Protective effects of polyphenols-enriched extract from Huangshan Maofeng green tea against CCl₄-induced liver injury in mice

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1. Introduction

The liver is a vital organ that plays a pivotal role in metabolism and detoxification of various endogenous and exogenous harmful substances [1]. It is well known that free radicals cause cell damage through the mechanisms involving in lipid peroxidation with subsequent tissue injury, especially liver injury [2]. Various xenobiotics are known to cause hepatotoxicity, one of which is carbon tetrachloride (CCl₄). CCl₄-induced toxicity depends on dose and duration of exposure, or time of observation, and at low doses, transient effects prevail, such as loss of Ca²⁺ sequestration, impairment of lipid homeostasis, release of noxious or beneficial cytokines, and apoptotic events. Other effects, with higher doses or longer exposure, are more serious, permanent, and develop over a longer period of time, such as fatty degeneration, fibrosis, cirrhosis, and even cancer [3]. Recently, the use of active dietary ingredients and traditional herbs, which are believed to be harmless and free from serious adverse reactions, has increased all over the world for the prevention and treatment of liver diseases [3,4]. 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) [4]. In this regard, the polyphenols derived from Huangshan Maofeng green tea is expected to play a promising role in protection against liver injury induced by CCl₄.

Tea (Camellia sinensis), a popular aromatic beverage worldwide, is well known for its color, flavor, and taste. Green tea from different plant resources is widely consumed as ancient as 2000 years in Asian countries [5], and have drawn increasing attention as dietary supplements for their excellent antioxidant activities and the other health-promoting effects [6]. Several studies have found that green tea can offer protection against oxidative damage to red blood cells induced by various of inducers (e.g., H₂O₂), and tea ingestion is also shown to enhance antioxidant capacity in human plasma [7]. A wide variety of antioxidant phytochemicals presented in tea are of particular interest and crucial importance because they are recognized as the universal antioxidant defense ingredients against the hepatotoxicity, carcinogenic effects and vascular diseases of free radicals [8]. Therefore, natural tea polyphenol-rich fraction is emerged as the excellent resource for the treatment of oxidative stress-based liver injury.

Huangshan Maofeng green tea is a new cultivar produced in Huangshan region of Anhui province in southern China with short sunshine-hour. It is claimed that drinking Huangshan Maofeng green tea can promote health and alleviate the severity of many disorders [9]. However, there is no scientific study available to describe the potential effect of Huangshan Maofeng green tea or
its extracts against liver damage. It is well known that CCl₄ can produce highly reactive trichloromethyl-free radicals (‘CCl₃ or CCl₂, O’O’), 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 [9]. In this regard, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzyme activities were used to serve as the parameters of the hepatotoxic extent in the mice [10]. In addition, the increase in the level of lipid peroxidation, and the decrease in antioxidant defense system are obviously seen along with the liver damage induced by CCl₄ [2]. Therefore, the aim of this study was to investigate the protective effects of the polyphenols-rich extract (HMTP) from Huangshan Maofeng green tea on CCl₄-induced hepatic damage in mice, including the effects of HMTP on the biochemical determinations of serum ALT, AST and ALP activities, and the antioxidant levels in the liver combined with hepatic histopathological observations in vivo, as well as its free radical scavenging activity in vitro. Furthermore, the main tea polyphenols present in HMTP were identified and quantified by high-performance liquid chromatography (HPLC) to gain an insight into the compounds responsible for its antioxidant and hepatoprotective effects.

