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Protective Effects of the Flavonoid-Rich Fraction from Rhizomes of *Smilax glabra* Roxb. on Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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Abstract Hepatoprotective agents could prevent tissue damage and reduce morbidity and mortality rates; such agents may include folkloric or alternative treatments. The present study evaluated the protective effects of the flavonoid-rich fraction from rhizomes of *Smilax glabra* Roxb. (SGF) on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. Sprague-Dawley male rats were orally treated with SGF daily and received CCl_4 intraperitoneally twice a week for 4 weeks. Our results showed that SGF at doses of 100, 300 and 500 mg/kg significantly reduced the elevated activities of serum aminotransferases (ALT and AST), alkaline phosphatase and lactate dehydrogenase and the level of hepatic thiobarbituric acid–reactive substances compared to the CCl_4-treated group. Moreover, SGF treatment was also found to significantly increase the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and glutathione compared with CCl_4-induced intoxicated liver. Histopathologic examination revealed that CCl_4-induced hepatic damage was markedly reversed by SGF. The results suggest that SGF has hepatoprotective and antioxidant properties in CCl_4-induced liver injury in rats.

Keywords *Smilax glabra* Roxb. · Flavonoids · Membrane breakdown · Hepatoprotection · Carbon tetrachloride · Antioxidant

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Introduction

Smilax glabra Roxb. (Liliaceae) is a traditional Chinese herb, referred to as *tufuling* in Chinese medicine (Xia et al. 2010). The rhizome of *S. glabra* has been used in folk medicine for the treatment of brucellosis (Chu and Ng 2006), syphilis (Galhena et al. 2012), acute and chronic nephritis (Chen et al. 2000) and metal poisoning, such as from lead, mercury and cadmium (Ng and Yu 2001; Xia et al. 2010). In many Asian countries *S. glabra* is commonly used clinically to treat liver diseases, and a few studies have indicated that it could inhibit human hepatoma HepG2 and Hep3B cell growth (Thabrew et al. 2005; Sa et al. 2008; Galhena et al. 2012). *S. glabra* extract (SGE) could inhibit HepG2 and Hep3B cell growth by causing cell-cycle arrest at either the S phase or the S/G_2 transition and induce apoptosis, as evidenced by a DNA fragmentation assay (Sa et al. 2008). Moreover, the decoction comprised of *S. glabra* and two other herbs could significantly inhibit the formation of paw edema in rats bearing early hepatocarcinogenic changes (Galhena et al. 2012).

Some researchers have isolated and identified several flavonoids (Chen et al. 1999), phenolics (Ng and Yu 2001) and phenylpropanoid glycosides (Chen et al. 2000) from rhizomes of *S. glabra*. Among these, smitilbin, engeletin,

astilbin, eurryphin and resveratrol could protect against hepatocyte damage from liver nonparenchymal cells through selectively producing dysfunction of nonparenchymal cells with an essential requirement of rhamnose (Chen et al. 1999; Ooi et al. 2004).

Carbon tetrachloride (CCl_4) is a hepatotoxic agent that is widely used to induce liver injury in experimental animals in order to evaluate the antioxidant properties of possible hepatoprotective agents (Pinto et al. 2012). During hepatotoxicity, cytochrome P450 metabolizes CCl_4 to trichloromethyl radical ($^{\bullet}CCl_3$) and trichloromethyl peroxy radical ($^{\bullet}OOCCl_3$) (Szymonik-Lesiuk et al. 2003). These free radicals lead to the peroxidation of fatty acids found in the phospholipids making up the cell membranes. Lipid peroxide radicals, lipid hydroperoxides and lipid breakdown products develop in this process; and each constitutes an active oxidizing agent (Cengiz et al. 2013). Consequently, cell membrane structures and intracellular organelle membrane structures are completely broken down and finally induce corresponding health problems (Preethi and Kuttan 2009).

The general strategy for prevention and treatment of liver damage includes reducing the production of reactive metabolites and inhibiting the generation of free radicals using antioxidants (Bansal et al. 2005). The methanol extract of *S. glabra* rhizomes induced an increase of antioxidant activities in V79-4 cell culture (Ooi et al 2004). In a previous study (Xia et al. 2010), we reported that SGE could significantly increase the glutathione (GSH) content and alanine aminotransferase (ALT), superoxide dismutase (SOD) and catalase (CAT) activities in lead-exposed rats.

