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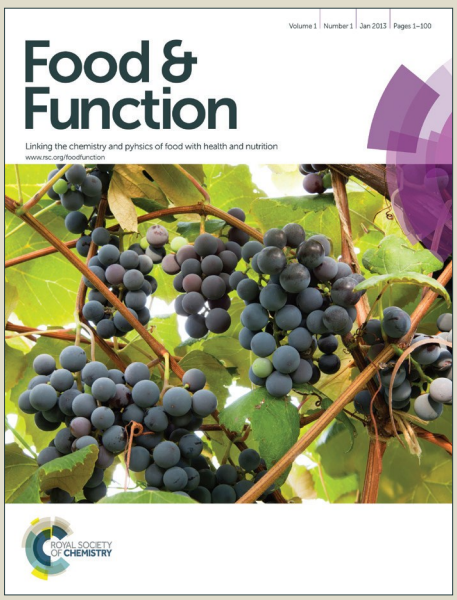

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ARTICLE

Protective effects of *Chaenomeles thibetica* extract against carbon tetrachloride-induced damages via MAPK/Nrf2 pathway

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Chaenomeles thibetica, as a type of fruit in Chaenomeles, is commonly cultivated and used as Mugua in China and as liquor, candy, and functional food in Tibet. Total phenol, flavonoid, and proanthocyanidin contents were measured in *C. thibetica* extract (CTE). CTE had a positive effect on free radical scavenging and anti-lipid oxidation *in vitro*. The protective effects of CTE against carbon tetrachloride (CCl_4)-induced oxidative damage *in vivo* were also measured. Results of the antioxidative enzymes indicated that CTE can increase the activities of catalase, superoxide dismutase, and glutathione contents and reduce the level of malondialdehyde in rats. The levels of alanine aminotransferase, aspartate transaminase, alkaline phosphatase, and total bilirubin were significantly reversed by CTE compared with the elevated levels in the CCl_4 group. Besides, CTE could reversed the cell viability of HepG2 intoxicated with CCl_4 via phosphorylation of mitogen-activated protein kinases (MAPKs), activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and increasing the expression of phase II detoxification enzymes. These effects may expand the applications of *C. thibetica* and offer alternative food with antioxidant and hepatoprotective function in food industry.

Introduction

Five wild *Chaenomeles* species exist in the world, namely, *Chaenomeles speciosa* (Sweet) Nakai, *Chaenomeles thibetica* T. T. Yu, *Chaenomeles cathayensis* (Hemsl.) Schneid., *Chaenomeles sinensis* (Thouin) Koehne, and *Chaenomeles japonica* (Thunb.) Lindl. ex Spach.[1,2] These five wild *Chaenomeles* species are commonly cultivated, and their fruits were used as Mugua in China and as liquor, candy, and medicine in Tibet.[3,4] The *Chaenomeles* species is extensively applied in the food industry because of its numerous biological activities, such as antimicrobial, anti-influenza, antioxidant, antihyperglycemic, and antihyperlipidemic.[5-8] Among the five species, the contents of polyphenols and two triterpenes (oleanolic acid and ursolic acid) in *C. thibetica* are relatively higher than other species. So, *C. thibetica* was used to detect more activities. If the application of *C. thibetica* can be expanded, it may be helpful for the development of edible folk herbs in food industry.

The liver is an organ with various functions, such as the synthesis, secretion, and metabolism of xenobiotics.[9] When eliminating xenobiotics, free radicals may be produced. For example, carbon tetrachloride (CCl_4) is metabolized to the trichloromethyl radical ($\cdot CCl_3$) and proxy trichloromethyl radical ($CCl_3OO^{\cdot}$) by cytochrome P450 in the liver.[10] Thus, CCl_4 at a high dosage could cause liver injury and failure through cellular necrosis, oxidative stress, and inflammation.[10] So, CCl_4–induced hepatoxicity was a common animal model for investigating the potential hepatoprotective acivity.[11-13] Therefore, the use of antioxidants with free radical scavenging effect may prevent or alleviate such liver injury.

Xenobiotics may exacerbate some diseases by oxidative and electrophilic stress, such as liver injury/hepatic failure. Cells are induced to develop some adaptive responses by intrinsic and extrinsic oxidants and electrophiles to counteract these environmental stresses.[14] The redox sensitive transcription factor nuclear E2-related factor 2 (Nrf2) regulates antioxidant defence systems with the production of endogenous antioxidant defences and detoxifying enzymes.[15] Normally, Nrf2 is tethered in the cytoplasm by the Kelth-like ECH-associated protein 1 (Keap1) for subsequent proteasomal degradation.[16] Moreover, c-Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK) and P38 are the main mitogen-activated protein kinases (MAPKs) which could be phosphorylated by chemopreventive agents or antioxidants to separate Nrf2 and Keap1. Following the phosphorylation, free Nrf2 is translocated to nucleus and activated the expression of Phase II detoxifying enzymes, such as glutathions-S-transferase (GST), quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1), etc.[17]

In this study, we mainly focused on the protective effects of *C. thibetica* extracts (CTE) against CCl_4-induced damages. Furthermore, we measured the expression of phosphorylated MAPKs (p-JNK, p-ERK, p-P38), Nrf2 and some detoxifying enzymes (GST, NQO1) to clarify the antioxidant pathway of CTE. This study may expand the application of *C. thibetica*, which could be

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considered as functional food against oxidative damage and hepatotoxicity.