2. Materials and methods

2.1. Materials and chemicals

The green tea of Huangshan Maofeng, a geographically specific C. sinensis, was purchased from the supermarket of the Fourth military medical university, which was harvested in the Huangshan region of Anhui province, China. Pure standards of epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), chlorogenic acid, caffeic acid, rutin (quercetin-3-O-rhamnoside), quercetin, hyperoside and caffeine were purchased from Wako Pure Chemical Industries Ltd. Co. (Osaka, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), potassium ferricyanide [K₃Fe(CN)₆], and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). Nitroblue tetra-zolium (NBT), nicotinamide adenine dinucleotide (NADH), and phenazine methosulfate (PMS) were the products of Applichem (Darmstadt, Germany). Biphenyldicarboxylate pills (BP) and CCl₄ were obtained from Zhengjiang Wanbang Pharmaceutical Co. (Wenling, China) and Tianjin Tianli Chemical Reagent Co. (Tianjin, China), respectively. Assay kits of ALT, AST, ALP, Malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Acetoneitrile and methanol were purchased from Honeywell (USA). Deionized water was prepared using a Millipore Milli-Q-Plus system (Millipore Corp., Bedford, MA, USA). All the other chemicals used in the study were of the analytical grade.

2.2. Extraction and isolation of HMTP

The polyphenolics were isolated from Huangshan Maofeng tea as previous procedure [6]. The green tea of Huangshan Maofeng (200 g) was dried in a domestic microwave at 55 °C for 3 h and mashed into powers. Samples were extracted under reflux using 75% methanol (1:10 w/v) at 80 °C for 3 h. After that, the supernatant was poured-out, and the residue was re-extracted under the same condition for three cycles. The extracted solution was incorporated, filtered and centrifuged at 3000g for 10 min to get rid of insoluble substance. Subsequently, the ethanolic extract was concentrated to 5% of the original volume with a vacuum rotary evaporator, and then it was extracted for 3 times using ethyl acetate in a 1:3 ratio (v/v) at room temperature. Finally, the ethyl acetate was dislodged using a rotary evaporator under reduced pressure and the remaining matter was lyophilized in the freeze-dry apparatus (Yuhua, China) to get the refined HMTP.

2.3. Determination of total polyphenols and total flavonoids

Total polyphenols in the extracts were estimated with the Folin–Ciocalteau method as gallic acid equivalents (GAE), expressed as milligrams of gallic acid per gram of extract [6]. Briefly, appropriately diluted standard solutions or 2 mL extracts were mixed with 2 mL of Folin–Ciocalteau reagent and allowed to react for 3 min. After the addition of 6 mL of 10% Na₂CO₃, the mixture was allowed to stand at 25 °C for 2 h. Absorbance was measured at 760 nm, and total polyphenol contents were calculated as GAE from a calibration curve, \( A = 8.0833C + 1.5063 \) (\( R^2 = 0.9953 \), 24–72 μg of gallic acid). The data were presented as the average of triplicate analyses. In addition, total flavonoids of the extracts were measured as rutin equivalents (RE) using a modified colorimetric method [6]. Different concentrations of extracts or standard solutions (2 mL) were mixed with 5% NaNO₂ solution (2 mL), and then 2 mL of 10% Al(NO₃)₃ solution was added and allowed to stand for 5 min. The resultant reaction solution was mixed with 10 mL of 4% NaOH solution, and 30% ethanol was immediately supplied to the final volume of 50 mL, followed by a thorough mixture and a further stand for 15 min. Absorbance of the mixture was determined at 380 nm versus blank water, and all the determinations were carried out in triplicate. Calibration curve of rutin was prepared with the same procedure. The concentration of flavonoids was calculated as RE according to the following linear equation \( A = 26.7676C - 0.2986 \), \( R^2 = 0.9933 \) (8–48 μg of rutin). The content of flavonoids was calculated as RE based on the calibration curve. The data were presented as the average of triplicate assays.

2.4. HPLC chemical analysis of HMTP

The chemical compositions of HMTP were determined by HPLC according to previously publishing method [11] using a Shimadzu LC-2010A system equipped with an UV–vis detector (Shimadzu, Kyoto, Japan). In brief, the HPLC analysis was performed on a reversed-phase HPLC column (4.6 mm i.d. × 250 mm, 5 μm, Venusil, USA). The separation was carried out by changing the scale of mobile phase A (0.5% methanoic acid/50% acetonitrile/50% water) to mobile phase B (water, containing 0.5% methanoic acid), and the gradient was as follows: 0–10 min, 75% B; 10–30 min, linear gradient from 75% to 50% B; 30–45 min, linear gradient from 50% to 30% B; 45–55 min, linear gradient from 30% to 10% B. Elution was carried out at a flow rate of 1.0 mL/min, and the wavelength for UV detection was 280 nm. The injection volume was 20 μL. All the separations were completed at 30 °C. Data is average values for three experiments.