Although the inhibitory activity of *S. glabra* on hepatoma cell growth was investigated in vitro (Thabrew et al. 2005; Sa et al. 2008), scientific studies of *S. glabra*'s usefulness with respect to liver injury induced by CCl_4 in rats are lacking. Therefore, the present study evaluated the protective effects of the flavonoid-rich fraction from rhizomes of *S. glabra* Roxb. (SGF) on CCl_4-induced hepatotoxicity in Sprague-Dawley male rats.

Materials and Methods

Plant Material and the Preparation of SGF

The rhizome of *S. glabra* was purchased from a local vendor of Chinese medicinal herbs and identified by the herbalist of Zhejiang Chinese Medical University. A voucher sample was prepared and deposited at the herbarium of Zhejiang Chinese Medical University. SGE was prepared as described previously (Xia et al. 2010). Briefly, the rhizome was air-dried, ground and extracted three times with ethanol/water (60:40, v/v) at 80 °C for 2 h each; then, the extract was filtered through Whatman No. 1 filter paper, and the ethanol from the extract was removed under vacuum. Then, the residue was dissolved in distilled water and further fractionated with *n*-hexane, ethyl acetate and *n*-butanol. Finally, the solvents from the fractionated extracts were removed under vacuum and the residues lyophilized. The dry powder of the ethyl acetate fraction as SGF was chosen for the current study.

Total flavonoid content in SGF was measured as described previously (Xia et al. 2011) and calculated as rutin equivalents (milligrams per gram).

Animals

Sixty male Sprague-Dawley rats (190 ± 10 g) were obtained from SLAC Laboratory Animals (Shanghai, China). Rats were acclimated to the experimental facility for 1 week and housed in stainless steel cages in a room with a 12 h dark/light cycle, an ambient temperature of 23 ± 1 °C and relative humidity of 55 ± 5 %. Rats were allowed standard laboratory food and water (Xia et al. 2010). Our University Animal Care and Use Committee approved the protocols for the animal study, and the animals were cared for in accordance with the ethical guidelines of Zhejiang University.

Experimental Design

Animals were randomly divided into six groups, with each consisting of 10 rats. Group I received only vehicle, olive oil (3 ml/kg) and 20 % DMSO (3 ml/kg). Animals of groups II, III, IV and V received CCl_4 3 ml/kg (30 % in olive oil, v/v) intraperitoneally (ip) twice a week for 4 weeks. Group II was treated with CCl_4 only, while groups III, IV and V were treated with 3 ml/kg of SGF dissolved in 20 % DMSO at dose levels of 100, 300 and 500 mg/kg by oral gavage, respectively, per day for 28 days. Animals of group VI were only given SGF (500 mg/kg) daily by oral gavage. At the end of the experimentation period, 24 h after the last treatment, all animals were anesthetized with CO_2, weighed and killed. Blood samples were collected from all animals from the retro-orbital venous plexus for biochemical variable analysis. Liver samples were dissected out, washed immediately with ice-cold saline to remove as much blood as possible and immediately stored at −70 °C until analysis. An extra sample of liver was excised and fixed in 10 % formalin solution for histopathologic analysis. Sections (5 μm thick) were cut and stained with hematoxylin and eosin for histological examination.

Liver damage was assessed by estimation of serum activities of ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) using commercially available test kits from by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The results were expressed as units per liter.

The liver supernatant was used as a source to assay enzymatic markers of oxidative stress, including SOD, CAT, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-*S*-transferase (GST) activities. We also determined GSH, thiobarbituric acid-reactive substances (TBARS) and the total protein content. GPx, GR and GST were determined using commercially available test kits from by Nanjing Jiancheng Bioengineering Institute.

SOD, CAT, GSH and TBARS were determined according to the methods described by us and others (Xia et al. 2010; Ellman 1959; Marklund and Marklund 1974; Saxena and Flora 2004), which are briefly reviewed below.

The level of GSH in hepatic supernatant was determined according to the method of Ellman (1959). Supernatant (0.02 ml) was added to 9 ml of distilled water. Then, 1 ml of phosphate buffer (pH 8.0) was added. Subsequently, 0.02 ml 5,5′-dithiobis(2-nitrobenzoic acid) was added to 3.0 ml of this solution. The results were expressed as the contents (nanomoles of GSH) per milligram protein.

