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Protective Effects of Fermented Filtrate from *Antrodia camphorata* in Submerged Culture against CCl_4-Induced Hepatic Toxicity in Rats

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The protective effects and the possible mechanisms of dry matter of fermented filtrate (DMF) from *Antrodia camphorata* in submerged culture (ACSC) on H_2O_2-induced cytotoxicity in HepG2 and carbon tetrachloride (CCl_4)-induced hepatotoxicity in Sprague−Dawley rats were investigated. The results showed that the inhibitory effect of DMF and its crude triterpenoids on lipid peroxidation occurred in a dose−response manner in an AAPH/linoleic acid system. When HepG2 cells were pretreated with DMF at the concentration of 0.10 mg/mL for 4 h and then induced by 1 h of treatment with H_2O_2 (100 μM), lipid peroxidation was significantly ($p < 0.05$) decreased, as measured by the formation of malondialdehyde. The oral pretreatment with DMF [0.25 and 0.50 mg/kg of body weight (bw)] for 5 consecutive days prior to the administration of a single dose of 40% CCl_4 (0.10 mL/100 g of bw, ip) significantly prevented the increase in serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and liver lipid peroxidation ($p < 0.05$). Histopathological evaluation of the rat liver revealed that DMF reduced the incidence of liver lesions, including neutrophil infiltration, hydropic swelling, and necrosis induced by CCl_4 in rats. Moreover, reduced glutathione (GSH)-dependent enzymes (glutathione peroxidase, glutathione reductase, and glutathione *S*-transferase) and the GSH/GSSG ratio were significantly improved in the oral pretreatment DMF of rats ($p < 0.01$). The results suggest that DMF may play a role in preventing oxidative damage in living systems by up-regulating hepatic GSH-dependent enzymes to preserve the normal GSH/GSSH ratio and scavenging free radicals formed during CCl_4 metabolism.

KEYWORDS: ***Antrodia camphorata*; submerged culture; hepatotoxicity; lipid peroxidation; GSH/GSSH ratio; crude triterpenoids**

INTRODUCTION

A causal relationship between the presence of oxidants and the generation of lipid hydroperoxides may be an important etiological mechanism of many diseases, such as cardiovascular disease, aging, and cancer (*1*). Liver injury has often been induced in laboratory animals by the administration of carbon tetrachloride (CCl_4), which requires bioactivation by the cytochrome P450 system in the liver, yielding the reactive metabolite trichloromethyl radical ($CCl_3^{\bullet}$). This free radical can abstract a hydrogen atom from the microsomal membrane of liver cells and produce lipid hydroperoxides (*2*). These hydroperoxides can decompose to alkoxy ($RO^{\bullet}$) and peroxy ($ROO^{\bullet}$) free radicals that can oxidize other cell components, resulting in changes in enzyme activity or the generation of mediators [i.e., malondialdehyde (MDA) and reactive oxygen species]. These subsequently cause a rise in intracellular Ca^{2+}, depleting GSH or releasing iron to produce severe hepatocellular damage in rats (*3*−*5*).

Antrodia camphorata is known as niu-chang-chih or niu-chang-ku, and the host plant, niu-chang, is the Chinese common name for *Cinnamomum kanehirai*, which is an endangered species in Taiwan. Ku in Chinese means mushroom, and chih means *Ganoderma*-like fungus. It is a new species of the genus *Antrodia* (family Polyporaceae, Aphyllophorales) that is parasitic on the inner cavity of the endemic species *C. kanehirai* Hay (*6*). *A. camphorata* was previously identified as a new *Ganoderma* species, *Ganoderma camphoratum*, in 1990 due to their similar characteristics (*7*). Traditionally, it is used as a Chinese remedy for food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver cancer (*8*). Preliminary pharmacological studies revealed that zhankuic acid (a type of steroid acid) in the fruiting bodies of *Antrodia cinnamomum* is cytotoxic to P 388 murine leukemia at 4 μg/mL and is anticholinergic as well as antiserotonergic as tested on guinea pig ileum preparation at 10 μg/mL (*9*). In addition, methanol extracts from dry mycelium and fresh fruiting bodies of *A. camphorata* had similar antioxidant activity, whereas those from dry fruiting bodies did not have antioxidant ability (*10*).

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Because the growth rate of the natural *A. camphorata* in the wild is very slow and it is difficult to cultivate in a greenhouse, the fruiting bodies are rare and very expensive. Therefore, using a submerged cultured method to obtain useful cellular materials or to produce effective substances from cultured mycelia might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies (*11*). Recently, we have been found that the dry matter of fermented filtrate (DMF) had the strongest antioxidant activity in different solvent extracts of *A. camphorata* in submerged culture (ACSC), and phenolic compounds, polysaccharides, and triterpenoids might be the major antioxidants in DMF. To further understand the antioxidant activity of DMF from ACSC in living systems and the possible mechanisms involved in this protection, we investigate its potential protection against H_2O_2-induced cytotoxicity in HepG2 and carbon tetrachloride (CCl_4)-induced hepatotoxicity in Sprague−Dawley (SD) rats