2.5. Evaluation of in vitro antioxidant activity of HMTP

2.5.1. Assay for ferric-reducing antioxidant power (FRAP)

The FRAP of HMTP was measured according to our validated method [6]. Briefly, 2.5 mL of pH 6.6 phosphate buffer and 2.5 mL of 1% K₃Fe(CN)₆ were added to the tested HMTP solution (1.0 mL) at various concentrations (25–130 μg/mL). The mixture was put into 50 °C water bath for 20 min when mixed uniformly, and then 2.5 mL 10% TCA was added to each mixed solution, followed by a mixture and further centrifugation at 3000g for 10 min. The absorbance at 700 nm was measured immediately. Increased absorbance of the reaction mixture indicates a greater reducing power.
2.5.2. Scavenging activity on DPPH radicals

The assay was performed as described by the method [12]. In brief, 1.0 mL of HMTP solution at various concentrations (50–200 μg/mL) was placed in a cuvette, and 3.0 mL of 0.1 mM DPPH in aqueous methanol was added. After 20 min, absorbance at 517 nm was measured using a spectrophotometer. All measurements were made in triplicate, and DPPH-scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: scavenging activity against DPPH (\% \text{Abs}) = \left[1 - \frac{\text{Abs of sample}}{\text{Abs of blank}}\right] \times 100.

2.5.3. Scavenging activity on superoxide anion radicals

The reducing activity on superoxide anion (\text{O}_2^-) was measured as previously described [4] with some modifications. Briefly, in the PMS-NADH system various concentrations (0–300 μg/mL) of HMTP (1.0 mL), 1.0 mL NBT, 1.0 mL NADH and 0.4 mL PMS were successively added into test tube. The mixture was shaken adequately and left to stand at room temperature for 5 min before detecting absorbance at 560 nm with the visible spectrometer. Scavenging capability was calculated according to the following formula: suppression rate (\%) = \left[1 - \frac{\text{Abs of sample}}{\text{Abs of blank}}\right] \times 100.

2.5.4. Hydroxyl radicals-scavenging capacity

The scavenging activity of HMTP on hydroxyl radical (HO•) was determined according to a previous procedure [12]. 1.0 mL of HMTP solution at various concentrations (0–500 μg/mL) was added to a mixture solution of 1.0 mL FeSO₄, 1.0 mL salicylic acid–ethanol and 1.0 mL H₂O₂. The resultant mixture was incubated for 60 min at 37 °C and then the absorbance at 510 nm was measured. The experiments were performed in triplicate and the percentage of HO•–scavenging activity was calculated according to the following equation: scavenging activity (\%) = \left[1 - \frac{\text{Abs of control}}{\text{Abs of sample}}\right] \times 100.

2.6. Animals and experimental design of the CCl₄-induced hepatotoxicity

Sixty Kun-ming mice (18–22 g) were obtained from the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). The mice were randomly divided into six groups with each containing 10 animals, and maintained in a temperature-controlled environment (22 ± 2 °C) and a humidity of 55 ± 5% with light period 07:00 a.m. to 20:00 p.m. and free access to water and standard rodent chow (40% corn flour, 26% wheat flour, 10% bran, 10% fish meal, 10% bean cake, 2% mineral, 1% coarse, and 1% vitamin complex, Qianmin Feed Factory) for 15 days prior to the start of the experiment. All the animals received humane care according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China. HMTP was dissolved in 5% sodium carboxymethylcellulose (CMCNa).

