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Hepatoprotective Effects of Apple Polyphenols on CCl_4-Induced Acute Liver Damage in Mice

Jingyu Yang, Yan Li, Fang Wang, and Chunfu Wu*

Department of Pharmacology, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China

In this study, the hepatoprotective effects of apple polyphenols (AP, Appjfnol) against CCl_4-induced acute liver damage in Kunming mice as well as the possible mechanisms were investigated. Mice were treated with AP (200, 400, and 800 mg/kg, ig) for seven consecutive days prior to the administration of CCl_4 (0.1%, intraperitoneally). The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH) concentrations in the hepatic homogenate, and histopathological changes in mouse liver sections were determined. Levels of ferrous sulfate-L-cysteine ($FeSO_4$-L-Cys)-induced lipid peroxidation and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were also determined in vitro. AP significantly prevented the increase in serum ALT and AST levels in acute liver injury induced by CCl_4 and produced a marked amelioration in the histopathological hepatic lesions coupled to weight loss. The extent of MDA formation was reduced; the SOD activity was enhanced, and the GSH concentration was increased in the hepatic homogenate in AP-treated groups compared with the CCl_4-intoxicated group. AP also exhibited antioxidant effects on $FeSO_4$-L-Cys-induced lipid peroxidation in rat liver homogenate and DPPH free radical scavenging activity in vitro. These results indicate that AP has a significant protective effect against acute hepatotoxicity induced by CCl_4 in mice, which may be due to its free radical scavenging effect, inhibition of lipid peroxidation, and its ability to increase antioxidant activity.

KEYWORDS: Apple polyphenols (AP); hepatoprotective effects; CCl_4; antioxidant activity; lipid peroxidation

INTRODUCTION

Liver disease is a serious health problem because the liver is an important organ for the biotransformation and detoxification of endogenous and exogenous harmful substances. Steroids, vaccines, and antiviral drugs, which have been used to treat liver diseases, have potential adverse effects, especially when administered long-term (*1*). It is, therefore, necessary to develop new more effective and safer drugs for the treatment of liver disease.

It is well-known that free radicals cause cell damage through mechanisms involving covalent binding to macromolecules and lipid peroxidation with subsequent tissue injury (*2*), especially liver injury (*3*, *4*). It has been reported that a number of polyphenolic compounds extracted from food or fruit can protect against liver injury, because they exhibit one or a combination of antioxidant, antifibrotic, immunomodulatory, or antiviral activities (*5*, *6*). In recent years, therefore, there has been a substantial increase in the use of so-called complementary dietary polyphenols and alternative therapies to treat patients with liver disease.

Apples have been part of the human diet since ancient times, and they are one of the most commonly consumed fruits worldwide. The main classes of polyphenols in apple are flavonoids, including quercetin, (−)-epicatechin and (+)-catechin, procyanidins, and anthocyanidins; dihydrochalcones such as phloretin and phloridzin; and other polyphenolic compounds such as chlorogenic acid (*7*). A number of studies have shown that apple polyphenols (AP) have a variety of pharmacological functions, including antioxidant (*8*) and antiallergic (*9*) activities, with little adverse effect (*10*). However, there is no scientific report available to support the hepatoprotective activity of AP as a potential source of natural polyphenols.

Liver injury induced by CCl_4 is the most intensively studied system for xenobiotic-induced oxidative hepatotoxicity (*11*). It is well-known that alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum enzyme activities served as parameters to demonstrate the extent of hepatotoxicity in the mice. Furthermore, the increase in the level of lipid peroxidation, the decrease in SOD activity, and the breakdown of the GSH-dependent antioxidant defense system are obviously seen along with the liver damage induced by CCl_4 (*12*,*13*). Therefore, the aim of this study was to investigate the protective effects of AP on CCl_4-induced hepatic damage, including the effects of AP on the biochemical determinations of the levels of serum ALT and AST and the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH) in liver homogenate

*To whom correspondence should be addressed: Department of Pharmacology, Shenyang Pharmaceutical University, Wenhua Rd. 103, Shenyang 110016, P. R. China. Telephone and fax: +86 24 23843567. E-mail: wucf@syphu.edu.cn or chunfuw@gmail.com.

