

Hepatoprotection of *Chlorella* against Carbon Tetrachloride-induced Oxidative Damage in Rats

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Abstract. The effects of 80% ethanolic *Chlorella* extracts (GPE) on carbon tetrachloride (CCl₄)-induced hepatic damage were investigated in Sprague-Dawley rats. The rats were orally treated with GPE (0.5 g/kg body weight) or silymarin (0.2 g/kg body weight) over four consecutive weeks with administration of CCl₄ (20% CCl₄, 0.5 ml/rat twice a week). The GPE had a significant protective effect against liver injuries, as well as oxidative stress induced by CCl₄, resulting in reduced lipid peroxidation and improved serum biochemical parameters such as aspartate aminotransferase and alanine aminotransferase. The reduced levels of glutathione, vitamin C, superoxide dismutase, and catalase in the CCl₄-treated rats were significantly increased by treatment with GPE. Furthermore, the activity of GPE was comparable to the standard drug silymarin. In conclusion, *Chlorella* may be useful as a hepatoprotective agent against chemical-induced liver damage in vivo.

Chlorella, unicellular green algae, has been found to contain highly nutritious substances and to exert various biological effects. Glycoproteins derived from *Chlorella vulgaris* have exhibited antitumor (1) and immunomodulatory effects (2-3). Sulaiman *et al.* have shown that *C. vulgaris* has a chemopreventive effect on liver cancer induced by ethionine in rats (4). Several reports have indicated that the bioactive component polysaccharides from *C. pyrenoidosa* were responsible for its great potential antitumor and immunomodulatory activities (5-8). Cherng and Shih found that the administration of *C. pyrenoidosa* was able to prevent dyslipidemia in rats and hamsters after a chronic high fat diet

treatment (9). Furthermore, *Chlorella* is also widely applied as an important ingredient within the food industry (10-12). In recent years, studies on the antioxidant abilities of *Chlorella* have been of interest to many researchers. Increasing evidence indicates that free radical damage is related to carcinogenesis, mutagenesis, aging and atherosclerosis (13-14). Research data have also revealed that oxidative stress such as hydrogen peroxide (H₂O₂), free radical formation and lipid peroxidation, is associated with changes in antioxidant level and mild oxidative stress may also induce the regulation of antioxidative enzymes (15). Antioxidants, such as glutathione (GSH), vitamin C and vitamin E, and antioxidative enzymes such as superoxide dismutases (SODs), catalase and glutathione peroxidases (GPXs) play protective roles when counteracting oxidative stress (16).

The industrial solvent carbon tetrachloride (CCl₄) is a potent environmental hepatotoxin. It is widely used to induce oxidative stress and liver damage in animals. The liver injury induced by CCl₄ results from free radicals and lipid peroxidation that cause hepatic cell damage. CCl₄ can be metabolized to free radical intermediates by cytochrome P450 in the hepatocytes; these free radical intermediates then can initiate lipid peroxidation to affect cell integrity and, finally, result in cell damage due to oxidative stress (17-20). Therefore, the main objective of this study was to investigate the antioxidative activity and potential protective effects of *Chlorella* on CCl₄-induced oxidative stress in rats. The protective activity of *Chlorella* was also compared with silymarin, which has been used for over 20 years in clinical practice for the treatment of toxic liver disease (21).

Materials and Methods

Materials. Ascorbic acid, bovine serum albumin (BSA), cumene hydroperoxide, α,α -diphenyl- β -picrylhydrazyl (DPPH), hydrogen peroxide (H₂O₂), GSH, glutathione reductase, pyrogallol, reduced β -nicotinamide adenine dinucleotide phosphate (β -NADPH), thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), meta-phosphoric acid (MPA),

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cysteine, butylated hydroxytoluene (BHT), methanol, vitamin E, dithiothriol (DTT), monobromobimane (MbBr), silymarin and Triton® X-100 were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The bicinchonic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL, USA). All reagents used were of analytical grade or purer.

Preparation of 80% ethanolic extract of chlorella (GPE). Dried, powdered chlorella (*C. sorokiniana*) was purchased from the Taiwan Chlorella Manufacturing Co. Ltd. (Taipei, Taiwan, ROC). Chlorella was extracted with 80% ethanol (50-fold) by stirring for 2 h. After filtering, the extracting solvent was evaporated at 40°C by a vacuum condenser and the remaining residue was dried by a vacuum freeze-dryer. The yield of the dried GPE was 14.0%. The extract was sealed in plastic bottles and stored at -70°C.

Measurement of the total phenolic content and DPPH radical-scavenging activity. The total phenol content was analyzed using Folin-Ciocalteu's reagent (22). Gallic acid was used as the standard for the calibration curve, and the total phenolic contents were expressed as mg gallic acid equivalents per gram of tested extracts. The DPPH radical-scavenging activity of the extract was measured according to the method of Chung *et al.* (23). The capability to scavenge DPPH radical was calculated by the following equation: scavenging effect (%) = $(1 - [\text{absorbance of sample at 517 nm} / \text{absorbance of control at 517 nm}]) \times 100\%$.