Mice from the normal (negative control) and CCl₄-intoxicated groups were given the single dose of physiological saline (0.3 mL, intragastrically, ig) once daily. In the positive group, animals were treated with single dose of 400 mg/kg bw clinical hepatoprotective drug BP (0.3 mL, ig) once daily. In low-, medium- and high-dose of HMTP-treated groups, mice received HMTP at 200, 400, or 800 mg/kg bw (0.3 mL, ig) once daily, respectively. All administrations were conducted for 21 consecutive days. After the last treatment, mice were fasted for 6 h. All mice except those in the normal group were given simultaneously a 0.8% CCl₄/peanut oil mixture (v/v, 0.3 mL) by intraperitoneal injection, whereas the normal group received peanut oil alone. After this, all the mice were fasted for 12 h, but drink water ad libitum was susceptible. At the end of the experimental period, all animals were fully anesthetized by the inhalation of ether and weighed, and then sacrificed to obtain blood and livers.

2.7. Preparation of serum and hepatic tissue samples

The samples of blood were drawn by cardiac puncture and centrifuged at 3000g for 10 min after solidification in order to separate the serum. The serum was stored in the fridge at −20 °C for subsequent analysis. Liver tissues were carefully excised (cleaning the extraneous tissues) and washed with ice-cold normal saline to clear away blood thoroughly, and then refrigerated at −80 °C. In line with the records of the body weight and the liver weight of every mouse, we worked out the consequence of the hepatosomatic index (HI) on the base of the following formula: HI = liver weight/body weight × 100%. The liver tissue homogenate was prepared by automatic homogenate machine and centrifuged at 3000g for 10 min, and then stored at −20 °C for further analysis.

2.8. Determination of ALT, AST and ALP activities in serum

The level of liver injury was estimated by measuring serum enzyme activities of ALT, AST and ALP, using corresponding commercially available diagnostic kits, and the results were expressed in U/L.

2.9. Measurement of MDA, GSH-Px and SOD in liver homogenate

Antioxidant components in the hepatic tissue samples were also analyzed in the experimental mice. The liver tissue was homogenated by an automatic homogenizer. During the preparation, 0.5 g of each hepatic tissue was homogenated in ninefold (w/v) cold normal saline, and centrifuged at 2000g for 10 min. The supernatant was used for the MDA, GSH-Px, and SOD assays, reflected as common indexes of antioxidant status of tissues, and all of these biochemical makers were measured by using commercial kits according to instructions, and the results were expressed as nmol/mg prot., mg/g prot., and U/mg prot., respectively. Total protein contents in homogenates were determined by the method of Coomassie Brilliant Blue [6].

2.10. Histopathological examinations

A portion tissue obtained from liver was preserved in 4% buffered paraformaldehyde phosphate solution, and then embedded in paraffin. The paraffin blocks were sliced into 4–6 μm sections and stained with hematoxylin-eosin (H&E) according to a standard procedure. Finally, the sample was analyzed by assessing the morphological changes under a light microscope.

2.11. Statistical analysis

Linear regression analysis was performed at least in triplicate and all the results were presented as means ± SD (standard deviation). Differences between variables were analyzed using one-way analysis of variance (ANOVA) and the results were analyzed by SPSS for windows. P-values of <0.05 were considered to be statistically significant.

3. Results

3.1. Chemical analysis of the polyphenols in HMTP

To evaluate the bioactive compounds of Huangshan Maofeng tea, we successively extracted the polyphenols-enriched HMTP with a multistep purification procedure. With this method, the
extraction yield of polyphenols and flavonoids from Huangshan Maofeng green tea can reach approximately 25.4% and 16.6% (w/w) of the dried tea, respectively. The total phenolic contents of HMTP determined using the Folin–Ciocalteu method were 44.9 μg GAE/mg extract, while the contents of total flavonoids were 37.3 μg RE/mg extract, suggesting that HMTP is a polyphenol-enriched extract.