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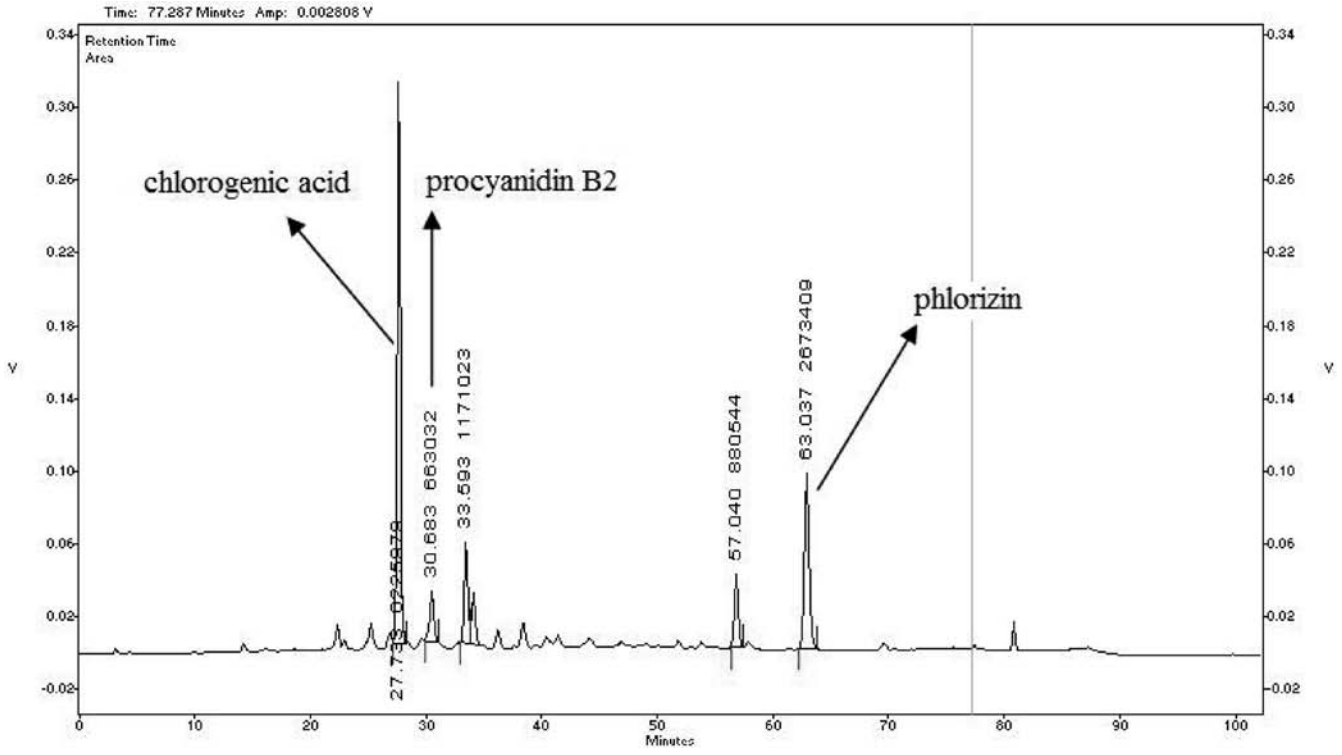

Figure 1. Profiles of apple polyphenol extract determined by reversed phase HPLC.

combined with hepatic histopathological observations in vivo and its free radical scavenging activity in vitro.

MATERIALS AND METHODS

Polyphenol Extraction. For the preparation of apple polyphenols (AP, Appjfnol), apple pericarp was extracted twice with 30−50% ethanol for 2 h under reflux and was filtered with a stainless steel wire sieve. The filtrate was concentrated in vacuo and then subjected to polyamide column chromatography (14−30 mesh) with an EtOH/H_2O solvent system. The ethanol eluant was collected and concentrated between 50 and 80 °C and with a degree of vacuum between −0.04 and 0.08 MPa. Then it was spray-dried to powder at an entrance temperature of 150−195 °C. The products were mixed to ensure they were uniform, and the impurities were eliminated by 60−100 mesh sieves.

Polyphenolic Content and HPLC Analysis. The total phenolic content was determined according to the method described by Singleton et al. (*14*). The sample was accurately weighed and dissolved in a 100 mL brown volumetric flask with distilled water by ultrasound. The standard solution of catchin and chlorogenic acid (0.04 and 0.06 mg/mL, respectively) was prepared. Both of these solutions were filtered through a 0.45 μm syringe membrane filter. The standard and the sample (0.40 mL each) were mixed with the Folin-Ciocalteu phenol reagent (0.5 mL) and Na_2CO_3 [1.5 mL, 20% (w/v)]. The absorbance at 760 nm was measured 20 min after incubation at 30 °C using a Cary ultraviolet−visible spectrophotometer (Varian). The amount of total phenolics was calculated.

HPLC separation of polyphenols was performed by reversed phase chromatography on a 250 mm × 4.6 mm C_{18} 5 mm column (Kromasil), using an L-6200 intelligent pump (Hitachi Ltd.) equipped with an L-4200 UV−vis detector (Hitachi) fixed at 280 nm. The column was eluted at a flow rate of 1.0 mL/min with an acetonitrile/water/glacial acetic acid (80:19.6:0.4) mixture (A) and acetonitrile (B) as the mobile phase; the gradient was changed as follows: 100% A from 0 to 3 min, 96% A from 3 to 6 min, 90% A from 6 to 15 min, 85% A from 15 to 30 min, 77% A from 30 to 50 min, 75% A from 50 to 60 min, 70% A from 60 to 66 min, 50% A from 66 to 80 min, 20% A from 80 to 83 min, 100% A from 83 to 85 min, and 100% A from 85 to 105 min. A 10 μL sample was analyzed. The main *o*-diphenols were identified on the basis of the retention times of authentic standard references: chlorogenic acid, procyanidin B2, EGCG, and phlorizin. The polyphenol profiles of AP analyzed by reversed phase HPLC are shown in **Figure 1**. The total amount of polyphenols in Appjfnol was 81.29%. It contained 17.08% chlorogenic acid, 7.31% procyanidin B2, 5.17% phloridzin, and < 0.2% EGCG. The extract specifications are summarized in **Table 1**.