Treatment of animals. Fifty male weanling Sprague-Dawley rats were obtained and fed commercial chow diets (Fwusow Industry Co., LTD, Taiwan). They were randomly divided into five groups with ten animals in each group (A: control, B: GPE, C: CCl₄, D: GPE+CCl₄ and E: silymarin+CCl₄). All the animals were maintained in a controlled environment (12 h light/dark cycles at 22±2°C and 50±10% humidity). Food and tap water were supplied *ad libitum*. The body weight and food intake of each rat was recorded daily throughout the experiment. The control group (A) was gavaged with 0.5 ml of normal saline, 0.9% (w/v) NaCl, daily over four consecutive weeks. The GPE group (B) was gavaged with GPE, dissolved in 0.5 ml normal saline, at a dose of 0.5 g/kg body weight/day over four consecutive weeks. The CCl₄ group (C) was gavaged with 0.5 ml 20% CCl₄ in olive oil/rat, on Mondays and Thursdays and also administered 0.5 ml normal saline daily over four consecutive weeks. The GPE+CCl₄ group (D) and silymarin+CCl₄ group (E) were gavaged with GPE (0.5 g/kg body weight) or silymarin (0.2 g/kg body weight) daily over four consecutive weeks and received CCl₄ in the same manner as the C group. The A and B groups, rats not administered CCl₄, received 0.5 ml olive oil at the same time points.

Tissue sampling and preparation. After four weeks of feeding, food was withheld overnight and the rats were decapitated the next morning. The blood was collected directly into sterile tubes. The serum was prepared by centrifugation at 805 ×g for 10 min at 4°C and analyzed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The livers were excised, weighed and immediately homogenized in an ice-cold 0.05 mole/l phosphate buffer (pH 7.4) using a Potter-Elvehjem-type homogenizer with a teflon pestle. One portion of this tissue homogenate (0.3 g/ml) was used for assaying the levels of vitamin C, vitamin E and reduced GSH, while the remaining portion was centrifuged at 12,000 ×g, 4°C for 10 min (Universal 16R freeze Centrifuge; Hettich,

Tuttlingen, Germany). The resulting supernatant was used to determine activities of SOD and GPX, protein contents and lipid peroxidation. This animal research and all the procedures were reviewed and approved by the Animal Research Ethics Committee at Providence University, Taichung, Taiwan.

Determination of biochemical markers of hepatic injury. The levels of AST and ALT in the serum samples were determined by enzymatic methods using an automatic analyzer (Synchron CX-7 Systems; Beckman, Fullerton, CA, USA) at a commercial analytical service center (Lian-Ming Co., Taiwan, ROC).

Lipid peroxidation measurement. Lipid peroxides are unstable and decompose to form MDA, thus MDA levels are widely used as an indicator of lipid peroxidation. The thiobarbituric acid-reactive substances (TBARS) method was used to estimate tissue MDA level spectrophotometrically at 535 nm (24). TEP was used as a standard and the results were calculated as nanomoles of MDA per mg of tissue protein.

Antioxidants measurement. The liver vitamin C content was stabilized by MPA and cysteine solution, then determined by high-performance liquid chromatography (HPLC) through a 5 µm Econosphere C-18 reversed-phase cartridge column (Merck, Darmstadt, Germany) with an electrochemical detector using a BSA PM-80 isocratic pump (Bioanalytical System, West Lafayette, USA) (25). The eluant was 40 mM sodium acetate/EDTA solution at a flow rate of 0.6 ml/min.

The vitamin E (α-tocopherol) standard and the tissue cytosols were diluted in 0.25% BHT/0.2% ascorbate/methanol solution prior to HPLC analysis. The tissue vitamin E and GSH contents were measured by HPLC through a 5 µm Econosphere C-18 reversed-phase cartridge column (Merck) with a fluorimetric detector using the Hewlett Packard 1100 Series System (Agilent Technologies, Waldbronn, Germany) (26-27). For vitamin E separation, 10 mM sodium acetate/methanol (A solution) and 75% methanol (B solution) were used as the eluants at a flow rate of 1.2 ml/min. Thirty microliters of sample mixture were injected into the C-18 column. To achieve the separation, the gradient used was 100% A solution for 0-10 min, 100% B solution for 10-15 min and 100% A solution for 15-19 min. The tissue GSH was reduced by DTT/ phosphate solution and the MbBr derivative produced prior to HPLC analysis. A 30 mM TBA/ methanol solution was used as the eluant at a flow rate of 1 ml/min.

Determination of the antioxidative enzyme activities. GPX activity was determined spectrophotometrically at 340 nm by an enzyme-coupled method with glutathione reductase, utilizing cumene hydroperoxide as substrate at 30°C (28). The rate of decrease in the NADPH concentration was observed at 340 nm over a 3 min period at 30 s intervals. One unit of GPX activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol of NADPH/min/ml.

SOD activity was determined spectrophotometrically at 325 nm based on the SOD-mediated decrease in the rate of pyrogallol autoxidation under alkali conditions (29). One unit of SOD activity was defined as half the rate of reduction of pyrogallol autoxidation over a 1 min period at 15 s intervals.