To further characterize composition information, HMTP was also subjected to compositional determination of monomeric phenolic compounds by HPLC technique. A representative HPLC chromatogram of authentic standards is shown in Fig. 1A, and the polyphenol profile of HMTP analyzed by HPLC is illustrated in Fig. 1B. The identification of polyphenolic compounds was performed according to the retention time ($t_R$) obtained from authentic standards under identical HPLC conditions. As depicted in Fig. 1B, eight peaks corresponding to authentic standards were identified in the order of caffeine (4.7 min), chlorogenic acid (7.1 min), EC (9.5 min), caffeic acid (14.2 min), EGC (16.1 min), EGCG (24.3 min), rutin (25.2 min) and ECG (30.5 min). In this study, linear regression was assessed for the content calculation, and the assay had excellent linearity between $Y$ (peak area of the standard polyphenols) and $X$ (concentration of the polyphenols) with the correlation coefficients ($R^2$) in the range of 0.9951–0.9997, and the quantitative data were calculated from their respective calibration curves (Table 1). As shown in Fig. 1B and Table 1, EGC (271.2 μg/mg) was identified to be presented in the highest level in HMTP, followed by rutin (239.3 μg/mg) and EC (89.3 μg/mg), and only traces of caffeine (5.20 μg/mg), caffeic acid (5.13 μg/mg), EGCG (4.72 μg/mg), chlorogenic acid (4.49 μg/mg), and ECG (3.44 μg/mg) were detected. The results obtained in this analysis clearly indicate that HMTP is a good source as a natural flavonoid antioxidant agent.

3.2. Antioxidant effects of HMTP in vitro

In this study, the antioxidant capacity of HMTP was firstly estimated via DPPH, O$_2•$ and HO• systems and FRAP assay. As shown in Fig. 2A, HMTP exhibited a certain degree of ferric-reducing antioxidant power in a dose-dependent manner. The scavenging activity of HMTP on DPPH was 19.7%, 32.3%, 40.3%, 52.4%, 59.8% and 64.3% in the broad range of 50–200 μg/mL (Fig. 2B), respectively. Similarly, HMTP also showed the significant scavenging effects by 7.8%, 16.2%, 23.1%, 27.8%, 36.1%, 46.2% and 50.8% against O$_2•$ in a concentration-dependent manner (30–300 μg/mL, Fig. 2C).
at the concentrations of 120–500 μg/mL also exerted obvious scavenging effects (13.2%, 23.1%, 34.5%, 40.8%, 45.5%, 52.8% and 56.1%) against HO•, respectively (Fig. 2D). The present data indicate that HMTP has potential to be explored as a strong antioxidant, although it is inferior to the reference BHT in antioxidant capacity (Fig. 2).

3.3. Effects of HMTP administration on body weight, liver weight and HI in mice

After the treatment with HMTP for 21 days, the change in body weight, liver weight and HI of the tested mice is summarized in Table 2. It was found that the CCl4-treated mice gained significantly body weight \((p < 0.05)\), liver weight \((p < 0.01)\) and HI \((p < 0.05)\) in comparison with the normal mice. However, the CCl4-induced increase in the liver weight and HI could be decreased by the pretreatment with HMTP at the high dosage of 800 mg/kg bw \((p < 0.05)\), respectively. As shown in Table 2, a similar effect was also observed with the pretreatment of the positive drug BP at 400 mg/kg bw.

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Equation of regression ((Y = aX + b))</th>
<th>(R^2)</th>
<th>Contents ((\mu g/mg))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>(Y = 2 \times 10^{4}X - 36,389)</td>
<td>0.9974</td>
<td>5.20</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>(Y = 1 \times 10^{5}X + 27,539)</td>
<td>0.9993</td>
<td>4.49</td>
</tr>
<tr>
<td>EC</td>
<td>(Y = 3 \times 10^{4}X - 727,692)</td>
<td>0.9968</td>
<td>88.3</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>(Y = 2 \times 10^{5}X - 24,523)</td>
<td>0.9957</td>
<td>5.13</td>
</tr>
<tr>
<td>EGC</td>
<td>(Y = 2 \times 10^{5}X - 232,494)</td>
<td>0.9851</td>
<td>271.2</td>
</tr>
<tr>
<td>EGCG</td>
<td>(Y = 1 \times 10^{5}X + 12,204)</td>
<td>0.9997</td>
<td>4.72</td>
</tr>
<tr>
<td>Rutin</td>
<td>(Y = 4 \times 10^{5}X - 140,846)</td>
<td>0.9982</td>
<td>239.3</td>
</tr>
<tr>
<td>ECG</td>
<td>(Y = 1 \times 10^{5}X + 21,394)</td>
<td>0.9995</td>
<td>3.44</td>
</tr>
</tbody>
</table>