MATERIALS AND METHODS

Materials. ACSC was obtained from the Biotechnology Center, Grape King Inc., Chungli, Taiwan. Human hepatocyte cell line (HepG2, CCRC 60025) was purchased from the Food Industry Research and Development Institute, Hsin-chu, Taiwan. Linoleic acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase assay kit, and butylated hydroxytoluene (BHA) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide, Tween 20 (polyoxyethylenesorbitan monolaurate), sodium borate, sodium dihydrogen phosphate, anhydrous dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Shimakyu Co. (Osaka, Japan). Trolox (Hoffman-La Roche) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethyl sulfoxide (DMSO) was obtained from Fluka Chemie (Buchs, Switzerland). Protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.), medium for cell culture (GIBCO BRL, Grand Island, NY), and other reagents or dishes (Nunc, Roskilde, Denmark) were obtained from the indicated suppliers.

Culture Conditions of *A. camphorata*. *A. camphorata* hyphae were separated from the fruiting bodies and inoculated into a culture medium composed of 2.5% cornstarch, 2% sucrose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.3% $MgSO_4$, 0.3% $(NH_4)_2SO_4$, and 0.05% citric acid in distilled water and adjusted to the initial pH range of 5.3−5.5. Each shaking flask culture was carried out in a 2 L Erlenmeyer flask containing 1 L of medium and incubated at 27−30 °C for 7 days. Thereafter, 3.5 L of shaking flask cultures was inoculated into a 500 L fermented tank containing 350 L of culture medium and then cultured at 27−30 °C for 7 days with a 0.5 vvm aeration rate [aeration volume/medium volume (L)/min] by shaking at 50 rpm with a rotary shaker to obtain a mucilaginous medium containing mycelia. Residual sugar concentration detected by using the phenol−H_2SO_4 method was ∼0.1 g/L after cultivation for 7 days. The mycelia were collected by means of centrifugation (4 °C, 8000 rpm for 15 min), washed with distilled water, and finally freeze-dried to a powder form. The yield of mycelia in submerged culture was 1.1 g of dry weight of mycelia/100 g of ACSC.

Preparation of DMF from ACSC. One liter of mycelial extracellular medium was filtered and concentrated 6-fold by a rotary evaporator working under vacuum and then freeze-dried to a powder form. The yield of DMF powder was 5.17 g.

Determination of Crude Triterpenoids. The triterpenoid was determined by modifying the method of Chen et al. (*12*). DMF (10 g) was extracted with 95% ethanol (2 L) for 24 h. The mixture was filtered, and the filtrate was evaporated to dryness with a rotatory evaporator. The residue was extracted with $CHCl_3/H_2O$ (1:1, v/v) three times. The $CHCl_3$ layer was further extracted with saturated $NaHCO_3$ (100 mL) three times, and the alkaline solution was collected and acidified with 6 N HCl to a pH of 3−4. Then, the solution was extracted with $CHCl_3$ (100 mL) three times. The solvent was evaporated by a rotary evaporator working under vacuum to obtain a brown acidic-$CHCl_3$-soluble material (crude triterpenoids, 0.40 g in yield).

Effect of DMF on Lipid Peroxidation. The inhibition of lipid peroxidation was determined using the method of Liegeois et al. (*13*). An aqueous solution of linoleic acid was prepared as follows: linoleic acid (0.25 mL) was added dropwise to 5 mL of 0.05 M borate buffer, pH 9, containing 0.25 mL of Tween 20. The resulting dispersion was clarified by adding 1 mL of 1 N sodium hydroxide. The volume was adjusted to 50 mL with additional borate buffer. Thirty microliters of a 16 mM linoleic acid dispersion was added to a quartz cuvette containing 2.81 mL of 0.05 M phosphate buffer, pH 7.4, prethermostated at 40 °C. The oxidation reaction was initiated at 37 °C by adding of 150 μL of 40 mM AAPH solution (which was freshly prepared in 0.05 M phosphate buffer, pH 7.4). Then, oxidation was carried out in the presence of 20 μL of DMF (0−0.50 mg/mL). The rate of peroxidation at 37 °C was monitored by recording the increase in absorbance at 234 nm caused by conjugated diene hydroperoxides. Inhibition peroxidation percentage = $[1 - (A_{\text{sample,234nm}} - A_{\text{blank,234nm}})/(A_{\text{control,234nm}} - A_{\text{blank,234nm}})] \times 100$.

Cell Culture and Treatment. HepG2 cells were cultured in complete MEM [containing 10% fetal bovine serum (FBS), 100 units/mL of pencillin, 100 mg/mL of streptomycin, pH 7.4] at 37 °C in 5% CO_2. The effect of DMF on cytotoxicity was tested by treating cells with different concentrations of DMF in MEM medium for 24 h. The inhibitory effect of DMF on H_2O_2-induced lipid peroxidation was determined by preincubating cells with different concentrations of DMF in MEM medium for 4 h and then further incubated cells for 1 h with the addition of H_2O_2 (100 μM) to medium.

