

## Chemical Composition and Hepatoprotective Effects of Polyphenol-Rich Extract from *Houttuynia cordata* Tea

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**ABSTRACT:** This study was designed to investigate the antioxidant activity, hepatoprotective effect, and phenolic composition of the ethyl acetate fraction (EAF) extracted from *Houttuynia cordata* tea. EAF was shown to exhibit strong ferric-reducing antioxidant power (FRAP) and scavenging activity against DPPH radical in vitro, and the antioxidant effects were further verified by suppressing CCl<sub>4</sub>-induced oxidative stress in mouse liver at three tested doses of EAF (250, 500, and 1000 mg/kg bw). Pretreatment with EAF (1000 mg/kg bw) prior to CCl<sub>4</sub> administration significantly ( $p < 0.001$ ) decreased the CCl<sub>4</sub>-elevated levels of serum AST, ALT, alkaline phosphatase, total bilirubin, and hepatic MDA in mice and prevented the increases in GSH, SOD, and CAT caused by CCl<sub>4</sub>. HPLC analysis revealed that three predominantly polyphenolic compounds present in EAF were quercitrin (111.7 μg/mg), quercetin (43.8 μg/mg), and hyperoside (29.1 μg/mg). These results combined with liver histopathology indicate that EAF possesses a significant protective effect against acute hepatotoxicity induced by CCl<sub>4</sub>, which may be due to the strong antioxidant activity of phenolic components.

**KEYWORDS:** *Houttuynia cordata*, tea polyphenols, antioxidant activity, hepatoprotective effects, HPLC

### INTRODUCTION

The liver plays a pivotal role in metabolism and detoxification of various components entering the body, and hepatic injury associated with these metabolic dysfunctions can result in many disorders ranging from transient elevation of liver enzymes to life-threatening hepatic fibrosis, liver cirrhosis, and even hepatocellular carcinoma.<sup>1,2</sup> Therefore, it is of great importance to develop hepatoprotective agents to protect people from liver injury. However, the number of investigated medicines used successfully is limited, and some of them have potential adverse effects, especially when administered chronically or subchronically.<sup>3</sup>

In recent years, the use of active dietary ingredients and traditional herbs, which are believed to be harmless and free from serious adverse reactions, for the prevention and treatment of liver diseases has increased all over the world.<sup>2–6</sup> Within these natural phytoconstituents, dietary polyphenols such as flavonoids contain a number of phenolic hydroxyl groups and have been demonstrated to be the key ingredients responsible for the beneficial effect, which is mainly due to their scavenging activity against reactive oxygen species (ROS).<sup>5–7</sup> It is widely recognized that ROS can cause cell damage via the mechanism involving lipid peroxidation with subsequent tissue injury, especially liver injury.<sup>7</sup> For this reason, it is of the highest priority to find natural antioxidants, especially polyphenol-rich fractions, for preventing or attenuating toxic liver injury today.

As widely consumed beverages around the world, herbal teas from different plant resources have drawn increasing attention as dietary supplements for their excellent antioxidant activities and other health-promoting effects.<sup>8–14</sup> *Houttuynia cordata* Thunb. is a food plant widely distributed throughout

southeastern Asia. Green leaves and young roots of *H. cordata* are popularly consumed as high-quality agricultural vegetables among peasants in southern China and Thailand.<sup>15,16</sup> The stems and leaves of *H. cordata* are also used as folk tea alone or in combination with other herbs.<sup>15,16</sup> Studies have shown that *H. cordata* contains a wide range of polyphenols such as rutin, quercetin, hyperoside, quercitrin, and chlorogenic acid, which have been considered to be responsible for the antioxidant activity.<sup>15,17</sup> In addition to health promotion, *H. cordata* is also increasingly popular for adjuvant therapy because it has been shown to possess a variety of pharmacological functions of antiviral, antibacterial, antileukemic, and antiallergic activities, as well as effects on refractory hemoptysis and malignant pleural effusion.<sup>18–20</sup> However, there is no scientific study available to describe the potential effect of *H. cordata* against liver damage and what kinds of compounds it is related to.

In the present study, we compared the antioxidant effects of various solvent fractions extracted from Chinese *H. cordata* folk tea. Furthermore, the protective effects of the selected polyphenol-rich fraction on CCl<sub>4</sub>-induced hepatic damage were investigated in mice. Moreover, the main tea polyphenols present in ethyl acetate extract of *H. cordata* tea were identified and quantified by RP-HPLC to gain an insight into the compounds responsible for its antioxidant and hepatoprotective effects.

