



The *in vivo* antioxidant and antifibrotic properties of green tea (*Camellia sinensis*, Theaceae)

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ABSTRACT

The *in vivo* antioxidant and antifibrotic properties of green tea (*Camellia sinensis*, Theaceae) were investigated with a study of carbon tetrachloride (CCl₄)-induced oxidative stress and hepatic fibrosis in male ICR mice. Oral administration of green tea extract at doses of 125, 625 and 1250 mg/kg for 8 weeks significantly reduced ($p < 0.05$) the levels of thiobarbituric acid-reactive substances (TBARS) and protein carbonyls in the liver by at least 28% compared with that was induced by CCl₄ (1 mL/kg) in mice. Moreover, green tea extract administration significantly increased ($p < 0.05$) the activities of catalase, glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) in the liver. Our study found that oral administration of green tea extract prevented CCl₄-induced hepatic fibrosis, as evidenced by a decreased hydroxyproline level in the liver and a reduced incidence of hepatic fibrosis by histological observations. These results indicate that green tea exhibits potent protective effects against CCl₄-induced oxidative stress and hepatic fibrosis in mice by inhibiting oxidative damage and increasing antioxidant enzyme activities.

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1. Introduction

Trichloromethyl free radicals, which are metabolites of CCl₄, are capable of binding to DNA, lipids, proteins or carbohydrates, eventually leading to membrane lipid peroxidation (Recknagel, Glende, Dolak, & Waller, 1989). Increased lipid peroxidation is generally believed to be an important underlying cause of the initiation of oxidative stress in various tissue injuries as well as cell death and the progression of many acute and chronic diseases (Halliwell, 1997). In experimental studies, CCl₄ is commonly used as a toxin to induce hepatic oxidative damage and fibrosis because CCl₄-induced liver oxidative damage responses in animals are superficially similar to human liver oxidative damage (Hsu et al., 2008). Therefore, CCl₄-induced hepatic oxidative damage and fibrosis have been extensively used in animal models to evaluate the therapeutic potential of drugs and dietary antioxidants. Epidemiological and experimental studies have demonstrated that natural antioxidants such as carotenoids, tocopherols and flavonoids can effectively prevent and cure oxidative stress-related diseases

(Vitaglione, Morisco, Caporaso, & Fogliano, 2004). Therefore, growing attention has been given to phytochemicals that are characterised as natural antioxidants.

Green tea (*Camellia sinensis*, Theaceae) is one of the most popular beverages in the world; it is associated with many cultures in Asia, particularly China and Japan. Frank et al. (2009) showed that daily consumption of green tea is safe and has no adverse effects on human health. Because green tea contains abundant bioactive substances, it has been reported to have beneficial biological effects. Most of the beneficial effects of green tea are attributed to its polyphenols, mainly catechins and catechin derivatives, including (–)-epigallocatechin-3-gallate (EGCG), epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and (–)-gallocatechin gallate (GCG) (Wang, Provan, & Helliwell, 2003).

Green tea catechins have been reported to possess various physiological and pharmacological properties, including effective antioxidants that scavenge free radicals (Higdon & Frei, 2003), antifungal activities, antibacterial activities (Friedman, 2007) and anticancer activities (Bode & Dong, 2009). In various experimental models, green tea catechins have been shown to protect against cisplatin-induced nephrotoxicity and to delay memory regression in aged mice (Khan et al., 2009; Unno et al., 2007). The functional activities of green tea catechins have been reported *in vitro* and *in vivo*; specifically, EGCG has been a research focus in recent years

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due to its high antioxidant activity and its presence in relatively large amounts in green tea. Although EGCG is the most abundant and best studied green tea polyphenol, it seems that green tea's preventive effects are stronger with a mixture of tea catechins, such as polyphenon E (Poly E; a decaffeinated green tea catechin mixture), or green tea extracts than with EGCG alone (Bode & Dong, 2009; Fu et al., 2009). In preventing oxidative stress-related pathology, natural antioxidants in complex mixtures that are ingested with the diet are more efficacious than pure compounds due to their particular interactions and synergisms (Vitaglione et al., 2004).

Because of the strong antioxidant activity of green tea, we hypothesised that green tea administration might be useful for preventing various types of oxidative damage induced by oxidative stress. Therefore, the aims of this study were to determine the catechin content of green tea and to evaluate the antioxidant and antifibrotic properties of green tea *in vivo*. Silymarin, an antioxidant flavonoid complex that is isolated from milk thistle seeds (*Silybum marianum*, Compositae), was used as a reference antioxidant (Hsu et al., 2008). The levels of hepatic GSH, protein carbonyls, thiobarbituric acid-reactive substances (TBARS) and antioxidant enzymes such as catalase, SOD, GSH-Px and glutathione reductase (GSH-Rd) were determined to evaluate the intracellular antioxidant status in mice after green tea administration. Additionally, to evaluate the effects of green tea on toxin-induced hepatic fibrosis, hydroxyproline levels and histopathologic examinations of hepatocyte fibrosis were determined.

2. Materials and methods

2.1. Chemicals

Gallic acid (GA), (+)-gallocatechin (GC), (–)-epigallocatechin (EGC), (+)-catechin (C), (–)-epigallocatechin gallate (EGCG), (–)-epicatechin (EC), (–)-gallocatechin gallate (GCG) and (–)-epicatechin gallate (ECG) standards and silymarin were purchased from Sigma Chemical Company (St. Louis, MO, USA); the purity of all standards were greater than 95%. Analytical grade orthophosphoric acid and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Deionised water was prepared using a Mill-RQ and Milli Q-UV water purification system (Millipore Co., Ltd., Taipei City, Taiwan). All other chemicals and reagents used were obtained from local sources and were of analytical grade.

