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# Antioxidative and Hepatoprotective Effects of *Antrodia camphorata* Extract

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*Antrodia camphorata* (*A. camphorata*) is well-known in Taiwan as a traditional Chinese medicine. The purpose of this study was to evaluate the ability of *A. camphorata* extracts to protect against oxidative stress in vitro and against carbon tetrachloride ($CCl_4$)-induced hepatic injury in vivo. An extract of *A. camphorata* inhibited nonenzymatic iron-induced lipid peroxidation in rat brain homogenates with an $IC_{50}$ value about 3.1 mg/mL. It also scavenged the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The dose of the *A. camphorata* extract resulting in a decrease of 0.20 in the absorbance of DPPH was about 31 ± 0.7 $\mu$g/mL. Furthermore, an *A. camphorata* extract dose-dependently (250−1250 mg/kg) ameliorated the increase in plasma aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) levels caused by chronic repeated $CCl_4$ intoxication in mice. Moreover, *A. camphorata* extract significantly improved the $CCl_4$-induced increase in hepatic glutathione peroxidase, reductase, and $CCl_4$-induced decrease in superoxide dismutase activities. It also restored the decrement in the glutathione content and catalase activity of hepatic tissues in $CCl_4$-intoxicated mice. Furthermore, it also dose-dependently inhibited the formation of lipid peroxidative products during $CCl_4$ treatment. Histopathological changes of hepatic lesions induced by $CCl_4$ were significantly ameliorated by treatment with an *A. camphorata* extract in a dose-dependent manner. These results suggest that *A. camphorata* extract exerts effective protection against chronic chemical-induced hepatic injury in vivo, by mediating antioxidative and free radical scavenging activities.

**KEYWORDS:** ***Antrodia camphorata*; antioxidant; free radical scavenger; carbon tetrachloride; hepatotoxicity**

## INTRODUCTION

Many studies have shown that reactive oxygen species (ROS), including oxygen free radicals, are causative factors in the etiology of degenerative diseases, including some hepatopathies (*1*, *2*). Carbon tetrachloride ($CCl_4$) is most frequently used as a chemical inducer of experimental liver cirrhosis (*3*). It has been suggested that hepatic necrosis caused by $CCl_4$ involves bioactivation by the microsomal cytochrome P450-dependent monooxygenase system, resulting in the formation of trichloromethyl free radicals and ROS that initiate lipid peroxidation and protein oxidation (*4*). ROS have also been shown to modify and damage proteins, carbohydrates, and DNA in both in vitro and in vivo models (*5*). Therefore, biomembrane and bioactive molecules are disturbed or inactivated by aggressive oxidative stress. Furthermore, hepatic microsomes, mitochondria, and the nuclei of hepatocytes are also impaired by peroxidative products, with hepatocytes ultimately being destroyed and becoming necrotic (*6*). According to in vitro and in vivo studies, several classical antioxidants have been shown to protect hepatocytes against lipid peroxidation or inflammation, thereby preventing the occurrence of hepatic necrosis (*7*, *8*).

*Antrodia camphorata* (*A. camphorata)* is well-known in Taiwan as a traditional Chinese medicine. It is rare and expensive because its grows only on the inner heartwood wall of the endemic evergreen *Cinnamonum kanehirai* and cannot be cultivated. It has been used as a remedy for drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer (*9*). However, there are as yet no published data documenting the antioxidative and hepatoprotective effects of *A. camphorata*.

In the present study, we examined and compared the relative inhibitory activities of an *A. camphorata* extract with silymarin in chronic $CCl_4$-induced liver injury in mice. Concurrently, we

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also evaluated the inhibitory activities of *A. camphorata* on free radical scavenging activity and lipid peroxidation in vitro.

## MATERIALS AND METHODS

**Materials.** Thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl, carbon tetrachloride, silymarin, hydrogen peroxide, sodium azide, 5,5′-dithiobis (2-nitro benzoic acid) (DTNB), 2-thiobarbituric acid, and other reagents used in the study were obtained from Sigma Chemical (St Louis, MO). Diagnostic kits for assaying alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) were also purchased from Sigma.

**Preparation of Aqueous Extracts from *A. camphorata*.** *Antrodia camphorata* was obtained from Well Shine Biotechnology Development Co. (Taipei, Taiwan). Air-dried *A. camphorata* samples were ground and then shaken with phosphate-buffered saline (PBS) at the ratio of 1:25 (w/v) at 25 °C for 10 h, and then centrifuged at 3000*g* for 10 min, followed by passing through a 0.2-$\mu$m pore size filter. The stock solution was stored at −5 °C before use.

**Antioxidative Activity in Rat Brain Homogenates.** Rat brain homogenates were prepared from the brains of freshly killed Wistar rats, and the homogenate's peroxidation in the presence of ferrous ions was measured as we previously described (*10*). Tetramethoxypropane was used as a standard, and the results were expressed as nanomoles (nmol) of malondialdehyde equivalents per milligram protein of the supernatant of rat brain homogenates.