Experimental

Chemicals

The compound α,α-diphenyl-β-picrylhydrazyl (DPPH) was procured from Sigma-Aldrich (St. Louis, MO, USA). The compound 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was procured from Fluka (Menlo Park, CA, USA). Gallic acid (GA), rutin (RU), catechin (CA), ascorbic acid (vitamin C), and butylated hydroxytoluene (BHT) were procured from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals used for the analysis were of AnalaR grade and were obtained from the China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, China). The antibodies of β-actine, p-JNK, NQO1 and GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies of p-ERK and Nrf2 were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody of p-P38 was purchased from Abcam (Cambridge, UK).

Plant materials

Specimens of *C. thibetica* fruits were collected from the Tibetan Traditional Medicine Pharmaceutical Factory (Lhasa City, Tibet AR, People's Republic of China) and authenticated using references and authoritative books by the corresponding author (Wuhan University, Wuhan, China).[2] Voucher specimen (no. 722) has been deposited in the Institute of Traditional Chinese Medicine and Natural Products, School of Pharmaceutical Sciences, Wuhan University. The plant material was air-dried indoors at room temperature prior to extraction.

Preparation of experimental samples

C. thibetica fruits were ground to powder form (50 g, size less than 0.25 mm) and extracted in 70% ethanol at room temperature for 24 h.[18] Then, the solvent was removed by rotary evaporation at 50 °C. The residues were lyophilized, and the resulting dry powder was stored at 4 °C.

Determinations of total phenolic, flavonoid, and proanthocyanidin contents

The total phenolic content was measured by using the Folin–Ciocalteu reagent method.[19] The total phenolic content was calculated and compared with the standard curve of GA, which served as a standard chemical.

The total flavonoid content of the extract was determined by colorimetric assay.[20] The extract (5 mL) was added to a 10 mL flask, and then 5 mg/mL $NaNO_2$ solution (0.5 mL) was added. After mixing, the mixture was kept for 6 min at room temperature, and then 100 mg/mL $Al(NO_3)_3$ solution (0.5 mL) was added to the flask, mixed well, and kept for 6 min at room temperature. Finally, 40 mg/mL NaOH solution (4 mL) was added, mixed well, and kept for 15 min. Absorbance was measured at 510 nm. The total flavonoid content was calculated and compared with the calibration curve of RU, which served as a standard chemical.

Proanthocyanidin determination was based on the previously described procedure.[21] Extract solution (0.5 mL) was mixed with 3 mL of a 40 mg/mL vanillin–methanol solution and 1.5 mL of hydrochloric acid, and then the mixture was kept for 15 min in the dark. Absorbance was measured at 500 nm and was used to calculate the final result expressed as milligrams of CA equivalents per gram dry extract.

HPLC- ESI-MS analyses of CTE

HPLC- ESI-MS analyses were conducted on the Shimadzu high-performance liquid chromatography (HPLC) system (Tokyo, Japan) equipped with binary solvent manager, SPD-20A UV detector, and LTQ Orbitrap XL™ (Thermo Fisher Scientific, Waltham, MA, USA). The Xtimate™ C18 column (4.6 mm × 250 mm, 5 μm) was used. A linear gradient elution of water containing 0.1% acetic acid (A) and acetonitrile (B) was used (18% solvent B for 12 min, 30% solvent B for 8 min, and 50% solvent A for 10 min). The flow rate was 1.00 mL/min, the column temperature was 25 °C, the injection volume was 20 μL, and the detection wavelength was 254 nm.

By solvent splitting, 20% eluent was allowed to flow into the mass spectrometry (MS) instrument with an electrospray source in the positive ion mode. The MS analysis conditions were as follows: sheath gas was 30 psi and auxiliary gas was 10 psi, desolvation temperature was 275 °C, capillary voltage was 4,500 V, and scan range (*m/z*) was 100 to 1,000. The HPLC and MS instruments were connected to each other by the UV cell outlet.

