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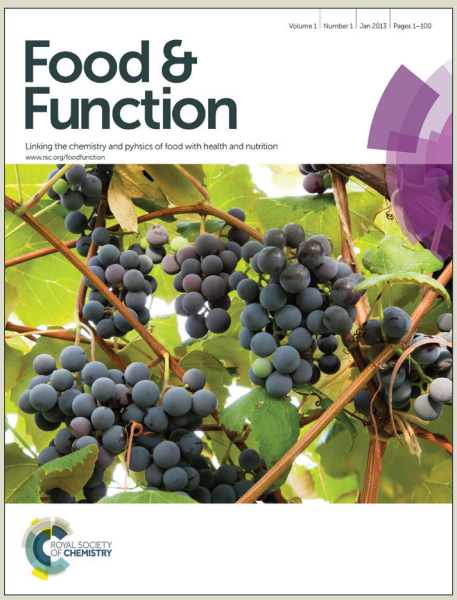

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Differential Protective Effects of Polyphenols Extracts from Apple Peels and Fleshes against Acute CCl_4-Induced Liver Damage in Mice

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Abstract

The present study reports the phenolic profiles, antioxidant and hepatoprotective properties of Red Fuji Apple peel polyphenolic extract (APP) and its flesh polyphenolic extract (AFP) against the CCl_4-induced acute hepatic damage in mice. It was found that the polyphenol and flavonoid contents of APP were significant higher than that of AFP. APP was shown to exhibit stronger *in vitro* antioxidant activities than AFP in a dose-dependent manner. Administration of APP at 250 and 500 mg/kg·bw in mice ahead of CCl_4 injection was further shown to exhibit stronger *in vivo* protective effects than that of AFP, which could observably antagonize the CCl_4-induced increase in serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities, hepatic malondialdehyde level, and prevent the CCl_4-caused decrease in antioxidant superoxide dismutase and glutathione peroxidase activities, compared to CCl_4-treated mice ($p<0.05$). This finding demonstrates that the polyphenolic extract from apple, especially its peel, can be explored as chemopreventive or chemotherapeutic agent against oxidative stress-related liver disorders.

Keywords: Apple polyphenols, Peels and fleshes, HPLC, Antioxidant activity, Hepatoprotective effect

1. Introduction

Carbon tetrachloride (CCl_4), an industrial solvent, is a potent hepatotoxic agent. It is widely used in animal models for induction of acute liver injury.[1] The hepatotoxicity caused by CCl_4 is divided in two phases: during phase I, cytochrome P450 metabolises CCl_4 to trichloromethyl radical ($\cdot CCl_3$) and trichloromethyl peroxy radical ($\cdot OOCCl_3$) that are assumed to initiate free radical-mediated lipid peroxidation.[2] In phase II, CCl_4 is known to reduce antioxidant enzymes or their substrates (e.g. superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidase, and glutathione), and induce oxidative stress.[1] Even though there has huge amounts of advances in modern medicines which have been used to treat liver diseases, they are occasionally inadequate and can have serious adverse effects, especially when administered for a long time.[3-4] Therefore, the search for new natural plant-derived compounds that can safely and effectively block or reverse liver injury remains a priority.[5]

Accumulating evidence suggests that daily consumption of fruits and vegetables is associated with reduced risk of chronic diseases.[6] Compared to other tree fruits, apples (*Malus domestica* Borkh) contain a higher content of bioactive phytochemicals, which seem to be responsible for most of the reported health benefits.[5] Studies conducted in the past few years have also demonstrated that apples are one of the main contributors of nutritional phytochemicals in the human diet, and are proved to be a good, safe, and low-cost natural source of different classes of phenolic compounds.[7-8] The main classes of polyphenols in apple are flavonoids, including quercetin, (-)-epicatechin, (+)-catechin, procyanidins, anthocyanidins, dihydrochalcones (e.g. phloretin and phloridzin), and other polyphenolic compounds such as chlorogenic acid. These compounds could provide a chemical basis for health benefits of apple peel and flesh in folk medicines.[9-10] As a result, an urgent need is felt to develop new strategies based on apple

flavonoid products as chemopreventive or chemotherapeutic agents to lower the risk of chronic diseases, including liver disease.