Catalase activity was determined spectrophotometrically utilizing H₂O₂ as substrate (30). The rate of H₂O₂ dismutated to H₂O and

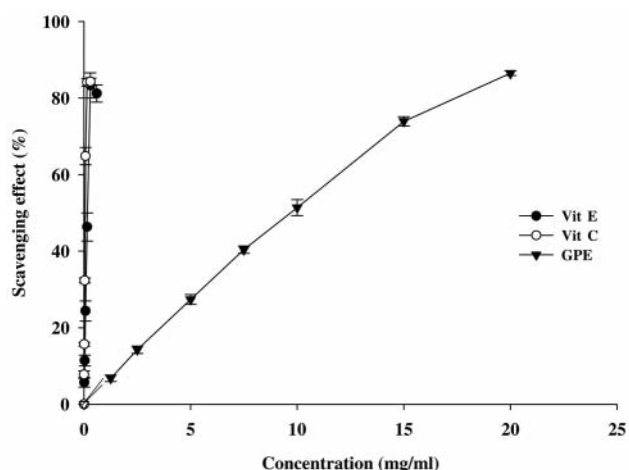


Figure 1. Antioxidant activity of GPE determined by the DPPH method. Each value represents the mean±S.D (n=3).

O₂ is proportional to the catalase activity. The decrease in the amount of H₂O₂ was observed at 240 nm over a 1 min period at 15 s intervals. One unit of catalase activity was defined as 1 mmol H₂O₂ remaining per minute. The protein content of the tissue cytosols was determined based on the Biuret reaction (31) of a BCA kit using BSA as standard. The specific activity of the enzyme was expressed as unit/mg protein.

Statistical analysis. All the results except those for AST and ALT activities, body weight and daily food intake were normalized to cellular protein content. The mean values were compared by analysis of variance (ANOVA) with the Scheffe method for comparing groups (32). A significance level of 5% was adopted for all the comparisons.

Results

Total phenolic content and scavenging effect on DPPH radical. The total phenolic content of GPE was 26.67±0.92 mg gallic acid equivalent/g. Figure 1 shows the dose-response curve of the DPPH radical-scavenging activity of GPE, which increased with the concentration. The half-inhibition concentration (IC₅₀) for the DPPH radical-scavenging activity of GPE, vitamin E and vitamin C was 9.95±0.53 mg/ml, 0.18±0.02 mg/ml and 0.05±0.00 mg/ml, respectively. To obtain 80% DPPH scavenging activity, the concentrations of GPE, vitamin E and vitamin C needed were 17.08, 0.29 and 0.14 mg/ml respectively. In other words, to reach a similar extent of DPPH scavenging effect, the concentration required for chlorella extracts was considerably higher than that required for vitamin E or vitamin C.

Effects of GPE on the body weight gain, food efficiency and relative liver weight. Table I shows the daily body weight

Table I. Effect of GPE on daily body weight gain and feed efficiency in rats treated with CCl₄.

Group	Daily body weight gain (g/day/rat)	Feed efficiency (g gain/g feed)
A	5.43±0.41	0.22±0.03
B	5.72±0.66	0.23±0.02
C	2.03±0.29*	0.10±0.02*
D	2.40±0.37*.#	0.13±0.02*.#
E	2.79±0.49*.#	0.12±0.01*

The data are the mean±S.D. from ten rats. A: Control, B: GPE, C: CCl₄, D: CCl₄ + GPE, E: CCl₄ + silymarin. *Significantly different from A group, *p*<0.05. #Significantly different from C group, *p*<0.05.

Table II. Effect of GPE on relative weight and protein content of liver in rats treated with CCl₄.

Group	Relative weight (g/100 g body weight)	Protein content (mg/g liver)
A	2.88±0.13	162.94±4.46
B	3.00±0.15	164.37±5.60
C	3.84±0.15*	138.88±6.10*
D	3.41±0.27*.#	147.78±7.40*.#
E	3.44±0.30*.#	147.70±3.06*.#

The data are the mean±S.D. from ten rats. A: Control, B: GPE, C: CCl₄, D: CCl₄ + GPE, E: CCl₄ + silymarin. *Significantly different from group A, *p*<0.05. #significantly different from group C, *p*<0.05.

gain and feed efficiency of the rats in each group. There was a significant decrease (*p*<0.05) in the daily body weight gain and feed efficiency of the CCl₄-treated rats when compared with the control group. The administration of GPE (0.5 g/kg body weight) or silymarin (0.2 g/kg body weight) over four weeks significantly reversed the CCl₄ effects, inducing body weight gain and improved feed efficiency.

Table II shows the relative weight and total protein content of the livers of the rats in each group. There was a significant increase (*p*<0.05) in the relative weights and a significant decrease (*p*<0.05) in the total protein contents of the livers of the CCl₄-treated rats when compared with those of the control group. The administration of GPE or silymarin over four consecutive weeks significantly reduced the relative liver weight and increased the total protein levels in the liver when compared with the rat group that received CCl₄ only. However the administration of GPE or silymarin with CCl₄ resulted in lower total protein and higher relative weights of the liver when compared with the control (*p*<0.05). Feeding of GPE alone did not affect the daily body weight gain, feed efficiency, relative weight or total protein content of the liver during the experimental period.