In vitro antioxidant activity

DPPH radical scavenging assay

DPPH free radical scavenging activity was measured based on the method of Huang et al., wherein 2.7 mL 0.2 mM DPPH in ethanol was mixed with 0.3 mL test samples in ethanol at different concentrations (0-1000 μg/ml).[21] Each mixture was shaken vigorously and kept at room temperature for 1 h. BHT and vitamin C served as references. Absorbance was measured at 517 nm with a Beckman spectrophotometer. Radical scavenging activity was calculated as follows:

$$\text{Scavenging rate} = \left[\left(A_s - A_i\right)/A_s\right] \times 100,$$

where A_s is the absorbance of pure DPPH and A_i is the absorbance of DPPH with various samples. The IC_{50} value, as a concentration required to scavenge 50% of the radical, was calculated according to the curve between concentrations and scavenging rates.

ABTS radical scavenging assay

The scavenging ability of the ABTS radical cation ($ABTS^+$) was determined based on the method of Huang et al.[21] The $ABTS^+$ solution was prepared from the reaction of 7 mM ABTS (5 mL) and 2.45 mM (88 μL) potassium persulfate after incubation and kept at

room temperature for 16 h in the dark. Then, the solution was diluted with 70% ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. The $ABTS^+$ solution (2.7 mL) was mixed with 0.3 mL CTE (0-200 μg/ml). The reaction mixture was allowed to stand for 30 min and the absorbance was measured at 734 nm. BHT and vitamin C served as references. Radical scavenging rates and IC_{50} values were calculated by using the previously presented equation of DPPH.

Reducing power assay

The Fe^{3+} reducing power of the samples was determined by a previously described method with slight modifications.[22] Test samples (0.2 mL) with various concentrations were mixed with 1.0 mL phosphate buffer (0.2 M, pH 6.6) and 1.0 mL potassium ferricyanide (1%, w/v). Then, the mixtures were incubated at 50 °C for 20 min, terminated by adding 500 μL trichloroacetic acid (10%, w/v), and centrifuged at 1,000×*g* for 10 min. Afterward, 2 mL of the supernatant was mixed with 2 mL distilled water and 0.4 mL ferric chloride solution (0.1%, w/v) for 10 min. The absorbance was measured at 700 nm. The extract concentration providing 0.5 of absorbance was also calculated.[23] BHT and vitamin C served as references.

β-Carotene bleaching assay

Antioxidant activity assay of CTE was conducted based on the method of Chen et al.[24] β-Carotene (4 mL; 0.3 mg/mL chloroform), 40 mg linoleic acid, and 400 mg Tween 80 were mixed in a flask. Subsequently, the mixture was removed by rotary evaporation at 40 °C for 10 min. The residue was dissolved in 100 mL distilled water and agitated to form an emulsion. The emulsion (3 mL) was added to a tube containing 0.2 mL CTE. BHT served as the reference for comparative purposes. The absorbance was measured every 30 min at 470 nm. All determinations were conducted in triplicate. Antioxidant activity coefficient (AAC) was calculated as follows:

$$AAC=[(A_{A(120)}-A_{C(120)})/(A_{C(0)}-A_{C(120)})] \times 1000$$

Where $A_{A(120)}$ is the absorbance of sample at 120 min, $A_{C(120)}$ is the absorbance of control at 120 min, $A_{C(0)}$ is the absorbance of control at 0 min.[25-26]

Protective effect against CCl_4-induced damage

CCl_4-induced damage

Sprague–Dawley (SD) rats (170 ± 20 g) were purchased from the Laboratory Animal Center of Wuhan University, Wuhan, China. All rats were housed in a regulated environment at 25 ± 1 °C under 12 h light/12 h dark conditions with a relative humidity of 30 to 60% and maintained on a standard pellet diet (rat feed purchased from the Laboratory Animal Center of Wuhan University, Wuhan, China) with water ad libitum. The animals were allowed to acclimatize to the room conditions for 2 days. The study received clearance from the Institutional Animal Ethical Committee of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Wuhan University, Wuhan, China.

Rats were divided into six groups consisting of six animals in each group. Rats in Group I (control group) were given distilled water containing 0.3% sodium carboxymethylcellulose (CMC-Na) (1 mL/kg body weight, p.o.) for 5 days and olive oil (1 mL/kg body weight, s.c.) on Days 2 and 3.[18] Rats in Group II (model group) received 0.3% CMC-Na (1 mL/kg body weight, p.o.) for 5 days and a 1:1 mixture of CCl_4 and olive oil (2 mL/kg body weight, s.c.) on Days 2 and 3. Rats in Group III (positive control group) were treated with the standard drug silymarin (100 mg/kg body weight, p.o.) daily for 5 days and received the same CCl_4–olive oil mixture on Days 2 and 3 as the model group, 30 min after the administration of silymarin. Silymarin was replaced by 400, 200, and 100 mg/kg body weight of CTE in Groups IV, V, and VI (test groups), following the same procedure as Group III. On Day 6, animals were sacrificed by bleeding. The livers were dissected for biochemical characterization.