Animals. Kunming mice of both sexes (weighing 20−25 g) and male Wistar rats (weighing 200−220 g), obtained from the Experimental Animal Center of Shenyang Pharmaceutical University, were used. They were allowed free access to water and food [composed of 40% corn flour, 26% wheat flour, 10% bran, 10% fish meal, 10% bean cake, 2% mineral, 1% coarse, and 1% vitamin complex (Qianmin Feed Factory)]. All the animals were housed in a room maintained at a temperature of 23 ± 3 °C and a relative humidity of 50 ± 10% with artificial lighting from 8:00 a.m. to 8:00 p.m. for 1 week before and during the experiments. All the experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

Chemicals. The following agents and reagents used were obtained from the sources indicated: Bifendate Pills (BP, Zhejiang Medicine Co., Ltd., Xinchang Pharmaceutical Factory); CCl_4 (Chongqing Chuandong Chemical Co., Ltd.); bean oil and diagnostic kits for alanine aminotransferase (ALT, Beijing BHKT Clinical Reagent Co., Ltd.); aspartate aminotransferase (AST) (Changchun Huili Biotech Co., Ltd.); malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH) (Nanjing Jiancheng Bioengineering Institute); thiobarbituric acid (TBA) (Sigma Chemical Co.); L-Cys, ascorbic acid (AA), $FeSO_4$, and DPPH free radical (Sigma Chemical Co.); and absolute ethanol (99.5%). All other chemicals and solvents were of analytical grade and commercially available.

CCl_4-Induced Hepatotoxicity Model (15). Mice were randomly divided into six groups of 10 animals (five males and five females) each. In the control group and CCl_4-intoxicated group, animals were given a single dose of distilled water (0.2 mL/10 g, ig) daily. In the Bifendate Pills (BP) group, animals received reference drug BP (200 mg/kg, 0.2 mL/10 g, ig) once daily. In the test groups, animals were given 200, 400, and 800 mg of AP/kg (0.2 mL/10 g, ig) once daily. All administrations were conducted for seven consecutive days. On the seventh day, all mice except those in the control group were given simultaneously a CCl_4/bean oil mixture (0.1:100, intraperitoneally) 1 h after the last administration, while the control group received bean oil alone. Then all the animals were fasted for 16 h and were subsequently tested for the following analysis.

Table 1. Specifications of Apple Polyphenols

parameter	specification	method
total amount of procyanidins	80 g/100 g	UV−vis spectrophotometry
amount of phlorizin	4.0−8.0 g/100 g	reversed phase HPLC
amount of chlorogenic acid	15.0−20.0 g/100 g	reversed phase HPLC
amount of procyanidin B2	≥4.0 g/100 g	reversed phase HPLC
amount of EGCG	≤0.4 g/100 g	reversed phase HPLC
amount of ash	0.4%	ignition at 550 °C
amount of total heavy metals as lead	<10 mg/kg	sodium sulfide colorimetric method
amount of arsenic	<0.3 mg/kg	atomic absorption spectroscopy
microbiological analysis, total plate count	<1000 colony-forming units (CFU)/g	GB/T4789.2-2008
yeast and mold	<25 CFU/g	GB/T4789.15-2008
coliform	≤40 MPN/100 g	GB/T4789.3-2008
pathogenic bacterium	not found	GB/T4789.4,5,10-2008

Table 2. Effect of AP on Body Weight Gain in Mice[a]

		body weight (g)						
group	dose (mg/kg)	day 1	day 2	day 3	day 4	day 5	day 6	day 7
AP	0	23.78 ± 0.61	26.10 ± 0.54	27.14 ± 0.60	26.70 ± 0.54	28.38 ± 0.74	28.84 ± 0.75	28.44 ± 0.82
	200	24.28 ± 0.64	25.98 ± 0.58	25.83 ± 0.62	25.61 ± 0.73	25.86 ± 0.79[c]	25.80 ± 0.83[c]	25.16 ± 0.69[c]
	400	23.42 ± 0.50	25.11 ± 0.49	25.52 ± 0.74[b]	25.47 ± 0.87	25.50 ± 0.76[c]	25.93 ± 0.86[c]	26.17 ± 0.76[b]
	800	23.11 ± 0.38	24.81 ± 0.46[b]	24.75 ± 0.52[c]	24.48 ± 0.59[b]	25.32 ± 0.70[c]	25.05 ± 0.76[c]	25.06 ± 0.59[c]
BP	200	22.57 ± 0.76	24.63 ± 0.70[b]	25.43 ± 0.77[b]	26.00 ± 0.81	26.83 ± 0.83	27.16 ± 0.86	27.28 ± 0.95

[a] Animals were given orally either AP (0, 200, 400, or 800 mg/kg) or Bifendate Pills (BP) (200 mg/kg) once daily for 7 consecutive days. The body weight was monitored each day. Values are expressed as means ± the standard error of the mean of 10 mice in each group (except that there were 20 mice in the group treated with 0 mg of AP/kg because it contained the control and CCl_4-intoxicated group). [b] $P < 0.05$. [c] $P < 0.01$ compared with the control group.

Effect of AP on Body Weight. During the whole experiment, the general condition and body weight were monitored.

ALT and AST Assays for Monitoring Liver Function. Blood was taken for analysis of the serum ALT and AST levels, which were determined by the method of Reitman and Frankel (*16*) with commercially available diagnostic kits.