Hepatic tissue lipid peroxidation was measured by shaking the 2 ml of liver homogenate (5 %, w/v) in 150 mM KCl, 0.025 M Tris–HCl buffer (pH 7.5) for 30 min at 37 °C and measuring the malondialdehyde formed with the thiobarbituric acid reaction. The amount of TBARS was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

SOD activity in the hepatic supernatant was measured using the method described by Marklund and Marklund (1974). The reaction mixture was composed of supernatant with 0.2 M pyrogallol, 1 mM EDTA and 50 mM Tris–HCl (pH 8.2), in a final volume of 1 ml. The results were expressed as units per minute per milligram of protein.

CAT activity in the hepatic supernatant was assayed according to the method described by Aebi (1974). The reaction mixture contained supernatant with 10 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0), in a final volume of 1 ml. The rate of decomposition of H_2O_2 was measured.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. All statistical analyses were performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL). Significant differences among the treatment means were determined using analysis of variance and Duncan's multiple range tests. Results were considered statistically significant at $p < 0.05$.

Table 1 Effect of the flavonoid-rich fraction from rhizomes of *Smilax glabra* (SGF) on liver weights in CCl_4-intoxicated rats

Groups	Relative liver weight (g/100 g)
Normal control	3.73 ± 0.20
CCl_4-treated	5.24 ± 0.29[a,**]
SGF100 + CCl_4	4.51 ± 0.23[b,*]
SGF300 + CCl_4	4.08 ± 0.21[b,**]
SGF500 + CCl_4	3.80 ± 0.20[b,**]
SGF500	3.64 ± 0.18[b,**]

SGF100 SGF 100 mg/kg, oral; *SGF300* SGF 300 mg/kg, oral; *SGF500* SGF 500 mg/kg, oral

* $p < 0.05$, ** $p < 0.01$

[a] Compared to the normal control group

[b] Compared to the CCl_4-treated group

Results

Total flavonoid content was estimated as 547 ± 28.5 mg rutin equivalents/g dry weight of SGF. Therefore, the high content of flavonoids in SGF has strong antioxidant potential to protect the damage in CCl_4-treated rats.

The relative liver weights of each group of rats are shown in Table 1. The results showed a significant increase ($p < 0.05$) of relative weight, by nearly 40 %, for CCl_4-treated rats compared to the normal control group. In contrast, rats that received the indicated dose of SGF showed a significant decrease ($p < 0.05$) in liver weight compared to the CCl_4-treated group.

Results in Table 2 revealed a significant elevation of serum ALT, AST, ALP and LDH activities in CCl_4-treated group compared to normal controls ($p < 0.05$), indicating that CCl_4 induced significant damage to the hepatic cells. Treatment of rats with SGF at 100, 300 and 500 mg/kg markedly reduced ($p < 0.05$) serum ALT, AST, ALP and LDH activities in a dose-dependent manner compared to the CCl_4-treated group. These results suggested the potential of SGF in protecting against liver injury on CCl_4 induction.

The histopathological changes induced by CCl_4 treatment and by SGF are shown in Fig. 1. Compared with the liver tissues of the normal controls, the liver tissue in the CCl_4-treated rats had extensive injuries, characterized by slight to severe necrosis of hepatocytes, cell swelling, disruption of membranes and contraction of the nucleus. Treatment with SGF at 100, 300 and 500 mg/kg ameliorated the CCl_4-

Table 2 Effect of the flavonoid-rich fraction from rhizomes of *Smilax glabra* (SGF) on serum activities of ALT, AST, ALP and LDH in CCl_4-intoxicated rats

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)
Normal control	32.1 ± 1.74	96.4 ± 4.75	103.2 ± 5.01	491.7 ± 26.43
CCl_4-treated	73.1 ± 4.12[a,**]	214.7 ± 11.02[a,**]	242.7 ± 11.64[a,**]	1,187.5 ± 59.26[a,**]
SGF100 + CCl_4	63.5 ± 3.18[b,*]	186.9 ± 9.48[b,*]	212.9 ± 10.33[b,*]	992.7 ± 51.42[b,*]
SGF300 + CCl_4	47.3 ± 2.33[b,**]	148.4 ± 8.12[b,**]	168.5 ± 8.72[b,**]	749.5 ± 35.69[b,**]
SGF500 + CCl_4	34.9 ± 1.85[b,**]	115.9 ± 5.81[b,**]	124.5 ± 6.14[b,**]	553.0 ± 27.64[b,**]
SGF500	31.2 ± 1.62[b,**]	97.2 ± 4.53[b,**]	102.9 ± 5.03[b,**]	490.6 ± 25.11[b,**]

SGF100 SGF 100 mg/kg, oral; *SGF300* SGF 300 mg/kg, oral; *SGF500* SGF 500 mg/kg, oral