2.2. Material

Green tea powder made from natural tea leaves (*Camellia sinensis*) was obtained from AGV Co., Ltd. (Chiayi City, Taiwan). According to the manufacturer's information, the green tea powder was prepared by adding 5 g of tea leaves to 500 mL of boiling water and then steeping the leaves for 30 min. The extraction solution was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 500 mL of boiling water and filtered, and the two extraction solutions were combined to obtain the green tea extract solution. The green tea extract solution was placed in a freezer at -80°C for one day and then dried in a freeze dryer (-42°C , below 133×10^{-3} mbar) for 48 h. After freeze drying, the samples were pulverised to form a powder and immediately stored at -20°C for further analysis. In accordance with the company-provided general analysis, the green tea extract comprised 70.05% water, 0.84% protein, 0.36% lipid, 28.26% carbohydrate and 0.49% ash.

2.3. Determination of catechin contents in green tea extract

The catechin contents of the green tea extract were analysed with a high-performance liquid chromatography system (Waters

e2695, Waters Co., Milford, MA, USA) that was fitted with a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, photodiode array detector and a C18 reversed phase column (250×4.6 mm, 5- μm particle size; Gemini 5 μ C18 110 Å, Phenomenex®) (Torrance, CA, USA), as described previously (Wang et al., 2003). The mobile phases consisted of 0.1% orthophosphoric acid in deionised water (v/v; eluent A) and 0.1% orthophosphoric acid in methanol (v/v; eluent B). The mobile phase gradient was as follows: 0–5 min, 20% eluent B; 5–7 min, linear gradient of 20–24% eluent B; 7–10 min, 24% eluent B; 10–20 min, linear gradient of 24–40% eluent B; 20–25 min, linear gradient of 40–50% eluent B. The post-run time was 5 min. The elution was performed at a solvent flow rate of 1 mL/min. Catechins were detected with a diode array detector, and chromatograms were recorded at 280 nm. The column temperature was maintained at 30°C . The samples were injected using a manual injection valve (10 μL injection volume). Peaks were identified by comparing their retention times and UV spectra in the 200–400 nm range with authentic standards.

2.4. Animals

Male ICR mice (20 ± 2 g) were obtained from the Animal Department of BioLASCO Taiwan Company and were quarantined and allowed to acclimate for a week prior to experimentation. The animals were handled under standard laboratory conditions with a 12-h light/dark cycle at a temperature of $25 \pm 2^{\circ}\text{C}$ and a relative humidity of $55\% \pm 5\%$ in a controlled room. The basal diet used in these studies, PMI Nutrition International, LLC, Certified Rodent LabDiet 5001, is a certified feed with appropriate analyses performed by the manufacturer and provided to WIL Research Laboratories, LLC. Food and water were available *ad libitum*. Our Institutional Animal Care and Use Committee approved the protocols for the animal study, and the animals were cared for in accordance with institutional ethical guidelines.

2.5. Treatment

The animals were randomly divided into 6 groups of 10. Group I served as the normal control and was orally administered distilled water daily with intraperitoneally (i.p.) administered olive oil (1 mL/kg body weight) twice per week for 8 weeks. To induce oxidative stress and hepatic fibrosis (*in vivo*), animals of Groups II, III, IV, V and VI were i.p. administered 1 mL/kg body weight of CCl_4 (20% in olive oil) twice per week for eight weeks. After CCl_4 administration, Group II served as the CCl_4 control and was orally administered distilled water daily. Group III served as the positive control and was orally administered silymarin (200 mg/kg) daily for 8 weeks. Groups IV, V and VI were orally administered green tea powder dissolved in distilled water at doses of 125, 625 and 1250 mg/kg, respectively, daily for 8 weeks. At the end of the experiment, the animals were sacrificed by CO_2 administration. Liver samples were dissected and washed immediately with ice-cold saline to remove as much blood as possible, then they were immediately stored at -80°C for further analysis. The largest right lobe of each liver was excised and fixed in a 10% formalin solution for histopathologic analyses.

2.6. Measurement of lipid peroxidation

The quantitative measurement of lipid peroxidation was performed by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) in the liver according to the method reported by Hsu et al. (2008). The amount of TBARS formed was quantitated by their reaction with thiobarbituric acid (TBA) and used as an index of lipid peroxidation. In brief, samples were mixed

with a TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 N hydrochloric acid. The reaction mixtures were placed in a boiling water bath for 30 min and centrifuged at 1811g for 5 min. The supernatant was collected, and its absorbance was measured at 535 nm with an ELISA plate reader (μ Quant, BioTek, VT, USA). The results were expressed as nmol/g protein using the molar extinction coefficient of the chromophore ($1.56 \times 10^{-5} \text{ M}^{-1}\text{cm}^{-1}$).

2.7. Measurement of protein carbonyls

Oxidative damage to proteins was quantified by the carbonyl protein assay, which is based on the reaction with dinitrophenylhydrazine, as described previously (Levine et al., 1990). Briefly, proteins were precipitated by adding 20% trichloroacetic acid and redissolved in 10 mM dinitrophenylhydrazine to give a final protein concentration of 1–2 mg/ml, with 2 N hydrogen chloride added to the corresponding sample aliquot reagent blanks. The absorbance was measured at 370 nm with an ELISA plate reader. The data were expressed as nmol of carbonyls/mg protein.

2.8. Measurement of SOD, catalase, GSH-Px and GSH-Rd activities and GSH levels

Liver homogenates were prepared in cold Tris–HCl (5 mmol/L, containing 2 mmol/L EDTA, pH 7.4) using a homogeniser. The unbroken cells and cell debris were removed by centrifugation at 10,000g for 10 min at 4 °C. The supernatant was used immediately for the SOD, catalase, GSH-Px, GSH-Rd and GSH assays. The activities of these enzymes and the level of GSH were determined according to the Randox Laboratories Ltd kit instructions (Antrim, UK).