Effect of DMF on HepG2 Cell Growth (MTT Test). The tetrazolium dye colorimetric test (MTT test) is used to monitor cell growth indirectly, as indicated by the conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically (*14*). Briefly, the HepG2 cell numbers were counted by hemocytometer and seeded in 96-well microplates (2×10^4 cells/well in 200 μL of complete MEM medium) for 12 h. Cells were then washed with PBS and coincubated with DMF (0.05−0.50 mg/mL) for 24 h. The medium (100 μL) was removed at the end of incubation; then 10 μL of MTT (5 mg/mL) was added to each well, and incubation was allowed to continue for 45 min. Finally, 250 μL of DMSO was added to each well and incubated for 2 h. The plate was read by a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany) at a wavelength of 570 nm.

Determination of Cytotoxicity: Lactate Dehydrogenase (LDH) Leakage. LDH is frequently utilized in in vitro toxicity studies as an indicator of cell viability as a function of cell membrane integrity (*15*). HepG2 cell injury was quantitatively assessed by the measurement of LDH, released from damaged or destroyed cells, in the extracellular fluid 24 h after the experiment. An aliquot of medium was removed from culture dishes after 24 h of exposure to the DMF (0.05−0.50 mg/mL) and was analyzed for LDH leakage into the culture medium by using commercial kits (Sigma Chemical Co.). The total LDH activity was determined after cells were disrupted thoroughly by sonication. The percentage of LDH leakage was then calculated to reflect the cytotoxicity. LDH leakage was expressed by the percentage of total activity (activity in the medium)/(activity in the medium + activity of the cells) × 100).

Measurement of Lipid Peroxidation: Malondialdehyde (MDA) Formation. HepG2 cells were pretreated with DMF and then with H_2O_2 as described above. Determination of MDA by thiobarbituric acid (TBA) was used an index of the extent of lipid peroxidation (*16*). BHT (10 μL, 50 mM) was added into hemolysate or liver tissue homogenate (1 mL) to terminate the peroxidation reaction and then was mixed with 1 mL of 7.5% (w/v) cold trichloroacetic acid (TCA) to precipitate proteins. The supernatant was reacted with 1 mL of 0.8% (w/v) TBA in a boiling water bath for 45 min. After cooling, the lipid peroxidation product (MDA) was assayed according to an improved TBA fluorometric method at 555 nm with excitation at 515 nm using 1,1,3,3-tetraethoxypropane as the standard. The protein concentration was determined using a standard commercial kit (Bio-Rad Laboratories Ltd.). The results were expressed as MDA formation per milligram of protein.

Animal Treatment. Male Sprague−Dawley rats (180 ± 20 g) were used for the experiments. The rats were provided with food and water

ad libitum and divided into five groups (six rats/group). To study the protective effect against the CCl_4-induced hepatotoxicity, DMF [0.10, 0.25, and 0.50 g/kg of body weight (bw)] was given daily by gastric tube to the animals for 5 consecutive days. On day 6, CCl_4 in olive oil (2:3, v/v) was injected (0.1 mL/100 g of bw, ip) to each animal, and 18 h later the rats were sacrificed under anesthesia; blood samples were collected for the assays of ALT and AST. The livers were excised from the animals and assayed for the GSH level, GSH-dependent enzymes activity, MDA formation, and pathological histology, according to the procedures described below. All experimental procedures involving animals were conducted in accordance with National Institutes of Health (NIH) guidelines. This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University of Taiwan, Taichung.

Hepatotoxicity Assessment. Hepatic enzymes AST and ALT were used as biochemical markers for early acute hepatic damage. Serum activities of AST and ALT were determined by using the colorimetric method of Reitman and Frankel (*17*).

Pathological Histology. Immediately after removal of hepatic tissues, they were fixed in 10% buffered formaldehyde, processed for histological examination according to the conventional method, and stained with hematoxylin and eosin (H&E). The morphology of any lesions observed was classified and registered.

Assay of Glutathione *S*-Transferase (GST). Liver homogenates were prepared by homogenizing tissue in 0.075 M NaCl containing 3 mM EDTA, pH 7.4, to obtain a 10% solution. The samples were immediately centrifuged (12000 rpm for10 min) at 4 °C to obtain the homogenate of liver tissue. The GST activity was determined spectrophotometrically according to the method of Dierickx (*18*). GST activity was measured using 0.5 mL of liver homogenate, 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM glutathione as substrates in a final volume of 1 mL of 0.1 M sodium phosphate buffer, pH 6.5, at 37 °C. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance−time curve at 340 nm for 5 min. Enzyme activity was expressed as micromoles per minute per milligram of protein. The protein content of liver homogenates was determined using a Bio-Rad protein assay kit.

Assay of Glutathione Peroxidase (GPx). The GPx activity was determined spectrophotometrically according to the method of Mohandas et al. (*19*). The following solutions were pipetted into a cuvette: 0.1 mL of homogenate and 0.8 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GSH reductase, and 1 mM GSH. This mixture was preincubated for 5 min at 37 °C. Thereafter, the overall reaction was initiated by adding 0.1 mL of prewarmed 2.5 mM H_2O_2, and then the decrease in absorbance from the slope of the initial linear portion of the absorbance−time curve at 340 nm was monitored for 5 min. The nonenzymic reaction rate was correspondingly assayed by replacing the homogenate sample by buffer. GP_X activity could be expressed as nanomoles of NADPH per minute per milligram of protein.