* $p < 0.05$, ** $p < 0.01$

[a] Compared to the normal control group

[b] Compared to the CCl_4-treated group

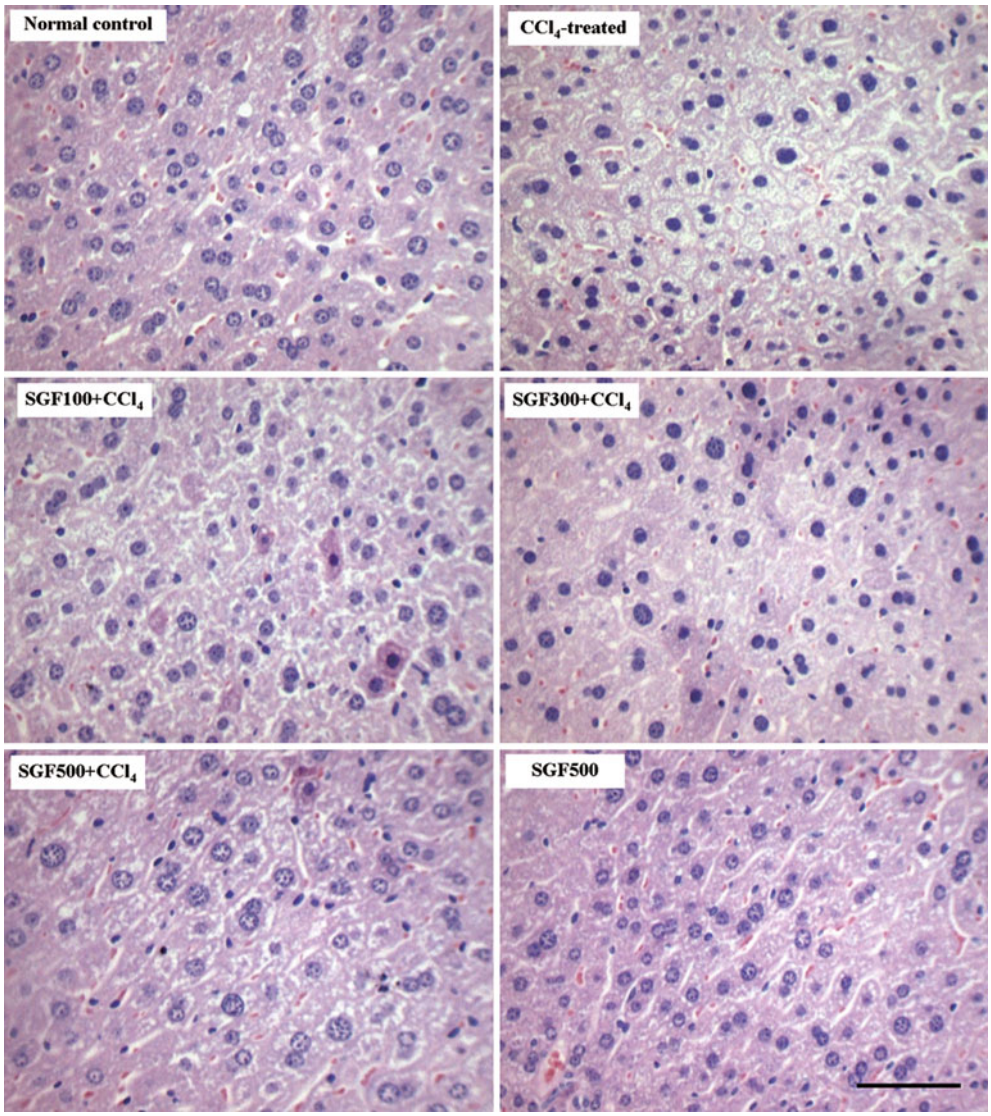

Fig. 1 Effects of the flavonoid-rich fraction from rhizomes of *Smilax glabra* Roxb. (SGF) on liver histopathology stained with hematoxylin and eosin. *Normal control* received only vehicles (olive oil and DMSO); *CCl_4-treated* received CCl_4 3 ml/kg (30 % in olive oil), ip; *SGF100 + CCl_4* received SGF (100 mg/kg) + CCl_4, oral; *SGF300 + CCl_4* received SGF (300 mg/kg) + CCl_4, oral; *SGF500 + CCl_4* received SGF (500 mg/kg) + CCl_4, oral; *SGF500* received SGF (500 mg/kg), oral. Scale bar = 50 μm

induced liver injury and markedly diminished the histological alterations.