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**Table 1. Contents of Total Phenolics and Total Flavonoids of Various Solvent Fractions from *H. cordata* Tea and Their DPPH Radical-Scavenging Effect and Ferric-Reducing Antioxidant Power (FRAP)**

fraction	total phenolics ( $\mu\text{g GAE}/\text{mg fraction}$ )	total flavonoids ( $\mu\text{g RE}/\text{mg fraction}$ )	EC <sub>50</sub> ( $\mu\text{g}/\text{mL}$ )	FRAP <sup>a</sup>
DEF	87.2 $\pm$ 0.6	54.6 $\pm$ 0.4	128.7 $\pm$ 8.5	0.440 $\pm$ 0.007
CF	26.5 $\pm$ 0.3	24.1 $\pm$ 0.3	430.2 $\pm$ 11.0	0.175 $\pm$ 0.008
EAF	408.6 $\pm$ 0.9 <sup>b</sup>	339.2 $\pm$ 1.0 <sup>b</sup>	41.3 $\pm$ 3.4 <sup>b</sup>	1.065 $\pm$ 0.008 <sup>b</sup>
BF	37.8 $\pm$ 0.3	28.7 $\pm$ 0.6	285.8 $\pm$ 7.5	0.283 $\pm$ 0.007
RF	29.0 $\pm$ 0.5	25.6 $\pm$ 0.7	369.5 $\pm$ 7.8	0.176 $\pm$ 0.006

<sup>a</sup>Absorbance at a concentration of 600  $\mu\text{g}/\text{mL}$ . <sup>b</sup> $p < 0.05$ , compared to the other fractions.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Gallic acid, rutin, hyperoside, quercitrin, and quercetin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ], and trichloroacetic acid (TCA) were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). Assay kits of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malonaldehyde (MDA) were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). All other chemicals were of the highest grade available.

**Preparation of Various Solvent Fractions from *H. cordata* Tea.** The *H. cordata* tea (stems and leaves, a Chinese green tea) was purchased from Guilin Gexianweng Pharmaceutical Co., Ltd., and the tea as powder was extracted under reflux with 70% ethanol (1:10 w/v) at 60 °C. After 4 h, the supernatant was decanted, and the residue was re-extracted under the same conditions. The combined extracts were cooled to room temperature, filtered, and centrifuged. Subsequently, the ethanolic extract was concentrated to 5% of the original volume with a rotary evaporator under reduced pressure. After that, the concentrated solution was sequentially extracted three times using diethyl ether (DEF), chloroform (CF), ethyl acetate (EAF), and *n*-butanol (BF) in a 1:3 ratio (v/v) at room temperature, and the residue was considered as an individual fraction (RF). Finally, the solvents in each fraction were removed using a rotary evaporator.

**Determination of Total Phenolics and Total Flavonoids.** Total phenolic compounds in the various extracts were estimated by the Folin–Ciocalteu method as gallic acid equivalents (GAE), expressed as milligrams of gallic acid per gram of extract.<sup>21</sup> Briefly, aliquots of 2 mL extracts or standard solutions were mixed with 1 mL of Folin–Ciocalteu reagent and allowed to react for 3 min. After the addition of 1 mL of 10%  $\text{Na}_2\text{CO}_3$ , the mixture was allowed to stand at 25 °C for 2 h. Absorbance was measured at 760 nm, and total phenolic contents were calculated as GAE from a calibration curve,  $A = 0.0183C + 0.0847$  ( $r^2 = 0.9993$ , 5–50  $\mu\text{g}$  of gallic acid). The data were presented as the average of triplicate analyses.

In addition, the total flavonoids of the extracts were measured as rutin equivalents (RE) using a modified colorimetric method.<sup>22</sup> Appropriately diluted extracts or standard solutions (1 mL) were mixed with 5%  $\text{NaNO}_2$  solution (0.2 mL). After 6 min, 0.2 mL of 10%  $\text{AlCl}_3$  solution was added and allowed to stand for another 6 min. Subsequently, the reaction solution was mixed with 0.6 mL of 4% NaOH solution, and 60% ethanol was immediately supplied to the final volume of 10 mL, followed by a thorough mixture and a further stand for 10 min. Absorbance of the mixture was determined at 510 nm versus blank water, and all determinations were carried out in triplicate. Rutin calibration curve was prepared in ethanolic solutions with the same procedure. The concentration of flavonoids was calculated as RE according to the following linear equation based on the calibration curve  $A = 0.9755c - 0.0053$ ,  $r^2 = 0.9990$  ( $c$ , 0.1–1.4 mg of rutin).

**Measurement of in Vitro Antioxidant Activities.** The ferric-reducing antioxidant power (FRAP) of different fractions from *H. cordata* tea was determined according to the validated method as

described previously.<sup>23</sup> The ability of the fractions to scavenge DPPH radical was determined by using a modified method,<sup>23</sup> and the percentage of scavenging activity was plotted against the sample concentration to obtain the EC<sub>50</sub>, defined as the concentration of sample necessary to cause 50% inhibition.

**Animals and Experimental Design.** Kunming mice of both sexes (18–22 g) were obtained from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China), and the study was approved by the Medical Ethics Committee of the University. Animals were acclimatized for at least 7 days prior to use and maintained in a temperature-controlled environment (22  $\pm$  2 °C) with a 12 h light–dark cycle and free access to water and standard rodent chow. All animals were treated in accordance with the Guidelines of the Principle of Laboratory Animal Care (NIH Publication, revised 1985). The animals were randomly divided into five groups with each consisting of 10 mice. Polyphenol-rich EAF was suspended in a 2% Tween-80 aqueous solution and administered intragastrically (ig) at 250, 500, and 1000 mg/kg body weight (dose of EAF/bw) once daily for 8 consecutive days. The animals from the normal and  $\text{CCl}_4$ -intoxicated groups were also given the same volume of vehicle for 8 consecutive days. Three hours after the final treatment, the mice in the  $\text{CCl}_4$ -intoxicated group and EAF-protective group were treated with  $\text{CCl}_4$  (20 mg/kg bw, ip) dissolved in soybean oil (0.1%, v/v). In the normal group, animals were given a single dose of Tween aqueous. After all of the animals were fasted for 20 h, they were sacrificed for obtaining blood and livers.