Assay of Glutathione Reductase (GR). The GR assay monitored the oxidation of NADPH consumed in the reduction of glutathione disulfide (GSSG) by the change in absorbance at 340 nm (*20*). The following solutions were pipetted into a 1 cm spectrophotometric cuvette: 0.1 mL of homogenate and 0.9 mL of 0.10 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH. This mixture was preincubated for 5 min at 37 °C. Then reactions were started by the addition of 0.1 mL of homogenate, and the decrease in absorbance from the slope of the initial linear portion of the absorbance−time curve at 340 nm was monitored for 5 min.

GSH/GSSG Ratio. A small portion of rat liver was removed for the GSH assay according to the method of Reed et al. (*21*). In brief, 100 μL of 10% perchloric acid (PCA) was added to liver homogenates (400 μL) as a precipitant of protein. The mixtures were vortex-mixed and centrifuged (4 °C, 15000 rpm for 15 min). The pellet was dissolved in 1 N NaOH to analyze protein content. The supernatants (200 μL) were decanted into other centrifuge tubes, treated with 20 μL of 100 mM iodoacetic acid (IAA), and then neutralized with an excess of sodium bicarbonate (dry powder, ~20 mg). The mixtures were allowed to react using a wave rotor for 15 min in the dark at room temperature to form the *S*-carboxymethyl derivative of GSH. A 0.2 mL volume of 3% 2,4-dinitrofluorobenzene (DNFB) was added to the reaction mixtures and was allowed to react in the dark at room temperature for 8 h to form the *N*-(2,4-dinitrophenyl) derivatives of *S*-carboxymethyl-GSH and GSSG. The mixtures were centrifuged (15000 rpm for 15 min), and aliquots of the supernatants were used for the determination of GSH and GSSG by HPLC analysis. The HPLC system consisted of a Hitachi l-6200 pump, a Hitachi D-2500 chromatointegrator, a Hitachi L-4200 UV−vis detector, and a 3-aminopropyl column (200 × 4 mm, 5 μm). The mobile phase consisted of (A) water/methanol (1:4, v/v) and (B) 2 M sodium acetate, pH 4.6/methanol (36:64, v/v). The 3-aminopropyl column was eluted by a 30 min gradient from 75% (A) to 5% and then isocratically maintained for 15 min at a flow rate of 1.2 mL/min and UV detection at 365 nm.

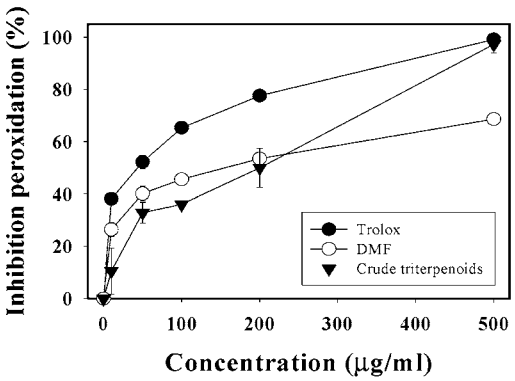

Figure 1. Antioxidant activities of Trolox as well as dry matter of filtrate (DMF) and its crude triterpenoids from *A. camphorata* in submerged culture (ACSC) as measured by conjugated diene formation in an AAPH/linoleic acid system at 20 min of incubation. Inhibition peroxidation % = $[1 - (A_{sample,234nm} - A_{blank,234nm})/(A_{control,234nm} - A_{blank,234nm})] \times 100$. Each value is the mean ± standard deviation (SD) ($n = 3$).

Statsistical Analysis. The values of mean and standard deviation (mean ± SD) and 95% confidence intervals (CI) of means to verify the statistical significance of all parameters were calculated. If necessary, data were tested by two-way ANOVA. p values of <0.05 were assumed to be statistically significant. All data are expressed as the means of three measurements.

RESULTS

Effect of DMF on Lipid Peroxidation. Figure 1 shows the antioxidant activities of DMF and its crude triterpenoids, as measured on the basis of conjugated diene formation in the AAPH/linoleic acid system and compared with Trolox. The inhibitory effect of DMF (54%) on lipid peroxidation was approximately equivalent to that of its crude triterpenoids but less than that of Trolox (78%) at the concentration of 0.20 mg/mL. As shown in **Figure 1**, there was a concentration-dependent increase in the antioxidant activity of DMF, and the inhibitory activity was 69% at a concentration of 0.50 mg/mL. Crude triterpenoids in DMF also showed a dose-dependent ($r = 0.97$) inhibition of AAPH-induced lipid peroxidation with an IC_{50} value of 0.21 mg/mL. The antioxidant activity of crude triterpenoids from DMF was equal to Trolox (99%) at the concentration of 0.50 mg/mL.