2.9. Measurement of hydroxyproline levels

The hydroxyproline levels in the livers were determined according to the method of Jamall, Finelli, and Que Hee (1981) with minor modifications. The liver samples were weighed and completely hydrolysed in 6 M HCl. After hydrolysis, the samples were derivatised using chloramine T solution and Ehrlich's reagent, and the optical density was measured at 558 nm. A standard calibration curve was prepared using *trans*-4-hydroxy-L-proline.

2.10. Histopathologic evaluation

The livers were preserved in neutral buffered formalin and processed for paraffin embedding following standard microtechniques. Four-to-five-micron sections of livers were stained with Masson's trichrome for hepatocyte fibrosis and observed under the microscope (IX71S8F-2, Olympus, Tokyo, Japan).

2.11. Statistical analysis

All values are expressed as the mean \pm SD. Comparisons between groups were performed using a one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison tests using the statistical software SPSS (Drmarketing Co., Ltd. New Taipei City, Taiwan). Statistically significant differences between groups were defined as $p < 0.05$.

3. Results and discussion

3.1. Catechins in green tea powder

The potential health implications of green tea leaf extract and related products require a detailed analysis of the catechin

contents of these substances. Table 1 shows the catechin composition in the green tea extract that was used for this study. The results show that eight catechins in the green tea extract could be separated simultaneously within 30 min and that the amount of total catechins in the extract was 259,082 $\mu\text{g/g}$. EGCG, ECG and EGC were the major identified catechins, comprising 57.53%, 13.78% and 10.61% of the total catechins, respectively. Other significant catechins in the extract were (+)-gallo catechin (GC), (+)-catechin (C), EC and GCG; a phenolic acid, gallic acid, was also identified (Table 1). We recently showed that EGCG, EGC and ECG were the principal catechins in green tea extract, comprising 81.03% of the total catechins (Hsu, Tsai, Chen, Huang, & Yen, 2011). Our results are in agreement with previous studies that reported that green tea leaves were rich in catechins, with EGCG, EGC and ECG accounting for 80.9–87.7% of the total catechins (Wang et al., 2003).

3.2. In vivo antioxidant potential

3.2.1. Effects of green tea on lipid peroxidation

In animal models of toxin-induced organ oxidative damage, one of the principal mechanisms of oxidative damage is lipid peroxidation by toxin-biotransformed metabolites, which generates a variety of relatively stable toxic products, many of which are aldehydes (Recknagel et al., 1989). The measurement of TBARS is a well-established method for screening and monitoring lipid peroxidation. Biological specimens contain a mixture of TBARS, including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS are expressed in terms of malondialdehyde (MDA) equivalents. MDA is the major reactive aldehyde that is produced during the peroxidation of biological membrane polyunsaturated fatty acids (Vaca, Wilhelm, & Harms-Rihdsahl, 1988). An increase in TBARS levels indicates enhanced lipid peroxidation, leading to tissue damage and a failure of the antioxidant defence mechanisms to prevent the formation of excessive free radicals (Recknagel et al., 1989). In the present study, CCl_4 -induced toxicity caused a significant increase (53%) in liver TBARS levels compared to the control group (Fig. 1). However, treatment with green tea extract significantly reversed these changes. The administration of green tea extract at a dose of 125 mg/kg significantly decreased the rate of increased TBARS by 42% compared to the CCl_4 -treated group ($p < 0.05$). Similar results were found for 625 and 1250 mg/kg of green tea extract, but there were no significant differences among the tested doses. The positive control drug, silymarin, at a dose of 200 mg/kg also inhibited the elevation in TBARS levels following CCl_4 administration (Fig. 1). None of the green tea extract treatments showed significant differences ($p > 0.05$) in TBARS levels compared to treatment with silymarin, indicating that all tested doses of green tea extract inhibited lipid peroxidation in a manner that was comparable to that of silymarin. These findings are consistent with a report demonstrating that green tea plays a protective role and reduced hepatic TBARS levels following

Table 1
Catechin contents in green tea used on this studies in mice.

Compound	Content ($\mu\text{g/g}$)
Gallic acid (GA)	1319.32 \pm 15.07
(+)-gallo catechin (GC)	6949.75 \pm 130.15
(–)-epigallo catechin (EGC)	27496.56 \pm 968.35
(+)-catechin (C)	5145.81 \pm 111.67
(–)-epigallo catechin gallate (EGCG)	149060.64 \pm 2476.10
(–)-epicatechin (EC)	19541.71 \pm 523.78
(–)-gallo catechin gallate (GCG)	13830.30 \pm 514.64
(–)-epicatechin gallate (ECG)	35737.78 \pm 129.58
Total catechin (sum of above-mentioned eight catechins)	259081.88

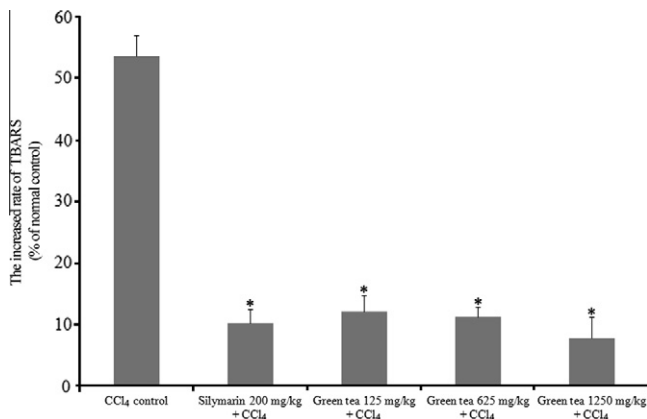


Fig. 1. Effects of green tea on liver TBARS in CCl₄ intoxicated mice. Values are the mean \pm SD for ten mice; * p < 0.05 compared with CCl₄-treated control group.

CCl₄-induced oxidative damage (Xiao, Lu, Shen, & Wu, 2002). Chen et al. (2004) reported that the green tea polyphenol EGCG significantly decreased CCl₄-induced lipid peroxidation in rat livers.