Biochemical determinations

Liver homogenates (10.0%, w/v) were prepared with 50 mM cold potassium phosphate buffer (pH 7.4) and then centrifuged at 5,000 rpm for 8 min. The supernatant was collected for further analysis. All treatments were conducted at 4 °C. The antioxidant indices of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) were determined. The activities of glutamate pyruvate transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin (TB) were measured by using commercial reagent kits obtained from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) based on the instruction manuals.

Histopathologic examination

Liver tissue samples were fixed with 10% formalin for 24h, embedded in paraffin. The sections were cut into 5 μm thick in rotary microtome and stained with haematoxylin-eosin (HE) dye.

Hepatoprotective activity and protein expression of CTE in HepG2

Cell viability

Human hepatoblastoma HepG2 was obtained from the China Center For Type Culture Collection (CCTCC, Wuhan, Hubei, China), and cells were maintained in RPMI-1640 medium with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin antibiotics and then incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The cell viability was determined by MTT assay.[27] Cells were cultured in 96-well plates at 10^5 cells/well in 100 μl, and incubated for 24 h. All wells were intoxicated with CCl_4 (1%) except the control group for 2h. Then, treatment groups were incubated with CTE (62.5, 125, 250 μg/ml) and Silymarin (100μg/ml) with serum-free 1640 medium containing 0.1% dimethylsulfoxide (DMSO, v/v). After 24 h, 20 μl of MTT (5 mg/ml) was added to each well, and the plates were incubated at 37 °C for 4 h. The solution was removed and added with 100 μl/well of DMSO. The absorbance in each well was

measured at 492 nm using a 96-well plate reader. The percentage viability was calculated using the following formula:

Cell viability (%) = ($Abs_{control}$ −Abs_{sample}) × 100 / $Abs_{control}$.

Western blot analysis for protein expression in HepG2

Protein expressions of p-P38, p-JNK, p-ERK, Nrf2, NQO1 and GST in HepG2 cells were determined using Western blotting.[28] Briefly, total protein were extracted with RIPA buffer containing protease inhibitors. The proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane. Then, the membrane was blocked with 5% nonfat milk solved in TBS buffer for 1 h and incubated with different primary antibodies. Signals were developed by incubating the membrane with enhanced chemiluminescence Plus Western Blotting Detection Reagents and exposed on X-Omat Blue XB-1 Film (Kodak, Rochester, NY) for autoradiography.

Statistical analysis

All experiments were conducted in triplicate, and the results were reported as the means ± standard deviations. The data were analyzed using one-way ANOVA. The statistically significant effects were analyzed, and their means were compared using Tukey's multiple range test. A $p < 0.05$ value was considered statistically significant.

Results and discussion

Determinations of total phenolic, flavonoid, and proanthocyanidin contents

Table 1 Contents of total phenolics, flavonoids, and proanthocyanidin and IC_{50} values of the free radical scavenging effect of CTE.

Sample	Total phenolics (mg GAE/g extract)	Total flavonoids (mg RUE/g extract)	Proanthocyanidin (mg CAE/g extract)	IC_{50} values (μg/mL)	
				DPPH	ABTS
CTE	252.7 ± 5.5	22.5 ± 1.07	7.28 ± 0.38	148.17 ± 0.62	25.67 ± 2.48
BHT	–	–	–	163.90 ± 2.02	17.55 ± 0.16
Vitamin C	–	–	–	144.93 ± 0.75	16.72 ± 0.27

CTE, *C. thibetica* extract; BHT, butylated hydroxytoluene.

The results of total phenolic, flavonoid, and proanthocyanidin content determinations were shown in Table 1. Phenols were considered the main bioactive compounds and have been the focus of considerable attention.[3] Total phenolic content of CTE was 252.7 ± 5.5 mg gallic acid equivalent (GAE)/g of extract, of which GA served as the reference. Flavonoids, as a large group of polyphenolic compounds, have a significant contribution to biological activities, especially in free radical-induced chronic disease.[29] The content of total flavonoid was determined to be 22.5 ± 1.07 mg rutin equivalent (RUE)/g of extract compared with RU. Proanthocyanidin plays an important role in determining the nutritional quality of food products derived from plant sources.[30] CTE has 7.28 ± 0.38 mg of catechin equivalent (CAE)/g dry extract. Similar results were obtained in a previous study that investigated the polyphenols and triterpenes from *Chaenomeles* fruits.[3]

HPLC-UV-ESI-MS analysis

The chemical composition of CTE was analyzed via HPLC-UV-ESI-MS. The chromatogram of CTE was obtained at 254 nm (Fig. 1). The results of HPLC-MS and tentative identification are shown in Table 2. These compounds exhibited quasi-molecular ions $[M + H]^+$, $[M + Na]^+$ or $[M + K]^+$. These compounds were identified as citric acid (**1**), apigenin (**2**), agrimophol (**3**), roseoside (**6**), myricetin-3-*O*-rhamnoside (**7**), β-sitosterol (**8**), oleanolic acid ester (**9**), and quercetin pentoside (**11**) based on previous reports, which are presented in Table 2.[31-38] Main chemicals in CTE had been deduced. These identified compounds, especially citric acid, myricetin-3-O-rhamnoside, β-sitosterol, oleanolic acid ester, and quercetin pentoside, were reported to exhibit antioxidant activities.[39-43] Therefore, it was undoubted that CTE possessed a protective effect against oxidative damage.