Determination of MDA, GSH, and SOD Activity. Livers were excised immediately after the animals were sacrificed. The livers, except a portion of the left lobe to be used for histopathological sections, were frozen quickly and stored at −80 °C until the preparation of hepatic homogenates for the determination of levels of MDA, SOD, and GSH.

Levels of MDA, GSH, and SOD in the liver homogenate were estimated using commercially available diagnostic kits. The level of MDA, a measure of lipid peroxidation, was measured by the thiobarbituric acid (TBA) reaction method. The nonprotein liver GSH level was measured by the method of Moron et al. (*17*). The activity of SOD was measured by the method of McCord (*18*). The protein concentration was measured by the method of Lowry.

Histopathological Examinations. A portion of the left lobe of the liver was preserved in a 10% formalin solution for histopathological sections. The fixed tissues were embedded in paraffin; sections 3−5 μm thick were obtained, deparaffinized, dehydrated in ethanol (50−100%), and cleared with xylene. The extent of CCl_4-induced damage, including cell necrosis, steatosis, hyaline regeneration, and ballooning degeneration, was evaluated by assessing the morphological changes in liver sections stained with hematoxylin and eosin (H&E) under an Olympus light microscope. The images were examined with an Olympus BX50 light microscope with corresponding magnification. The data were analyzed by the image analysis system MetaMorph Offline (UIC/OLYMPUS).

$FeSO_4$-L-Cys-Stimulated Lipid Peroxidation Assay in Vitro (15). The liver homogenate of a male rat was used. The protein content was determined using the Lowry method. The reaction mixture, composed of 1 mL of liver homogenate [10% (w/v)], 1 mL of a solution with various AP concentrations, 0.05 mL of 1 mM $FeSO_4$, and 0.02 mL of 10 mM L-Cys, was incubated for 30 min at 37 °C. Then it was mixed with 1 mL of 0.67% TBA and 2 mL of a 20% trichloroacetic acid solution and heated in a boiling water bath for 10 min. Ascorbic acid (AA) was taken as a reference drug. After the mixture had cooled under running water, the absorbance was measured at 532 nm.

DPPH Free Radical Scavenging Activity (19). The free radical scavenging activity of AP was measured using the stable radical DPPH. A solution of DPPH (0.1 mM) in ethanol was prepared, and 900 μL of this solution was added to 100 μL of an AP solution at different concentrations (10^{-6} to 10 mg/mL). The resulting solution was thoroughly mixed by vortexing and left in the dark, and then the absorbance was measured at 517 nm at different times until the reaction reached a steady state. AA was used as a reference drug. The scavenging activity was determined by comparing the absorbance with that of the blank (0%) which contained only DPPH and solvent. When the reaction reached a plateau, the AP IC_{50} value was calculated from the results.

Statistical Analysis. The data are expressed as means ± the standard error of the mean. Differences among means were analyzed by one-way ANOVA. A $P < 0.05$ value was considered statistically significant (SPSS version 13.0, SPSS).

RESULTS

Effect of AP on the Increase in Body Weight. The changes in mouse body weight during the 7 day treatment with AP are summarized in **Table 2**. The amount of diet intake was not reduced during the AP treatment (data not shown). Mouse body weights were significantly suppressed on day 5 ($P < 0.01$), day 3 ($P < 0.05$), and day 2 ($P < 0.05$) after the treatment with 200, 400, and 800 mg of AP/kg, respectively. BP (200 mg/kg), the positive control, showed a slight suppressive effect only on days 2 and 3.

Effects of AP on the Serum ALT and AST Activity. The results of the hepatoprotective effect of AP on the serum ALT and AST activity are shown in **Figure 2**. In the CCl_4-intoxicated group, serum ALT and AST activities were 289 ($P < 0.001$) and 138 units/L ($P < 0.001$), respectively, whereas these values were only 65 and 54 units/L, respectively, in the control group. These data indicated that CCl_4 significantly increased both of these two enzyme activities. Moreover, the elevated levels of serum ALT and AST were significantly reduced ($P < 0.05$) in the groups pretreated with AP (400 and 800 mg/kg), and this reduction was dose-dependent.

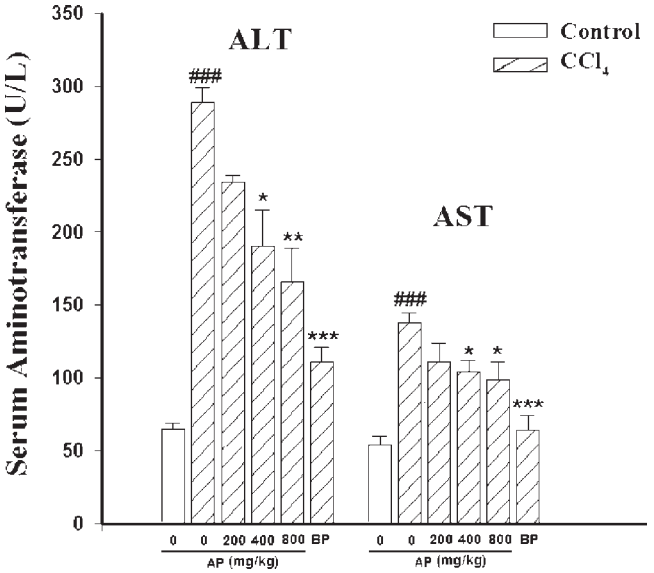

Figure 2. Effects of AP on serum ALT and AST activity after CCl_4 treatment in mice. Animals were given orally either AP (0, 200, 400, or 800 mg/kg) or Bifendate Pills (BP) (200 mg/kg) once daily for 7 consecutive days prior to the single administration of CCl_4 (0.1%, ip), and then serum ALT and AST activities were determined. Values are expressed as means ± the standard error of the mean of 10 mice in each group. ###$P < 0.001$, compared with the control group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, compared with the CCl_4-intoxicated group.