The majority of recent studies focus on the differential properties of the component profiles and amount of apple flavonoids among cultivars or species.[11-12] It is also suggested that the combination of polyphenols naturally found in fruits and vegetables is the most favorable for human's healthy benefits, including antioxidant and antiproliferative activities.[13] Interestingly, apple peels have recently been proved to exhibit more potent antioxidant effects than apple fleshes, which suggests that apple peels provided the major portion of bioactive flavonoids.[11] Nevertheless, there is limited information about the diversities of polyphenolic contents or bioactivities between peels and fleshes of apple[12] and the hepatoprotective activities of apple peels and fleshes with differential polyphenols have never been examined.

Therefore, the main endeavor of the present study was to investigate the potential antioxidant and hepatoprotective activities of the peel polyphenolic extract (APP) and flesh polyphenolic extract (AFP) from Red Fuji apple, which is one of the main varieties cultivated in northern China and stands out for its distinctive sensory characteristics, especially for its flavor, crunchy texture and long storage life.[14-15] For this purpose, methanolic extracts of the apple peels and fleshes were prepared, and their phenolic profiles were determined by high-performance liquid chromatography (HPLC). Furthermore, *in vitro* antioxidant activities of APP and AFP were measured using $DPPH^{\bullet}$, $HO^{\bullet}$, and $O_2^{\bullet-}$ systems and ferric-reducing antioxidant power assay, and *in vivo* protective effects of APP and AFP against CCl_4-induced liver oxidative stress damage in mice were also assessed by measuring the serum and hepatic parameters as well as the histological observation. All of these findings demonstrate that the polyphenolic extract from apple, especially its peel, could therefore be a suitable chemopreventive or chemotherapeutic

agent against oxidative stress-related liver disorders.

2. Materials and methods

2.1. Chemicals and reagents

CCl_4 was purchased from Tianjin Tianli Chemical Reagent Co. (Tianjin, China). Bifendate pills (BP) were obtained from Zhengjiang Wanbang Pharmaceutical Co. Ltd. (Wenling, China). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), and phenazine methosulphate (PMS) were provided from Applichem (Darmstadt, Germany). Ascorbic acid (Vit. C) was from Sigma (St. Louis, MO). Potassium ferricyanide [$K_3Fe(CN)_6$] and trichloroacetic acid (TCA) were purchased from Sigma (Sigma Aldrich GmbH, Steinheim, Germany). Haematoxylin and eosin (H & E) and oil red O were the products of Shanghai Lanji Technological Development Co. Ltd. (Shanghai, China). Diagnostic kits used for the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), superoxide dismutase (SOD), malonaldehyde (MDA), and glutathione peroxidase (GSH-P*x*) were obtained from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Chlorogenic acid, caffeic acid, epicatechin, rutin, hyperoside, quercitrin, quercetin, and phloretin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile and methanol were the products of TEDIA (Fairfield, OH, USA). All other chemicals were analytic reagents.

2.2. Preparation of polyphenols-extracts from apples

Red Fuji apples were purchased in local retail shop (Xi'an, China). All apples were peeled (1-2 mm thickness) with a hand peeler. The peels and fleshes of the apples were grated with a juicer, and the tissue homogenates were respectively lyophilized (FD-1, Henan Yuhua

Instrument Co., China). The dried peels and fleshes were separately milled into fine powders and stored at 4°C in the dark overnight before extraction. After several pre-experiments to compare the efficacy of conventional agitation extraction and the ultrasonic-assisted extraction, the ultrasonic-assisted extraction was finally used to isolate polyphenol fraction as described previously with minor adjustments.[16] In brief, 1.0 g powdered apple peels or apple fleshes were mixed with 20 mL of 80% methanol in a 250 mL conical flask, and extracted in an ultrasonic cleaning bath (KQ-250V, Kun-Shan Ultrasonic Instruments Co., Ltd, Kunshan, China). 2% Ascorbic acid was used in the extraction of the APP and AFP to inhibit the activities of polyphenol oxidase enzyme.[17] Ultrasound equipment was operated at a frequency of 40 kHz, 250 W of power, and temperature of 35°C for 20 min. The filtered extract from three extractions was combined and condensed with temperature below 40°C to remove most of the methanol, followed by a supplement of deionized water (200 mL), and evaporated continuously to remove all methanol. The residual aqueous solution (about 100 mL) containing polyphenols was freeze-dried and yielded yellow powder, defined as APP or AFP from apple peels and fleshes.