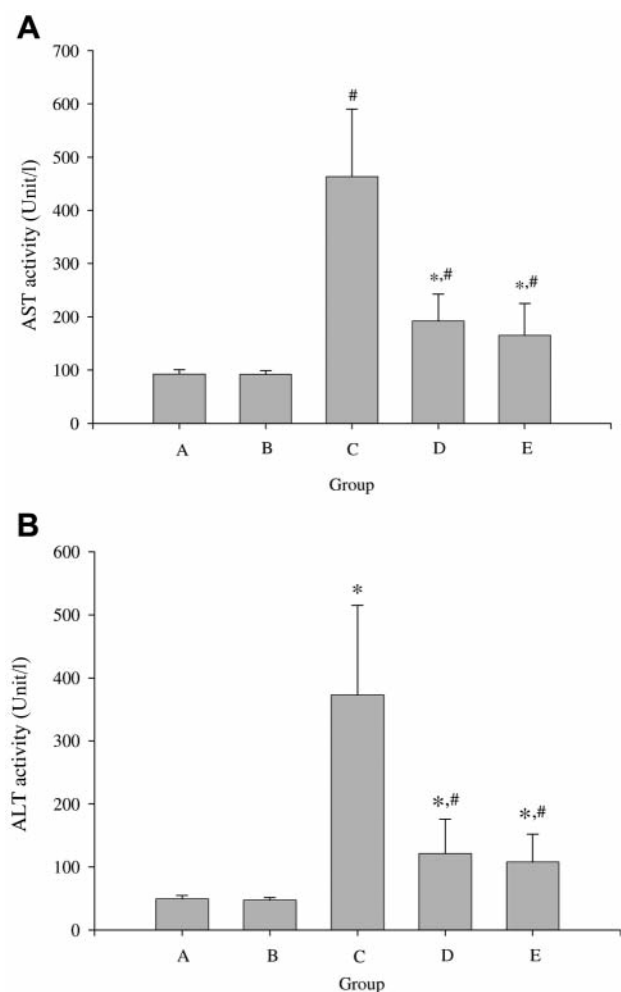


Figure 2. Effect of GPE over four consecutive weeks on serum AST (A) and ALT (B) activity in rats treated with CCl_4 . The data are the mean \pm S.D. from ten rats. A: Control, B: GPE, C: CCl_4 , D: CCl_4 + GPE, E: CCl_4 + silymarin. *Significantly different from group A, $p < 0.05$; #significantly different from group C, $p < 0.05$.

Effect of GPE on CCl_4 -induced liver toxicity. The treatment with CCl_4 significantly ($p < 0.05$) elevated the serum AST and ALT activities compared with the control group (Figure 2). GPE or silymarin administration with the CCl_4 treatment significantly ($p < 0.05$) lowered the serum AST and ALT activities as compared with the CCl_4 treatment group, although the AST and ALT activities were still higher compared with the control ($p < 0.05$). GPE treatment alone did not affect ($p > 0.05$) the serum AST and ALT activities.

Effect of GPE on CCl_4 -induced lipid peroxidation levels. Lipid peroxidation levels in the liver are expressed as MDA content in Figure 3. The treatment with CCl_4 significantly ($p < 0.05$) elevated the liver MDA levels compared with the control group. GPE or silymarin administration with CCl_4

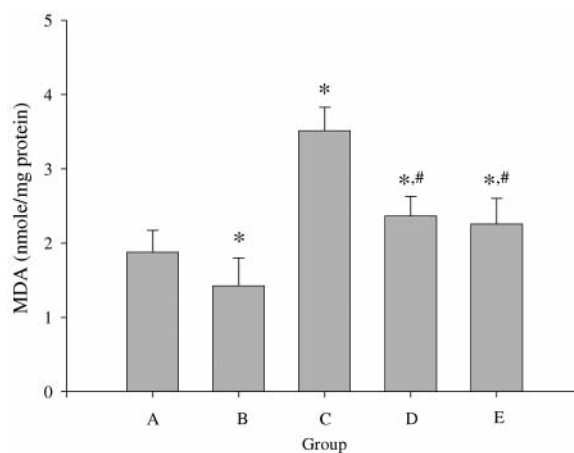


Figure 3. Effect of GPE over four consecutive weeks on lipid peroxidation in the liver of rats treated with CCl_4 . The data are the mean \pm S.D. from ten rats. A: Control, B: GPE, C: CCl_4 , D: CCl_4 + GPE, E: CCl_4 + silymarin. *Significantly different from group A, $p < 0.05$; #significantly different from group C, $p < 0.05$.

treatment significantly ($p < 0.05$) lowered the MDA levels as compared with the CCl_4 treatment group, while the MDA levels were still higher compared with the control ($p < 0.05$). GPE treatment alone resulted in significantly ($p < 0.05$) lower MDA levels in the liver tissues than in the control group.

Effect of GPE on antioxidant content in CCl_4 -induced oxidative stress. Figure 4 shows the hepatic vitamin C, vitamin E and GSH levels of the rats in each group. There was a significant decrease ($p < 0.05$) in the hepatic vitamin C and GSH levels of the CCl_4 -treated rats when compared with those of the control group. GPE or silymarin administration with CCl_4 treatment significantly ($p < 0.05$) increased the hepatic vitamin C and GSH levels as compared with the CCl_4 treatment group. GPE or silymarin administration with the CCl_4 treatment significantly ($p < 0.05$) increased the hepatic GSH levels as compared with the control group. However, no difference ($p > 0.05$) in hepatic vitamin E level was found between any groups. Nor did GPE treatment alone affect ($p > 0.05$) the hepatic antioxidant levels during the experimental period.

