cells (HSCs) and reducing the accumulation of collagen in liver tissues.

2. Materials and methods

2.1 Materials

2.1.1 Standard compounds in C. tinctoria

Chlorogenic acid, marein, flavanomarein, okanin, isookanin and 3, 5-di-*O*-caffeoyl quinic acid were purchased from Nanjing Jingzhu Biotechnology Ltd. (Nanjing, China). Lutein was purchased from Sinopaharm Chemical Reagent Co., Ltd. (Shanghai, China). All the standard compounds had a purity of ≥ 98%. Detailed information such as catalog numbers is listed in Supplement Materials.

2.1.2 Reagents

Acetonitrile, methanol, acetic acid and formic acid were of grade of high performance liquid chromatography (HPLC) and purchased from Merk KGaA (Darmstadt, Germany). HPLC water was prepared with a Millipore water purification system (Billerica, MA, USA). ACS reagent grade hexanes, ethyl acetate, butanol and 95% of aqueous ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade anhydrous ethanol, sodium hydroxide, sodium nitrite and aluminum trichloride were purchased from Shanghai General Chemical Reagent Factory (Shanghai, China).

2.1.3 Antibodies

Information of the following antibodies, namely, *α*-smooth muscle actin (*α*-SMA), TGF-β1, extracellular signal-regulated kinases1/2 (ERK1/2), small mothers against decapentaplegic 1 (Smad1), p-Smad1, Smad2, p-Smad2 and secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Abcam (Cambridge, MA, USA). Alex-488 conjugated goat anti-rabbit immunoglobin G (IgG) secondary antibody was purchased from Bio-SWAMP (Wuhan, China). Glyceralaldehyde 3-phosphate dehydrogenase (GAPDH) and p-ERK1/2 were from Cell Signaling Technology (Danvers, MA, USA). Other secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Reagents such as hydrogen peroxide and diaminobenzidine (DAB) for immunohistochemistry were purchased from Zhongshan Jinqiao Biotechnology (Beijing, China). Supplement Materials listed detailed information such as catalog numbers and others.

2.2 Extraction of flavonoids and analyses

2.2.1 Extraction

C. tinctoria was purchased from Yining, Ili Kazakh Autonomous Prefecture, Xinjiang Province, China. Dried flowers of *C. tinctoria* Nutt. (100 g) were crushed and extracted with 1 L of 70% aqueous ethanol under reflux for 2 h. Then, the resulted mixture was filtered upon cooling and the extraction process was repeated twice more. The filtrates from the triplicate extractions were combined and concentrated *in vacuo* to yield crude extract of *C. tinctoria*, which was re-suspended in water (150 mL) and extracted with 200 mL each of hexanes, ethyl acetate and *n*-butanol, respectively. The *n*-butanol layer was separated and concentrated to dryness *in vacuo* to yield marein-rich CF (5.2 g, yellow solid). The yielded CF was analyzed on a reversed-phase HPLC (Fig. 2) and then supplemented to liver fibrotic mice.

2.2.2 Major flavonoids of CF by HPLC analyses

The analysis of marein-rich *C. tinctoria* extract was performed on a Dionex UltiMate 3000 LC Module system including an LPG-3400 pump, a VWD-3400 UV-Vis detector, and a WPS-3000 SL auto-sampler from Sunnyvale, CA, USA. A Luna C_{18} reverse phased column (4.6 mm × 150 mm, 3 μm) with particle porous of 100 Å (Phenomenex, Torrance, CA, USA) was equipped for analysis. Mobile phases consisted of water with 0.1% trifluoroacetic acid (TFA) (solvent A) and acetonitrile (solvent B) with a flow rate of 1.0 mL/min. Detection wavelength was at 280 and 385 nm. A linear gradient elution program was used from initial 10% B, increased to 25% B over 12 min, to 45% B in next 12 min, and to 75% B in 8 min. Every single peak area of standard compounds used for the quantification was developed in the linear range for each standard compound. The sample solutions of CF were prepared in a concentration of 1 mg/mL in 60% aqueous ethanol. The analysis of each sample was performed in triplicate.

2.3 Animals experiment

Six-week old female Sprague-Dawley rats (SD rats) with an average body weight (BW) of 150−200 g were divided into 5 experimental groups with 8 rats in each group. The rats were fed with a normal rodent chow 5001 and *ad libitum* access to water, maintained at (25 ± 1) ℃ and a relative humidity of 50% − 60% on a cycle of 12 h light/12 h dark throughout the entire experiment. BW of the rats was recorded once every 7 days, and clinical symptoms and physical conditions were also observed. All animal experiments were strictly followed the Guidelines for the Care and Use of Laboratory Animals, Ministry of Science and Technology, China. The approval number of this animal study was SCXK(E) 2017-0012, from the Animal Care and Scientific Committee of Huanggang Normal University, Hubei, China.