Cytotoxicity of DMF in HepG2 Cells. The cytotoxicity of DMF in HepG2 cells was evaluated on the basis of its effect on cell growth (MTT assay) and LDH leakage. The MTT assay showed that DMF exhibited no toxic effect on HepG2 up to a concentration of 0.50 mg/mL (**Table 1**). Consistent with the results of the MTT assay, cells treated with DMF at the concentration of 0.05−0.50 mg/mL for 24 h did not show an

Table 1. Cytotoxicity of Dry Matter of Filtrate (DMF) from *A. camphorata* in Submerged Culture (ACSC) on Cell Growth (MTT Test) and LDH Leakage in HepG2 Cell Line

concn (mg/mL)	cell viability[a] (%)	LDH leakage[b] (%)
control	100.0 ± 0.0	3.8 ± 0.8
0.05	97.2 ± 5.1	3.0 ± 1.2
0.10	94.3 ± 9.9	3.0 ± 0.3
0.20	108.5 ± 9.1	3.4 ± 0.6
0.50	99.6 ± 8.7	4.1 ± 0.6

[a] Cells were precultured in 96-well microplates (2×10^4 cells/well in 200 μL of complete MEM medium) for 12 h and then incubated with DMF for 24 h. [b] Cells were cultured in a 10-cm dish (4×10^6 cells/dish in 10 mL of complete MEM medium) for 12 h and then incubated with DMF for 24 h. Data are presented as means ± SD ($n = 6$). The percentage of cell growth in the control group was treated as 100%.

Table 2. Effect of Dry Matter of Filtrate (DMF) from *A. camphorata* in Submerged Culture (ACSC) on H_2O_2-Induced Lipid Peroxidation in HepG2 Cell Line

treatment	MDA (nmol/mg of protein)
control	0.55 ± 0.03
H_2O_2 (100 μM)	0.75 ± 0.01
DMF[a]: 0.05 mg/mL + H_2O_2	0.72 ± 0.10
0.10 mg/mL + H_2O_2	0.60 ± 0.03*
0.20 mg/mL + H_2O_2	0.52 ± 0.02**

[a] HepG2 cells were pretreated with various concentrations of DMF before the addition of H_2O_2 as described iunder Materials and Methods. *, $p < 0.05$; **, $p < 0.01$, compared with the group treated with H_2O_2 alone ($n = 4$).

Table 3. Relative Liver and Kidney Weights of CCl_4-Treated Rats with or without Gavage with Dry Matter of Filtrate (DMF; 0.10, 0.25, and 0.50 g/kg of Body Weight) from *A. camphorata* in Submerged Culture (ACSC) for 5 Days

	relative organ wt[a] (g/100 g of bw)	
treatment	liver	kidney
control (0.9% NaCl)	3.65 ± 0.20	0.88 ± 0.06
40% CCl_4 (0.1 mL/100 g of bw)	3.88 ± 0.42	0.77 ± 0.09
DMF: 0.10 g/kg of bw + 40% CCl_4[b]	4.02 ± 0.25	0.82 ± 0.17
0.25 g/kg of bw + 40% CCl_4	3.92 ± 0.30	0.80 ± 0.10
0.50 g/kg of bw + 40% CCl_4	3.85 ± 0.46	0.82 ± 0.11

[a] Data are presented as means ± SD from six rats ($n = 6$). [b] DMF was dissolved in 0.9% NaCl solution.

obvious increase in LDH leakage (**Table 1**). Therefore, DMF at the concentration of 0.05−0.20 mg/mL was used for the following cell culture experiments.

Effect of DMF on Lipid Peroxidation in HepG2 Cells Induced by H_2O_2. Lipid peroxidation has been recognized as a potential mechanism for cell injury. When HepG2 cells were exposed to 100 μM H_2O_2 alone, the concentration of MDA, an index of lipid peroxidation, was found to be increased. **Table 2** shows that pretreatment of DMF (0.10 and 0.20 g/mL) to the cells significantly decreased the formation of MDA ($p < 0.05$ and $p < 0.01$, respectively), as compared to the group treated with H_2O_2 alone. The results revealed that the inhibitory effect of DMF on H_2O_2-induced lipid peroxidation in HepG2 was in a dose-dependent manner (**Table 2**).

Effect of DMF on the Relative Organ Weight of Rats. Table 3 shows the relative liver and kidney weights of rats in each group. There is no significant difference in the relative liver and kidney weights of CCl_4-treated rats with or without gavage of DMF (0.10−0.50 g/kg of bw) for 5 consecutive days when compared with controls.

Table 4. Histological Injury Score of Liver under Different Doses of Dry Matter of Fermented Filtrate (DMF) from *A. camphorata* in Submerged Culture (ACSC)

	injury score[a]		
group	neutrophil infiltration	cell necrosis	hydropic degeneration
control (0.9% NaCl)	0	0	0
40% CCl_4 (0.1 mL/ 100 g of bw)	3	2	4
DMF: 0.10 g/kg of bw + 40% CCl_4[b]	2	1	2
0.25 g/kg of bw + 40% CCl_4	2	1	2
0.50 g/kg of bw + 40% CCl_4	0	0	1

[a] Livers were scored for hepatic injury via light microscopy: 0 = no visible cell damage; 1 = focal hepatocyte damage on <25% of the tissue; 2 = focal hepatocyte damage on <25–50% of the tissue; 3 = extensive, but focal, hepatocyte lesions; 4 = global hepatocyte necrosis. [b] DMF was dissolved in 0.9% NaCl solution.