2.3. Assay for total phenolics and total flavonoids

Total phenolics in the dried extracts were estimated by the Folin-Ciocalteu method, expressed as milligrams of gallic acid equivalent (GAE) per 100g extract.[7] Briefly, 1.0 mL of measured solution and 1.0 mL of Folin-Ciocalteu reagent were mixed in a 10 mL volumetric tube and blended well. After 5 min, 3 mL of 30% sodium carbonate solution was added and mixed thoroughly. The absorbance was measured using a spectrophotometer at 760 nm wavelength after incubation for 2 h at room temperature. The measurement was compared to a standard curve of prepared gallic acid solutions: A =0.1217C + 0.01, R^2 = 0.9974 (10-40 μg of gallic acid). In addition, total flavonoids of the dried extracts were measured as rutin equivalent (RE)

using a modified colorimetric method.[18] In brief, aliquots of 1.0 mL extracts or standard solutions were mixed with 5% $NaNO_2$ solution (0.2 mL). After 6 min, 0.2 mL of 10% $AlCl_3$ solution was added and allowed to stand for another 6 min. Subsequently, the reaction solution was mixed with 0.6 mL of 4% NaOH solution, and 60% ethanol was immediately supplied to the final volume of 10 mL, followed by a thorough mixture and a further stand for 10 min. Absorbance of the mixture was determined at 510 nm versus blank water. The concentration of flavonoids was calculated compared to a standard curve of prepared rutin solutions: A = 9.9393C + 0.0045, R^2 = 0.9980 (10-60 μg of rutin) and expressed as milligrams of RE per 100 g extract.

2.4. HPLC analysis of polyphenolic compounds

A HPLC system (Shimadzu LC-2010A, Kyoto, Japan) equipped with an autosampler and an UV detector was used for the identification and quantification of various phenolic compounds in APP or AFP. The separation and analysis were performed on a C_{18} column (4.6 mm i.d. × 250 mm, 5 μm, Venusil, USA). The mobile phase A consisted of 0.5% formic acid in acetonitrile and water (50:50, v/v), and mobile phase B was water with 0.5% formic acid at a flow rate of 1.0 mL/min for a total run time of 60 min. The gradient program was as follows: 0-75% B in 10 min, 75-50% B in 20 min, 50-30% B in 15 min, 30-10% B in 10 min, and the injection volume was 20 μL. The detector was set at 280, 320 and 350 nm for simultaneous monitoring of the different groups of phenolic compounds, respectively. Finally, 280 nm was chose as the optimized wavelength to simultaneously analyze the individual polyphenol of APP and AFP. The individual compound was identified and quantified by comparison with standard solutions of known concentrations detected at 280 nm at 30°C. In our study, linear regression was used for the calculation of the quantitative result of the major active flavonoids in APP and AFP.

147 *2.5. In vitro evaluation of antioxidant activity of APP and AFP*

148 *2.5.1. Determination of DPPH radicals-scavenging effect*

149 The DPPH$^{\bullet}$-scavenging activity of APP or AFP was measured by the previously published
150 method.[19] Extract solution in range of 0.05-0.8 mg/mL was mixed with the solution of 0.1 mM
151 DPPH$^{\bullet}$ in methanol (3.0 mL). The mixture was shaken vigorously and allowed to stand for 1 h
152 before the absorbance was measured at 517 nm. Vit. C was used as positive control. Radical
153 scavenging activity was calculated as the following equation: DPPH$^{\bullet}$-scavenging activity = [1-
154 $(A_{sample} - A_{background})/A_{blank}] \times 100$.