After 7-day adaptive feeding, rats in the control group (Control) were intraperitoneally (IP) injected corn oil (0.1 mL/100 g BW) and all other rats received IP of 40% CCl_4 in corn oil at a dosage of 0.1 mL/100 g BW. Rats in Control and CCl_4-treated groups (CCl_4) were orally gavaged daily with ddH_2O. Other 3 groups of rats treated with CCl_4 were gavaged daily with i) 20 mg/kg BW of silymarin daily (Silymarin), ii) CF of 100 mg/kg BW of rats (CF-100), and iii) CF of 300 mg/kg BW of rats (CF-300), respectively. Upon 8 weeks of continuous administration (CCl_4, silymarin, CF-100 and CF-300), the experimental rats were fasted for 24-h then sacrificed by CO_2 asphyxiation. Samples of blood and liver were immediately collected for biochemical and histopathological analysis, respectively.

2.4 Biochemical analysis

Samples of rat blood were placed at room temperature for one hour prior to serum separation on a centrifuge at 1 000 r/min for 10 min. The resulted serum samples were then used to determine the

levels of AST, ALT, GSH, SOD and MDA using commercial test kits purchased from Jiancheng Bioengineering Institute (Nanjing, China). The value of absorbance was measured on a MK3 microplate reader (ThermoFisher, Waltham, MA, USA).

2.5 Histological and immunohistochemistry study

Liver samples were separated from the left lobe of each rat liver, fixed in 10% neutral buffered formalin (24 h), embedded in paraffin, sliced to sections of 5 μm of thickness, then stained with hematoxylin and eosin (H&E) for general pathological observation, and also stained with Sirius red for collagen fiber detection.

The sample histopathological profiles were observed at 200× magnification under an Olympus BH_2 optical microscope (Hino, Japan). The degree of hepatic fibrosis was independently evaluated by three pathologists in reference to the METAVIR scoring system, i.e. S0 (no observed fibrosis), S1 (perisinusoidal or portal fibrosis observed), S2 (formation of fiber septum and hepatic lobular structure preservation), and S3 (fiber septal formation and hepatic lobular structure destruction) and early cirrhosis[21].

2.6 Real-time (RT)-PCR

From the liver tissue, total RNA was extracted after homogenization using TRIzol reagent (detailed Cat# and manufacturer in Supplement Materials). Then mRNA was reverse-transcribed to complementary DNA (cDNA) with a commercial Advantage RT-for-PCR Kit according to the manufacturer's instruction. RT-PCR was performed on a Bio-Rad Real-Time PCR System using SYBR Green Master Mix with primers listed in Supplemtent Materials. Expression levels were calculated using delta delta threshold cycle ($2^{-\Delta\Delta Ct}$) method.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected and centrifuged at 277 × *g* for 10 min. Serum was then collected after centrifugation. The contents of TNF-α, TGF-β and IL-17 in serum were determined according to manufacturer's instruction (Bio-Swamp, Wuhan, China). The optical density (OD) value of each well was read immediately at 450 nm using a LabSystems MK3 microtiter plate reader (Helsinki, Finland).

2.8 Western blotting

Immunoblotting analysis was performed following a previous report[22]. The liver tissue was homogenized after radio-immunoprecipitation assay (RIPA) lysis buffer from Biovision Inc. (Milpitas, CA, USA) was added in the amount of 150−250 μL/20 mg tissue. The RIPA buffer consisted of 1% PMSF and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA). Proteins were extracted from the yielded liver lysates by gel electrphrosis then transferred to Millipore polyvinylidene fluoride membranes (0.45 μm, Billerica, MA, USA), which were then incubated with primary antibodies against α-SMA, TGF-β, ERK1/2, p-ERK1/2, Smad1/2, p-Smad1/2, GAPDH and HRP-conjugated antibodies at 4 ℃ overnight. The intensities of protein bands were detected using an enhanced chemiluminescence image analyzer of Tanon-5200 (Tanon, Shanghai). Quantifications of all protein were adjusted for the corresponding β-actin level.

2.9 Statistical analysis

SPSS 16.0 software (SPSS Inc., Chicago) was used for statistical analysis. All results were represented as mean ± standard error of the mean (SEM). Data were analyzed using Student's *t* test for 2 groups, one-way ANOVA, and Dunnett's test for three groups. A *P* value of less than 0.05 ($P < 0.05$) was regarded statistically significant. Graph bars were quantified by ImageJ 1.42 software analysis (NIH, Bethesda, USA).