Table 5. Effect of Dry Matter of Fermented Filtrate (DMF) from *A. camphorata* in Submerged Culture (ACSC) on Serum ALT and AST Activities and Hepatic MDA in Rats Treated with CCl_4[a]

treatment	ALT (units/L)	AST (units/L)	MDA (nmol/mg of protein)
control (0.9% NaCl)	196 ± 19	78 ± 19	0.95 ± 0.28
40% CCl_4 (0.1 mL/100 g of bw)	392 ± 81	298 ± 83	1.72 ± 0.47
DMF: 0.10 g/kg + 40% CCl_4	261 ± 75	94 ± 26*	1.38 ± 0.13
0.25 g/kg + 40% CCl_4	298 ± 60*	91 ± 21*	1.01 ± 0.18*
0.50 g/kg + 40% CCl_4	230 ± 36*	82 ± 10*	0.97 ± 0.18*

[a] Data are presented as means ± SD from six rats ($n = 6$). Asterisks indicate significant difference by comparison with the control group (CCl_4 alone), $p < 0.05$.

Pathological Histology of the Liver. Figure 2 shows that the treatment with CCl_4 caused neutrophil (inflammatory cell) infiltration, hydropic degeneration, and necrosis of hepatocytes in the portal region of rat livers. However, DMF pretreatment reduced the injury score of neutrophil infiltration as well as hydropic degeneration of the liver lesions and inhibited necrosis of liver cells (**Table 4**). Histological examination showed that the group pretreated with 0.50 g/kg of bw of DMF exhibited light hepatotoxicity (hydropic degeneration).

Effects of DMF on CCl_4-Induced Hepatotoxicity. Table 5 shows the effects of DMF on ALT and AST levels in serum and TBARS level in liver homogenate of rats treated with CCl_4. Similar to liver morphology, the acute hepatotoxicity reaction was significantly ($p < 0.05$) suppressed in all of the animals pretreated with 0.25 and 0.50 g/kg of bw of DMF. When a single dose of CCl_4 was given to rats by intraperitoneal (ip) injection, it caused elevations of serum AST and ALT and also an increased formation of MDA in liver (**Table 5**). DMF significantly decreased ($p < 0.05$) the activity of ALT and AST by 48 and 94%, respectively, when administered at a dosage of 0.25 g/kg of bw. As shown in **Table 5**, pretreatment with DMF at dosages of 0.10 and 0.25 g/kg of bw inhibited lipid peroxidation in liver homogenate of rat by 44 and 92%, respectively.

Effects of DMF on Hepatic GSH-Dependent Enzymes. Figure 3 shows the effects of DMF on hepatic GSH-dependent enzymes in CCl_4-induced liver damage of rats. The GSH-dependent enzyme activities, GST, GPx, and GR, in the rat liver from the CCl_4-induced damage group elicited 82, 50, and 86% reductions, respectively, compared to the untreated group. Pretreatment with DMF at a dose of 0.10 g/kg of bw for 5 consecutive days significantly increased the CCl_4-induced reduction of GSH-dependent enzyme activities in rat liver ($p < 0.05$). Liver GST, GPx, and GR activities of the untreated