**Measurement of Biochemical Parameters in Serum.** The serum was separated after clot formation by centrifugation at 4 °C. Liver damage was assessed by estimating enzymatic activities of serum ALT, AST, and ALP, as well as serum TB level using the corresponding commercial kits (Nanjing Jiancheng Institute of Biotechnology, China), respectively. The results of ALT, AST, and ALP were expressed as units per liter (U/L), and TB was expressed as milligrams per deciliter.

**Assay of Hepatic Levels of GSH, CAT, SOD, and MDA.** Hepatic tissue was homogenized (10%, w/v) in ice-cold 50 mM phosphate buffer (pH 7.4) and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was used for the measurements of MDA, GSH, CAT, and SOD. Total protein contents were determined by Coomassie brilliant blue G250 (Sigma-Aldrich), using bovine serum albumin (Fluka, Steinheim, Germany) as a standard.

**Histopathological Analysis.** Portions of freshly obtained liver were fixed in 4% buffered paraformaldehyde phosphate solution for 24 h. The sample was then embedded in paraffin, sliced into 3–5  $\mu\text{m}$  sections, stained with hematoxylin–eosin (H&E) according to a standard procedure, and finally analyzed by light microscopy.

**RP-HPLC Analysis of Polyphenol-Rich Fraction EAF.** Chemical composition of the polyphenol-rich fraction EAF, demonstrated as the strongest antioxidant activity among investigated fractions, was determined using a HPLC method.<sup>17</sup> The analysis was performed using a reversed-phase HPLC column (4.6 mm i.d.  $\times$  250 mm, 5  $\mu\text{m}$ , Venusil, USA) on a Shimadzu LC-2010A HPLC system equipped with an UV–vis detector, an autosampler, and a Shimadzu Class-VP 6.1 chromatography workstation (Shimadzu, Kyoto, Japan). A gradient elution was performed by varying the proportion of solvent A (acetonitrile) to solvent B (water, containing 0.2% acetic acid), with a flow rate of 1.0 mL/min. The solvent gradient was as follows: 0–20 min from 10 to 20% A; 20–28 min from 20 to 26% A; 28–50 min

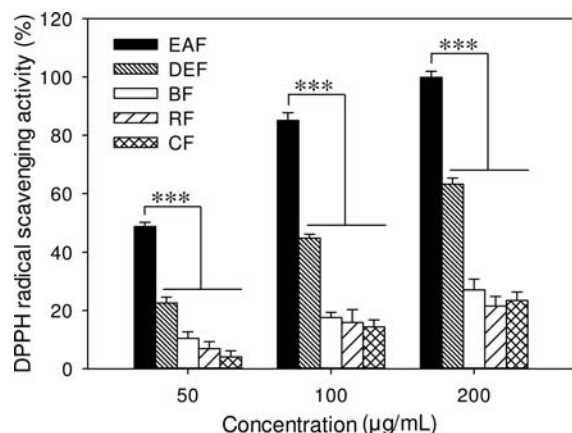
from 26 to 40% A. The wavelength for UV detection was 280 nm, and the injection volume was 20  $\mu\text{L}$ . All separations were performed at 30  $^{\circ}\text{C}$ .

**Statistical Analysis.** The experimental results were expressed as the mean  $\pm$  standard deviation (SD).  $\text{EC}_{50}$  values from in vitro data were calculated by regression analysis. The data were subjected to an analysis of variance (ANOVA,  $p < 0.05$ ) and Duncan's multiple-range tests (SPSS, version 13.0). A significant difference was judged to exist at a level of  $p < 0.05$ .

## RESULTS

**Total Phenolic and Total Flavonoid Contents in Different Fractions.** The *H. cordata* tea was extracted with 70% ethanol, and a 19.5% extraction yield of the tea powder was achieved. The further fractionation of the ethanol extract with different solvents in order of increasing polarity of diethyl ether, chloroform, ethyl acetate, *n*-butanol, and ethanol (residue part) led to five fractions, namely, DEF, CF, EAF, BF, and RF. As presented in Table 1, the total phenolic contents of DEF, CF, EAF, BF, and RF determined using the Folin–Ciocalteu method were found to be 87.2, 26.5, 408.6, 37.8, and 29.0  $\mu\text{g}$  GAE/mg fraction, respectively. Similarly, the contents of total flavonoids were of the same order: EAF > DEF > BF > RF > CF ( $p < 0.05$ ). This quantitative assay demonstrated that EAF contained the highest amount of total phenolics (408.6  $\mu\text{g}$  GAE/mg fraction) and total flavonoids (339.2  $\mu\text{g}$  RE/mg fraction), suggesting that EAF is a polyphenol-enriched fraction.