**Stable Free Radical Scavenging Action.** Stable radical scavenging activity was measured as described previously (*11*). 1,2-Diphenyl-2-picrylhydrazyl (DPPH), a stable nitrogen-centered free radical, was dissolved in ethanol to give a 100-$\mu$M solution. One microliter of ethanolic DPPH in a cuvette was added to the test compounds or vehicle solution. The decrease in absorption at 517 nm was correlated with the scavenging action of the test compounds (*11*), and the concentration of the antioxidant that induced a change of 0.20 in absorbance during a 30-min observation time was taken as an evaluation of antioxidative activity.

**Animals.** Male ICR mice, 5 weeks old and weighing 20−25 g, were used for the chronic $CCl_4$-induced liver injury model. The animals were maintained under a 12-h light/dark cycle in a temperature- and humidity-controlled room. Mice were fed with laboratory pellet chow and given water ad libitum.

**Chronic $CCl_4$-Induced Liver Injury in Vivo.** Mice were divided into six groups: (1) normal control (PBS); (2) vehicle (corn oil) plus $CCl_4$ treatment; *A. camphorata* extracts (3) (250 mg/kg), (4) (750 mg/kg), (5) (1250 mg/kg) plus $CCl_4$ treatment; and (6) silymarin (100 mg/kg) plus $CCl_4$ treatment. Mice were treated with sublethal doses of $CCl_4$ (40% $CCl_4$/olive oil, 0.1 mL/10 g body weight per day, *s.c.* twice per week) for 8 weeks to induce chronic chemical liver injury. Groups of mice were treated with *A. camphorata* extracts (250, 750, and 1250 mg/kg per day, respectively, *p.o.*, 4 days per week) or vehicle solution (corn oil) for 8 weeks. Control mice were treated with PBS for 8 weeks. Blood samples at 0.2 mL with heparin (10 U/mL) were collected from the tail vein at the end of the first, third, sixth, and eighth weeks. Blood and livers were obtained immediately after the animals were sacrificed. Livers were weighed and utilized for the following biological analyses. Liver homogenates (10%, w/v) were obtained in 50 mM phosphate buffer (pH 7.0) and stored at −80 °C within 2 weeks before analysis.

**Measurement of Plasma Transaminase Activities.** GPT and GOT activities in the plasma were measured using GPT and GOT EIA kits according to the manufacturer's instructions.

**Glutathione Reductase Assay.** Liver glutathione reductase (GSH Rd) was assayed by a reaction mixture containing 0.99 mL of 100 mM potassium phosphate buffer (pH 7.0), 1.1 mM $MgCl_2$, 5 mM oxidized GSSG, and 0.1 mM NADPH. Ten microliters of liver homogenate was added to trigger the NADPH conversion reaction. Changes in absorbance were monitored by a continuous-recording spectrophotometer (V-530, Jasco) at 340 nm for 5 min at 25 °C. The specific enzyme activity of GSH Rd was expressed as nmol NADPH/min per mg protein.

**Glutathione Peroxidase Assay.** Glutathione peroxidase (GSH Px) activity was expressed as nanomoles of NADPH oxidized to NADP per minute per milligram of protein, with a molar extinction coefficient for NADPH at 340 nm of $6.22 \times 10^6$ (*12*). Liver GSH Px was assayed in a 1-mL crystal cuvette containing 0.8 mL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM $NaN_3$, 0.2 mM NADPH, 1 U/mL GSH Rd, and 1 mM GSH. Five microliters of the liver homogenate and buffer was added to make a total volume of 0.9 mL. The reaction was initiated by the addition of 100 $\mu$L of 2.5 mM $H_2O_2$, and the conversion of NADPH to NADP was monitored with a spectrophotometer at 340 nm for 3 min. The specific enzyme activity of GSH Px was expressed as mg/protein.

**Determination of Superoxide Dismutase Activity.** A portion of the liver tissue was homogenized (10%, w/v) in a solution (0.32 M sucrose, 1 mM EDTA, and 10 mM Tris−HCl, pH 7.4) using a polytron homogenizer, after which it was centrifuged at 10 000 rpm (Beckman, Microfuge) at 4 °C for 30 min. The supernatant was assayed for superoxide dismutase (SOD) activity by following the inhibition of nitroblue tetrazolium (NBT) reduction. SOD activity was assayed by a reaction mixture containing 985 $\mu$L of 100 mM phosphate buffer (pH 7.4), 0.3 mM $K_2H_2$−EDTA, 0.5 mM NBT, and 0.1 mM xanthine. The mixture was preincubated for 3 min at 25 °C, and 10 $\mu$L of 0.02 U/mL xanthine oxidase was added to generate superoxide and induce NBT reduction. Changes in absorbance at 560 nm were recorded at 20-s intervals for 5 min. SOD activity was determined from a standard curve of the percentage inhibition of NBT reduction with standard SOD activity. Data are expressed as SOD units/mg protein as compared with the standard.