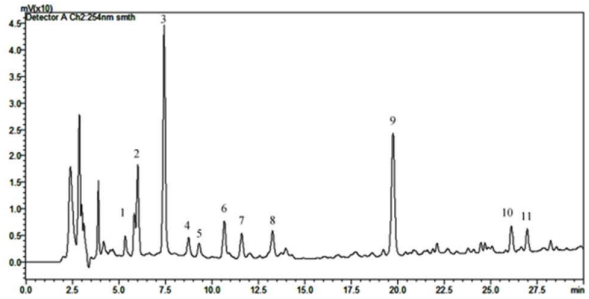

Figure 1. HPLC chromatogram of CTE at 254 nm. CTE, *C. thibetica* extract.

Table 2 Retention times and MS patterns of the compounds in CTE.

Peak	t_R (min)	*m/z*	Compounds	References
1	5.34	193[a]	Citric acid	31
2	6.00	293[b]	Apigenin	32
3	7.43	475[a]	Agrimophol	33
4	8.74	356	Unknown	
5	9.30	551	Unknown	
6	10.66	387[a]	Roseoside	34
7	11.59	481[b]	Myricetin-3-*O*-rhamnoside	35
8	13.26	453[c]	β-Sitosterol	36
9	19.74	588[a]	Oleanolic acid ester	37
10	16.10	437	Unknown	
11	26.95	435[a]	Quercetin pentoside	38

[a]$[M + H]^+$; [b]$[M + Na]^+$; [c]$[M + K]^+$.

***In vitro* antioxidant activity**

DPPH and ABTS are used as the main radicals for testing antioxidant activities and are considered stable free radicals.[22] In the DPPH radical scavenging assay, the IC_{50} (the concentration required to scavenge 50% of the radical) values of CTE, BHT, and vitamin C were 148.17 ± 0.62, 163.90 ± 2.02, and 144.93 ± 0.75 μg/mL, respectively. In the ABTS radical assay, the IC_{50} values of CTE, BHT, and vitamin C were 25.67 ± 2.48, 17.55 ± 0.16, and 16.72 ± 0.27 μg/mL, respectively. CTE showed an effect similar to that of BHT and vitamin C. However, in the ABTS radical assay, CTE was not as effective as BHT and vitamin C. An excellent free radical scavenging activity may hint a potential antioxidant activity.

Another index for evaluating antioxidant activity is reducing power. Reductive capability is the power to transform Fe^{3+} to Fe^{2+}, which can be reflected through the absorbance. The increased absorbance of the reaction mixture indicated the increased reducing power of the sample. As shown in Fig. 2A, CTE showed a relatively strong and dose-dependent reducing capability. The extract concentrations providing 0.5 of absorbance of vitamin C, BHT and CTE were 157, 296 and 412 μg/ml. So, the reductive capability might be the way of CTE against oxidative damage. The color of β-carotene, as an indicator, was bleached with the use of linoleic acid, which can generate a free radical with a hydrogen atom. The antioxidants could slow down the bleaching rate by neutralizing the free radical.[24] As shown in Fig. 2B, the color of solutions treated with CTE and BHT bleached slower than the control group. The AAC of BHT and CTE were 876.92±12.69 and 425.17 ± 69.21, respectively.

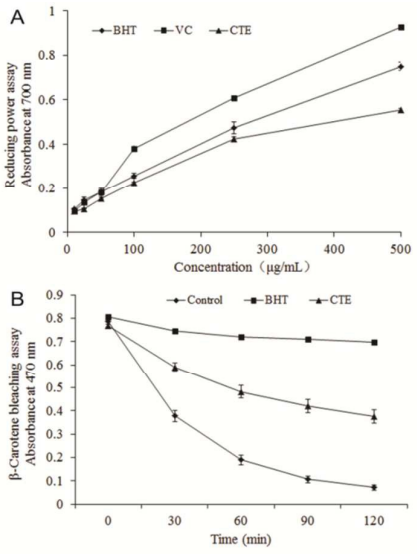

Figure 2. Antioxidant activity determined using the reducing power assay and β-carotene bleaching assay. (A) Reducing power assay. (B) β-Carotene bleaching assay. Values are expressed as the means ± standard deviations (n = 3 in each group). CTE, *C. thibetica* extract; BHT, butylated hydroxytoluene; VC, ascorbic acid.