3. Results

3.1 Preparation of marein enriched CF

The content of polyphenols, majorly flavonoids, in *C. tinctoria* is very rich and could reach more than 20% in dry weight[3,9]. Moreover, it has been reported that *C. tinctoria* possess multiple biological activities including antioxidant[4-5], hypertension[9-14], antiageing[6-7], acute liver injury[16] and others. Hence, *C. tinctoria* extracts have been prepared and tested in several animal models such as dementia[6], ageing[7], hyglycemia[9-14], and inflammation[11] among others. However, the preparation and use of a *C. tinctoria* flavonoid extract has been seldom reported except in studies that used small amounts in a single molecule dose (e.g. marein or flavonomarein). In this study, we have prepared a large quantity of marein-dominant flavonoids from *C. tinctoria* and then investigated its preventive effects on liver fibrosis and underlying mechanisms.

Main CF in *C. tinctoria* include marein, flavonomarein, and isookanin among others, but dominant ones are usually marein and flavonomarein (Fig. 1). The preparation of marein-enriched CF began with routine extraction of *C. tinctoria* with 70% aqueous ethanol and the resulting extract was fractionated with stepwise solvent extraction of hexanes, ethyl acetate, and *n*-butanol, respectively. The content of marein in our prepared flavonoid-rich *C. tinctoria* extract was 65.2% by HPLC analysis, which was the first time that a marein-dominant *C. tinctoria* extract was prepared. Other flavonoids identified were flanonomarein (20.3%) and isookain (2.1%). Also 2 phenolic acids were detected, that is, chlorogenic acid (3.3%) and 3,5-*O*-dicaffeoylquinic acid (4.1%). A few other minor contents were unidentified. The total flavonoids of the prepared marein-enriched CF were 87.6% measured by HPLC method (Fig. 2).

3.2 CF improved liver function and attenuated oxidative stress in CCl_4-treated SD rats

In general, rats treated with CCl_4 had a significant increase in food and water intake and also a greater body gain than normal rats in the control group. However, the increased body weight gain, and food and water intake in CCl_4 rats were significantly alleviated by CF treatment (CF-100, CF-300). Similarly, liver weight in CCl_4-treated rats was significantly higher than that in normal rats, but was reduced by CF treatment (data not shown).

Results showed the improvement of liver function and oxidative stress status in experimental rats after CCl_4 treatment (Table 1). Comparisons among all groups were evaluated using ANOVA. ALT and AST are 2 major enzymes reflecting liver function, hence recognized as biomarkers of liver damage. The serum levels of ALT and AST in rats treated with CCl_4 were significantly elevated by 5.7

and 6.5 times, respectively, compared to that of Control rats. However, the elevated serum levels of ALT and AST induced by CCl_4 were effectively alleviated by the CF application in a dose dependent manner ($P < 0.01$, $n = 8$). For instance, the levels of ALT were reduced by CF-100 and CF-300 by 32.2% and 50.8%, respectively, compared to the CCl_4-treated group. The levels of ALT were reduced by 36.9% (CF-100) and 62.4% (CF-300), respectively.

In vivo antioxidant enzymes and substances protect cells from oxidative damage induced by free radicals. In the present study, levels of GSH and SOD were measured and found that CCl_4-treatment resulted in significantly lower levels of GSH and SOD ($P < 0.01$). However, the addition of CF to the diet at the dosages of 100 and 300 mg/kg has dramatically elevated hepatic SOD activity, marked by the increased levels of SOD with 2.3 times (CF-100, $P < 0.01$) and 4.3 times (CF-300, $P < 0.01$), respectively, as shown in Table 1. As an *in vivo* antioxidant, GSH prevent damage from oxidative stress. Treatment with CCl_4 caused a significant decrease for the GSH level from 24.03 mg/g pro (Control group) to 5.66 mg/g pro in the CCl_4 group (Table 1). The supplementation of CF has recovered the level of GSH in 100 and 300 mg/kg BW by 55.3% and 126.5%, respectively ($P < 0.01$), compared to the CCl_4-treated model group.

MDA is the terminal product of lipid peroxidation and its elevated level in tissue represents a higher degree of oxidative stress. Hepatic level of MDA has increased significantly after CCl_4-treatment (Table 1), indicating severe oxidative stress. However, hepatic MDA levels in rats fed with CF significantly decreased by 22.9% in the CF-100 group and 63.6% in the CF-300 group (Table 1, $P < 0.01$), demonstrating the strong antioxidant effect of CF, which is in accordance with previous studies[4-5].