**Antioxidant Activities of Various Fractions.** The model system of scavenging DPPH free radical is employed to screen the antioxidant potential of DEF, CF, EAF, BF, and RF. As shown in Figure 1, all of the tested fractions showed a

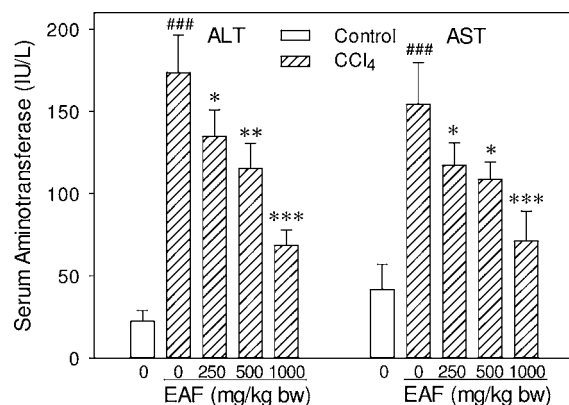


**Figure 1.** DPPH radical-scavenging activity of different solvent fractions from *H. cordata* tea. Data are presented as the mean  $\pm$  standard deviation of three replicates ( $n = 3$ ). \*\*\*,  $p < 0.001$ , statistically significant differences versus EAF group at the corresponding concentrations.

concentration-dependent scavenging activity against DPPH radicals. The scavenging activities of EAF on DPPH radicals were 48.5, 85.0, and 99.8% at concentrations of 50, 100, and 200  $\mu\text{g/mL}$ , respectively, which were significantly higher than that of the other fractions at the same concentrations ( $p < 0.001$ , Figure 1). Furthermore,  $\text{EC}_{50}$  values of the tested various fractions against DPPH radicals were achieved in the broad concentration range of 50–800  $\mu\text{g/mL}$ , and the results are summarized in Table 1. It was clear that the  $\text{EC}_{50}$  of EAF (41.3  $\mu\text{g/mL}$ ) was the lowest among the investigated fractions (DEF,

128.7  $\mu\text{g/mL}$ ; BF, 285.8  $\mu\text{g/mL}$ ; RF, 369.5  $\mu\text{g/mL}$ ; CF, 430.2  $\mu\text{g/mL}$ ), and the activity could be ranked as EAF > DEF > BF > RF > CF ( $p < 0.05$ ). Moreover, the ferric-reducing antioxidant power (FRAP) of the various tested fractions was also determined. As expected, all of the tested extracts as electron donor caused the reduction of  $\text{Fe}^{3+}$ /ferricyanide complex to  $\text{Fe}^{2+}$  complex, which could be monitored by measuring the enhanced formation of PerI's Prussian blue at 700 nm. As can be seen in Table 1, EAF at a high concentration of 600  $\mu\text{g/mL}$  exhibited the absorbance readings of 1.065, which was significantly superior to the tested other fractions (DEF, 0.440; BF, 0.283; RF, 0.176; CF, 0.175). The results from the DPPH and FRAP tests showed significant association of both total phenolic and flavonoid contents with antioxidant capacity (Figure 1 and Table 1). These results suggest that phenolics are major contributing compounds toward the antioxidant activity of the *H. cordata* tea, and polyphenol-rich EAF might have the potential to provide significant natural defense against oxidative damage.

**Effects of EAF on Serum ALT and AST Activities.** The results of the hepatoprotective effects of EAF on the enzymatic activities of serum ALT and AST are shown in Figure 2. In the

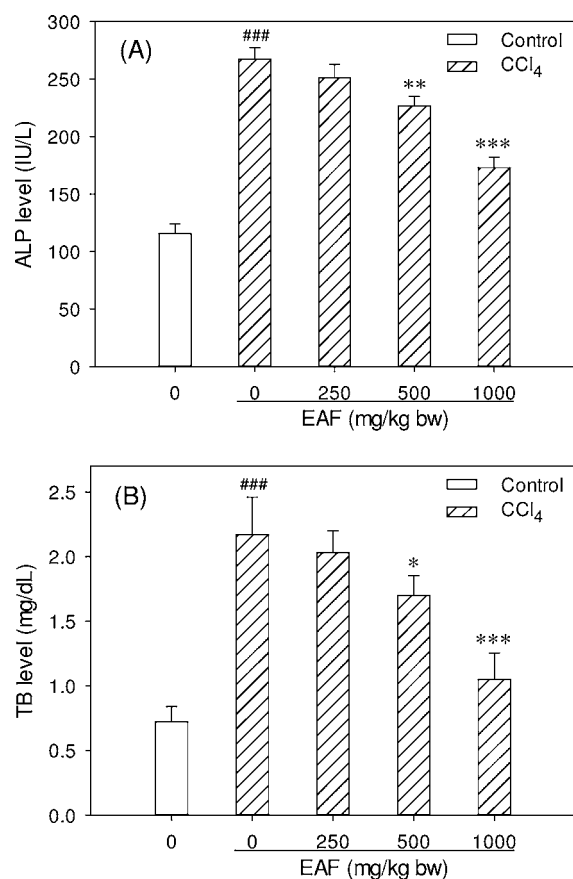


**Figure 2.** Effect of EAF on serum ALT and AST in  $\text{CCl}_4$ -intoxicated mice. Mice were administered intragastrically EAF (250, 500, and 1000 mg/kg bw) once daily for 8 consecutive days prior to the single administration of  $\text{CCl}_4$  (0.1%, ip). Values are expressed as the means  $\pm$  standard deviation of 10 mice in each group. ###,  $p < 0.001$ , compared with the normal group; \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , and \*\*\*,  $p < 0.001$ , compared with the  $\text{CCl}_4$ -intoxicated group.

normal group, serum ALT and AST activities were  $22.4 \pm 6.5$  and  $41.7 \pm 15.4$  IU/L, respectively. After  $\text{CCl}_4$  intoxication, serum ALT and AST activities showed remarkable increases to  $173.7 \pm 22.7$  ( $p < 0.001$ ) and  $154.3 \pm 25.4$  IU/L ( $p < 0.001$ ), respectively; the  $\text{CCl}_4$ -induced increases were significantly attenuated by pretreatment with EAF at 250, 500, and 1000 mg/kg bw ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ), respectively, and this reduction was dose-dependent (Figure 2).