3.2.2. Effect of green tea on protein carbonyls

Protein carbonyl groups are an important biomarker of protein oxidation, and the accumulation of protein carbonyls has been observed in several human diseases, including Alzheimer's disease, diabetes, arthritis and chronic liver diseases (Berlett & Stadtman, 1997). Protein carbonyl derivatives can be generated through the oxidative cleavage of proteins by either the α -amidation pathway or by oxidation of glutamyl side chains, resulting in the formation of a peptide in which the *N*-terminal amino acid is blocked by an α -ketoacyl derivative. The use of protein carbonyl groups as biomarkers of oxidative stress has some advantages, including the relatively early formation and relative stability of carbonylated proteins compared to other oxidation products (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003).

To evaluate the effects of green tea extract treatment on CCl₄-induced oxidative liver damage, protein carbonyl levels were determined in this study. Protein carbonyl levels were significantly higher in the CCl₄-treated group than in the control group (p < 0.05) (Fig. 2A). By contrast, green tea extract administration at doses of 125, 625 and 1250 mg/kg significantly decreased CCl₄-induced hepatic oxidative damage. The percentage of protein carbonyls following treatment with the lowest dose of green tea extract (125 mg/kg) was significantly lower (53%) than that of the CCl₄-treated group (p < 0.05). Similar results were also found after administration of 625 and 1250 mg/kg of green tea extract. However, green tea extract showed no dose-dependent protective effect against CCl₄-induced oxidative stress. Silymarin also inhibited the increase in protein carbonyl levels following CCl₄ administration (Fig. 2A). These findings are consistent with those of a previous *in vivo* report that showed that green tea extract reduced hepatic protein carbonyl levels following ethanol-induced hepatic oxidative damage (Augustyniak, Waszkiewicz, & Skrzydlewska, 2005), suggesting that the free radicals that were released in the liver were effectively scavenged by green tea. Several reports have suggested that habitual green tea consumption can protect cells and tissues from oxidative damage by scavenging oxygen free radicals and significantly reduce the levels of carbonyl groups caused by ethanol in the brain tissues of young and adult rats (Higdon & Frei, 2003; Skrzydlewska, Augustyniak, Michalak, & Farbiszewski, 2005).

3.2.3. Effect of green tea on GSH levels

The intracellular antioxidant status is maintained in an equilibrium in mammalian cells. Dietary supplementation with extra

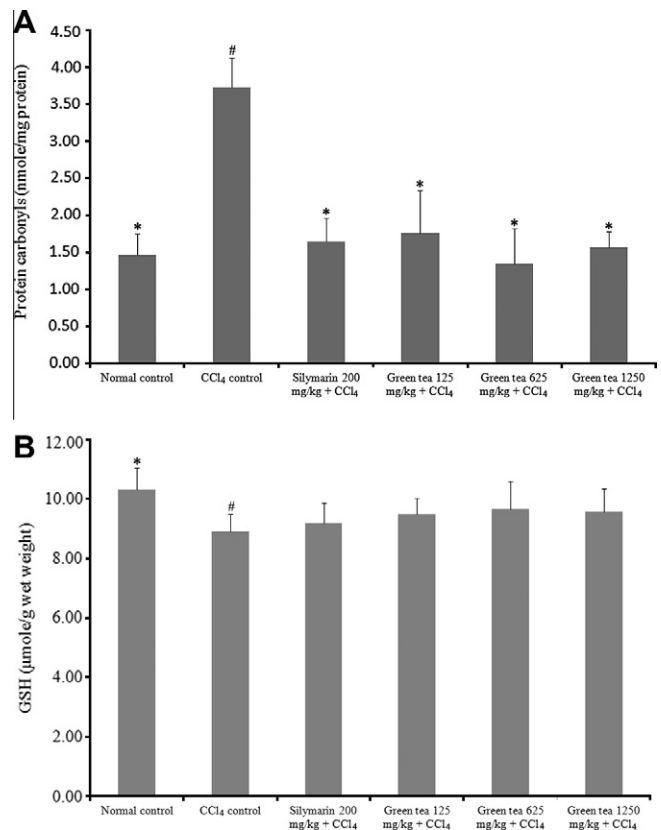


Fig. 2. Effects of green tea on liver protein carbonyls (A) and GSH (B) in CCl₄ intoxicated mice. Values are the mean \pm SD for ten mice; * p < 0.05 compared with normal control; # p < 0.05 compared with CCl₄-treated control group.

natural antioxidants mainly aids the intracellular antioxidant defence system, which includes nonenzymatic antioxidants (e.g., GSH, bilirubin and vitamin E) and enzymatic antioxidants such as SOD, catalase, GSH-Px and GSH-Rd in protecting cells and organs against ROS-induced oxidative damage (Halliwell, 1997). GSH acts as a nonenzymatic antioxidant in the detoxification pathway that reduces H₂O₂, hydroperoxide and xenobiotic toxicity. GSH is readily oxidised to glutathione disulphide (GSSG) upon reaction with xenobiotic compounds, which may then cause a decrease in GSH levels. GSSG is either rapidly reduced by GSH-Rd and NADPH or utilised in the endoplasmic reticulum to aid protein folding processes. Eventually, GSSG is recycled by protein disulphide isomerase to form GSH. Because of these recycling mechanisms, GSH is an extremely efficient intracellular buffer for oxidative stress (Ting et al., 2011). Therefore, it appears that GSH conjugation is essential to decrease the toxic effects of CCl₄. In the present study, CCl₄ treatment significantly decreased GSH levels in the liver compared to the control group (p < 0.05). However, the GSH levels were not significantly different between the CCl₄-treated group and the green tea extract-treated groups. Similar observations were found in animals that were treated with silymarin (Fig. 2B).