All of the aforementioned assays indicated that CTE showed a clear antioxidant activity *in vitro*. Besides, dose-dependent manners were always observed. It might be related to the different active constituent contents, such as total phenolic, flavonoid and proanthocyanidin.

***In vivo* protective effect against CCl_4-induced damage**

CCl_4 was used to cause liver injury by LPO and accelerate free radical derivatives to measure the effect of antioxidant and liver protection *in vivo*. CCl_4 can be accumulated in hepatic cells and metabolized to the CCl_3 radical by liver cytochrome P450-dependent monooxygenases.[21] The free radical reacted with oxygen to form trichloromethyl peroxyl radical ($CCl_3OO^{\cdot}$), which could induce lipid peroxides. Then, MDA was produced and caused damage to the hepatic tissue and the *in vivo* oxidative system. However, antioxidants may be used to protect against the liver injury caused by CCl_4 by a defense system, such as SOD, CAT, and GSH, which convert ROS into nontoxic compounds. SOD and CAT are two important enzymes in preventing oxidative damage. SOD was used to catalyze and convert the superoxide radical into H_2O_2 and O_2. Moreover, CAT was used to metabolize hydrogen peroxide into oxygen and water and protect tissues against reactive hydroxyl species in the defense system.[44] GSH is a tripeptide containing glutamic acid, cysteine, and glycine, which exist in almost every cell of our bodies. GSH also helps maintain the normal function of the immune system and has antioxidation and detoxification effects.

In this study, antioxidant activity of CTE was measured in the liver. The results are shown in Table 3. MDA formation was significantly increased in the model group compared with that in the control group ($p < 0.001$). The positive control (silymarin) and CTE groups showed a significantly reversed tendency toward oxidative damage. The effect of CTE at a concentration of 400 mg/kg was similar to that of silymarin. When measuring total superoxide dismutase (T-SOD) and CAT, comparable results were obtained. The activities of T-SOD and CAT were decreased in the model group. However, CTE increased the decreased levels to a relatively normal condition compared with the control group. The results of GSH content indicated that CCl_4 decreased GSH content (the model group); otherwise, the treatment groups showed an effectively reversed condition. The results of the aforementioned parameters indicated that CTE has a significant antioxidant effect that can prevent oxidative damage *in vivo* effectively.

CCl_4 can also cause hepatic damage. Deficiency of metabolic enzymes that are located in intracellular structures results from changes in the endoplasmic reticulum.[45] Therefore, AST, ALT, ALP, and TB were measured to evaluate hepatoprotective activity. In this study, the high levels of AST and ALT caused by CCl_4 were significantly decreased in the treatment groups (Table 3). ALP and TB were also measured to reflect the pathological alteration in biliary flow. The levels of the two parameters were significantly increased in the model group, but CTE treatment reduced the levels (Table 3). Thus, we can conclude that CTE has the potential ability to stabilize CCl_4-induced biliary dysfunction in the rat liver. Thus, CTE exhibited potential hepatoprotective activity.

Based on the histopathological observations of liver tissue samples, the control group showed a normal cellular architecture with distinct hepatic cells and no histologic abnormalities (Fig.3A).

In contrast, the administration of CCl_4 caused severe damage. The liver sections showed hepatocyte fat deposition, hepatocyte swelling, vacuole formation in the cells, and loss of cellular boundaries (Fig. 3B). When treated with silymarin and CTE, the histologic abnormalities were turned to recover (Fig.3C, 3D, 3E, 3F). These groups showed a relatively normal lobular pattern with a mild degree of fatty change and vacuole in cells.

mitochondria of living cells to water-insoluble formazan, which deposits in cells; this process does not occur in dead cells. The cell viability can be reflected by dissolving formazan in DMSO and obtaining the absorbance at 492 nm. After incubating the cells in different CTE concentrations for 24 h, cell viability significantly reversed in a dose-dependent manner from the trend which was induced by CCl_4 (Fig. 4A). At the concentration of 250 μg/ml, the cell viability was similar with control group.

Table 3 Antioxidant and hepatoprotective activities of CTE in CCl_4-intoxicated rats.