155 *2.5.2. Assay for hydroxyl radicals-scavenging activity*

156 The HO$^{\bullet}$-scavenging ability of APP or AFP was determined as described previously.[20] The
157 mixture which contained different concentrations of APP or AFP samples, 1.0 mL $FeSO_4$, 1.0
158 mL salicylic acid ethanol and 1.0 mL H_2O_2 was then incubated at 37°C for 60 min. Absorbance
159 was measured at 510 nm. The percentage of HO$^{\bullet}$-scavenging activity of APP or AFP was
160 calculated according to the following formula: scavenging activity against HO$^{\bullet}$(%) =1-
161 $[(A_{sample}-A_{blank})/A_{control}] \times 100$.

162 *2.5.3. Measurement of superoxide anion radicals-scavenging activity*

163 $O_2^{\bullet-}$-scavenging activity was measured according to the previous method with slight
164 modification.[20] Briefly, the mixture containing indicated concentrations of APP or AFP sample
165 and NBT (100 μM), NADH (300 μM) was prepared in a total volume of 1.0 mL Tris-HCl buffer
166 (50 mM, pH 8.2). The reaction was started by adding PMS (30 μM) to the mixture for 10 min at
167 25°C, followed by absorbance analysis at 560 nm. The scavenging effect was calculated as
168 follows: suppression rate (%) = $(1-A_{sample}/A_{blank}) \times 100$.

169 *2.5.4. Assessment of ferric-reducing antioxidant power*

The reducing power of various extracts was determined as described previously.[19] Various concentrations of APP or AFP (0.2 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) solution of $K_3Fe(CN)_6$. After incubation at 50°C for 20 min, 2.5 mL of a 10% (w/v) TCA solution was added, and the resultant mixture was centrifuged at 3000g for 10 min. The upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%), and absorbance was then measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power.

2.6. Experimental animals

Experiments were performed in Male Kunming mice with an average weight of 20 ± 2 g, which were purchased from Laboratory Animal Center of Fourth Military Medical University (Xi'an, China). All the experiments were approved by Committee on Animal Care and Use of this university. All animals were acclimatized to the laboratory environment for one week before experiments were conducted. They were housed in spacious cages, which were environmentally controlled under the conditions (humidity: 55% ± 5%; temperature: 22 ± 2°C) with a 12 h light-dark cycle and all mice have free access to tap water and rodent chow.

2.7. Experimental design

After all mice were adapted to the controlled condition for one week, mice were randomly divided into seven groups with each of 10. Animals from the first group (normal) and the second group (a CCl_4 hepatotoxicity injury model) were given the same volume (0.4 mL) injection of saline for 14 consecutive days. Mice from the third group (positive control) were injected intragastrically (ig) with reference drug BP (250 mg/kg·bw) once daily over the 14 consecutive days prior to CCl_4 intoxication. Groups of 4-7 were served as the test groups. Mice were administered 250 and 500 mg/kg·bw APP or AFP in distilled water for 14 days, respectively. On

the fifteenth day, all the mice except those in the normal group were simultaneously intoxicated intrapertoneally (ip) with the single dose of CCl_4 (10 mL/kg·bw, 0.8% in peanut oil) 12 h after the last administration, while the mice from the normal group were injected with peanut oil (10 mL/kg·bw) alone.

At the end of the experiment, all of the animals were anaesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), and then the animals were sacrificed by cervical dislocation. Blood was withdrawn into a syringe from the abdominal aorta, and mouse liver was immediately removed and washed by ice-cold physiological saline.[21] Blood samples were separated for serum aliquots by centrifugation at 3000g for 10 min at 4°C and stored at -20°C for later biochemical analysis within two weeks. The liver tissues were immediately excised. One part of the liver samples was immediately stored at -20°C until analysis, and another part was excised and fixed in 10% formalin solution for histopathological analysis.

2.8. Estimation of serum ALT, AST and ALP

Liver damage and protection were assessed by estimating the serum enzymatic activities of ALT, AST and ALP using commercially available diagnostic kits according to the instructions. The enzymic activities were expressed as units per litre (U/L).