3.2.4. Effect of green tea on hepatic antioxidant enzyme activities

A decrease in enzymatic antioxidant activities is related to an increase in lipid peroxide or free radical production following CCl₄-induced liver damage (Hsu et al., 2008). However, the activities of antioxidant enzymes play important roles in protecting cells or organs against oxidative damage. In the present study, SOD, catalase, GSH-Px and GSH-Rd were measured as indices of the antioxidant status of tissues. Fig. 3 shows that significant decreases in hepatic catalase, GSH-Px and GSH-Rd activities were observed in

the CCl₄-treated group compared to the control group ($p < 0.05$). There were significant increases ($p < 0.05$) in catalase, GSH-Px and GSH-Rd activities in the green tea extract-treated groups at doses of 125, 625 and 1250 mg/kg compared to the CCl₄-treated group. However, the different doses of green tea showed no dose-dependent protective effects against CCl₄-induced decreases in antioxidant enzyme activities. A similar observation was found in animals that were treated with silymarin. In addition, the maximum dose of green tea (1250 mg/kg) led to significantly higher SOD levels ($p < 0.05$) compared to the CCl₄-treated group. When the different doses of green tea were compared to silymarin, there were no significant differences ($p > 0.05$) in the antioxidant enzyme activities, suggesting that the abilities of all tested doses of green tea extract to restore and/or maintain the activity of antioxidant enzymes were comparable to that of positive control drug silymarin.

SOD is an extremely effective defence enzyme that converts the dismutation of superoxide anions into hydrogen peroxide (H₂O₂). Catalase is a haemeprotein in all aerobic cells that decomposes H₂O₂ to oxygen and water. GSH-Px plays a major role in the detoxification of xenobiotics in the liver and metabolises H₂O₂ and hydroperoxides to nontoxic products and stops the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane (Hsu et al., 2008). GSH-Rd is a cytosolic hepatic enzyme that is involved in the detoxification of xenobiotics by their conjugation with GSH (Baudrimont, Ahouandjivo, & Creppy, 1997). The activities of these enzymatic antioxidants are reduced by lipid peroxides or ROS, which results in decreased activities of these enzymes under conditions of CCl₄ toxicity. Therefore, the

results of the present study indicate that catalase, GSH-Px and GSH-Rd activities were significantly enhanced by the administration of green tea extract to CCl₄-treated mice, suggesting that green tea extract has the ability to restore or maintain the activities of antioxidant enzymes in CCl₄-damaged livers.

3.3. In vivo antifibrotic potential

3.3.1. Effect of green tea on hydroxyproline levels

Hydroxyproline, a major component of the protein collagen, is produced from the hydroxylation of the amino acid proline by the enzyme proline hydroxylase, which occurs before the completion of polypeptide chain synthesis. The level of hydroxyproline reflects the level of collagen in liver tissue. Recent studies have indicated that an increase in hydroxyproline levels in the liver indicates enhanced hepatic fibrosis, which is associated with the exacerbation of lipid peroxidation and the depletion of antioxidant status after treatment with CCl₄ (Jamall et al., 1981; Tsukamoto, Matsuoka, & French, 1990). The levels of hydroxyproline in the CCl₄-treated group were significantly higher than those in the control group ($p < 0.05$) (Fig. 4), suggesting CCl₄-induced fibrosis in the liver. In contrast to the CCl₄-treated group, the groups that were treated with 125, 625 and 1250 mg/kg of green tea extract showed significantly decreased ($p < 0.05$) hydroxyproline levels (18%, 21% and 23%, respectively). A similar result was found in animals that were treated with silymarin. Additionally, the differences in the levels of hydroxyproline did not reach statistical significance ($p > 0.05$) between the green tea extract treatment groups and the silymarin treatment group, indicating that there was no

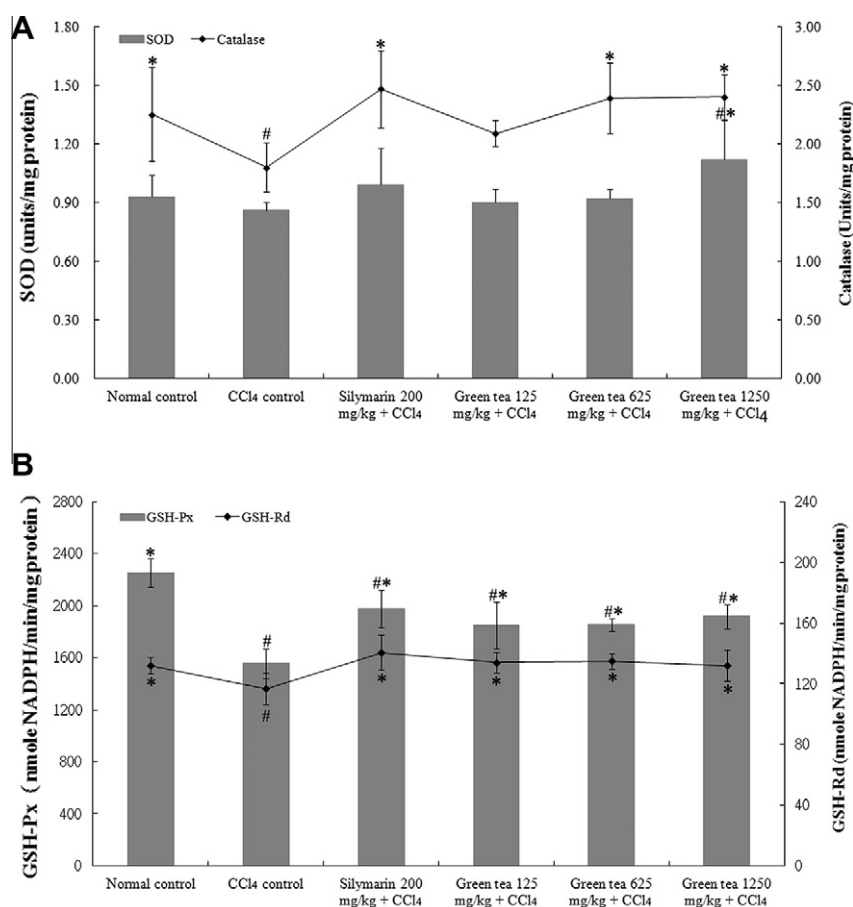


Fig. 3. Effects of green tea on liver antioxidant enzymes in CCl₄ intoxicated mice. (A) SOD and catalase. (B) GSH-Px and GSH-Rd. Values are the mean \pm SD for ten mice; # $p < 0.05$ compared with normal control; * $p < 0.05$ compared with CCl₄-treated control group.