**Catalase Assays.** Liver homogenates (10%, w/v) were centrifuged (10 000 rpm) at 4 °C for 10 min. Supernatant (5 $\mu$L) was added to a crystal cuvette containing 0.995 mL of a 30-mM $H_2O_2$ solution prepared in potassium phosphate buffer. Change in the absorbance was monitored at 240 nm for 1 min. Commercially available catalase was used as a standard. Using the reaction time interval ($\Delta t$) of the absorbance ($A_1$ and $A_2$), the following equation was generated to calculate the rate constant ($K$): $K = (2.3/\Delta t) \times \log(A_1/A_2)$ (*13*). The specific activity of the enzyme was expressed as $K$/mg protein.

**Determination of Glutathione Content.** GSH was determined by titration with DTNB as described previously (*14*). The glutathione content was determined using a calibration curve prepared with an authentic sample. An aliquot of lysate was used for determination of the protein content. GSH values were expressed as $\mu$mol/mg protein.

**Measurement of Hepatic Lipid Peroxidation.** The content of malonaldehyde (MDA) formation in liver homogenate was determined using the thiobarbituric acid (TBA) method (*11*). Briefly, 250 $\mu$L of liver homogenate (10%, w/v) was first mixed with the same volume of 50 mM potassium phosphate buffer. Then 20 $\mu$L of an ice-cold trichloroacetic acid (TCA) solution (4% [w/v] in 0.3 N HCl) and 200 $\mu$L of TBA-reactive substance reagent (0.5% [w/v] TBA in 50% [v/v] acetic acid) were also added. Samples were boiled and cooled, extracted with *n*-1-butanol, and centrifuged in a microcentrifuge for 10 min at 10 000 rpm. The butanol layer containing the TBA-reactive substances (TBARS) was read at 532 nm. The results were expressed as absorbance (532 nm)/mg protein.

**Determination of Protein Contents.** Protein contents of brain and liver homogenates were determined by the Bio-Rad method (*15*) using bovine serum albumin as a standard.

**Liver Histology.** Mice were sacrificed at the end of the eighth week, and the livers were freshly and quickly obtained. Liver slices were made from a part of the left and central lobes, and immediately fixed in a 10% buffered formalin phosphate solution, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

**Statistical Analysis.** The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman−Keuls method. A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

**Inhibition of Lipid Peroxidation in Rat Brain Homogenates.** *Antrodia camphorata* extract was tested for its ability to inhibit nonenzymatic lipid peroxidation in rat brain homogenates stimulated by ferrous ion. The *A. camphorata* extract concentra-

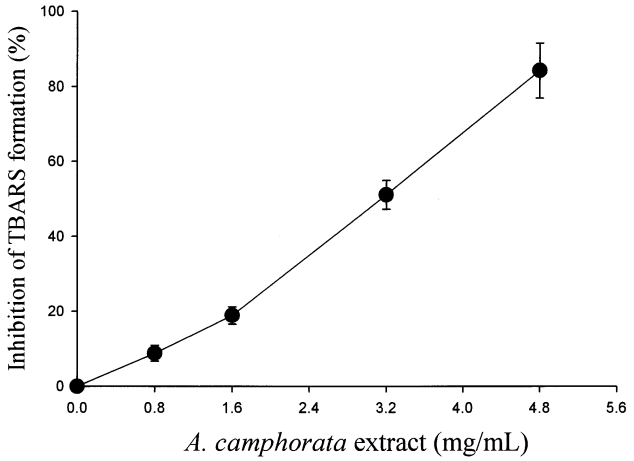


**Figure 1.** Inhibitory effects of the *A. camphorata* extract on iron-dependent lipid peroxidation in rat brain homogenates. Brain homogenates were treated with various doses of an *A. camphorata* extract (0.8–4.8 mg/mL) or vehicle control (PBS) at 37 °C for 30 min followed by the addition of 200 $\mu$M $Fe^{2+}$. Results are expressed as percent inhibition of TBARS formation vs vehicle control (PBS). Data are presented as the means ± SEM ($n = 4$).

tion-dependently (0.8−4.8 mg/mL) inhibited ferrous-induced lipid peroxidation (**Figure 1**). The *A. camphorata* extract did not interfere with the TBA test because the color formation was not changed if the extract was added after incubation with TBA reagents. The $IC_{50}$ of the *A. camphorata* extract to inhibit lipid peroxidation was about 3.1 mg/mL.

**Interaction with Stable Free Radical (DPPH)-Scavenging Action.** The change in absorbance produced by reduced DPPH was used to evaluate the ability of test compounds to act as free radical scavengers. DPPH decolorization was increased by the *A. camphorata* extract in a concentration- and time-dependent manner (data not shown). The concentration of the *A. camphorata* extract resulting in a decrease of 0.20 in the absorbance of DPPH was approximately 31 ± 0.7 $\mu$g/mL ($n =$ 4, data not shown). This result showed that an *A. camphorata* extract was a good scavenger to interact with the nitrogen-centered stable free radical, DPPH.