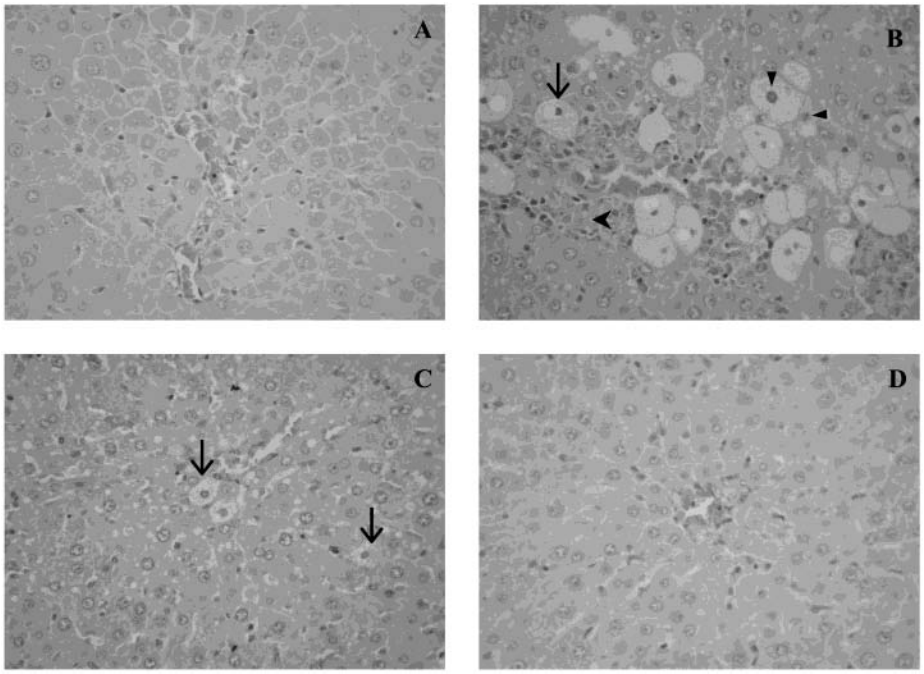

Figure 2. Effect of DMF from ACSC on CCl_4-induced liver damage of SD rats: (A) blank control group ($n = 6$); (B) animals treated with 40% CCl_4 (0.1 mL/100 g of bw) showed severe neutrophil infiltration (big arrowhead), hydropic degeneration (arrow), and necrosis (small arrowheads); (C) animals pretreated with 0.25 g/kg DMF and then with CCl_4 showed moderate neutrophil infiltration; (D) animals pretreated with 0.50 mg/kg DMF and then with CCl_4 showed more normal morphology than other CCl_4-treated groups; hematoxylin/eosin staining; magnification ×400.

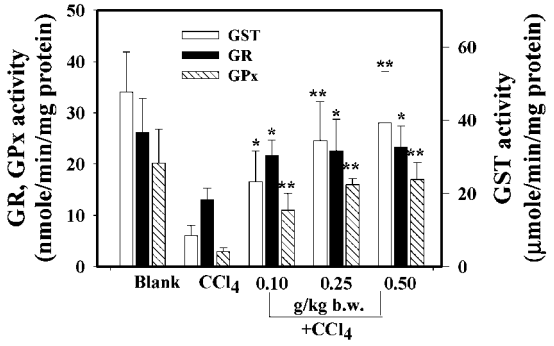

Figure 3. Effect of DMF from ACSC on hepatic GSH-dependent enzymes in CCl_4-induced liver damage of SD rats. GST, GSH *S*-transferase; GR, GSH reductase; GPx, GSH peroxidase. The data are presented as means ± SD from six rats ($n = 6$). Asterisks indicate significant difference by comparison with the control (CCl_4 alone) as determined by ANOVA: *, $p < 0.05$; **, $p < 0.01$. DMF was dissolved in 0.9% NaCl solution.

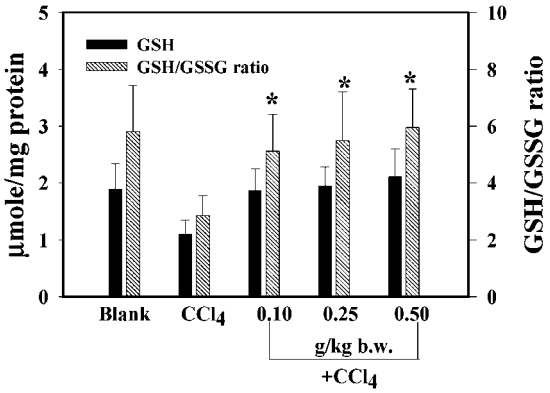

Figure 4. Effect of DMF from ACSC on the content of GSH and GSH/GSSG ratio in SD rat liver that was induced by CCl_4. Bars represent mean ± SD ($n = 6$). Values are significantly different by comparison with the control (CCl_4 alone), $p < 0.01$.

group did not show any significant difference ($p > 0.05$) in response to diet supplementation of 0.50 g/kg of bw of DMF.

Effects of DMF on GSH/GSSG Ratio. GSH is an important cellular antioxidant, and it plays a major role in protecting cells against oxidative stress. **Figure 4** shows the effect of DMF on the content of GSH and on the GSH/GSSG ratio in rat liver that was induced by CCl_4. The CCl_4-treated group showed a significantly reduced GSH level and GSH/GSSG ratio in the rat liver ($p < 0.01$). However, the groups with DMF pretreatment had the harmful effect of CCl_4 reduction. The GSH level and GSH/GSSG ratio in the group treated with 0.10 g/kg of bw of DMF was significantly higher than the group treated with CCl_4 alone, and there were no significant differences compared to the untreated group ($p > 0.01$).

DISCUSSION

Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanisms of their actions. To clarify the mode of action of DMF, in vitro lipid peroxidation experiments were carried out first. Our experimental results show that DMF and its crude triterpenoids had a strong scavenging activity on peroxyl radical ($ROO^{\bullet}$) generated in an AAPH/linoleic acid system (**Figure 1**). Moreover, DMF inhibited the H_2O_2-induced lipid peroxidation in HepG2 (**Table 2**). Therefore, DMF may have a protective effect against CCl_4-induced hepatotoxicity in rats by scavenging free radicals formed during CCl_4 metabolism and by enhancing the level of antioxidant defense systems to inhibit lipid peroxidation of liver cells.

Triterpenoid compounds, which are widely distributed in *Ganoderma*, were previously considered to play an important role for hepatoprotective, anti-HIV, and antihypertension activities and the prevention of platelet aggregation and histamine release in different model systems (*22*). In addition, Shiao et al. (*23*) found that ganoderic acid and ganodermic acid of *G. lucidum* (a type of triterpenoids) exhibited inhibitory activity

on cholesterol synthesis. In addition, Zhu et al. (*24*) reported that triterpenoid isolated from mushrooms exhibited effective antioxidant activity against lipid peroxidation in liver mitochondria induced by Fe^{2+}−ascorbic acid. Our previous study showed that the TEAC value of crude triterpenoids in DMF was ~3.0 mM (*25*). As shown in **Figure 1**, the inhibitory effect of crude triterpenoids in DMF on lipid peroxidation was in a dose−response manner. Thus, we suggested that crude triterpenoids might be important antioxidants in DMF. However, the mode of antioxidant action needs further investigation.