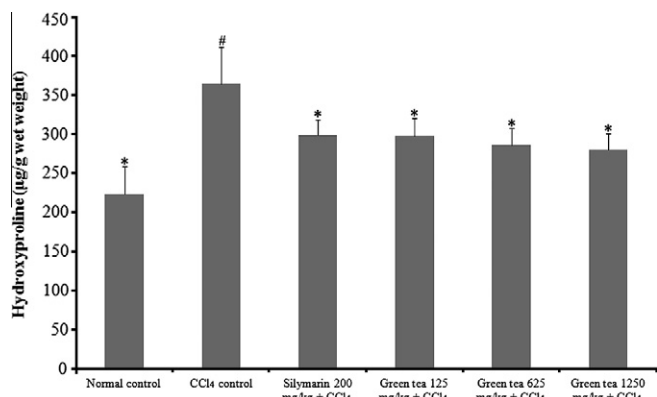


Fig. 4. Effects of the green tea on hydroxyproline in CCl₄-intoxicated mice. Values are the mean \pm SD for ten mice; # p < 0.05 compared with normal control; * p < 0.05 compared with CCl₄-treated control group.

difference between green tea extract treatment and silymarin treatment with respect to hepatic fibrosis. The hydroxyproline levels found in the present study are supported by biochemical observations that the tested doses of green tea extract provide a level of protection comparable to that of silymarin.

Zhen et al. (2007) reported that green tea polyphenols arrest the progression of hepatic fibrosis in a rat model by inhibiting oxidative damage, as evidenced by decreased hydroxyproline levels in

the rat livers. These results are in agreement with our findings that green tea extract administration caused a significant decrease in hydroxyproline levels compared to the CCl₄ treatment group, suggesting that green tea extract has the ability to protect against CCl₄-induced hepatic fibrosis in mice.

3.3.2. Effects of green tea on histopathologic characteristics

In the histological examinations in this study, hepatocyte fibrosis was evaluated by Masson's trichrome stain. In clinical diagnoses and experimental examinations, the detection of liver fibrosis often depends on the microscopic detection of collagen fibres, and Masson's trichrome stain is a routine staining technique for detecting collagen fibres in liver tissue (Bondini, Kleiner, Goodman, Gramlich, & Younossi, 2007). The use of a routine staining technique has the advantage that experienced pathologists can reliably and consistently identify liver damage in formalin-fixed, paraffin embedded tissue sections.

The histopathologic observations also provided important evidence supporting the oxidative damage analysis and hydroxyproline assay. Histopathologic changes in fibrosis occurred in CCl₄-treated mouse livers, and their prevention by treatment with green tea extract was observed, as shown in Fig. 5. The collagen of these fibrotic tissues had a blue colour when stained by Masson's trichrome. In the normal control animals, the liver sections showed normal hepatic cells without fibrosis (Fig. 5A). The livers of mice that were treated with CCl₄ showed extensive accumulation of thick fibrotic tissue, resulting in the formation of continuous

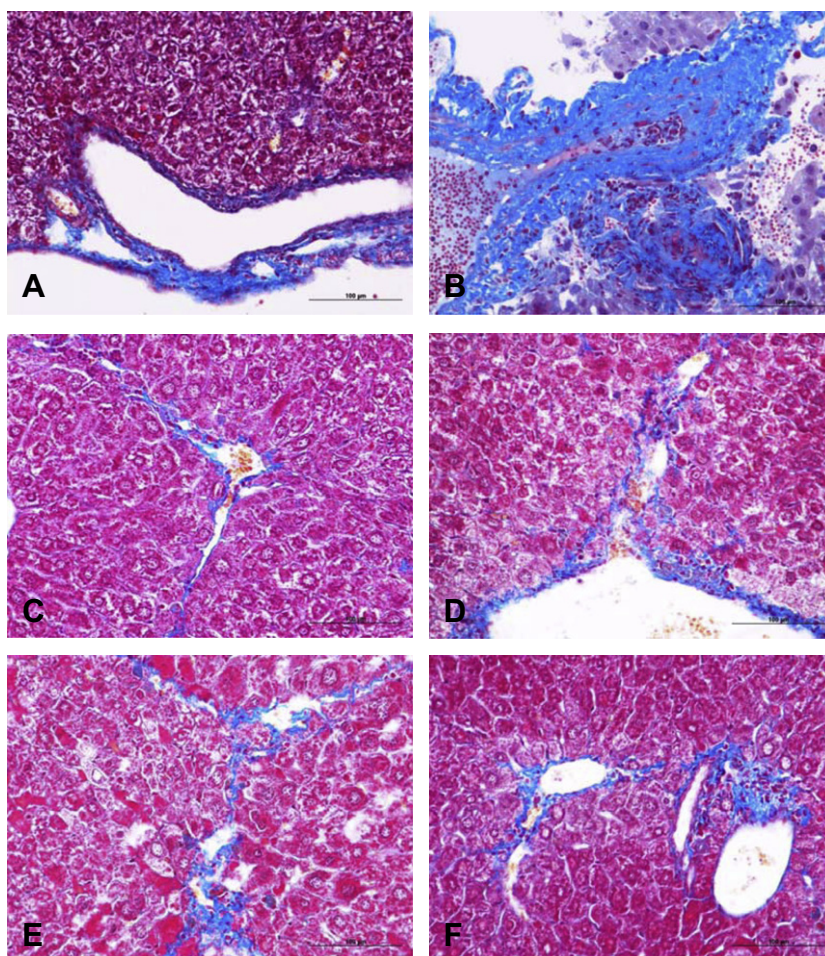


Fig. 5. Histopathological changes of fibrosis occurred in CCl₄-intoxication and prevention by the treatment with green tea (Masson trichrome stain, 200 \times). (A) Normal control, (B) CCl₄ control, (C) silymarin 200 mg/kg + CCl₄, (D) green tea 125 mg/kg + CCl₄, (E) green tea 625 mg/kg + CCl₄, (F) green tea 1250 mg/kg + CCl₄.