3.3 Histopathological analysis of liver tissue

We further investigated the protective effects of CF on CCl_4-triggered liver injury using H&E staining and Sirius red staining on slides of liver tissue (Fig. 3). Results showed that in the Control group of rats, the liver color and liver lobules were normal; collagen fibers around the portal area and central vein were scarce; hepatic sinusoids were not dilated; hepatocytes were able to be identified clearly and evenly distributed; and no fibroplasia was observed in the hepatic lobules of rats in the Control group. In contrast, rats treated with CCl_4 developed liver damage expressed by hepatocellular lesions, inflammation and liver fibrosis (Fig. 3A), marked by necrotic, shedding and inflammatory liver cells, sinusoid fibrotic liver cells around the central vein, and fibrous septum formation and hyperplasia in the central vein and tube area. Treatment of CCl_4-injured rats with CF and silymarin effectively ameliorated the whitening liver surface (Fig. 3B), decreased degree of patchand bridging necrosis, and reduced inflammatory cells that infiltrated into the portal area; and fibrillar collagen sediment was rarely found in the portal/periportal area (Fig. 3B), indicating that as effective as silymarin, CF demonstrated an efficient protective effect on liver injury and fibrosis (Fig. 3C).

3.4 CF decreased levels of proinflammatory cytokines and growth factor

One of the chief driving forces of fibrogenesis is the proinflammatory cytokines that are released from injured hepatocytes and also from nonparenchymal cells. It has been revealed that monocyte production of TNF-α was a critical downstream response to the IL-17 produced by Th17 cells[23]. IL-17 has a prominent role in activating TGF-β signalling pathways in HSCs as it induces upregulation of TGF-β receptor II and enhances activation of downstream kinases[24]. We further detected the serum levels of TNF-α, IL-17 and TGF-β1 with ELISA assay. The CCl_4 treatment triggered a dramatic increase in the level of TNF-α in serum (Fig. 4). The serum TNF-α level was increased 9.2-fold comparing to the Control group, but decreased significantly after CF supplementation, with a 1.3- and 2.1-fold reduction at dosages of 100 and 300 mg/kg ($P < 0.01$), respectively. It has been revealed that

Table 1
Protective effect of CF on liver function and oxidative stress in CCl_4 treated SD rats.

Group	ALT (U/L)	AST (U/L)	GSH (mg/g pro)	SOD (U/g pro)	MDA (nmol/g pro)
Control	16.1 ± 1.7^a	23.8 ± 2.7^a	24.03 ± 0.50^a	120.17 ± 14.00^a	6.38 ± 0.19^a
CCl_4	90.0 ± 4.1^b	155.8 ± 6.6^b	5.66 ± 0.90^b	13.50 ± 2.25^b	64.94 ± 2.18^b
Silymarin/CCl_4	23.6 ± 2.9^c	44.0 ± 2.9^c	19.56 ± 1.01^c	87.77 ± 9.22^c	16.30 ± 1.34^c
CF-100/CCl_4	62.1 ± 6.9^d	98.3 ± 6.9^d	8.79 ± 0.07^d	31.54 ± 4.03^d	43.20 ± 2.94^d
CF-300/CCl_4	44.3 ± 2.7^e	58.6 ± 2.7^e	12.82 ± 0.71^e	58.52 ± 0.95^e	23.64 ± 0.99^e

Note: MDA, SOD, GSH in serum measured by individual commercial kits. Data shown was mean ± standard deviations ($n = 3$). Within the same column, values with different letters (a-e) show significant difference ($P < 0.01$).