Effect of GPE on antioxidative enzyme activities in CCl_4 -induced oxidative stress. As shown in Figure 5, the administration of CCl_4 caused a significant ($p < 0.05$) decrease of hepatic SOD and catalase activities in the rats when compared with those of the control group. GPE or silymarin administration with CCl_4 treatment significantly ($p < 0.05$) increased the hepatic SOD and catalase activities compared with the CCl_4 treatment group. Both GPE and

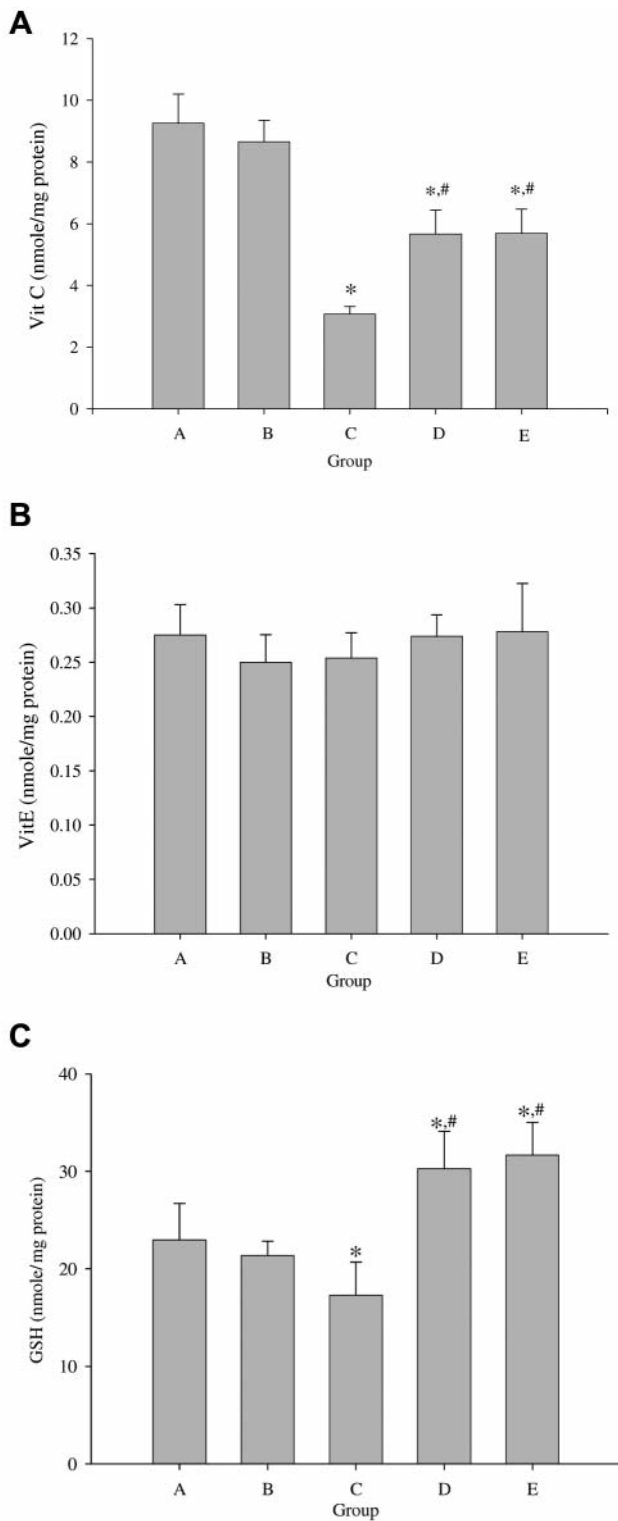


Figure 4. Effect of GPE over four consecutive weeks on vitamin C (A), vitamin E (B) and GSH (C) levels in the liver of rats treated with CCl₄. The data are the mean±S.D. from ten rats. A: Control, B: GPE, C: CCl₄, D: CCl₄ + GPE, E: CCl₄ + silymarin. *Significantly different from group A, $p < 0.05$; #Significantly different from group C, $p < 0.05$.