3.4. Effects of HMTP on serum ALT, AST and ALP activities

The hepatoprotective effects of HMTP on the enzymatic activities of serum ALT, AST and ALP are displayed in Fig. 3A–C, respectively. A single application of CCl4 led to a significant elevation of serum ALT and AST activities from 32.0 ± 2.8 and 25.0 ± 4.5 U/L in untreated normal mice to 67.8 ± 6.8 and 61.0 ± 3.3 U/L in CCl4-treated mice \((p < 0.01)\), respectively (Fig. 3A and B). Interestingly, in the mice treated with 400 mg/kg bw of HMTP, the activity of ALT and AST decreased to 53.2 ± 5.2 U/L and 46.6 ± 2.9 U/L, and at 800 mg/kg bw, the corresponding value was 36.4 ± 5.0 U/L and 27.8 ± 3.5 U/L, compared to CCl4-intoxicated mice, respectively \((p < 0.05, p < 0.01)\), and this protection effect can be performed in a dose-dependent manner. However, there was no statistical significance at low dosage of 200 mg/kg bw. Meanwhile, BP as positive drug was also effective in inhibiting the raise in serum ALT and AST activities at dosage of 400 mg/kg bw \((p < 0.05\) vs CCl4-intoxicated mice). In addition, the application of CCl4 also resulted in a significant increase in ALP activity from only 44.3 ± 4.8 U/L protein of the normal mice to

![Fig. 2](image-url) In vitro antioxidant effects and free radical scavenging activities of HMTP. (A) Ferric-reducing antioxidant power (FRAP) of HMTP (50–200 μg/ml) and BHT. (B) DPPH•-scavenging activities of various concentrations of HMTP (50–200 μg/mL) and BHT. (C) O2•−-scavenging capacities of HMTP (30–300 μg/mL) and BHT. (D) HO•-scavenging effects of HMTP (120–500 μg/mL) and BHT. Data are presented as means ± SD (n = 3).
94.6 ± 3.2 U/L protein (p < 0.01). As expected, ALP activity was significantly reduced to 70.2 ± 3.8 U/L protein (p < 0.05) in the mice pretreated with HMTP at 400 mg/kg bw, and a further alleviation was dose-dependently observed at 800 mg/kg bw HMTP, which was close to 48.5 ± 5.2 U/L protein of the positive agent BP (p < 0.05, Fig. 3C).

3.5. Effects of HMTP on hepatic MDA, GSH-Px, and SOD levels

Fig. 3D–F shows the effects of HMTP on MDA, GSH-Px, and SOD activities in hepatic tissues. In the CCl4-intoxicated group, MDA level was 4.87 ± 0.45 nmol/mg protein (p < 0.05), whereas these value was only 2.46 ± 0.32 nmol/mg protein in the normal mice (Fig. 3D), suggesting that CCl4 significantly caused lipid peroxidation. As expected, the elevated level of MDA was significantly reduced to 3.61 ± 0.49 nmol/mg protein (p < 0.05) in the mice pretreated with HMTP at 400 mg/kg bw, and a further decrease was observed at 800 mg/kg bw HMTP, which was close to 2.51 ± 0.34 nmol/mg protein of the positive agent BP (p < 0.01, Fig. 3D). As shown in Fig. 3E and F, the hepatic GSH-Px and SOD activities were also significantly decreased upon oral administration of CCl4 from 8.23 ± 0.45 mg/g protein and 199 ± 17.1 U/mg protein to 6.23 ± 0.34 mg/g protein and 159 ± 13.2 U/mg protein in the mice pretreated with HMTP at 100 mg/kg bw (p < 0.01, Fig. 3E), and to 4.23 ± 0.24 mg/g protein and 109 ± 11.1 U/mg protein in the mice pretreated with HMTP at 200 mg/kg bw (p < 0.01, Fig. 3F).
protein of untreated normal mice to 2.35 ± 0.76 mg/g protein and 70.5 ± 14.3 U/mg protein (p < 0.01), respectively. However, the pretreatment of HMTP remarkably elevated GSH-Px and SOD activities, especially when the dosage increased to 400 and 800 mg/kg bw. At a dosage of 400 mg/kg bw, the activities of GSH-Px and SOD increased to 6.81 ± 0.83 mg/g protein (p < 0.05) and 160 ± 18.3 U/mg (p < 0.05), and at 800 mg/kg bw, the corresponding value was 8.14 ± 0.51 mg/g protein (p < 0.05) and 193 ± 19.2 U/mg (p < 0.01), respectively. As a positive hepatoprotective control, BP drug also significantly inhibited the change of hepatic MDA, GSH-Px and SOD levels.