Groups	Antioxidant activity - Liver				Hepatoprotective activity - Serum			
	MDA (nmol mg^{-1} prot)	T-SOD (U mg^{-1} prot)	CAT (U mg^{-1} prot)	GSH (mg g^{-1} prot)	AST (U L^{-1})	ALT (U L^{-1})	ALP (U L^{-1})	TB (mg dL^{-1})
Control	2.30±0.84	175.30 ± 5.19	80.14±5.42	6.39±1.03	18.92±2.09	9.67±1.64	122.90±30.72	0.10±0.04
Model	4.02±0.94$^{\#\#\#}$	136.04±24.98$^{\#\#\#}$	46.97±8.96$^{\#\#\#}$	4.38±1.67$^{\#\#}$	27.66±8.42$^{\#\#}$	14.38±2.90$^{\#\#\#}$	185.52±39.85$^{\#\#}$	0.32±0.01$^{\#\#\#}$
Silymarin	2.15±0.45***	155.50 ± 8.47*	58.50±8.39**	5.02±1.22	19.53±4.51**	9.81±2.55**	127.39±31.13**	0.25±0.03*
CTE 100	2.85 ± 0.60*	164.43±11.28**	56.61±7.84*	5.64±0.51	23.03±4.82	9.83±1.61**	145.08±49.18*	0.27±0.09
CTE 200	2.89 ± 1.06*	165.65 ±24.94**	55.48±5.08*	5.58±0.58	20.59±3.78*	9.50±1.63**	123.76±8.87**	0.23±0.05**
CTE 400	2.09±0.78***	163.95 ± 8.35**	57.25±6.21*	6.26±0.64*	19.30±6.45*	9.59±2.41**	133.88±30.49*	0.24±0.02*

Values are expressed as the means ± standard deviations (n = 6 in each group). $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$, compared with the model group (CCl_4-intoxicated group). $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, compared with the control group. CTE 100, *C. thibetica* extract at concentration of 100 mg kg^{-1}; CTE 200, *C. thibetica* extract at concentration of 200 mg kg^{-1}; CTE 400, *C. thibetica* extract at concentration of 400 mg kg^{-1}.

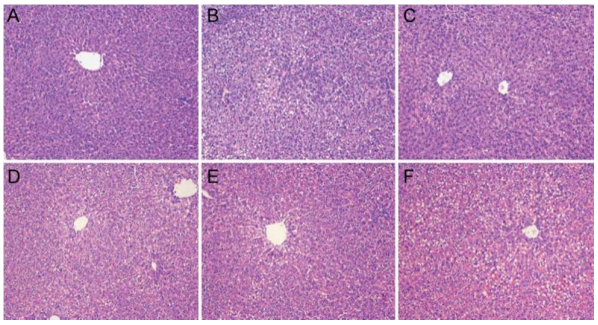

Figure 3. Photomicrographs of the rat liver sections (hematoxylin and eosin staining, 100×). (A) Control, (B) Model, (C)Silymarin, (D) *C. thibetica* extract at concentration of 100 mg kg^{-1}, (E) *C. thibetica* extract at concentration of 200 mg kg^{-1}, (F) *C. thibetica* extract at concentration of 400 mg kg^{-1}.

Protective effects and protein expression in HepG2

The influence of CTE exposure in HepG2 cells was analyzed. Exogenous MTT can be deoxidized by succinate dehydrogenase in

To further clarify the signaling pathways, we analyzed the MAPKs expression upon treatment of HepG2 cells with CTE. CTE treatment enhanced the phosphorylation-dependent activation of signaling components, such as p-JNK, p-ERK and p-P38 (Fig. 4B). The MAPK family plays an important role in regulating cell proliferation and death in response to stress. From the results, we may conclude that CTE can up-regulate the expression of p-JNK, p-ERK and p-P38, especially p-ERK. When the MAPKs were phosphorylated, the Nrf2 was stimulated and separated form Keap1, then transferred to nucleus. Nrf2 is an important transcription factor in regulating the oxidative stress. In our study, the expression of Nrf2 was up-regulated by different concentrations of CTE (Fig. 4C). Then, the free Nrf2 was combined with antioxidant response element (ARE). The Phase II detoxifying enzymes were activated, such as NQO1 and GST. NQO1 is a cytosolic flavoprotein which can be used for catalyzing two-electron reduction and detoxification of quinones and other redox cycling endogenous and exogenous chemicals.[46] GST is another detoxifying enzyme and used for maintaining optimal cellular levels of reduced GSH. In our study, CTE could reverse the expression of NQO1 down-regulated by CCl_4. Though the expression of GST was low in HepG2, we could also conclude that CTE with high concentrations can up-regulate the expression (Fig. 4C). The results might be helpful to interpret TB contents. As

shown in Table 3, TB contents of treatment groups still remained at an elevated level compared with the control group. It is reported that HO-1 could catalyse the rate-limiting step to degrade free heme into bilirubin, which could be served as endogenous antioxidant.[47] Besides, HO-1 was regulated by Nrf2. So, HO-1 derived bilirubin might be increased by the high expression of Nrf2. It might be used to interpret the high level of TB contents in treatment groups.