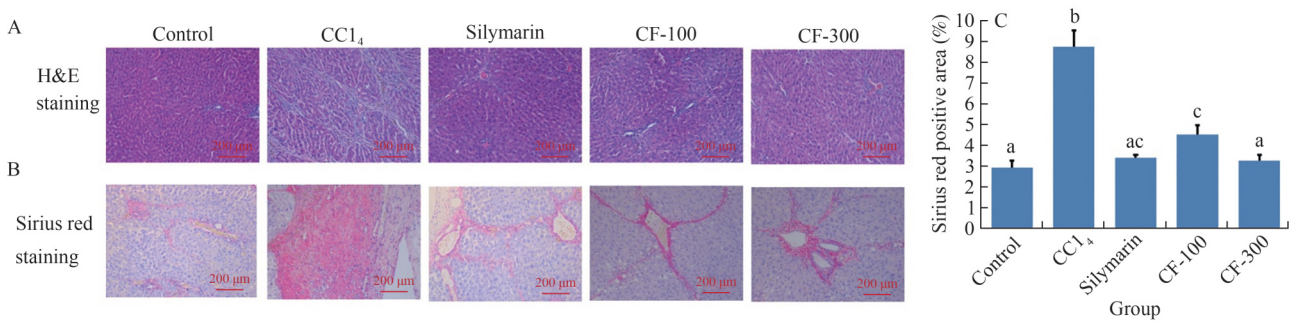


Fig. 3 (A) H&E staining (200× magnifications) on liver tissue; (B) Sirius red staining (200× magnifications) on liver tissue; (C) quantitative analysis of Sirius red staining using Image Pro-Plus 6.0. All slides are analyzed in 5 different typically fields. Results are the mean ± SEM; $n = 5$. Different letters (a-c) represent the significant differences in the same indexes among different groups when $P < 0.01$.

monocyte generation of TNF-α was a crucial downstream response to IL-17 produced by Th17 cells[23]. The 3.7-fold increase of serum IL-17 after CCl_4 treatment was also significantly decreased by CF supplementation in a dose dependent manner, i.e. a reduction of 1.4-fold in CF-100 *vs*. 1.9-fold in CF-300 group of rats ($P < 0.01$) (Fig. 4).

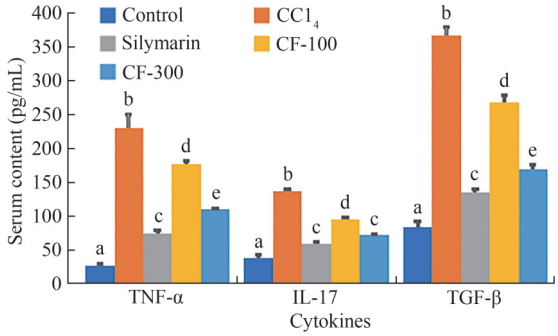


Fig. 4 CF alleviated CCl_4-induced proinflammatory cytokines and growth factor. Values are the mean ± SEM, $n = 5$; Different letters (a-e) are the significant differences of the same indexes among different groups when $P < 0.01$.

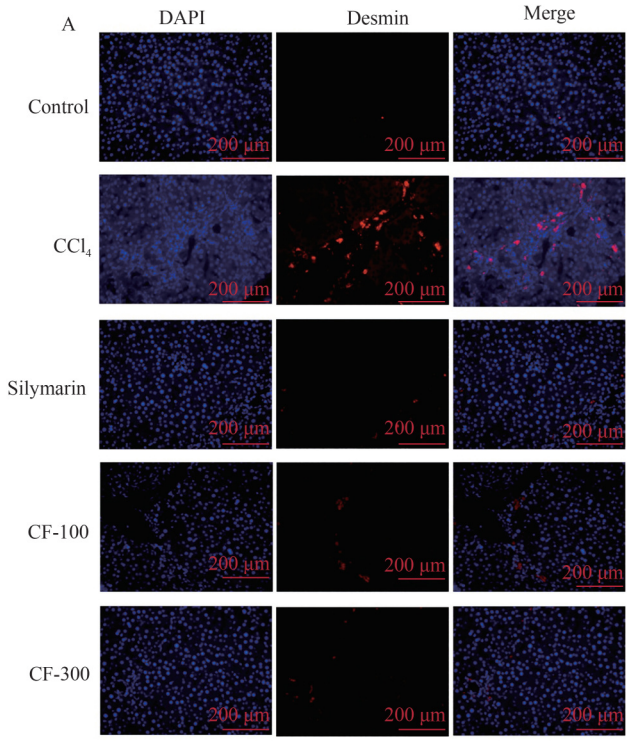
TGF-β1 is a key growth factor that stimulates activation and proliferation of HSCs[24], also a cytokine that mediates tissue inflammation and drives extracellular matrix (ECM) production in the liver. In the present study, TGF-β1 in serum and the tissue was measured respectively by ELISA and Western blotting. Results showed that after CCl_4 stimulation, the expression levels of TGF-β1 increased by 4.4- and 2.6-fold in serum and the liver, respectively, comparing to that of Control rats. But CF treatment of rats stimulated by CCl_4 significantly suppressed the elevated levels of TGF-β1 in serum and the liver (Fig. 4). CF treatment decreased the expression levels of serum TGF-β to 3.2-fold (CF-100), and 2.0-fold (CF-300), respectively, comparing to the Control group, indicating that CF effectively inhibited the hepatic fibrosis induced by CCl_4 treatment in a dose-dependent manner.