3.6. Histological observations of mouse livers

As depicted in Fig. 4, the histological observations supported the results obtained from serum and hepatic biochemical assays. H&E stained analysis of the liver from untreated normal mice showed intact central vein and hepatic cords with healthy hepatocytes and thin sinusoidal spaces (Fig. 4A). However, CCl₄-treated mice showed central vein disruption, ballooned lipid laden hepatocytes and dilated sinusoidal spaces (Fig. 4B), revealing extensive liver lesions. However, 200 or 400 mg/kg bw HMTP-treated mice showed moderate hypertrophy of hepatocytes with relatively intact central vein and marginal distortions of sinusoids (Fig. 4C and D). As illustrated in Fig. 4E and F, HMTP at 800 mg/kg bw and BP at 400 mg/kg bw showed the ideal hepatoprotection with intact central vein and well-marked hepatic cords, respectively, suggesting that HMTP could protect the liver from acute CCl₄-induced histopathological alteration.

4. Discussion

Green tea is continually reported to have a wide range of health benefits, and its polyphenols have been considered as the promising active ingredients responsible for the effects [13]. However, previous studies have not still connected the hepatoprotective effects to the phenolic constituents of Chinese Huangshan Maofeng green tea. In this study, we for the first time explored the hepatoprotective efficacy of polyphenol-rich extract from Huangshan Maofeng green tea. Here, HMTP was characterized as polyphenols-enriched fraction with EGC (271.2 μg/mg) being the main catechin, followed by rutin (239.3 μg/mg), and EC (89.3 μg/mg). It is
well known that the polyphenolic extracts from many types of plants, such as green tea and black soybean, have high efficiency in reducing oxidative damage and lipid peroxidation and scavenging free radicals [14]. In our hands, the assays for FRAP and scavenging DPPH, HO· and O2· appeared to have a concentration-dependent antioxidant effect of HMTP in vitro, and this effect was further verified by testing the antioxidant potential of HMTP in an animal model with experimental CCl4-induced oxidative stress, suggesting that the existence of main EGC, rutin and EC in HMTP might be the main contribution for the hepatoprotective effect.

CCl4-induced hepatotoxicity is the most commonly used experimental model system to evaluate the hepatoprotective activity of plant extracts and drugs [1]. It is also known that the active metabolites of CCl4, trichloromethyl free radicals (CCl3) or trichloromethyl peroxy radicals (CCl3O2·), can react with liver to induce lipid peroxidation and cause hepatic cell death, resulting in an elevated of serum enzyme AST, ALT and ALP, and an increased incidence and severity of histopathological hepatic lesions [2]. The present study revealed a significant increase in the activities of AST, ALT and ALP on exposure to CCl4, indicating considerable hepatocellular injury. Administration of HMTP attenuated the CCl4-induced increases in serum AST, ALT and ALP enzyme activities, and caused a subsequent recovery towards normalization comparable to the untreated normal mice. This protective effect of HMTP was further confirmed by the histopathological examinations (Fig. 4). It is reported that the reason of histological changes of HMTP was further confirmed by the histopathological examination in hepatotoxicity induced by CCl4 are apoptosis, necrosis, steatosis and mononuclear cell infiltration in lobular area or portal septa [15]. In our study, HMTP pretreatment prior to CCl4 had a significant contributor to the inhibition of the histological alteration.