**Effects of EAF on the Levels of Serum ALP and TB.** As depicted in Figure 3, panels A and B, in  $\text{CCl}_4$ -intoxicated mice the contents of serum ALP and TB sharply increased by 2.3- and 3.0-fold in comparison with that of the normal group ( $115.6 \pm 8.4$  IU/L and  $0.72 \pm 0.12$  mg/dL), respectively ( $p < 0.001$ ). The pretreatment of EAF dose-dependently reduced the ALP and TB levels caused by  $\text{CCl}_4$ , especially when the dosage increased to 500 and 1000 mg/kg bw ( $p < 0.05$ ,  $p < 0.001$ ). At a dosage of 500 mg/kg bw, ALP and TB levels decreased to  $226.6 \pm 8.3$  IU/L and  $1.70 \pm 0.15$  mg/dL, and at





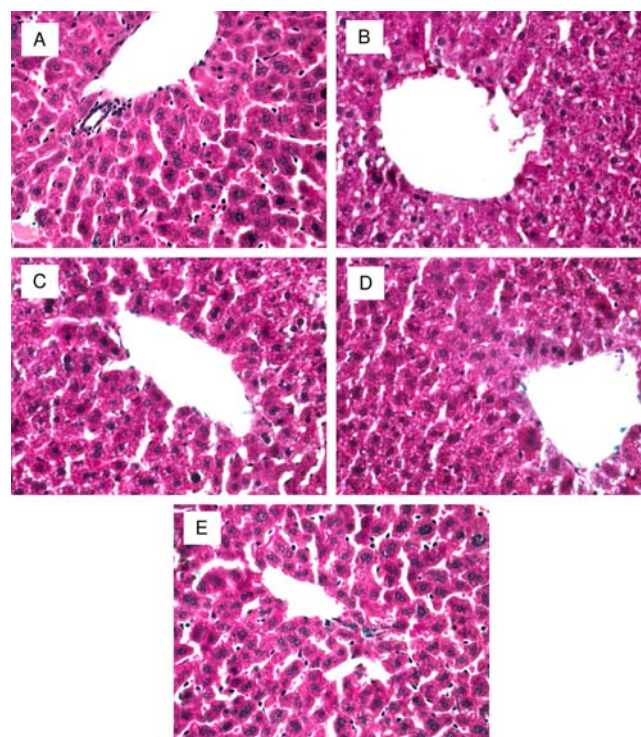
**Figure 3.** Effect of EAF on serum levels of ALP (A) and TB (B) in CCl<sub>4</sub>-intoxicated mice. Mice were treated intragastrically with EAF (250, 500, and 1000 mg/kg bw) once daily for 8 consecutive days prior to the single administration of CCl<sub>4</sub> (0.1%, ip). Values are expressed as the mean  $\pm$  standard deviation of 10 mice in each group. <sup>###</sup>,  $p < 0.001$ , versus the normal group; <sup>\*</sup>,  $p < 0.05$ , <sup>\*\*</sup>,  $p < 0.01$ , and <sup>\*\*\*</sup>,  $p < 0.001$ , versus the CCl<sub>4</sub>-intoxicated group.

1000 mg/kg bw, the corresponding values were  $172.8 \pm 9.2$  IU/L and  $1.05 \pm 0.21$  mg/dL, respectively. However, pretreatment with EAF at a low dose of 250 mg/kg bw led to a slight decrease in the levels of serum ALP and TB, but there was no statistical significance ( $p > 0.05$ ).

**Effects of EAF on Hepatic Levels of GSH, SOD, CAT, and MDA.** A single acute application of CCl<sub>4</sub> in mice also caused characteristic hepatotoxicity in antioxidant parameters of liver tissue, as indicated by the significant decrease in GSH, SOD, and CAT from  $11.37 \pm 0.71$  nmol/mg protein,  $114.3 \pm 5.3$  U/mg protein, and  $87.5 \pm 6.4$  U/mg protein in untreated normal group to  $4.05 \pm 0.82$  nmol/mg protein,  $58.3 \pm 6.5$  U/

mg protein, and  $36.7 \pm 8.7$  U/mg protein, and the remarkable increase in the content of oxidative mark MDA from  $0.92 \pm 0.18$  to  $2.85 \pm 0.49$  nmol/mgprot, respectively ( $p < 0.001$ , Table 2). Administration of EAF at doses of 250, 500, and 1000 mg/kg bw once daily for 8 consecutive days prior to the single administration of CCl<sub>4</sub> (0.1%, ip) effectively protected against a decrease in hepatic GSH, SOD, and CAT, considered as an index of the antioxidant status of tissues. As presented in Table 2, there was a significant increase in these antioxidant enzyme activities and a sharp decrease in MDA level in mice treated with EAF at doses of 500 ( $p < 0.01$ ) and 1000 mg/kg bw ( $p < 0.001$ ) relative to the CCl<sub>4</sub> only treatment.