fibrotic septa, nodules of regeneration, and noticeable alterations in the central vein compared to the normal control (Fig. 5B). The lesions of silymarin-treated mice were present to a lesser degree (Fig. 5C) than those found in the CCl₄-treated group. A lesser degree (mild to trace) of hepatocyte fibrosis was observed in the livers of green tea extract-treated mice at doses of 125, 625 and 1250 mg/kg (Fig. 5D–F). These animals showed less thick fibrotic tissue, which resulted in less pronounced destruction of the liver architecture compared to the CCl₄ treatment group. Similar results in other studies confirmed that drinking water with 0.1% EGCG, the major and most active green tea catechin, significantly decreased CCl₄-induced hepatocyte fibrosis in rat livers (Yasuda et al., 2009). The histological observations of fibrosis also supported the results of the liver hydroxyproline assay.

The histological observations of fibrosis also showed that there was no difference between the green tea extract-treated groups and the silymarin-treated group by histology. The results of the hepatic histopathologic examination are in accordance with biochemical observations that all tested doses of green tea provided the same level of protection as silymarin. Overall, the antifibrotic effect of green tea at all tested doses was comparable to that of silymarin, which was supported by the liver histopathology evaluations. According to the microscopic examinations, severe hepatic fibrosis induced by CCl₄ was substantially reduced by the administration of green tea extract, in accordance with the results of the oxidative damage analysis and the hydroxyproline assay.

The positive control compound, silymarin, which is an antioxidant flavonoid complex isolated from milk thistle seeds (*Silybum marianum*, Compositae), has been used to treat hepatotoxicity diseases in clinical practice for at least two decades. Silymarin has powerful free-radical scavenging properties and regulates intracellular GSH levels (Hsu et al., 2008). The mechanism by which silymarin protects against CCl₄-induced lipid peroxidation and hepatotoxicity involves decreasing the metabolic activation of CCl₄, acting as a chain-breaking antioxidant for scavenging free radicals or a combination of these effects (Lettéron et al., 1990). A considerable body of experimental work in animal models has demonstrated that silymarin reduces CCl₄-induced hepatotoxic effects by preventing lipid peroxidation (Hsu et al., 2008; Lettéron et al., 1990; Ting et al., 2011). In the present study, silymarin acted as a positive control, as evidenced by its ability to decrease the hepatic TBARS and protein carbonyl contents and increase catalase, GSH-Px and GSH-Rd activities in the liver compared to the CCl₄-treated group. Histopathologic changes in fibrosis also showed that silymarin reduces the incidence of liver lesions induced by CCl₄.

4. Conclusions

The results of this study demonstrated that green tea extract, which contains abundant catechins, can lead to the strong inhibition of CCl₄-induced oxidative damage in mice, as evidenced by its ability to increase the activities of antioxidant enzymes such as catalase, GSH-Px and GSH-Rd and decrease the TBARS and protein carbonyl contents in mouse liver tissue. We also showed that green tea extract protects against CCl₄-induced hepatic fibrosis, results that were supported by hydroxyproline assay and liver histopathologic evaluations. Among test doses of green tea exhibits antioxidant and antifibrotic effects on CCl₄-induced liver damages and the present study suggest that treatment with 125 mg/kg green tea extract exhibited optimal protection on the CCl₄-induced hepatotoxicity and the good antioxidant and antifibrotic effects is comparable with silymarin (200 mg/kg) in this study. Overall, the *in vivo* antioxidant and antifibrotic effects of green tea extract may be due to both the inhibition of lipid peroxidation processes and the induction of antioxidant enzymes activities. Oxidation is

involved in the pathogenesis of many diseases in which treatment with green tea is claimed to be effective. The inhibitory effects of dietary green tea may be useful as a protective agent against toxin-induced oxidative damage and fibrosis *in vivo*.