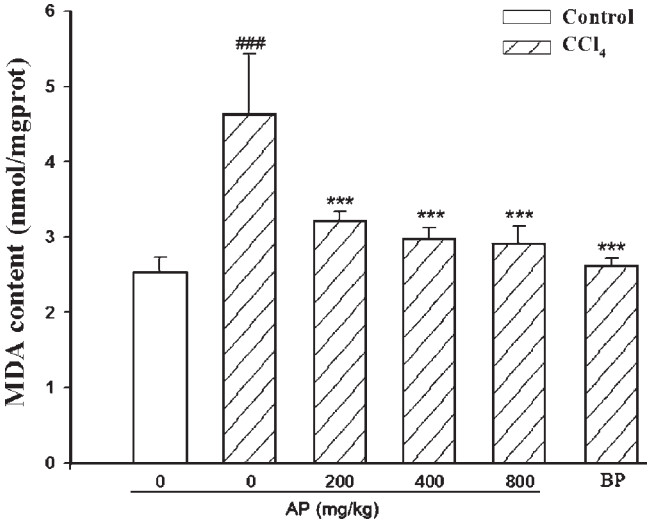

Figure 3. Effects of AP on the hepatic MDA content after CCl_4 treatment in mice. Animals were given orally either AP (0, 200, 400, or 800 mg/kg) or Bifendate Pills (BP) (200 mg/kg) once daily for 7 consecutive days prior to the single administration of CCl_4 (0.1%, ip), and then the levels of MDA in the liver homogenate were measured by the thiobarbituric acid (TBA) reaction method. Values are expressed as means ± the standard error of the mean of 10 mice in each group. ###$P < 0.001$, compared with the control group. ***$P < 0.001$, compared with the CCl_4-intoxicated group.

Effects of AP on Hepatic MDA, GSH, and SOD Activity. Lipid peroxidation is considered to be one of the principal causes of CCl_4-induced liver injury (*2*). Pretreatment of mice with AP effectively inhibited CCl_4-induced hepatotoxicity, as shown by the reduced level of hepatic MDA formation ($P < 0.001$); an index of the chain reaction of lipid peroxidation, this was found to be dose-dependent (**Figure 3**).

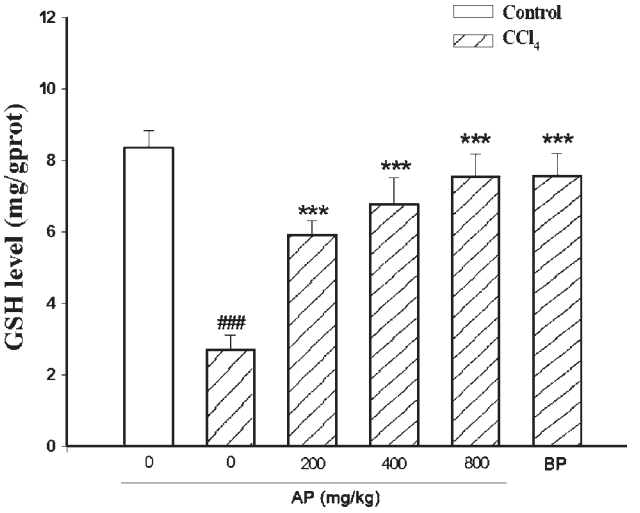

Figure 4. Effects of AP on the hepatic GSH level after CCl_4 treatment in mice. Animals were given orally either AP (0, 200, 400, or 800 mg/kg) or Bifendate Pills (BP) (200 mg/kg) once daily for 7 consecutive days prior to the single administration of CCl_4 (0.1%, ip), and then the GSH level in the liver homogenate was measured. Values are expressed as means ± the standard error of the mean of 10 mice in each group. ###$P < 0.001$, compared with the control group. ***$P < 0.001$, compared with the CCl_4-intoxicated group.

SOD is an extremely effective antioxidant enzyme. The increased production of free radicals caused by administration of CCl_4 is a major cause of the significantly reduced SOD activity. GSH is a nonenzymatic antioxidant and is the most important biomolecule for combating chemically induced toxicity. The data showed that the SOD activity significantly decreased ($P < 0.05$) and the GSH stores are markedly depleted ($P < 0.001$) in mice treated with CCl_4 compared with the control group. Pretreatment with AP significantly increased SOD activity ($P < 0.01$) (**Figure 4**) and reduced the extent of CCl_4-induced hepatic GSH depletion ($P < 0.001$) (**Figure 5**).

Histopathological Observations. Histological assessment was used to complete the study of the hepatoprotective effects of AP on CCl_4-induced acute liver damage (**Figure 6**). The histology of the liver sections of control animals showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins. The liver sections of CCl_4-intoxicated mice revealed extensive liver injuries, characterized by moderate to severe hepatocellular degeneration and necrosis around the central vein, fatty changes, inflammatory cell infiltration, ballooning degeneration, and the loss of cellular boundaries. However, the histopathological hepatic lesions were markedly ameliorated by pretreatment with AP. This was in good agreement with the results of serum aminotransferase activity (**Figure 2**) and hepatic oxidative stress levels (**Figures 3**−**5**).