**Plasma Transaminase.** Blood was collected at indicated time points for GPT and GOT determination after $CCl_4$ administration. Both plasma GPT and GOT levels were markedly increased to their maximum value (U/L) at 862 ± 58 and 1612 ± 252, respectively, at the end of the third week, and then decreased to 545 ± 25 and 809 ± 33 U/L by the eighth week (**Figure 2a** and b). In the normal group (PBS treatment), serum GPT levels were 64 ± 6 and 53 ± 4, and GOT levels were 142 ± 13, and 117 ± 14 U/mL at the end of the third and eighth weeks, respectively. On the other hand, *A. camphorata* extract treatment (250, 750, and 1250 mg/kg) produced dose-dependent reductions in GPT and GOT levels at the indicated time points. The *A. camphorata* extract (1250 mg/kg) markedly reduced the activities of GPT and GOT by about 79 and 74% at the eighth week for continuous treatment with $CCl_4$ as compared with those of the vehicle group (corn oil) (**Figure 2**). Silymarin (100 mg/kg) also significantly decreased the GPT and GOT levels to about 78% and 71% of the values measured in the vehicle groups (Figure 2).

**Glutathione Peroxidase and Reductase Activity. Figure 3** shows the changes in hepatic GSH peroxidase and reductase activities at 8 weeks in $CCl_4$-intoxicated mice. Activities of GSH peroxidase and reductase had increased by about 1.7- and 3.3-fold, respectively, at 8 weeks after $CCl_4$ administration as

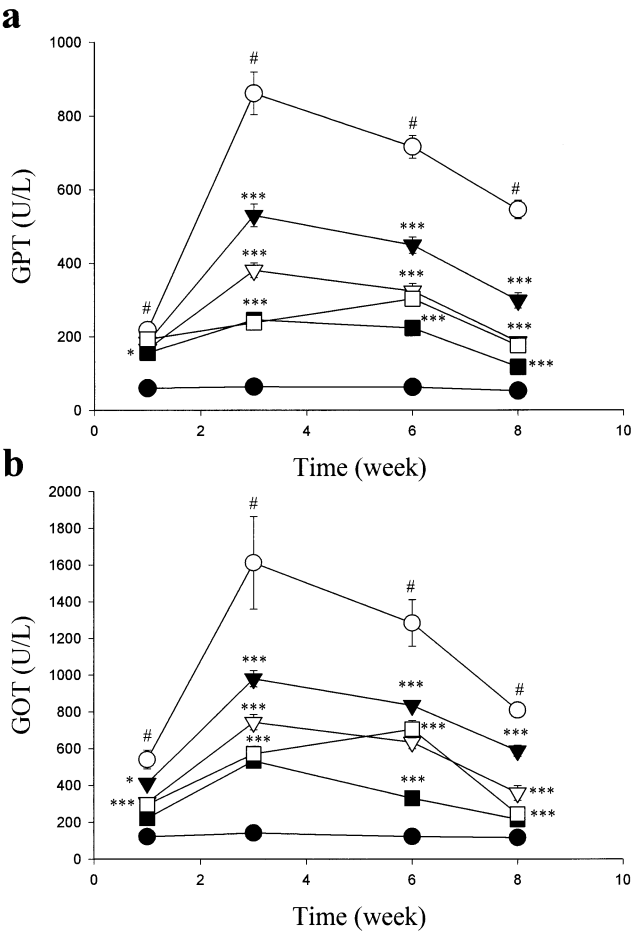


**Figure 2.** Effects of the *A. camphorata* extract and silymarin on plasma GPT and GOT formation in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (○, corn oil), various doses of an *A. camphorata* extract (▼, 250; ▽, 750; and ■, 1250 mg/mL), and silymarin (□, 100 mg/kg) for 8 weeks. Animals were treated with PBS without $CCl_4$ as the control group (●). Blood was collected at the end of the first, third, sixth, and eighth weeks. Plasma GPT and GOT levels were determined as described in Materials and Methods. Results are presented as the means ± SEM ($n = 10$); $^{\#}p < 0.001$ as compared with the normal control (without $CCl_4$ treatment); $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with the vehicle group (corn oil).

compared with those of the control group. On the other hand, the *A. camphorata* extract reduced both enzyme activities in a dose-dependent manner (250, 750, and 1250 mg/kg). However, the *A. camphorata* extract (250 mg/kg) slightly inhibited the elevated GSH peroxidase activity induced by $CCl_4$, but this did not reach statistical significance. At higher doses (750 and 1250 mg/kg), the *A. camphorata* extract markedly attenuated the activity of GSH peroxidase (**Figure 3a**). In addition, *A. camphorata* (250, 750, and 1250 mg/kg) also markedly inhibited the $CCl_4$-induced increase in GSH reductase activity. This enzyme activity was completely restored to the normal level by treatment with the *A. camphorata* extract at 1250 mg/kg (**Figure 3b**). As a positive hepatoprotective control, silymarin (100 mg/kg) also significantly restored GSH-related enzyme activities to the normal level under the same conditions (**Figure 3**).