**Histopathological Examination of Mice Liver.** Histopathological observation of the liver provided supportive evidence for the biochemical analysis, as presented in Figure 4. In the normal group, liver slices showed typical hepatic



**Figure 4.** Effects of EAF on the liver histological changes after CCl<sub>4</sub> treatment in mice (original magnification of 400 $\times$ ): (A) normal group; (B) CCl<sub>4</sub>-intoxicated group; (C) EAF (250 mg/kg bw) + CCl<sub>4</sub>; (D) EAF (500 mg/kg bw) + CCl<sub>4</sub>; (E) EAF (1000 mg/kg bw) + CCl<sub>4</sub>.

cellular architecture with well-preserved cytoplasm, prominent nucleus, and legible nucleolus (Figure 4A). The liver sections of

**Table 2.** Effects of EAF on Levels of Hepatic Superoxide Dismutase (SOD), Catalase (CAT), Glutathione (GSH), and Malondialdehyde (MDA)<sup>a</sup>

group	SOD (U/mg protein)	CAT (U/mg protein)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
control	$114.3 \pm 5.3$	$87.5 \pm 6.4$	$11.37 \pm 0.71$	$0.92 \pm 0.18$
CCl <sub>4</sub>	$58.3 \pm 6.5^b$	$36.7 \pm 8.7^b$	$4.05 \pm 0.82^b$	$2.85 \pm 0.49^b$
CCl <sub>4</sub> + EAF (250 mg/mL)	$67.4 \pm 8.9$	$46.4 \pm 9.1$	$5.92 \pm 0.57^{d,e}$	$2.31 \pm 0.22$
CCl <sub>4</sub> + EAF (500 mg/mL)	$78.8 \pm 5.7^c$	$50.3 \pm 4.1^{d,e}$	$6.26 \pm 0.59^{d,e}$	$2.07 \pm 0.18^{d,e}$
CCl <sub>4</sub> + EAF (1000 mg/mL)	$98.7 \pm 6.7^e$	$63.7 \pm 6.0^e$	$8.74 \pm 0.73^e$	$1.38 \pm 0.31^e$

<sup>a</sup>Values are expressed as the mean  $\pm$  standard deviation of 10 mice in each group. <sup>b</sup> $p < 0.001$ , compared to normal group. <sup>c</sup> $p < 0.05$ , as compared with CCl<sub>4</sub>-intoxicated group. <sup>d</sup> $p < 0.01$ , as compared with CCl<sub>4</sub>-intoxicated group. <sup>e</sup> $p < 0.001$ , as compared with CCl<sub>4</sub>-intoxicated group.

$\text{CCl}_4$ -intoxicated mice showed extensive liver injuries, characterized by severe hepatocellular degeneration and necrosis around the central vein, sinusoidal dilatation, loss of cellular boundaries, inflammatory cell infiltration, and cytoplasmic vacuolation (Figure 4B). Interestingly, the administration of EAF protected against this liver damage. EAF at a dose of 1000 mg/kg bw (Figure 4E) was more effective when compared with the two other doses (250 and 500 mg/kg bw) (Figure 4C,D). The administration of  $\text{CCl}_4$  along with EAF at 1000 mg/kg bw showed near-normal appearance (Figure 4E), suggesting that EAF could protect the liver from acute  $\text{CCl}_4$ -induced hepatic damage. This was in good agreement with the results of serum biochemical marks (e.g., aminotransferase) and hepatic oxidative stress levels.

#### Characterization of Polyphenolic Composition of EAF.

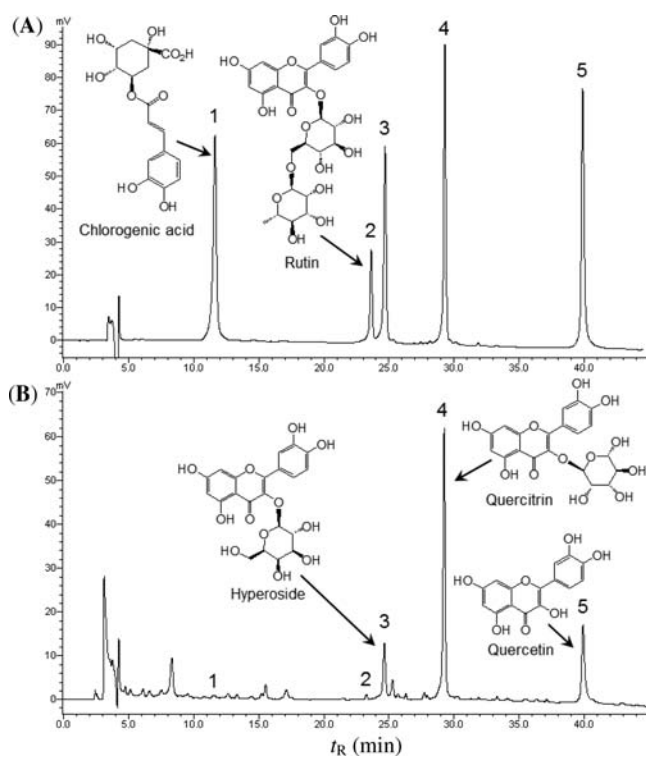
In this study, the main polyphenolic compounds present in EAF were identified and quantified to gain an insight into the compounds responsible for its antioxidant and hepatoprotective effects. HPLC profiles of polyphenolic compounds present in EAF are shown in Figure 5, and retention time ( $t_R$ ) and