It has been hypothesized that one of the principal causes of CCl4-induced liver injury is the formation of lipid peroxides by free radical derivatives of CCl4 [16]. Thus, the antioxidant activity or the inhibition of the generation of reactive oxygen species (ROS) is important in the protection against CCl4-induced hepatopathy [10]. It is well known that the body has an effective defense mechanism to prevent and neutralize ROS-induced damage, and a ROS-mediated lipid peroxidation mechanism has been implicated in the pathogenesis of various liver injuries and subsequent liver fibrogenesis in experimental animals [3]. In our study, HMTP pretreatment prior to CCl4 intoxication resulted in a reduction in MDA formation, compared to the CCl4-treated mice, and the inhibition of hepatic MDA was dose-dependent ( p < 0.05, Fig. 3D), indicating that HMTP prevented lipid peroxidation, which was deemed to be one of the chief causes of CCl4-induced liver injury [17].

In addition, live tissues are considered to be involved in congenital antioxidant defense mechanisms, and endogenous antioxidant enzymes, such as SOD, GSH-Px and catalase, are known to constitute a mutually supportive team of defense against ROS [18]. GSH-Px acts as an enzymatic antioxidant both intracellularly and extracellularly in conjunction with various enzymatic processes that reduces hydrogen peroxide (H2O2) and hydroperoxides, whereas the depletion of hepatic GSH-Px has been shown to be associated with an enhanced toxicity to chemicals, including CCl4 [19]. SOD can catalyze the clearance of the superoxide anion radicals and prevents the formation of H2O2 [20]. A decrease in the activity of these enzymes leads to deleterious effects, such as loss of integrity and function of cell membranes, which is regarded to connect with the accumulation of highly ROS [21]. In the current study, administration of CCl4 to mice led to a significant decrease in the antioxidant capacity of the liver by the reducing activity of the antioxidant enzymes. It was also worth noting that the pretreatment of HMTP boosted the antioxidant enzyme activities and consequently resisted the hepatic oxidative injury by preventing the drop of these indexes caused by CCl4 (Fig. 4). It has been reported that green tea polyphenols can prevent the loss of membrane permeability and reduce endogenous level of hydroxyl radicals, resulting in a large number of the oxidative damage elicited through lipid peroxidation and protein oxidation [22]. Several studies accredit the hepatoprotective effect of drugs and herbal agents to their antioxidant and free radical scavenging abilities [23]. Other hepatoprotective agents such as Sylimarin [24], vitamin E [25], Hippophae rhamnoides seed oil [26], Zizyphus spina-christi [27], Platycodon grandiflorum [28] and Phyllanthus niruri [29] have all been reported to prevent ROS-mediated lipid peroxidation by their free radical scavenging abilities. However, the effect of Huangshan Maofeng tea polyphenols on hepatoprotective bioactivity is unclear. Herein, the protective administration of HMTP to CCl4-treated mice remarkably kept hepatic lipid peroxides near to normal level, and this finding further underlined the importance of high content of polyphenols in HMTP for guarding intact endogenous cellular antioxidant defense systems.

In conclusion, the present results from this study clearly show that HMTP has a protective effect against in vitro oxidative stress, as well as acute oxidative hepatotoxicity induced by the administration of CCl4 in mice, and the hepatoprotective effect of HMTP may be due to the free radical scavenging effect, inhibition of lipid peroxidation, and increased antioxidant activity. The green tea of Huangshan Maofeng is found to be an abundant source of polyphenolic antioxidants and protects against the toxic effects of CCl4 in the liver. This study could provide a useful reference for the future exploitation of HMTP as a novel preventive and therapeutic measure for the treatment of oxidative stress-induced liver injury.

Conflict of Interest
The authors declare that there are no conflicts of interest.

Transparency Document
The Transparency document associated with this article can be found in the online version.

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