3.5 *CF depressed the activation of HSCs*

As an important source of growth factors in the liver, HSCs are the core in the pathological progression of liver fibrosis. Under chronic liver injury, HSCs are activated from quiescent retinoid-storing cells. The activation and proliferation of HSCs trigger cytokine release, high expression of α-SMA and desmin, accumulation of ECM and consequently the formation of liver fibrosis. In our investigation of the inhibitory effect of CF on CCl_4-triggered HSC activation, immunostaining analysis revealed that desmin was less detected in the CF group compared to the CCl_4 group (Fig. 5A). Moreover, mRNA levels of matrix metalloproteinases (MMPs), such as *MMP2* and *MMP9*, remarkably increased in the CCl_4-treated group of rats by 10.5 and 5.3 times, respectively. However, mRNA levels of these 2 MMPs were decreased significantly after CF intervention (Fig. 5B, $P < 0.01$).

3.6 *TGF-β/Smad pathway*

Previous study has demonstrated that TGF-β promoted over expression of fibrosis related α-SMA *via* ERK1/2[25]. Persistent



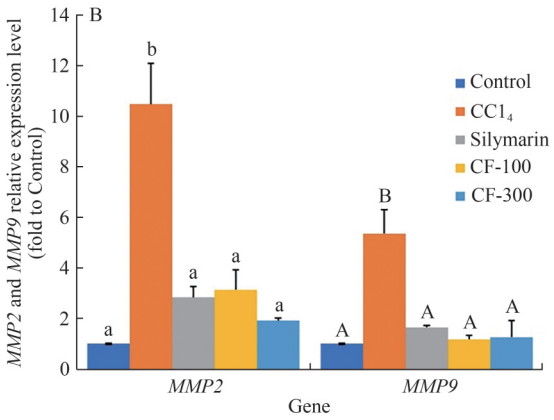


Fig. 5 CF down-regulated liver fibrogenic protein desmin and ameliorated *MMP2* and *MMP9* mRNA levelsof rats induced by CCl_4. (A) Levels of liver desmin were assessed using an immunofluorescence assay; (B) *MMP2* and *MMP9* mRNA levels in the liver were analyzed by RT-PCR. Using *GADPH* as internal reference, the expression value of Control group was set as 1, and the relative expression multiplier of the gene was calculated by $2^{-\Delta\Delta Ct}$ method. Values are expressed as mean ± SEM, $n = 5$; Different letters (a, b for *MMP2*; A, B for *MMP9*) represent the significance in the same indexes among different groups when $P < 0.01$.

activation of Smad1/2 and ERK1/2 signaling pathways can mediate the profibrotic response, hence TGF-β/Smad is a major signaling pathway leading to liver disease, and mediates liver fibrosis[26]. To reveal CF involved signaling pathways, we tested several core signaling pathway proteins including TGF-β, Smad1/2 and phosphorylation of ERK1/2 MAP kinases (Fig. 6). As shown in Fig. 6A, all tested proteins in rat liver were highly expressed when treated with CCl_4, comparing to the Control group of rats. However, CF treatment effectively down-regulated protein expression levels in the liver.

4. Discussion

Native to North America, *C. tinctoria* Nutt. has spread worldwide. In recent years, *C. tinctoria* has attracted attention because pharmacological studies have revealed that *C. tinctoria* and its extract possess strong antioxidant[4-5], antidiabetic[13-14] and hypotensive[5,8] effects chiefly due to its abundant flavonoids. *C. tinctoria* flavonoid compounds effectively inhibited angiotensin I-converting enzyme activity and prevented oxidative stress[5]. The ethyl acetate extract of *C. tinctoria* demonstrated anti-inflammatory effect in high glucose induced rat glomerular mesangial cells by significantly decreasing the expression levels of mRNA and protein of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1) and p-65, possibly through NF-κB signaling pathway[27]. However, the preventive effects of CF on chronic liver fibrosis remain unexplored. Marein is the most abundant flavonoid in *C. tinctoria*. In this study, we have successfully extracted CF enriched with marein whose content was as high as 65.2% in the CF, an 11.8-fold increase compared with a regular *C. tinctoria* extract (5.5% marein). This is the first reported *C. tinctoria* extract that had such a high content of marein.