**Superoxide Dismutase Activity in Liver Tissues.** Figure 4 shows the SOD activity of liver tissues. SOD activity in liver tissues was reduced by approximately half at 8 weeks in $CCl_4$-intoxicated mice compared with that of the control group (428 ± 14 vs 209 ± 15 U/mg). However, *A. camphorata* extract

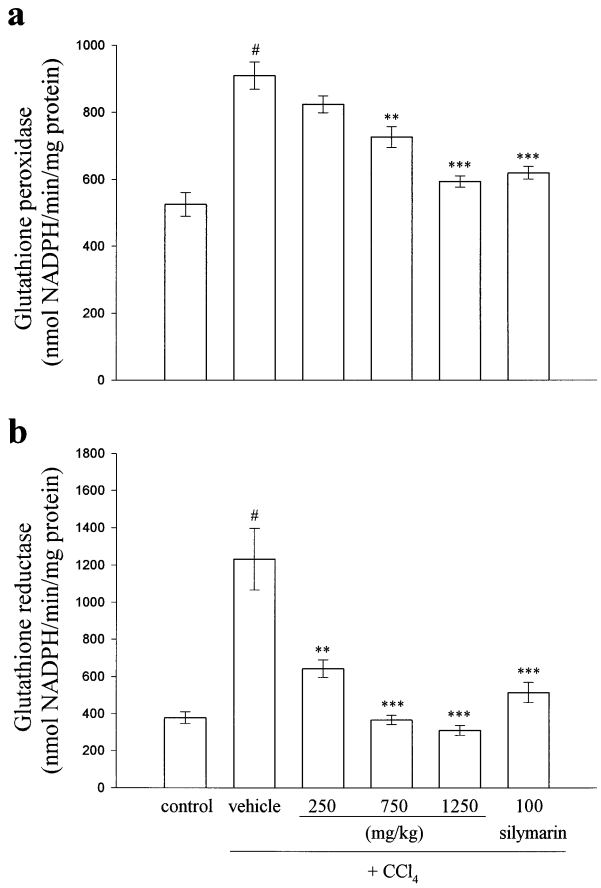


**Figure 3.** Effects of the *A. camphorata* extract and silymarin on hepatic glutathione peroxidase (a) and reductase (b) activities in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (corn oil), various doses of an *A. camphorata* extract (250, 750, and 1250 mg/mL), and silymarin (100 mg/kg) for 8 weeks. Animals were treated with PBS without $CCl_4$ as the control group. Homogenates of liver tissues were obtained from $CCl_4$-intoxicated mice at the end of the eighth week. Glutathione peroxidase and reductase activities were determined as described in Materials and Methods. Results are presented as the means ± SEM ($n = 10$); $^{\#}p < 0.001$ as compared with the normal control (without $CCl_4$ treatment), $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the vehicle group (corn oil).

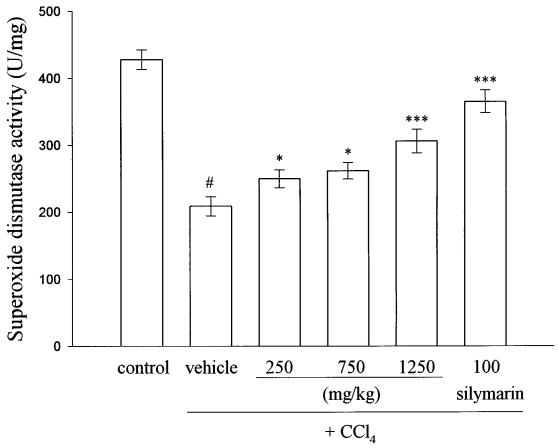


**Figure 4.** Effects of the *A. camphorata* extract on hepatic SOD activity in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (corn oil), various doses of an *A. camphorata* extract (250, 750, and 1250 mg/mL), and silymarin (100 mg/kg) for 8 weeks. Animals were treated with PBS without $CCl_4$ as the control group. Homogenates of liver tissues were obtained from $CCl_4$-intoxicated mice at the end of the eighth week. SOD activities were determined as described in Materials and Methods. Results are presented as the means ± SEM ($n = 10$); $^{\#}p < 0.001$ as compared with the normal control (without $CCl_4$ treatment), $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with the vehicle group (corn oil).