Effects of AP on $FeSO_4$-L-Cys-Induced Lipid Peroxidation. In vitro lipid peroxidation in a liver homogenate can proceed in a nonenzymatic way. It has been reported that L-Cys in the presence of Fe^{2+} stimulated lipid peroxidation in rat liver microsomes and mitochondria. To clarify the mode of action of AP, we conducted in vitro lipid peroxidation experiments. Consistent with the results of CCl_4-induced hepatic lipid peroxidation (**Figure 3**), AP produced a concentration-dependent inhibition of the $FeSO_4$-L-Cys-stimulated lipid peroxidation with an IC_{50} value of 0.11 mg/mL in liver homogenate (Figure.7). These results show that AP inhibits lipid peroxidation both in vivo and in vitro.

Effects of AP on DPPH Scavenging Activity. The results (**Figure 7**) show a significant reduction in the absorbance of the

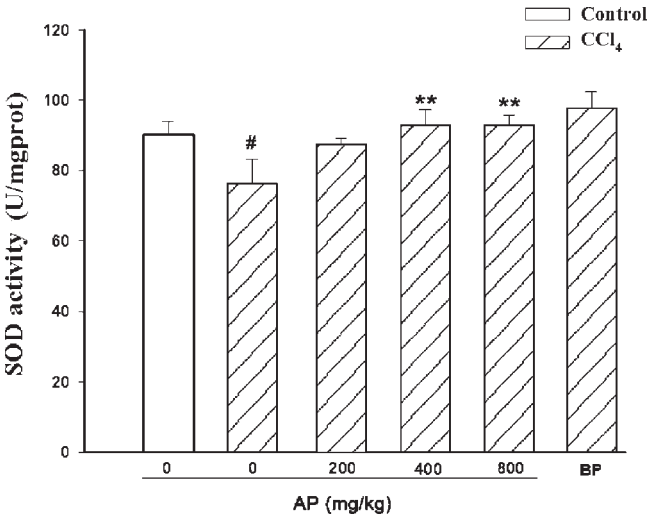

Figure 5. Effects of AP on the hepatic SOD activity after CCl_4 treatment in mice. Animals were given orally either AP (0, 200, 400, or 800 mg/kg) or Bifendate Pills (BP) (200 mg/kg) once daily for 7 consecutive days prior to the single administration of CCl_4 (0.1%, ip), and then the activity of SOD in the liver homogenate was measured. Values are expressed as means ± the standard error of the mean of 10 mice in each group. #$P < 0.05$, compared with the control group. **$P < 0.01$, compared with the CCl_4-intoxicated group.

reaction mixture which is both concentration-dependent and time-dependent. It also shows that a lower absorbance indicates a higher free radical scavenging activity. We found that 10^{-4} to 10 mg/mL AP possessed strong DPPH free radical scavenging activity. AP had an IC_{50} value of 2.46×10^{-3} mg/mL when the reaction reached a plateau (60 min), while AA had an IC_{50} value of 2.26 mg/mL (**Table 3**).

DISCUSSION

Hepatotoxicity induced by CCl_4 is the most commonly used model system for the screening of hepatoprotective activity of plant extracts and drugs (*11*). In this study, a significant increase in the level of AST and ALT in the serum was observed after administration of CCl_4, as reported previously (*12*, *20*). However, the increased levels of these enzymes were significantly decreased by pretreatment with AP, implying that the extract prevents liver damage, which is further confirmed by the reduced amount of histopathological injury.

Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by the free radical derivatives of CCl_4 and is responsible for cell membrane damage and the consequent release of marker enzymes of hepatotoxicity. In this study, significantly elevated levels of MDA, products of membrane lipid peroxidation, observed in CCl_4-treated mice indicated hepatic damage. Pretreatment of AP prevented lipid peroxidation which could be attributed to the radical scavenging antioxidant constituents.

The coordinate actions of various cellular antioxidants in mammalian cells are critical for the effective detoxification of free radicals. Among the cellular antioxidants, SOD has been studied extensively. SOD catalyzes the dismutation of the superoxide anion to H_2O_2 and O_2. Administration of CCl_4 to mice decreased the antioxidant capacity of mouse liver as evidenced by the decreased activity of the antioxidant enzymes, which is in agreement with earlier reports. AP pretreatment prevented the reduction in the antioxidant enzyme activities and the consequent oxidative damage to the liver. In fact, the multiple-dose pretreatment of AP alone significantly boosted the antioxidant enzyme activities (**Figure 8**).