The pathological process of liver fibrosis begins with the response to hepatic injury caused by factors like viral hepatitis, alcohol abuse, and/or chronic injury condition such as nonalcoholic steatohepatitis[27]. These factors trigger chronic inflammation and/or continuous oxidative stress and in response, ECM proteins are excessively synthesized and accumulated in different liver cells such as HSCs and periportal myofibroblasts among others[28]. The liver injury-stimulated imbalance of ECM protein over-synthesis and inhibited ECM degradation results in the loss of ECM dynamic equilibrium. Oxidative stress and inflammation activate HSCs, which in turn secrete cytokines including TGF-β and IL-6 among others that consequently enhances liver fibrosis. Therefore, the inhibition of TGF-β synthesis and its related signaling pathways could ameliorate heptafibrosis that is attested in experimental models[29]. CCl_4 was used to induce liver fibrosis in rats in this study. In the rat model, CCl_4 stimulates the *in vivo* production of free radicals, such as trichloromethyl ($\bullet CCl_3$) and trichloromethyl peroxyl ($\bullet OOCCl_3$) among others, lipid peroxidation and inflammation, which in turn causes endogenous cytokines to activate HSCs[30].

In our study, rats were given 40% CCl_4 in olive oil *via* an IP injection twice weekly for 8 weeks to cause severe hepatotoxicity illustrated by the increased levels of serum ALT and AST (Table 1) and liver injury from histological analysis (Fig. 4). At the same time, 2 different dosages of CF, i.e. marein-enriched CF (100 and 300 mg/kg bw) were administered orally to CCl_4-treated rats to investigate the protective effects of CF on hepatofibrosis. Oxidative

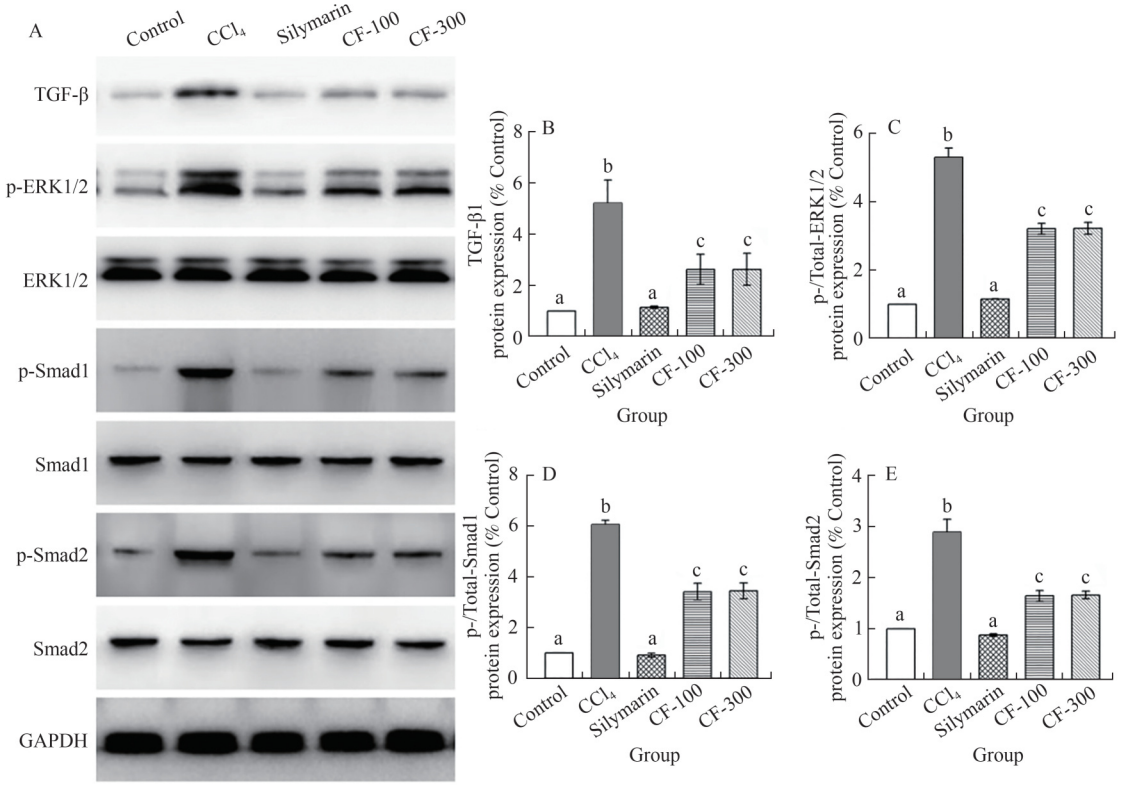


Fig. 6 CF attenuates the activation of HSCs in liver by restraining TGF-β/ Smad pathway. (A) Western blot detection of the expression levels of TGF-β, ERK1/2, p-ERK1/2, Smad1/2, p-Smad1/2 and GAPDH in rat liver, (B) TGF-β, (C) p-ERK1/2 and total ERK1/2, (D) p-Smad1 and total Smad1, (E) p-Smad2 and total Smad2 proteins expression in rat liver. Values are the mean ± SEM (n =5). Different letters (a-c) are the significant differences in the same indexes among different groups when $P < 0.01$.

stress is an important biomarker in the pathological process of liver fibrosis, hence we examined the antioxidant capacity of the livers in experimental rats. In the CCl_4-treated rats, levels of GSH and SOD were dramatically decreased, but CF administration of the CCl_4-treated rats remarkably increased the levels of GSH and SOD (Table 1). The elevated MDA levels were decreased dramatically after CF treatment in the model rats, indicating less lipid peroxidation in the liver of CCl_4-treated rats. Therefore, CF has effectively reduced the oxidative stress in the CCl_4-treated rats, hence protecting the liver from fibrogenesis.