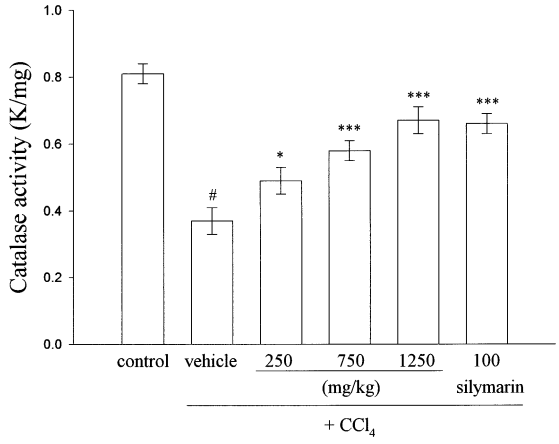


**Figure 5.** Effects of the *A. camphorata* extract on hepatic catalase activity in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (corn oil), various doses of an *A. camphorata* extract (250, 750, and 1250 mg/mL), and silymarin (100 mg/kg) for 8 weeks. Animals were treated with PBS without $CCl_4$ as the control group. Homogenates of liver tissues were obtained from $CCl_4$-intoxicated mice at the end of the eighth week. Catalase activities were determined as described in Materials and Methods. Results are presented as the means ± SEM ($n = 10$); $^{\#}p < 0.001$ as compared with the normal control (without $CCl_4$ treatment), $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with the vehicle group (corn oil).

administration (250, 750, and 1250 mg/kg) resulted in a significant and dose-dependent increase in SOD activity. Similarly, silymarin (100 mg/kg) also restored the SOD activity under the same conditions (Figure 4).

**Catalase Activity in Liver Tissues.** The catalase activity of liver tissues was 0.81 ± 0.03 K/mg in control mice (**Figure 5**). The enzyme activity decreased to 0.37 ± 0.04 K/mg at 8 weeks in $CCl_4$-intoxicated mice (**Figure 5**). The *A. camphorata* extract (250, 750, and 1250 mg/kg) dose-dependently increased the catalase activity of liver tissues at 8 weeks in $CCl_4$-intoxicated mice (**Figure 5**). In addition, both the *A. camphorata* extract and silymarin completely restored enzymatic activity to the normal level at the respective doses of 1250 and 100 mg/kg (**Figure 5**).

**Glutathione Content in Liver Tissues.** Figure 6 shows the changes in glutathione levels of liver tissues in $CCl_4$-intoxicated mice. Hepatic glutathione levels (nmol/mg protein) significantly decreased from 48.0 ± 2.1 in the control group to 20.7 ± 1.8 units in $CCl_4$-intoxicated mice at 8 weeks. At doses of 250−1250 mg/kg, the *A. camphorata* extract significantly increased the glutathione level in hepatic tissues. In addition, both the *A. camphorata* extract and silymarin almost completely restored the enzymatic activity to the normal level at the respective doses of 1250 and 100 mg/kg (**Figure 6**).

**In Vivo Lipid Peroxidation.** The hepatotoxicity of $CCl_4$ is thought to be due to lipid peroxidation. TBARS increased about 2.5-fold at 8 weeks in $CCl_4$-intoxicated mice compared with the that of control group (**Figure 7**). The *A. camphorata* extract (250, 750, and 1250 mg/kg) dose-dependently reduced the formation of TBARS in liver tissues in $CCl_4$-intoxicated mice (**Figure 7**). TBARS levels of liver tissues were almost completely restored to normal levels (0.36 ± 0.04 vs 0.29 ± 0.03)

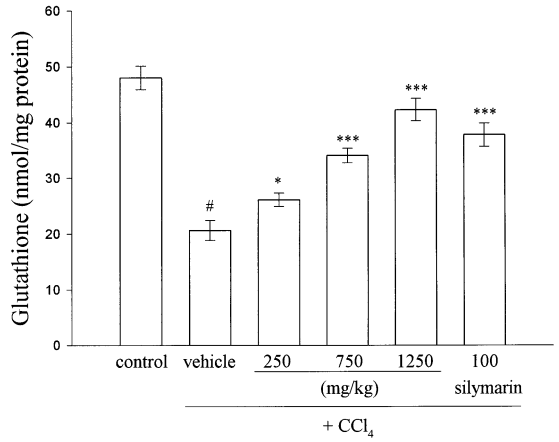


**Figure 6.** Effects of the *A. camphorata* extract on hepatic glutathione levels in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (corn oil), various doses of an *A. camphorata* extract (250, 750, and 1250 mg/mL), and silymarin (100 mg/kg) for 8 weeks. Animals were treated with PBS without $CCl_4$ as the control group. Homogenates of liver tissues were obtained from $CCl_4$-intoxicated mice at the end of the eighth week. Glutathione contents were determined as described in Materials and Methods. Results are presented as the means ± SEM ($n = 10$); $^{\#}p < 0.001$ as compared with the normal control (without $CCl_4$ treatment), $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with the vehicle group (corn oil).