Although the liver has a relatively rich supply of antioxidants, an increase of lipid peroxidation was observed when hepatocytes were exposed to CCl_4. CCl_4 is a classical hepatotoxicant that causes acute, reversible liver injury characterized by centrilobular hydropic degeneration and necrosis. Injury results initially from the metabolism of CCl_4 to $CCl_3^{\bullet}$, which initiates lipid peroxidation and covalently binds to essential macromolecules due to dysfunction of Ca^{2+}-ATPases and ATP depletion. Subsequently, this causes a rise in intracellular Ca^{2+} and H_2O to result in cell hydropic degeneration and necrosis. Necrosis of hepatocytes as indicated by the presence of pyknotic nuclei occurred frequently around the central vein as compared with the control. Secondary liver injury occurs from inflammatory processes initiated by the activation of kupffer cells, which release chemoattractants and activators of neutrophils (*26*). Thus, antioxidant activity and the inhibition of free radical generation are important in terms of protecting the liver from CCl_4-induced damage (*27*).

Zhu and Fung (*28*) also found that the ALT level in serum, as well as the level of TBARS in liver homogenate increased significantly in a manner both dose dependent and time dependent after CCl_4 treatment. Therefore, several hepatic enzymes in serum such as ALT and the level of TBARS in liver homogenate were used as the biochemical markers for early acute hepatic damage. The results of the present study demonstrate that pretreatment of rats with DMF had a markedly protective effect against CCl_4-induced hepatotoxicity in rats, as evidenced by decreased serum ALT and AST activities and lipid peroxidation (**Table 5**). Moreover, these protective effects were found to be dose dependent. DMF pretreatment at doses from 0.10 to 0.50 g/kg was found to significantly decrease CCl_4-induced hepatotoxicity. This phenomenon was confirmed by histological observation (**Figure 2**; **Table 4**).

GSH acts as an antioxidant both intracellularly and extracellularly in conjunction with various enzymatic processes that reduce hydrogen peroxide and hydroperoxide by oxidizing GSH to GSSG and other mixed disulfides. The ratio of GSH to GSSG would be expected to be a more sensitive marker of oxidative stress, because small increases in GSSG and decreases in GSH can appear to be more amplified by examining the ratio than by measuring either one separately (*29*). In addition, the GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. This system consists of GSH and an array of functionally related enzymes, of which GR is responsible for the regeneration of GSH, whereas GPx and GST work together with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides (*30*). Because GST increases the solubility of hydrophobic substances, it also plays an important role in the storage and excretion of xenobitics. Compounds that increase the activity of GST, which metabolizes toxic compounds to nontoxic, protect the liver. Thus, the levels of GSH and activities of the GPx, GST, and GR were used to monitor the peroxidative balance (*31*). Furthermore, induction of the hepatic GSH antioxidant system by chemopreventive agents has been reported in several studies (*31*, *32*).

Impaired activity of GST, GPx, and GR with increased lipid peroxidation and a marked decrease in the GSH/GSSG ratio have been observed in the liver of CCl_4-intoxicated rats (**Figures 3** and **4**; **Table 5**). Enhanced lipid peroxidation in rat liver reflects an excessive generation of free radicals exacerbated by a deficiency of hepatic antioxidant and detoxification systems. In contrast, reduced lipid peroxidation is associated with a significantly greater increase of the GSH-dependent enzymes and with a concomitant increase in the GSH/GSSG ratio in rats with oral pretreatment of DMF (**Figures 3** and **4**; **Table 5**). This is probably due to the decrease in the bioactivation of CCl_4 by pretreatment with DMF.

In conclusion, this in vitro experiment showed that DMF exhibited free radical scavenging activity in an AAPH/linoleic acid system. In addition, HepG2 pretreated with DMF significantly decreased the lipid peroxidation induced by H_2O_2. Administration of DMF ameliorated the effects of CCl_4-induced damages in hepatic lipid peroxidation and reduced GSH-dependent enzymes activity in rat liver. Our findings suggest that DMF might inhibit CCl_4-induced hepatotoxicity by upregulating hepatic GSH-dependent enzymes to preserve the normal GSH/GSSH ratio and free radical scavenging effect, thereby protecting against CCl_4-induced oxidative damage. Therefore, we suggest that DMF might be effective in lowering oxidative damage in living systems.

ABBREVIATIONS USED

ACSC, *Antrodia camphorata* in submerged culture; DMF, dry matter of filtrate; CCl_4, carbon tetrachloride; GSH, (reduced) glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; GR, glutathione reductase; GPx, glutathione peroxidase; MDA, malondialdehyde; TBARS, thiobarbituric acid reaction substances; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TEAC, Trolox equivalent antioxidant capacity.

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