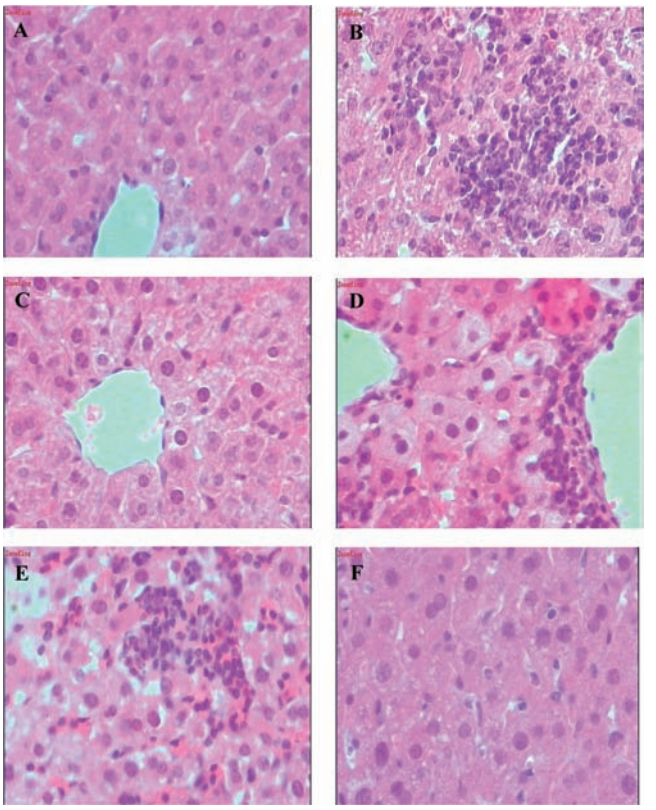

Figure 6. Effects of AP on the liver histological damage after CCl_4 treatment in mice. Animals were given orally either AP (0, 200, 400, or 800 mg/kg) or Bifendate Pills (BP) (200 mg/kg) once daily for 7 consecutive days prior to the single administration of CCl_4 (0.1%, ip), and then a portion of the left lobe of the liver tissues stained with hematoxylin and eosin (H&E) was used for histological assessment under a microscope. Representative photographs of liver sections stained with HE showing the pathological changes in hepatic tissues under microscopy (400×): (**A**) control group, (**B**) CCl_4-intoxicated group, (**C**) BP (200 mg/kg) and CCl_4 group, (**D**) AP (200 mg/kg) and CCl_4 group, (**E**) AP (400 mg/kg) and CCl_4 group, and (**F**) AP (800 mg/kg) and CCl_4 group.

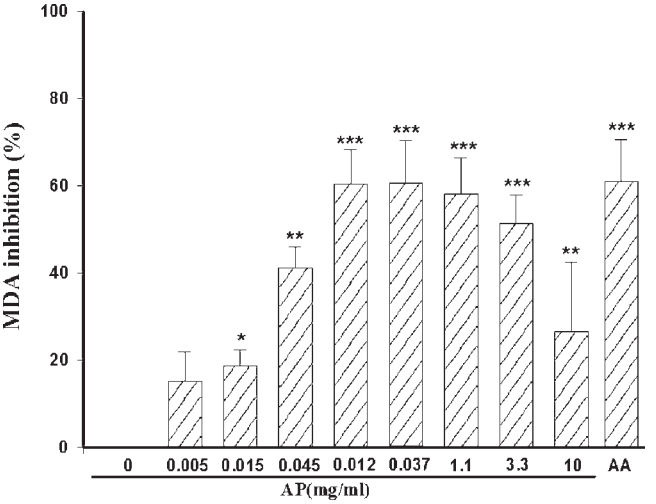

Figure 7. Effects of AP on MDA formation during liver microsomal lipid peroxidation induced by $FeSO_4$-L-Cys in vitro. Each value is expressed as the mean ± the standard error of the mean of three replicate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, compared with the control containing solvent only.

Table 3. Effects of AP on DPPH Radical Scavenging Activity (60 min)[a]

group	concentration	scavenging activity (%)	IC_{50} (mg/mL)
control	−	0	
AP	10^{-6} mg/mL	27.22	2.46×10^{-3}
	10^{-5} mg/mL	39.65	
	10^{-4} mg/mL	40.83[b]	
	10^{-3} mg/mL	47.63[b]	
	10^{-2} mg/mL	51.18[b]	
	10^{-1} mg/mL	63.31[b]	
	1 mg/mL	95.56[b]	
	10 mg/mL	94.67[b]	
AA	1 μM	9.54	2.26
	10 μM	38.21	
	100 μM	90.29[b]	

[a] Ascorbic acid (AA) was used as a reference drug. Each value is the mean of three replicate experiments. [b] $P < 0.05$, compared with the control containing DPPH and solvent only.

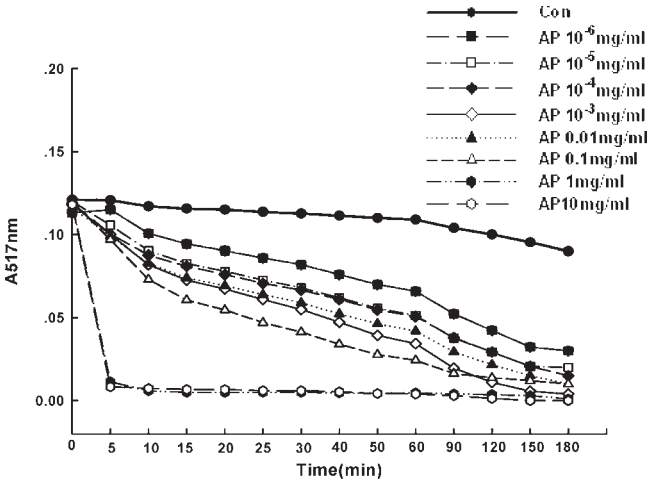

Figure 8. Kinetic behavior of AP on DPPH radical scavenging activity. The scavenging activity was determined by comparison of the absorbance with that of the blank (0%) that contained only DPPH and solvent. When the reaction reached a plateau, the AP IC_{50} value was calculated from the results.