HSCs store retinoids in a resting state and is localized in the space of Disse in the normal liver. Stimulation by CCl_4 triggers HSCs activation and proliferation, which is the core of the pathogenesis of liver fibrosis. Activated HSCs proliferate then undergo *trans*-differentiation to become activated myofibroblast-like cells, marked by more desmin production and excess secretion of extracellular matrix proteins such as α-SMA and growth factors such as TGF-β. Hence the inhibition of HSCs activation is of great importance in preventing liver fibrogenesis. The present study has demonstrated that CF treatment significantly decreased these elevated biomarkers resulting from CCl_4 injection, marked by the reduced amount of collagen accumulation from Sirius red staining (Fig. 4), significantly less desmin production, and decreased secretion of TGF-β and α-SMA (Fig. 5).

HSCs activation also triggered the production of MMPs, which could elevate hepatic fibrosis by cleaving the fibrillar extracellular matrix and cause the imbalance between the synthesis and degradation of ECM. In particular, the deposition of newly synthesized ECM from the early stage of liver fibrosis would destroy surrounding tissue and led to the continuous activation of HSCs, causing high expression of MMPs. Therefore, MMPs are considered as important biomarkers of the activation of HSCs in its early stage. Sirius red staining of liver biopsy showed that the rats in the CCl_4 group were in the early stages of liver fibrosis. The expression levels of MMP2 and MMP9 were elevated dramatically in the CCl_4-treated group of rats, comparing to that of the Control group (Fig. 6), but decreased significantly with CF treatment in a dose-dependent manner (Fig. 6, $P < 0.01$).

The progression of hepatic fibrosis has been closely related to TGF-β/Smad/ERK signaling pathway[22]. HSCs activation in CCl_4-induced liver fibrosis triggers high-level expression TGF-β, which in turn activates ERK1/2 phosphotylation. We have found that CF effectively reduced the phosphotylation of ERK1/2, which is also associated with the effective inhibition of α-SMA. Smad is a direct substrate of TGF-β receptor and its signaling is responsible for the major regulation of TGF-β effects. Activated TGF-β triggers the phosphorylation of Smad proteins including Smad1 and Smad2. In the present study, we further found that CF-treatment effectively inhibited the phosphorylation of Smad1 and Smad2, indicating the involvement of activated Smad1/2 in the pathological progression of liver fibrogenesis.

5. Conclusion

In summary, we have successfully prepared marein-dominant CF, in which the marein content was increased by 11.8 times to 65.2% compared to regular *C. tinctoria* extract. The administration of CF to CCl_4-treated SD rats dramatically attenuated hepatofibrogenesis and other heptocellular toxic effects induced by CCl_4. We further revealed that the mechanism of the efficacious effects of CF against hepatic fibrosis by increasing the activity of GSH and SOD and reducing the levels of MDA; inhibiting the activation of TGF-β and the production of α-SMA induced by TGF-β; alleviating the phosphorylation of ERK1/2 and Smad1/2, thus blocking the TGF-β/Smad signaling pathway and maintaining the collagen metabolic homeostasis in the liver. Therefore, the results of the present study have demonstrated that CF supplementation effectively suppressed CCl_4-induced hepatic fibrosis. Further isolation and investigation of pure marein and flavonomarein compounds for its anti-fibrotic effects and other biological activities is under way to elucidate the bioactivity and its associated mechanism of the main bioactive phytochemicals in *C. tinctoria* to be developed as a potential functional food.

Conflict of interest

Chi-Tang Ho is a senior editor, Shiming Li is a scientific editor , and Liwen Wang is a youth editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. 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.

Acknowledgements

This research was funded by the grant for Evaluation of Functional Property, Processing and Utilization of Xinjiang Characteristic Plant Resources (G2023046003L); the Xinjiang Uygur Autonomous Region Outstanding Youth Science Fund project (2024D01E11); National Nature Science Fund of China (82060788); and Hebei Natural Science Foundation (C2022204167).

Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2025.9250499.

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