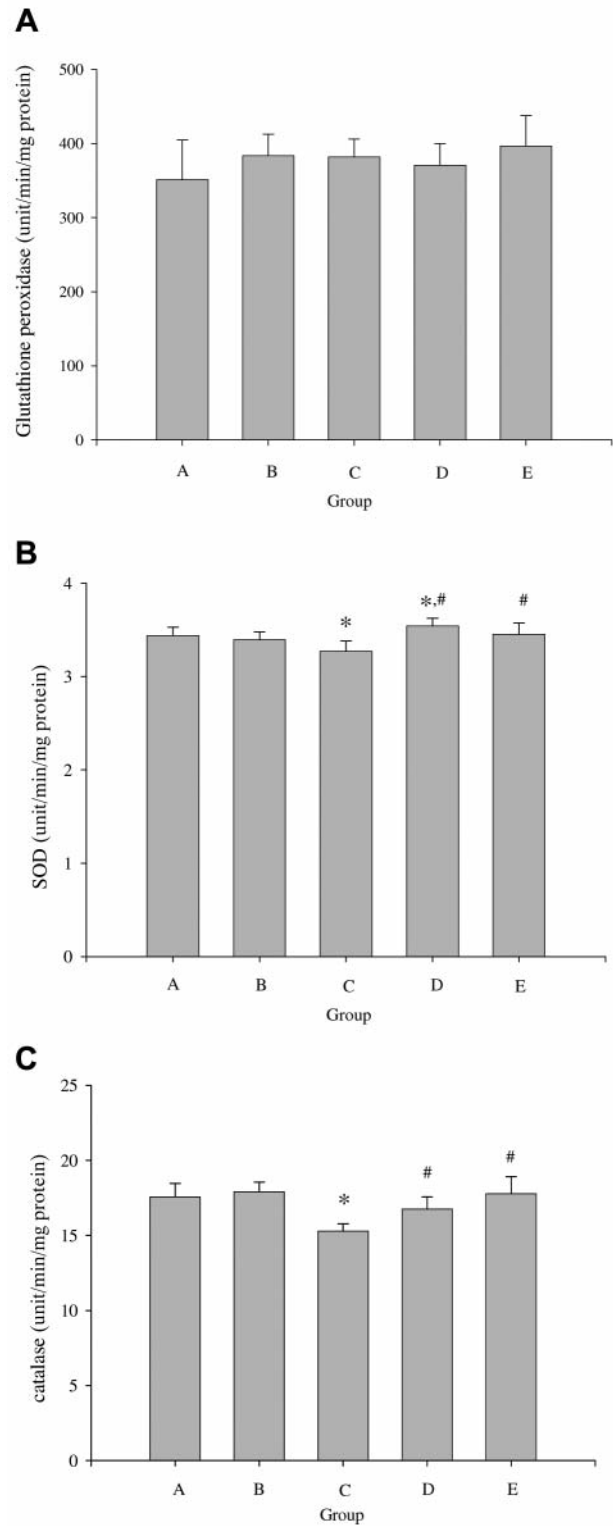


Figure 5. Effect of GPE over four consecutive weeks on glutathione peroxidase (A), SOD (B) and catalase (C) activity in the liver of rats treated with CCl₄. The data are the mean±S.D. from ten rats. A: Control, B: GPE, C: CCl₄, D: CCl₄ + GPE, E: CCl₄ + silymarin. *Significantly different from group A, $p < 0.05$; #Significantly different from group C, $p < 0.05$.

silymarin almost completely restored hepatic catalase activity to a normal level. Furthermore GPE or silymarin administration with CCl₄ treatment significantly ($p < 0.05$) increased the hepatic SOD activities as compared with the control group. However, no difference ($p > 0.05$) in hepatic GPX activities was found between any groups, nor did GPE treatment alone affect ($p > 0.05$) the hepatic antioxidative enzyme activity during the experimental period.

Discussion

Among therapeutics for liver diseases, protective drugs such as antioxidants have attracted more and more attention and proton radical-scavenging action is well known as an important mechanism of antioxidation. In this study, the *in vitro* analysis demonstrated an IC₅₀ for DPPH radical-scavenging activity of GPE was 9.95±0.53 mg/ml. Although the DPPH radical-scavenging ability of GPE was significantly less than that of vitamin E or vitamin C, it was evident that the extracts did show hydrogen-donating ability and could serve as free radical inhibitors or scavengers, possibly acting as primary antioxidants. Polyphenolic compounds such as flavonoids and anthocyanins are widely distributed in the human diet through vegetables, fruits, beans, cereals, tea, coffee, natural herbs and spice extracts, and they have been found to possess significant antioxidant activities (33-34) that are associated with a lower incidence and lower mortality rates from certain human diseases (35). In this study, the total phenolic compounds of GPE were 26.67±0.92 mg gallic acid equivalent/g and they may have contributed, in a substantial part at least, to the radical-scavenging activities of GPE.

The liver appears to be the primary organ for lipid peroxidation induced by CCl₄ treatment and the hepatic enzymes AST and ALT were used as biochemical markers to indicate early hepatic injury. The GPE and silymarin treatments had a markedly protective effect against CCl₄-induced hepatotoxicity in the rats, as evidenced by decreased serum AST and ALT activities and increased total protein concentration in the CCl₄-treated animals (Table II and Figure 2). The GPE treatment alone had no significant undesired actions on hepatocellular integrity.

The extent of lipid peroxidation, measured in terms of MDA, provides a good index of cell destruction because cells and tissues damaged by any mechanism tend to peroxidize more rapidly than normal cells. Therefore, the concentration of MDA may reflect the degree of oxidative stress (36). Tissue lipid peroxide levels were found to be significantly elevated in CCl₄-challenged rats (20, 37-38). In the present study, the administration of CCl₄ significantly induced while the concomitant administration of GPE or silymarin significantly reduced the MDA over-production in the liver tissue (Figure 3). The diminished MDA after co-treatment with the GPE and CCl₄ may be attributed to the antioxidant

activity of GPE by scavenging the radicals generated due to the metabolic transformation of CCl₄ in the liver.