GSH is the major nonenzymatic antioxidant and regulator of intracellular redox homeostasis, ubiquitously present in all cell types (*13*). Mechanistic studies on CCl_4-induced studies reveal that GSH conjugation plays a critical role in eliminating the toxic metabolites, which are the major cause of liver pathology. CCl_4 administration leads to a significant decrease in the glutathione level which can be an important factor in CCl_4 toxicity. The mechanism of hepatoprotection by AP against CCl_4 toxicity might be due to restoration of the GSH level at least.

In this study, AP pretreatment prior to CCl_4 intoxication resulted in a reduction in ALT and AST activities, lipid peroxidation, as well as enhancement of GSH and SOD activities in liver. There was no significant difference between males and females with respect to the protection of AP against CCl_4-induced acute liver damage.

The inhibition of free radical generation is important in terms of protecting the liver from CCl_4-induced damage (*20*). The DPPH free radical is a stable synthetic nitrogen radical that is known to remove labile hydrogen (*21*). Scavenging of DPPH radicals is one of the major antioxidation mechanisms by which the chain reaction of lipid peroxidation is inhibited (*22*). The result of the DPPH scavenging activity of AP suggested that AP exhibited a strong free radical scavenging effect directly which could have a beneficial action against pathological alterations caused by the generated free radical CCl_3 induced by CCl_4.

Phenolic agents occur widely and are found in many plants, including a variety of vegetables, fruits, and medicinal plants. Recently, the role of phenolic compounds of foods and beverages in the prevention of free radical-mediated diseases has attracted great interest. They possess a variety of antioxidant properties, which can be ascribed to a broad range of pharmacological activities. Inhibition of free radical-induced damage caused by antioxidant supplementation has become an attractive therapeutic strategy for reducing the risk of liver disease. From the overall results, it was concluded that AP exhibits free radical scavenging activity which could have a beneficial effect against oxidative liver damage induced by CCl_4. Since the phytochemical analysis of AP showed the presence of polyphenols, mainly chlorogenic acid, procyanidin B2, and phloridzin, and the antioxidant or hepatoprotective activities of such constituents are well-known (*23*−*25*), there has been speculation that these constituents might be responsible for the observed hepatoprotective effects.

It is worth noting that AP (200, 400, or 800 mg/kg) given orally to mice significantly prevented the increase in body weight compared with those in the control group. The body weight loss effect is normally seen with other polyphenols, such as green tea extract (*26*, *27*) and coffee (*28*). It is well-known that modest weight loss, 5−10% of the initial body weight, is associated with marked improvements in many risk factors (*29*). The finding of a body weight loss effect of AP in this study is thought to have no adverse effect to the body since AP significantly prevented the increase in body weight of ∼10%. It is reported that green coffee bean extract exerted an inhibitory effect on body weight gain in mice (*28*). Chlorogenic acid, the main component of this extract, had a weak effect on body weight loss. Since chlorogenic acid is also the main constituent of the apple polyphenols in this study, it is possible that it and/or other phenolic compounds in AP act synergistically to suppress the body weight gain. It is reported that apple polyphenols widely inhibit the expressions of genes involved in fatty acid synthesis (*30*), which might be partially responsible for the AP suppression of body weight gain. Clarifying the mechanism and key effective composition of AP needed further intact and systemic investigation.

However, the AP inhibition of body weight gain is inconsistent with previously reports about apple polyphenols (*9*, *30*, *31*). This discrepancy may be related to many factors. One is the different composition of apple polyphenols. This factor is possibly the most important issue for the different result since in Shoji's study they administered the apple polyphenols to rats orally only once (2000 mg/kg) but did not observe any effect on body weight. The second factor is the dose administered to animals and the duration. Compared with this study, Yoshioka reported the animals were treated with 0.1% apple polyphenols in drink water for 4 weeks. The dose (∼25−50 mg/kg for mice daily) was much lower than in our study (200−800 mg/kg).

In this study, AP was administered orally to mice for 7 consecutive days and shown to yield significant protection against acute liver damage. The range of the AP dose is 200−800 mg/kg, which is equivalent to ∼1.2−4.8 g of AP (polyphenol content of ∼1−4 g) for a 70 kg human adult. Thus, there is rational speculation that the smaller doses of AP could also exert marked protection if it was given for a period of time, for example, for ∼1 month. These data thus could be considered to be in accordance with the view that the total dietary intake of polyphenols should be approximately 1 g/day (*32*). Furthermore, the nutritional function of the smaller dose of AP given for a long period of time is under investigation.

In conclusion, we found that AP has a protective effect against acute hepatotoxicity induced by the administration of CCl_4 in mice and that the hepatoprotective effects of AP may be due to the free radical scavenging effect, inhibition of lipid peroxidation, and increased antioxidant activity. Further studies are needed to clarify whether the hepatoprotective effect is specific for CCl_4 and to identify the active constituent of AP. This study could serve as a useful reference to allow the future exploitation of AP as a novel preventive and therapeutic measure for the treatment of oxidative stress-induced liver injury.

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