GSH and TBARS are widely used as markers of free radical–mediated lipid peroxidation injury. Table 3 shows that CCl_4 treatment induced a significant decrease ($p < 0.05$) in the level of GSH in liver homogenates compared to control livers. Treatment of rats with SGF at 100, 300 and 500 mg/kg significantly increased ($p < 0.05$) the hepatic GSH level in a dose-dependent manner compared with the CCl_4-treated group. Hepatic TBARS content in the CCl_4-treated group was significantly ($p < 0.05$) higher than that in the normal control group. In contrast, rats that received the indicated dose of SGF showed a significant increase ($p < 0.05$) of the level of TBARS compared to the CCl_4-treated group.

Levels of SOD, CAT, GPx, GR and GST activities could be regarded as an index of the antioxidant status of the liver. The hepatic antioxidant enzymes SOD, CAT, GPx, GR and GST, measured in rats with CCl_4-induced liver damage, respectively, showed 46, 38, 45, 45 and 54 % of activity compared with the normal control group (Table 4). There was a significant increase ($p < 0.05$) in the activity of these enzymes in the SGF-treated groups at different doses compared to the CCl_4-treated group.

The nontoxic effect of SGF was also supported by the image in Fig. 1, which was in good correlation with the results of the serum aminotransferases and hepatic antioxidant enzyme activities.

Discussion

The liver is the main organ responsible for metabolism of both endogenous and exogenous compounds; therefore, it is also one of the first target organs for the toxic action of xenobiotics or their reactive metabolites (Szachowicz-Petelska et al. 2012). CCl_4-induced hepatic injury is commonly used as an experimental method to study the hepatoprotective effects of natural products and drugs (Cengiz et al. 2013). Oxidative stress and oxidative damage of cell components caused by CCl_4 are counteracted by compounds that have antioxidant properties. One of such potent antioxidant is *S. glabra*, which is known as an herb to treat various diseases in many Asian countries with a strong in vitro antioxidant capacity (Sa et al. 2008); but its in vivo antioxidant efficacy to CCl_4-treated rats has not yet been investigated.

This study was carried out to evaluate the protective effects of SGF on CCl_4-induced hepatotoxicity in rats. Increases in serum AST, ALT, ALP and LDH levels have been attributed to damaged structural integrity of the liver because these enzymes are released into the circulation after autolytic breakdown or cellular necrosis (Zhang et al. 2009). In the present study, we found that CCl_4 treatment significantly increased the activities of serum AST, ALT, ALP and LDH. Treatment with SGF in different doses significantly inhibited CCl_4-induced liver damage as evidenced by decreased serum aminotransferase, ALP and LDH activities.

The increased formation of reactive oxygen species and decreased antioxidant defense are defined as oxidative stress, which is widely recognized as an important feature of many diseases (Aydin et al. 2012). The antioxidant defense systems exist to prevent the formation of these increased reactive and free radicals. These include SOD-, CAT- and GSH-related enzymes (GPx, GR and GST). SOD is an exceedingly effective defense enzyme that converts the dismutation of superoxide anions into hydrogen peroxide (H_2O_2) (Reiter et al. 2000). CAT is mainly a heme-containing enzyme. The predominant subcellular localization of enzyme is in the peroxisomes, in which it catalyzes the dismutation of hydrogen peroxide to water and molecular oxygen (Aydin et al. 2012). GPx plays an important role in the detoxification of xenobiotics in the liver and catalyzes the reduction of H_2O_2 and hydroperoxides to nontoxic products (Hsu et al. 2008). GR is a cytosolic hepatic enzyme involved in the detoxification of a range of xenobiotic compounds by their conjugation with GSH (Naik and Panda 2007). GSTs catalyze the conjugation of GSH to a variety of compounds containing an electrophilic center and have been found in all tissues and organisms examined to date (Leaver and George 1998). In our study, the activity of antioxidant enzymes, such as SOD, CAT, GPx, GR and GST, significantly decreased in liver tissue of CCl_4-treated rats. However, administration of SGF significantly decreased the toxicity of CCl_4 and increased the activities of these antioxidant enzymes.