GSH is an important antioxidant in living organisms and exerts its protective effect in counteracting oxidative stress (39). In the present study, the decrease in the liver GSH and vitamin C levels induced by CCl₄ indicated that the antioxidative status of the liver was reduced (Figure 3). Moreover, the CCl₄-induced decrease in liver SOD activity may have reduced the dismutation of toxic superoxide anion radicals to less toxic H₂O₂ and resulting in increased hydroxyl radicals, producing more lipid radicals from unsaturated fatty acids to be oxidized to lipid peroxyl radicals (40). Furthermore, catalase activity was significantly reduced after CCl₄ treatment when compared to the control. Catalase plays a pivotal role in H₂O₂ catabolism and reduced catalase activity is linked to exhaustion of the enzyme as a result of oxidative stress caused by CCl₄. The maintenance of the GSH level and antioxidative status is crucial for cell viability and animal survival (41). The GPE administration exerted a protective effect by preventing the reduction of the levels of GSH and vitamin C and activities of SOD and catalase, as well as preventing the accumulation of lipid peroxides induced by CCl₄ in liver. The results indicated that GPE had an impact on improving hepatoprotection not only through its own radical scavenging activity, but also by boosting the host antioxidant status.

In conclusion, GPE has a significant protective effect in oxidative stress induced by CCl₄, resulting in reduced lipid peroxidation and improved serum biochemical parameters such as AST and ALT, as well as GSH, vitamin C, SOD, and catalase. Furthermore, the activity of GPE at a dose of 0.5 g/kg body weight was comparable to that of the standard drug silymarin (0.2 g/kg BW). Therefore, chlorella may be useful as a hepatoprotective agent against chemical-induced liver damage *in vivo*.

Acknowledgements

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References

- 1 Tanka K, Yamada A, Noda K, Hasegawa T, Okuda M and Shoyama Y: A novel glycoprotein obtained from *Chlorella vulgaris* strain CK22 shows antimetastatic immunopotential. *Cancer Immunol Immunother* 45: 313-320, 1998.
- 2 Hasegawa T, Kimura Y, Hiromatsu K, Kobayashi N, Yamada A, Makino M, Okuda M, Sano T, Nomoto K and Yoshikai Y: Effect of hot water extract of *Chlorella vulgaris* on cytokine expression patterns in mice with murine acquired immunodeficiency syndrome after infection with *Listeria monocytogenes*. *Immunopharmacology* 35: 273-282, 1997.