2.9. Measurment of MDA, SOD and GSH-Px in liver homogenate

Liver tissues were homogenized with cold physiological saline in a 1:9 ratio (w/v, liver: saline). The homogenates were centrifuged (2500g for 10 min) to collect supernatants for the subsequent determinations. The liver damage was assessed according to the hepatic measurements of MDA, SOD and GSH-P*x* activities, and they were assayed by using commercially available diagnostic kits according to the instructions. The results of MDA were expressed as nmol/mg protein, and the activities of SOD and GSH-P*x* were expressed as U/mg

protein.

2.10. Histopathological examination

Histology of the liver was examined through hematoxylin and eosin (H&E) and oil red O staining. Liver specimens fixed in 10% formalin were processed by routine histology procedures, and embedded in paraffin, sliced at 5-mm thickness, and stained with H&E dye and observed under a light microscope (DM-LB2, Leika ,Germany) for detection of hepatic damage, and images were recorded (400×). For oil red O staining, frozen liver sample was processed using cryostat (CM1950, Leika, Germany) and then stained. The slides were detected under the light microscope for observation and photograph.

2.11. Statistical analysis

All experiments were done in triplicate and results were reported as mean ± standard deviations (SD) for 10 mice in each group. The results were evaluated by one-way analysis of variance (ANOVA), and statistically significant effects were further analysed and means were compared using Duncan's multiple range test. Differences were considered significant at $p<0.05$.

3. Results

3.1. Total phenolic and total flavonoid contents in APP and AFP

APP and AFP were extracted from Red Fuji apple peels and fleshes with 80% aqueous methanol, and the methanol could isolate and enrich significant amounts of polyphenolic compounds.[12] With this method, the extraction yield of APP and AFP was 8.6% and 7.9%, respectively. The total phenolic contents of APP and AFP determined by Folin-Ciocalteu method were 557.5 ± 20.1 mg of GAE/100g of peel and 134.2 ± 10.4 mg of GAE/100g of flesh

239 respectively, which was in accordance with previous reports.[9] Similarly, the contents of total
240 flavonoids in APP and AFP were 282.1 ± 11.5 mg of RE/100 g of peel and 102.8 ± 9.1 mg of
241 RE/100 g of flesh, respectively. These quantitative assay indicated that apple peels contained
242 remarkably higher amount of total phenolics and total flavonoids than apple fleshes (p<0.01).

243 *3.2. HPLC analysis for polyphenolic composition of APP and AFP*

244 The main composition of the phenolic compounds in APP and AFP derived from apple peels
245 and fleshes was analyzed by a HPLC method according to the retention time (t_R) of authentic
246 standard references under identical conditions. As can be seen from Fig. 1A, the tested standard
247 polyphenols were completely separated from the baseline within 60 min. The peaks in the
248 chromatogram were identified in the order of chlorogenic acid, caffeic acid, epicatechin, rutin,
249 quercetin-3-o-galactoside (hyperoside), quercetin-3-rhamnoside (quercitrin), quercetin, and
250 phloretin with the t_R of 14.0, 19.2, 20.4, 29.8, 30.9, 35.2, 47.0 and 52.2. As shown in Fig. 1B,
251 APP derived from apple peel part contained significant amounts of polyphenolic compounds.
252 Eight polyphenols were identified, and rutin with the value of 65.3 mg/g was shown to be
253 present in the highest level in APP, followed by quercitrin, hyperoside, chlorogenic acid,
254 epicatechin, quercetin, caffeic acid, phloretin at the concentration of 23.7 mg/g, 8.7 mg/g, 4.1
255 mg/g, 0.7 mg/g, 0.5 mg/g, 0.4 mg/g, 0.1 mg/g, respectively. With regard to the flesh extract,
256 AFP was characterized by the presence of the same six polyphenols, namely 1.8 mg/g
257 chlorogenic acid, 0.03 mg/g caffeic acid, 0.1 mg/g epicatechin, 1.0 mg/g rutin, 1.2 mg/g
258 hyperoside, and 1.8 mg/g quercitrin. Relative high lipophilic aglycons of quercetin and phloretin
259 were not detected in AFP (Fig. 1C). The main compounds present in APP and AFP was listed in
260 Table 1. The linear regression was used for the calculation and the assay had excellent linearity
261 with the correlation coefficients (R^2) in the range of 0.9984-0.9999. As can be seen in Table 1,

the contents of the various polyphenols in APP were higher than that in AFP (p<0.01), and characteristic flavonoid profile between APP and AFP was different, which is suggested to exhibit differential biological effects.