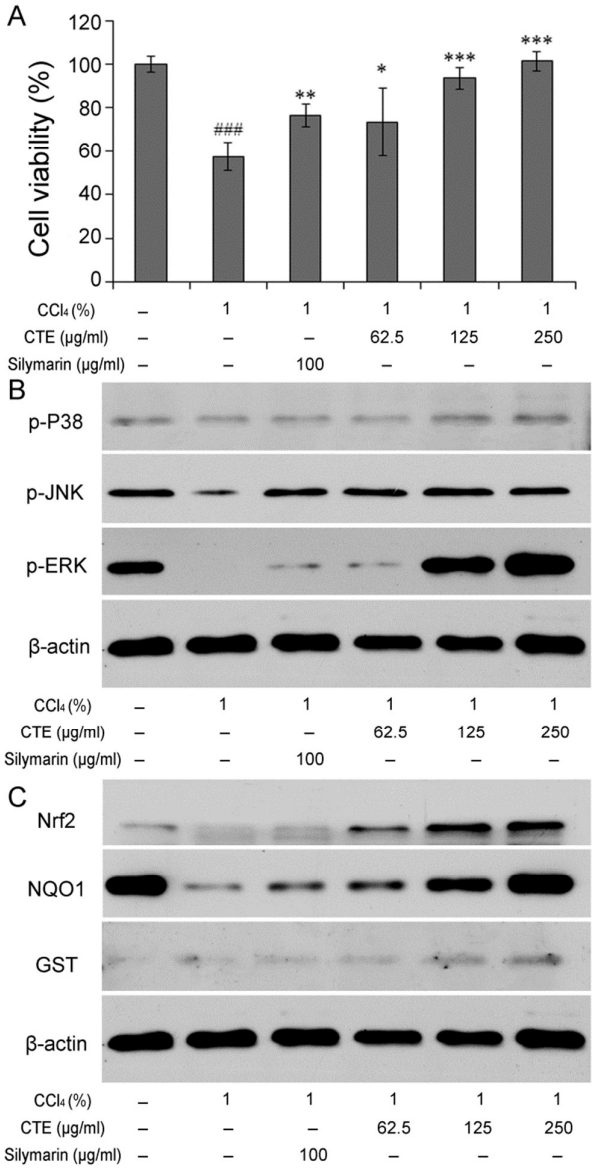

Figure 4. Cell viability and proteins expressions of CTE in HepG2. (A) Cell viability. (B) Expression of p-P38, p-JNK and p-ERK. (C) Expression of Nrf2, NQO1 and GST. CTE, *C. thibetica* extract.

These results indicated that CTE could up-regulate the expression of p-JNK, p-ERK, p-P38 which are involved in Nrf2-mediated expressions of NQO1 and GST. Besides, the protective effects of CTE in HepG2 might link with the phosphorylation of MAPKs, activation of Nrf2 pathway and up-regulation of NQO1 and GST. The action of CTE might be contributed by its main active compounds, apigenin, oleanolic acid and myricetin, which had been reported possessing the antioxidant/hepatoprotective effect via Nrf2 pathway.[48-52]

Conclusions

Basing on the results, we can conclude that CTE, which is rich in phenolic compounds, exhibited antioxidant effects *in vitro* and *in vivo*. To some extent, CTE could be used to prevent the CCl_4-induced damage via MAPK/Nrf2 pathway. These effects may be valuable for the application of *C. thibetica*, expound the mechanism of antioxidative activity, and provide functional food products with antioxidant and hepatoprotective potential.

Conflict of interest

The authors declared that there are no conflicts of interest.

Acknowledgments

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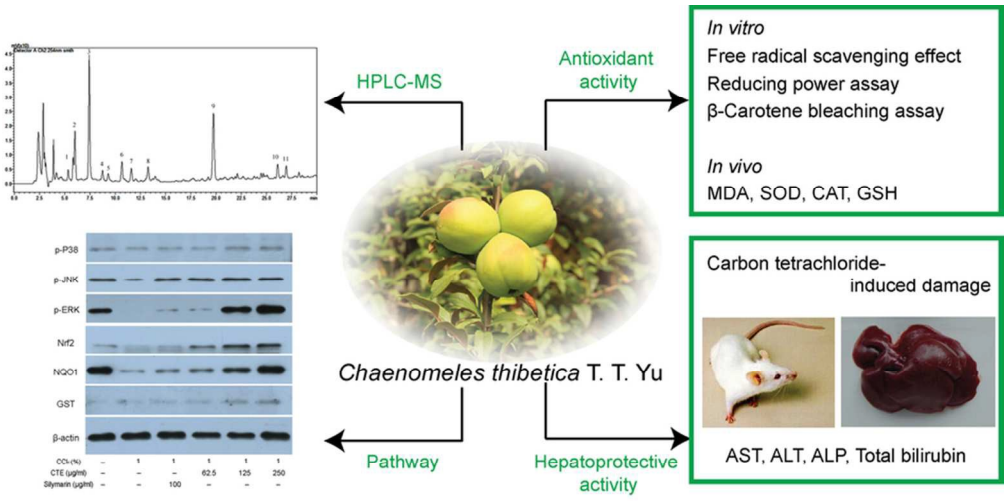

Chaenomeles thibetica extract possesses the antioxidant and hepatoprotective effects against carbon tetrachloride-induced damages via MAPK/Nrf2 pathway

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