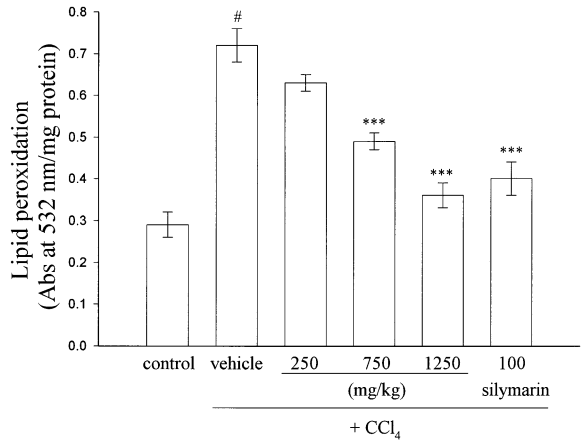


**Figure 7.** Effects of the *A. camphorata* extract on hepatic lipid peroxidation in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (corn oil), various doses of an *A. camphorata* extract (250, 750, and 1250 mg/mL), and silymarin (100 mg/kg) for 8 weeks. Animals were treated with PBS without $CCl_4$ as the control group. Homogenates of liver tissues were obtained from $CCl_4$-intoxicated mice at the end of the eighth week. TBARS formation was determined by the thiobarbituric conjugation method as described in Materials and Methods. Results are presented as the means ± SEM ($n = 10$); $^{\#}p < 0.001$ as compared with the normal control (without $CCl_4$ treatment), $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with the vehicle group (corn oil).

at a dose of 1250 mg/kg of the *A. camphorata* extract (**Figure 7**). Similarly, silymarin (100 mg/kg) markedly reduced TBARS formation in $CCl_4$-intoxicated mice.

**Hepatopathology.** Histopathological changes of necrotic, ballooning, and lipid-laden hepatocytes of liver sections were assessed at 8 weeks after $CCl_4$ administration (**Figure 8**). Typical intense centrilobular necrosis of hepatotoxicity was observed at 8 weeks in $CCl_4$-intoxicated mice (**Figure 8b**), at which time there was also a drastic increase in plasma transaminase activities (**Figure 2**). Marked macro- and microvesicular fatty

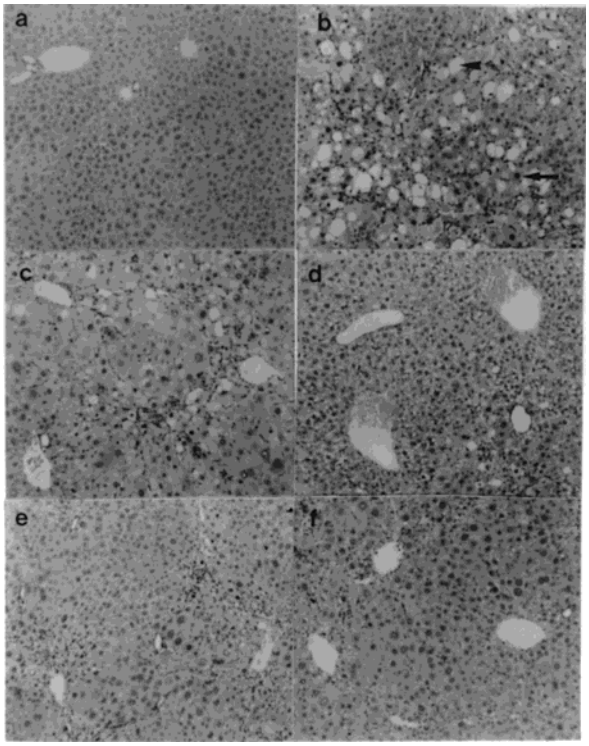


**Figure 8.** Effect of the *A. camphorata* extract on hepatic morphological analysis in $CCl_4$-intoxicated mice. $CCl_4$-intoxicated mice were treated with vehicle solution (corn oil), various doses of an *A. camphorata* extract (250, 750, and 1250 mg/mL), and silymarin (100 mg/kg) for 8 weeks. Livers were sectioned and stained with hematoxylin–eosin by standard techniques.: (a) a representative mouse with PBS treatment (control); (b) a representative mouse treated with $CCl_4$ alone. The hepatocytes show either necrotic cells (arrow) or abundant macro- and microvesicular fats (arrowhead); (c–f) Representative mice treated with 250, 750, and 1250 mg/kg of an *A. camphorata* extract or silymarin (100 mg/kg) plus $CCl_4$ treatment, respectively.

changes of hepatocytes around the central vein and parenchymal disarrangement were found. Ballooning degeneration and infiltration of inflammatory cells (such as hemosiderin-laden macrophages) were also found (**Figure 8b**), as compared to normal liver sections (**Figure 8a**). The hepatohistological changes induced by $CCl_4$ were markedly ameliorated by treatment with the *A. camphorata* extract in a dose-dependent manner (**Figure 8c**−**e**). Administration of the *A. camphorata* extract (1250 mg/kg) markedly decreased macro- and microvesicular changes, and they were rarely found in $CCl_4$-intoxicated mice treated with *A. camphorata* extract. Lipid-laden cells or lipid droplets were also rarely observed. In addition, ballooning-degenerated hepatocytes or inflammatory cells were markedly reduced (**Figure 8e**). Furthermore, sylimarin (100 mg/kg) also markedly reduced the alterations of necrotic, ballooned, and lipid-laden cells (**Figure 8f**).