3.3. In vitro antioxidant activities of APP and AFP

Firstly, we performed an antioxidant assay that evaluates the capacity of APP and AFP to scavenge the free radicals of DPPH$^{\bullet}$, HO$^{\bullet}$, and $O_2^{\bullet-}$, and enhance ferric-reducing antioxidant power *in vitro*, respectively (Fig. 2). As can be seen in Fig. 2A, APP and AFP were found to have the ability to scavenge DPPH$^{\bullet}$ at tested concentration range of 0.05-0.8 mg/mL. DPPH$^{\bullet}$-scavenging effect was 3.5%, 10.6%, 16.0%, 29.9%, and 55.8% for APP, which was significant higher than that of AFP (1.3%, 2.8%, 6.7%, 7.6%, and 14.2%), respectively (p<0.05). The assay for scavenging HO$^{\bullet}$ showed that the antioxidant effect of the polyphenols-enriched extract of apple peels was more potent than that of apple fleshes (Fig. 2B), where HO$^{\bullet}$-scavenging ability of APP was from 58.1 to 78.0% at the concentrations ranging from 0.05 to 0.8 mg/mL, while the effect of AFP was only from 45.3% to 67.2% following the treatment. Similarly, as shown in Fig. 2C, the $O_2^{\bullet-}$-scavenging activity of APP at 2.0 mg/mL was as high as 54.6%, which was almost 2.0-fold higher than that of same concentration of AFP (29.7%, p<0.05). Moreover, reducing capability was determined by monitoring the transformation of Fe^{3+} to Fe^{2+} in the presence of the extracts. As shown in Fig. 2D, APP in the range of 0.05-0.8 mg/mL resulted in a higher degree of ferric-reducing antioxidant power than that of AFP (p<0.05). Therefore, it was convinced that the polyphenolic extracts from apples possessed antioxidant property, and the effect of the polyphenols-enriched extracts from apple peels was observably superior to that from the fleshes.

3.4. Effects of APP and AFP on body weights and liver weights of mice

Table 2 summarizes the effects of APP and AFP on the body weights, liver weights and hepatosomatic index (HI) in experimental mice. As can be seen from Table 2, there was no significant alteration in the body weights among the mice of various treatment groups ($p > 0.05$). However, in comparison with the normal mice, CCl_4-treated mice gained a significant increase in liver weight ($p<0.05$), and a significantly elevated HI ($p<0.01$). Interestingly, the CCl_4-induced increases in the liver weight and HI could be well decreased by the pretreatment with APP and AFP at high dosage of 500 mg/kg·bw ($p<0.05$), respectively (Table 2). Additionally, a similar effect was also observed with the pretreatment of positive control BP at 250 mg/kg·bw.

3.5. Effects of APP and AFP on serum AST, ALT and ALP activities

The effects of oral administration of APP and AFP on CCl_4-induced elevation of serum AST, ALT, and ALP activities are shown in Fig. 3, application of a single dose of CCl_4 (0.8%, ip) in mice induced severe effects in hepatic injury as evidenced by a dramatic increase in serum AST (170.1%), ALT (176.9%), and ALP (213.3%) activities in comparison with untreated normal mice ($p<0.01$), respectively. However, the pretreatment of APP and AFP at doses of 250 and 500 mg/kg·bw once daily for 14 consecutive days dose-dependently reduced the CCl_4-induced elevation of serum ALT, AST and ALP activities ($p<0.05$), respectively. With the treatment of APP and AFP at high dosage of 500 mg/kg·bw in mice, AST activities significantly decreased to 81.3 ± 13.4 U/L and 126.9 ± 7.8 U/L from 170.1 ± 21.7 U/L of CCl_4-treated mice ($p<0.01$, Fig. 3A), and ALT activities were markedly reduced to 50.5 ± 16.3 U/L and 96.7 ± 12.5 U/L from 176.9 ± 14.4 U/L in CCl_4-intoxicated group, respectively ($p<0.01$, Fig. 3B). Similarly, ALP activities also decreased to 125.2 ± 12.5 U/L and 162.7 ± 19.7 U/L from 213.1 ± 20.3 U/L of CCl_4-injured mice following the treatment of APP and AFP at 500 mg/kg·bw, respectively