chlorogenic acid, rutin, hyperoside, quercitrin, and quercetin were baseline separated from each other with  $t_R$  of 11.7, 23.6, 24.7, 29.3, and 39.8 min, respectively, and the linear regression parameters of the calibration curves are shown in Table 3. As a consequence, good linearity (correlation coefficient  $R^2 > 0.9985$ ) between  $Y$  (peak area of the standards) and  $X$  (concentration of the standards) was achieved in the tested range. Several polyphenols were identified by comparison to the  $t_R$  of the authentic standards, and the quantitative data were calculated from their respective calibration curves (Table 3). As shown in Figure 5B and Table 3, quercitrin (111.7  $\mu\text{g}/\text{mg}$ , quercetin 3-*O*- $\beta$ -D-rhamnoside) was identified to be present in the highest level in EAF, followed by quercetin (43.8  $\mu\text{g}/\text{mg}$ , flavonol) and hyperoside (29.1  $\mu\text{g}/\text{mg}$ , quercetin-3-*O*-galactoside), and only traces of chlorogenic acid and rutin (quercetin-3-*O*-rhamnoglucoside) were detected. The results obtained in this analysis clearly indicate that *H. cordata* tea is a good source of quercetin and its glycosides as a natural antioxidant agent.

#### DISCUSSION

Naturally occurring phenolic compounds are widely distributed in green tea and herbal teas and have received widespread attention due to their healthy benefits.<sup>8–10,24</sup> To our knowledge there are no reports linking the hepatoprotective effects to the phenolic constituents of *H. cordata* tea from China. In the present study, both DPPH and FRAP assays appear to have a concentration-dependent antioxidant effect of phenolics in the different solvent extracts of diethyl ether, chloroform, ethyl acetate, *n*-butanol, and ethanol, which exerted a similar antioxidant effect on the aqueous extracts of *H. cordata*.<sup>15</sup> Among the tested fractions, the polyphenol-rich fraction EAF has the highest antioxidant capacities, reflecting the highest content of total phenolics and flavonoids found in this part. This effect of EAF was further verified by suppressing  $\text{CCl}_4$ -induced oxidative stress in the livers of mice, and all of the morphological changes observed in mice treated with  $\text{CCl}_4$  were also attenuated by pretreatment with EAF. Our finding contributes to the understanding of the strong positive relationship between total phenolic or flavonoid content in various extracts of *H. cordata* tea and antioxidant activities.

Hepatic injury of  $\text{CCl}_4$ -induced lipid peroxidation is a well-known experimental model to evaluate the therapeutic potential of drugs and dietary antioxidants.<sup>2,5,25–27</sup> The main cause of acute liver injury by  $\text{CCl}_4$  is free radicals, which are generated in its metabolism by the cytochrome P450 (CYP) system.<sup>28</sup> The content of CYP in liver is more abundant than that in any other organs, such as lung, kidney, and intestine.<sup>28</sup> By the activation of liver CYP,  $\text{CCl}_4$  produces the hepatotoxic metabolites trichloromethyl free radicals ( $\cdot\text{CCl}_3$  or  $\text{CCl}_3\text{OO}\cdot$ ), which immediately propagate a chain of lipid peroxidation events and finally lead to the breakdown of membrane structure and the consequent leakage of hepatic cell marker enzymes into the bloodstream.<sup>29</sup> In our study, significant increases in the



**Figure 5.** RP-HPLC profiles of the flavonoid standards (A) and the flavonoid compounds in EAF (B) at 280 nm. Peaks: 1, chlorogenic acid; 2, rutin; 3, hyperoside; 4, quercitrin; 5, quercetin.

abundance of the main compounds present in EAF are also presented in Table 3. As can be seen in Figure 5A, the standard

**Table 3.** Calibration Curves and Contents of the Polyphenolic Compounds in EAF from *H. cordata* Tea

no.	phenolic compound	content ( $\mu\text{g}/\text{mg}$ )	$t_R$ (min)	equation of regression ( $Y = aX + b$ )	$R^2$
1	chlorogenic acid	trace	$11.72 \pm 0.15$	$Y = 26373X + 1972$	0.9985
2	rutin	trace	$23.56 \pm 0.07$	$Y = 12421X + 16325$	0.9992
3	hyperoside	29.1	$24.71 \pm 0.12$	$Y = 17562X + 46611$	0.9995
4	quercitrin	111.7	$29.29 \pm 0.16$	$Y = 23241X + 43984$	0.9993
5	quercetin	43.8	$39.85 \pm 0.17$	$Y = 29007X - 21918$	0.9988

levels of serum ALT, AST, ALP, and TB were observed after administration of  $\text{CCl}_4$  as reported previously (Figures 2 and 3).<sup>2,27</sup> However, the increased levels of these enzymes were decreased by the administration of EAF (250–1000 mg/kg bw), implying that EAF may effectively protect the hepatocytes against the toxic effects of  $\text{CCl}_4$ . It is well-known that the increased activity of ALT enzyme is an indicator of the degree of cell membrane damage, and the elevated AST level is another indicator of mitochondrial damage.<sup>30</sup> On this basis, it is suggested that EAF not only stabilizes the hepatic cellular membrane but also has a protective effect on mitochondria.