## DISCUSSION

The phospholipid bilayers of cellular and subcellular membranes are undoubtedly major targets for free radicals. The compound that inhibits membrane phospholipid peroxidation seems to exert a pharmacological effect in the prevention of radical-induced oxidative pathological events (*16*). In this study, two in vitro antioxidative tests were used to assess the activities of the *A. camphorata* extract. Among cell-free systems, brain homogenates are usually chosen to evaluate antioxidant effects on lipid peroxidation (*11*). Rat brain homogenates exposed to

ferrous ion exhibit lipid peroxidation in air by a mechanism whose induction step may primarily involve site-bound iron-mediated decomposition of lipid hydroperoxides to yield alkoxy or peroxyl radicals, leading to the chain reaction of lipid peroxidation (*11*). In this system, the *A. camphorata* extract effectively inhibited lipid peroxidation. The DPPH tests provided direct evidence that the *A. camphorata* extract acted as a direct free radical scavenger.

It is well-known that free radicals derived from oxygen and other chemicals are important factors related to injury of the liver (*2*). ROS generation and lipid peroxidation are thought to be the common pathways inducing liver injuries by endotoxin, $CCl_4$, and tumor necrosis factor (TNF-α). The hepatotoxicity of $CCl_4$ results from reductive dehalogenation catalyzed by reduced cytochrome P450 which forms a highly reactive trichloromethyl free radical ($CCl_3$), and then forms the initiator of lipid peroxidation as the trichloromethyl peroxy radical ($CCl_3OO\cdot$) (*4*). Another important mechanism of cellular injury is the paracrine action of cytotoxic cytokines such as TNF-α (*17*). The expression of inflammatory cytokines such as TNF-α occurs during the actual period of hepatocellular injury in acute $CCl_4$-mediated liver damage (*18*). In this study, we also demonstrated that $CCl_4$ produced a marked increase in TNF-α levels ($47.8 \pm 8.2$ IU/mL, $n = 3$) compared with the control group ($6.3 \pm 1.8$ IU/mL, $n = 3$) (data not shown). An early mediator of hepatocyte injury and activation of Kupffer's cells is probably ROS. Thus, cells then manifest increased ROS, proteolytic enzymes, and inflammatory cytokines (e.g., IL-1, IL-2, and TNFα) which have been implicated in the induction of hepatocyte necrosis (*19*). Therefore, antioxidants may protect against $CCl_4$-induced hepatic hepatotoxicity through anti-peroxidation, free radical scavenging activity, and/or induction of defense enzyme expression (*20*).

In this study, the activities of GOT and GPT were rapidly elevated at 3−4 weeks after $CCl_4$ administration, and then had gradually decreased by 6−8 weeks. However, at the end of 8 weeks, hepatic necrosis seemed to be attenuated in $CCl_4$-intoxicated mice. This phenomenon may be explained by redox adaptation or autoprotection. The detailed mechanisms of this adaptation or autoprotection may occur through induction of genes of some defensive enzyme systems or by CYP2E1 inactivation (*21*, *22*). However, the detailed mechanisms are not fully understood and remain to be further resolved. In this study, we found that the *A. camphorata* extract markedly prevented hepatic necrosis in a dose-dependent manner. The hepatoprotective activity of the *A. camphorata* extract was also supported by histological examinations.

Some endogenous protective factors such as glutathione peroxidase and catalase are activated in the defense against oxidative cell injury by means of their being free-radical scavengers (*23*, *24*). The most important hepatic enzymes for the detoxification of lipid peroxide or ROS are glutathione peroxidase, glutathione reductase, and glutathione (*24*). Under oxidative stress, glutathione is largely consumed by glutathione-related enzymes, thereby resulting in induction of some intoxication (*22*). In the present study, chronic sublethal doses of $CCl_4$ enhanced the activities of glutathione-related enzymes, and decreased the glutathione content, whereas the *A. camphorata* extract reversed these effects. It is conceivable that the effect of the *A. camphorata* extract initially may be due to a reduction in hepatic peroxidative activities followed by inhibition of the activities of glutathione-related enzymes, thereby leading to restoration of the glutathione content in $CCl_4$-induced hepatotoxicity.

SOD and catalase are the major enzymes which catalyze ROS in most cells. Both enzymes play an important role in the elimination of ROS derived from the redox process of xenobiotics in liver tissues (*2*). It was suggested that catalase and SOD are easy inactivated by lipid peroxides or ROS (*25*). In this study, catalase and SOD were appreciably elevated by administration of the *A. camphorata* extract, suggesting that it can restore SOD enzymes and/or activate enzyme activity in $CCl_4$-damaged liver tissue. This result is consistent with a previous report which showed a significant restoration of SOD activity by treatment with an antioxidant in $CCl_4$-intoxicated animals (*26*).

In conclusion, the *A. camphorata* extract was effective in prevention of $CCl_4$-induced hepatotoxicity. It is conceivable that the *A. camphorata* extract exerts its hepatoprotective activity by, at least partly, scavenging free radical formation or by inhibiting inflammatory mediators in $CCl_4$-mediated lipid peroxidation. The inhibitory effects of a dietary *A. camphorata* extract may be useful as a hepatoprotective agent against chronic chemical-induced hepatotoxicity in vivo.

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