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References

- Augustyniak, A., Waszkiewicz, E., & Skrzydlewska, E. (2005). Preventive action of green tea from changes in the liver antioxidant abilities of different aged rats intoxicated with ethanol. *Nutrition*, *21*, 925–932.
- Baudrimont, I., Ahouandjivo, R., & Creppy, E. E. (1997). Prevention of lipid peroxidation induced by ochratoxin A in Vero cells in culture by several agents. *Chemico-Biological Interactions*, *104*, 29–40.
- Berlett, B. S., & Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *The Journal of Biological Chemistry*, *272*, 20313–20316.
- Bode, A. M., & Dong, Z. (2009). Epigallocatechin 3-gallate and green tea catechins: united they work, divided they fail. *Cancer Prevention Research*, *2*, 514–517.
- Bondini, S., Kleiner, D. E., Goodman, Z. D., Gramlich, T., & Younossi, Z. M. (2007). Pathologic assessment of non-alcoholic fatty liver disease. *Clinics in Liver Disease*, *11*, 17–23.
- Chen, J. H., Tipoe, G. L., Liong, E. C., So, H. S., Leung, K. M., Tom, W. M., Fung, P. C., & Nanji, A. A. (2004). Green tea polyphenols prevent toxin-induced hepatotoxicity in mice by down-regulating inducible nitric oxide-derived prooxidants. *The American Journal of Clinical Nutrition*, *80*, 742–751.
- Dalle-Donne, L., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta*, *329*, 23–38.
- Frank, J., George, T. W., Lodge, J. K., Rodriguez-Mateos, A. M., Spencer, J. P. E., Minihane, A. M., & Rimbach, G. (2009). Daily consumption of an aqueous green tea extract supplement does not impair liver function or alter cardiovascular disease risk biomarkers in healthy men. *The Journal of Nutrition*, *139*, 58–63.
- Friedman, M. (2007). Overview of antibacterial, antioxidant, antiviral, and antifungal activities of tea flavonoids and teas. *Molecular Nutrition & Food Research*, *51*, 116–134.
- Fu, H., He, J., Mei, F., Zhang, Q., Hara, Y., Ryota, S., Lubet, R. A., Chen, R., Chen, D. R., & You, M. (2009). Lung cancer inhibitory effect of epigallocatechin-3-gallate is dependent on its presence in a complex mixture (polyphenon E). *Cancer Prevention Research*, *2*, 531–537.
- Halliwell, B. (1997). Antioxidants and human disease: A general introduction. *Nutrition Reviews*, *55*, 44–52.
- Higdon, J. V., & Frei, B. (2003). Tea catechins and polyphenols: health effects, metabolism and antioxidant functions. *Critical Reviews in Food Science and Nutrition*, *43*, 89–143.
- Hsu, Y. W., Tsai, C. F., Chang, W. H., Ho, Y. C., Chen, W. K., & Lu, F. J. (2008). Protective effects of *Dunaliella salina* – A carotenoids-rich alga, against carbon tetrachloride-induced hepatotoxicity in mice. *Food and Chemical Toxicology*, *46*, 3311–3317.
- Hsu, Y. W., Tsai, C. F., Chen, W. K., Huang, C. F., & Yen, C. C. (2011). A subacute toxicity evaluation of green tea (*Camellia sinensis*) extract in mice. *Food and Chemical Toxicology*, *49*, 2624–2630.
- Jamall, I. S., Finelli, V. N., & Que Hee, S. S. (1981). A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Analytical Biochemistry*, *112*, 70–75.
- Khan, S. A., Priyamvada, S., Khan, W., Khan, S., Farooq, N., & Yusufi, A. N. K. (2009). Studies on the protective effect of green tea against cisplatin induced nephrotoxicity. *Pharmacological Research*, *60*, 382–391.
- Lettéron, P., Labbe, G., Degott, C., Berson, A., Fromenty, B., Delaforge, M., Larrey, D., & Pessayre, D. (1990). Mechanism for the protective effects of silymarin against carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice. Evidence that silymarin acts both as an inhibitor of metabolic activation and as a chain-breaking antioxidant. *Biochemical Pharmacology*, *39*, 2027–2034.
- Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., & Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*, *186*, 464–478.
- Recknagel, R. O., Glende, E. A., Jr., Dolak, J. A., & Waller, R. L. (1989). Mechanisms of carbon tetrachloride toxicity. *Pharmacology & Therapeutics*, *43*, 139–154.
- Skrzydlewska, E., Augustyniak, A., Michalak, K., & Farbiszewski, R. (2005). Green tea supplementation in rats of different ages mitigates ethanol-induced changes in brain antioxidant abilities. *Alcohol*, *37*, 89–98.
- Ting, H. C., Hsu, Y. W., Tsai, C. F., Lu, F. J., Chou, M. C., & Chen, W. K. (2011). The *in vitro* and *in vivo* antioxidant properties of seabuckthorn (*Hippophae rhamnoides* L.) seed oil. *Food Chemistry*, *125*, 652–659.
- Tsukamoto, H., Matsuoka, M., & French, S. W. (1990). Experimental models of hepatic fibrosis: A review. *Seminars in Liver Disease*, *10*, 56–65.
- Unno, K., Takabayashi, F., Yoshida, H., Choba, D., Fukutomi, R., Kikunaga, N., Kishido, T., Oku, N., & Hoshino, M. (2007). Daily consumption of green tea catechin delays memory regression in aged mice. *Biogerontology*, *8*, 89–95.
- Vaca, C. E., Wilhelm, J., & Harms-Rhdsahl, M. (1988). Interaction of lipid peroxidation product with DNA. A review. *Mutation Research*, *195*, 137–149.

- Vitaglione, P., Morisco, F., Caporaso, N., & Fogliano, V. (2004). Dietary antioxidant compounds and liver health. *Critical Reviews in Food Science and Nutrition*, *44*, 575–586.
- Wang, H., Provan, G. J., & Helliwell, K. (2003). HPLC determination of catechins in tea leaves and tea extracts using relative response factors. *Food Chemistry*, *81*, 307–312.
- Xiao, J., Lu, R., Shen, X., & Wu, M. (2002). Green tea extracts protected against carbon tetrachloride-induced chronic liver damage and cirrhosis. *Zhonghua Yu Fang Yi Xue Za Zhi*, *36*, 243–246 (in Chinese).
- Yasuda, Y., Shimizu, M., Sakai, H., Iwasa, J., Kubota, M., Adachi, S., Osawa, Y., Tsurumi, H., Hara, Y., & Moriwaki, H. (2009). (–)-Epigallocatechin gallate prevents carbon tetrachloride-induced rat hepatic fibrosis by inhibiting the expression of the PDGFR β and IGF-1R. *Chemico-Biological Interactions*, *182*, 159–164.
- Zhen, M. C., Wang, Q., Huang, X. H., Cao, L. Q., Chen, X. L., Sun, K., Liu, Y. J., Li, W., & Zhang, L. J. (2007). Green tea polyphenol epigallocatechin-3-gallate inhibits oxidative damage and preventive effects on carbon tetrachloride-induced hepatic fibrosis. *The Journal of Nutritional Biochemistry*, *18*, 795–805.