Table 3 Effect of the flavonoid-rich fraction from rhizomes of *Smilax glabra* (SGF) on hepatic GSH and TBARS concentrations in CCl_4-intoxicated rats

Groups	GSH (nmol/mg protein)	TBARS (nmol/g tissue)
Normal control	19.8 ± 1.45	168.4 ± 12.75
CCl_4-treated	9.1 ± 0.67[a,**]	317.6 ± 20.25[a,**]
SGF100 + CCl_4	12.2 ± 0.91[b,**]	275.6 ± 18.48[b,*]
SGF300 + CCl_4	16.4 ± 1.22[b,**]	222.9 ± 15.15[b,**]
SGF500 + CCl_4	19.3 ± 1.48[b,**]	186.3 ± 11.98[b,**]
SGF500	20.2 ± 1.51[b,**]	167.6 ± 9.58[b,**]

SGF100 SGF 100 mg/kg, oral; *SGF300* SGF 300 mg/kg, oral; *SGF500* SGF 500 mg/kg, oral

* $p < 0.05$, ** $p < 0.01$

[a] Compared to the normal control group

[b] Compared to the CCl_4-treated group

Table 4 Effect of the flavonoid-rich fraction from rhizomes of *Smilax glabra* (SGF) on hepatic antioxidant enzymes activity in CCl_4-intoxicated rats

Groups	SOD (U/min/mg protein)	CAT (μmol H_2O_2 consumed/min/mg protein)	GPx (U/mg protein)	GST (U/mg protein)	GR (U/g protein)
Normal control	19.8 ± 1.44	57.2 ± 3.63	120.1 ± 7.44	18.1 ± 1.28	3.79 ± 0.28
CCl_4-treated	10.6 ± 0.77[a,**]	35.4 ± 2.09[a,**]	65.2 ± 4.13[a,**]	9.8 ± 0.73[a,**]	1.71 ± 0.12[a,**]
SGF100 + CCl_4	13.9 ± 1.10[b,**]	40.5 ± 2.42[b,*]	77.5 ± 6.21[b,**]	10.7 ± 0.64	2.50 ± 0.15[b,**]
SGF300 + CCl_4	15.8 ± 1.25[b,**]	45.3 ± 2.98[b,**]	100.1 ± 7.07[b,**]	14.2 ± 1.06[b,**]	3.21 ± 0.22[b,**]
SGF500 + CCl_4	18.0 ± 1.31[b,**]	50.2 ± 3.24[b,**]	111.6 ± 7.13[b,**]	16.2 ± 1.21[b,**]	3.53 ± 0.24[b,**]
SGF500	19.6 ± 1.45[b,**]	57.5 ± 3.68[b,**]	121.5 ± 7.68[b,**]	18.0 ± 1.26[b,**]	3.81 ± 0.29[b,**]

SGF100 SGF 100 mg/kg, oral; *SGF300* SGF 300 mg/kg, oral; *SGF500* SGF 500 mg/kg, oral

* $p < 0.05$, ** $p < 0.01$

[a] Compared to the normal control group

[b] Compared to the CCl_4-treated group

In addition, treatment with SGF significantly elevated the GSH content in the liver of rats, suggesting that SGF could protect against the CCl_4-induced depletion of hepatic GSH. Moreover, the significant increase in the hepatic GR activity and GSH content confirm that treatment with SGF could effectively protect against the hepatic oxidative damage by GSH regenerated from glutathione disulfide (Pinto et al. 2012).

TBARS are major reactive aldehydes resulting from the peroxidation of polyunsaturated fatty acids. They are useful indicators of tissue damage, including a series of chain reactions (Khan et al. 2012). *S. glabra* is rich in flavonoids and phenolic compounds, providing protection from lipid peroxidation. Flavonoids and phenolics have high antioxidant capacity and have been shown to be effective antioxidants in inhibiting lipid peroxidation as well as potent radical scavengers (Xia et al. 2010). In this study, CCl_4-induced toxicity caused an increase of TBARS levels in the liver tissue compared to the normal control group. Treatment with SGF could reverse these changes and caused a significant decrease in TBARS levels compared to the CCl_4-induced hepatic toxicity in rats.

Histopathologic analysis in this study revealed that CCl_4-induced hepatic damage was markedly reversed by SGF. These data are in good agreement with the results for the activities of the serum aminotransferases ALP and LDH as well as that of hepatic antioxidant enzymes.

In conclusion, our results provide evidence for the effectiveness of SGF in prevention of CCl_4-induced oxidative stress and hepatic damage. This indicates the possibility of the use of this natural antioxidant in preventing disorders initiated by oxidative stress. Furthermore, SGF may be useful as a hepatoprotective agent against chemical-induced hepatotoxicity in vivo.

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