- 3 Hasegawa T, Noda K, Kumamoto S, Ando Y, Yamada A and Yoshikai Y: *Chlorella vulgaris* culture supernatant (CVS) reduces psychological stress-induced apoptosis in thymocytes of mice. *Int J Immunopharmacol* 22: 877-885, 2000.
- 4 Sulaiman S, Shamaan NA, Ngah WZW and Yusof YAM: Chemopreventive effect of *Chlorella vulgaris* in choline deficient diet and ethionine induced liver carcinogenesis in rats. *Int J Cancer Res* 2: 234-241, 2006.
- 5 Miyazawa Y, Murayama T, Ooya N, Wang LF, Tung YC and Yamaguchi N: Immunomodulation by unicellular green algae (*Chlorella pyrenoidosa*) in tumor-bearing mice. *J Ethnopharmacol* 24: 135-146, 1988.
- 6 Yang F, Shi Y, Sheng JC and Hu QH: *In vivo* immunomodulatory activity of polysaccharides derived from *Chlorella pyrenoidosa*. *Eur Food Res Technol* 224: 225-228, 2006.
- 7 Shi Y, Sheng J, Yang F and Hu Q: Purification and identification of polysaccharide derived from *Chlorella pyrenoidosa*. *Food Chem* 103: 101-105, 2007.
- 8 Sheng J, Yu F, Xin Z, Zhao L, Zhu X and Hu Q: Preparation, identification and their antitumor activities *in vitro* of polysaccharides from *Chlorella pyrenoidosa*. *Food Chem* 105: 533-539, 2007.
- 9 Cherng JY and Shih MF: Preventing dyslipidemia by *Chlorella pyrenoidosa* in rats and hamsters after chronic high-fat diet treatment. *Life Sci* 76: 3001-3013, 2005.
- 10 Gouveia L, Raymundo A, Batista A, Sousa I and Empis J: *Chlorella vulgaris* and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions. *Eur Food Res Technol* 222: 362-367, 2006.
- 11 Raymundo A, Gouveia L, Batista AP, Empis J and Sousa I: Fat mimetic capacity of *Chlorella vulgaris* biomass in oil-in-water food emulsions stabilized by pea protein. *Food Res Int* 38: 961-965, 2005.
- 12 Gouveia L, Batista AP, Miranda A, Empis J and Raymundo A: *Chlorella vulgaris* biomass used as colouring source in traditional butter cookies. *Innovat Food Sci Emerg Tech* 8: 433-436, 2007.
- 13 Halliwell B and Gutteridge JMC: Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219: 1-14, 1984.
- 14 Kehrer JP: Free radicals as mediators of tissue injury and disease. *Crit Rev Toxicol* 23: 21-48, 1993.
- 15 Han SK, Mytilineou C and Cohen G: L-DOPA up-regulates glutathione and protects mesencephalic cultures against oxidative stress. *J Neurochem* 66: 501-510, 1996.
- 16 Meister A: Glutathione deficiency produced by inhibition of its synthesis, and its reversal: applications in research and therapy. *Pharmacol Ther* 51: 155-194, 1991.
- 17 Recknagel RO and Glende EA Jr: Carbon tetrachloride hepatotoxicity: an example of lethal cleavage. *CRC Crit Rev Toxicol* 2: 263-297, 1973.
- 18 He SX, Luo JY, Wang YP, Wang YL, Fu H, Xu JL, Zhao G and Liu EQ: Effects of extract from *Ginkgo biloba* on carbon tetrachloride-induced liver injury in rats. *World J Gastroenterol* 12: 3924-3928, 2006.
- 19 Hsiao G, Shen MY, Lin KH, Lan MH, Wu LY, Chou DS, Lin CH, Su CH and Sheu JR: Antioxidative and hepatoprotective effects of *Antrodia camphorate* extract. *J Agric Food Chem* 51: 3302-3308, 2003.
- 20 Hung MY, Fu TYC, Shih PH, Lee CP and Yen GC: Du-Zhong (*Eucommia ulmoides* Oliv.) leaves inhibit CCl₄-induced hepatic damage in rats. *Food Chem Toxicol* 44: 1424-1431, 2006.
- 21 Messner M and Brissot P: Traditional management of liver disorders. *Drugs* 40: 345-357, 1990.
- 22 Sato M, Ramarathnam N, Suzuki Y, Ohkubo T, Takeuchi M and Ochi H: Varietal differences in the phenolic content and superoxide radical-scavenging potential of wines from different sources. *J Agric Food Chem* 44: 37-41, 1996.
- 23 Chung YC, Chang CT, Chao WW, Lin CF and Chou ST: Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *J Agric Food Chem* 50: 2454-2458, 2002.
- 24 Botsoglou NA, Fletouris DJ, Papageorgiou GE, Vassilopoulos VN, Mantis AJ and Trakatellis AG: Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food and feedstuff samples. *J Agric Food Chem* 42: 1931-1937, 1994.
- 25 Mitton KP and Trevithick JR: High-performance liquid chromatography-electrochemical detection of antioxidants in vertebrate lens: glutathione, α -tocopherol and ascorbate. *Meth Enzymol* 233: 523-539, 1994.
- 26 Yang CS, Chou ST, Lin L, Tsai PJ and Kuo JS: Effect of ageing on human plasma glutathione concentrations as determined by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr B* 674: 23-30, 1995.
- 27 Chou ST, Ko LE and Yang CS: Determination of tissue α -tocopherol in senescence-accelerated mice by high-performance liquid chromatography with fluorimetric detection. *Anal Chim Acta* 419: 81-86, 2000.
- 28 Paglia DE and Valentine WN: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 70: 158-169, 1967.
- 29 Marklund S and Marklund G: Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 47: 469-474, 1974.
- 30 Aebi H: Catalase. *In: Methods of Enzymatic Analysis*. Bergmeyer HU (ed.). Weinheim, Verlag Chemie, pp. 673-686, 1983.
- 31 Smith P, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BT and Klenk DC: Measurement of protein using bicinchoninic acid. *Anal Biochem* 15: 76-85, 1985.
- 32 Kleinbaum DG, Kupper LL and Muller KE: *Applied Regression analysis and Other Multivariable Methods*. Boston, PWS-Kent Publishing, pp. 361-374, 1998.
- 33 van Acker SA, van den Berg DJ, Tromp MNJL, Griffioen DH, van Bennekom WP, van der Vijgh, WJ and Bast A: Structural aspects of antioxidant activity of flavonoids. *Free Radic Biol Med* 20: 331-342, 1996.
- 34 Zheng W and Wang SY: Antioxidant activity and phenolic compounds in selected herbs. *J Agric Food Chem* 49: 5165-5170, 2001.
- 35 Hertog MGL, Hollman PCH and van de Putte B: Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J Agric Food Chem* 41: 1242-1246, 1993.
- 36 Yokozawa T, Cho EJ, Hara Y and Kitani K: Antioxidant activity of green tea treated with radical initiator 2,2'-azobis(2-amidinopropane)dihydrochloride. *J Agric Food Chem* 48: 5068-5073, 2000.
- 37 Ilavarasan R, Vasudevan M, Anbazhagan S and Venkataraman S: Antioxidant activity of *Thespesia populnea* bark extracts against carbon tetrachloride-induced liver injury in rats. *J Ethnopharmacol* 87: 227-230, 2003.

- 38 Kotamballi N, Murthy C, Jayaprakasha GK and Singh RP: Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using *in vivo* models. *J Agric Food Chem* 50: 4791-4795, 2002.
- 39 Yeh JY, Cheng LC, Ou BR, Whanger PD and Chang LW: Differential influences of various arsenic compounds on glutathione redox status and antioxidative enzymes in porcine endothelial cells. *Cell Mol Life Sci* 59: 1972-1982, 2002.
- 40 Fang YZ, Tang S and Wu G: Free radicals, antioxidants, and nutrition. *Nutrition* 18: 872-879, 2002.
- 41 Reed DJ: Glutathione: toxicological implication. *Annu Rev Pharmacol Toxicol* 30: 603-631, 1990.

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