Besides hepatic marker enzymes, the hepatic MDA level is commonly used as an indicator of liver tissue damage involving a series of chain reactions.<sup>31</sup> MDA is a major reactive aldehyde that appears during the final stages of lipid peroxidation of the polyunsaturated fatty acid of biological membrane. In our work, mice treated with  $\text{CCl}_4$  showed a striking increase in MDA levels as compared to the untreated normal mice ( $p < 0.001$ ). However, EAF at doses above 500 mg/kg bw could markedly prevent the increase in MDA formation (Table 2), which clearly demonstrated the ability of EAF to directly interact with ROS that might initiate lipid peroxidation.

In this study, EAF prevented lipid peroxidation, which could be attributed to the radical-scavenging antioxidant constituents. SOD and CAT are the major enzymes that play an important role in the elimination of ROS derived from the redox process in liver tissues,<sup>32</sup> where SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen.<sup>33</sup> Here, administration of  $\text{CCl}_4$  to mice sharply decreased antioxidant capacity of mouse liver as evidenced by inhibiting the enzymic activity of SOD and CAT, which is in agreement with earlier results.<sup>2,26</sup> However, the decreased levels of these enzymes were significantly increased by pretreatment with EAF (500 or 1000 mg/kg bw), suggesting that it could protect the two antioxidant enzymes or activate the enzyme activity in  $\text{CCl}_4$ -damaged liver tissue. It should also be noted that GSH is the major nonenzymatic antioxidant and regulator of intracellular redox homeostasis, ubiquitously present in all cell types.<sup>34</sup> Some studies reveal that GSH conjugation plays a critical role in eliminating the toxic metabolites, which are the major cause of liver pathology caused by  $\text{CCl}_4$ .<sup>35</sup> In our hands,  $\text{CCl}_4$  administration led to a significant decrease in GSH level of liver tissue. However, EAF pretreatment can significantly dispute the decrease of GSH and the consequent oxidative damage to the liver ( $p < 0.01$ , Table 2).

Histopathological examination can provide visual evidence for the hepatoprotective effects of investigated components. In agreement with the results of biochemical parameters assay in serum and liver tissue, administration of EAF reduced the histological alteration induced by  $\text{CCl}_4$  (Figure 4). The changes in  $\text{CCl}_4$ -intoxicated mice revealed severe confluence cytolysis, pyknosis, ballooning degeneration, and inflammatory cell infiltration, as reported previously.<sup>2,25,27</sup> This may be due to prevention of the toxic chemical reactions from the formation of highly reactive radicals because of the oxidative threat induced by  $\text{CCl}_4$ .

In this study, the main polyphenolic compounds present in EAF were identified and quantified by chromatographic analysis to gain an insight into the major active compounds responsible for its hepatoprotective effect. The HPLC assay clearly indicated that EAF contained the greatest concentration of quercitrin (111.7  $\mu\text{g}/\text{mg}$ , quercetin 3-*O*- $\beta$ -D-rhamnoside), where-

as other polyphenols such as quercetin (43.8  $\mu\text{g}/\text{mg}$ , flavonol) and hyperoside (29.1  $\mu\text{g}/\text{mg}$ , quercetin-3-*O*-galactoside) were also identified as abundant constituents (Figure 5). Previous studies have demonstrated that quercitrin, quercetin, hyperoside, and rutin could be potential therapeutic agents as they reduce oxidative DNA damage and lipid peroxidation and quench free radicals.<sup>36–39</sup> Quercetin was reported to be effective in several models of liver damage including  $\text{CCl}_4$ -induced cirrhosis in rats.<sup>38–40</sup> Quercetin derivatives such as quercitrin and rutin were demonstrated to be the main compounds in hepatoprotective extracts from plants,<sup>41–43</sup> and hyperoside was also shown to possess a protective effect against  $\text{CCl}_4$ -induced acute liver injury, which is likely due to the enhancement of the antioxidative defense system and suppression of the inflammatory response.<sup>25</sup> Therefore, the presence of quercitrin, quercetin, hyperoside, and other antioxidants in EAF from *H. cordata* tea may be the main contributing factor toward its hepatoprotective activity as dietary supplement.

In conclusion, the results from this study clearly demonstrate that polyphenol-rich EAF from *H. cordata* tea has a protective effect against  $\text{CCl}_4$ -induced acute hepatotoxicity in mice, as evidenced by the lowered tissue lipid peroxidation and elevated levels of enzymatic and nonenzymatic antioxidants in liver. The predominant individual polyphenolics in EAF were found to be quercitrin, quercetin, and hyperoside, responsible for antioxidant and hepatoprotective activities. The results indicate that the polyphenol-rich extract from *H. cordata* tea has a significant potential to allow future exploitation as a natural antioxidant and dietary source for the mitigation of oxidative stress-induced liver injury.

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### Notes

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