(p<0.05, Fig. 3C), suggesting that APP exhibited stronger protective effects on CCl_4-induced liver injury, relative to AFP (p<0.05). In this test, BP as a clinical hepatoprotective drug also significantly decreased the levels of serum ALT, AST and ALP activities (p<0.01 *vs* CCl_4-treated mice).

3.6. Effects of APP and AFP on hepatic MDA, SOD and GSH-Px levels

In vivo CCl_4-induced liver damage is well-known involved in the mechanism of lipid peroxidation,[2] and MDA is a key indicator of chain reaction of lipid peroxidation.[4] Fig. 3D showed the levels of peroxidation product MDA in liver tissues of normal and experimental mice. CCl_4 administration in mice caused a 2.0-fold increase in generation of MDA, as compared to untreated normal mice (p<0.01), indicating that CCl_4 caused notable liver peroxidation damage. However, the elevated MDA was alleviated dose-dependently after the administration of APP and AFP, respectively. At high dose of 500 mg/kg·bw, APP and AFP significantly reduced MDA levels from 4.3 ± 0.2 nmol/mg protein of CCl_4-intoxicated mice to 2.2 ± 0.1 nmol/mg protein and 3.4 ± 0.08 nmol/mg protein (p<0.01), respectively.

It is well known that free radical production from metabolism of CCl_4 significantly reduced antioxidant SOD enzymic activity,[22] and therefore, hepatic SOD status of experimental mice was also detected. As shown in Fig. 3E, hepatic SOD activity in CCl_4-treated mice obviously decreased by 34.1%, relative to normal mice (p<0.01). Interestingly, the administration of APP and AFP could protect against liver damage, and both were more effective at 500 mg/kg·bw than 250 mg/kg·bw. After the pretreatment of APP and AFP at 500 mg/kg·bw, SOD activities remarkably enhanced to 228.3 ± 4.7 U/mg protein and 183.4 ± 21.2 U/mg protein from 159.6 ± 20.4 U/mg protein of CCl_4-intoxicated mice (p<0.01), respectively. As depicted in Fig. 3F, hepatic GSH-P*x* activity in CCl_4-injuried mice was also inhibited to 85.9 ± 4.2 U/mg protein

from 210.1 ± 10.2 U/mg protein of normal mice (p<0.01). However, the pretreatment with APP and AFP could increase hepatic GSH-P*x* activity against the CCl_4-induced enzymatic inhibition, where 500 mg/kg·bw APP and AFP resulted in an elevation of up to 179.8 ± 10.9 U/mg protein and 128.8 ± 6.7 U/mg protein, respectively (p<0.01). As expected, the protective effect of APP was more significant than that of AFP (Fig. 3).

3.7. Histological findings

In this study, histopathological observations of H&E staining of the livers were performed to further support the evidence for the biochemical analysis. In comparison with hepatic cellular architecture of mouse tissues from the normal group (Fig. 4A), the liver sections in CCl_4-treated mice showed severe cellular degeneration, hepatocyte necrosis, cytoplasmic vacuolation, and the loss of cellular boundaries (Fig. 4B). However, hepatohistopathological changes induced by CCl_4 were ameliorated by the treatment with APP and AFP (Fig. 4C-F), and APP-treated mice showed a near normal appearance with well-preserved cytoplasm, prominent nuclei and legible nucleoli, suggesting that APP exerted higher hepatoprotective effect than AFP. Considering histological observations of the livers with oil red O staining, it was found that the liver tissues of normal mice showed no fatty changes with normal structure of central veins and radiating hepatic cords (Fig. 5A). On the contrary, the livers in the mice administrated with CCl_4 alone showed widespread deposition of lipid droplets inside the parenchyma cells (Fig. 5B). As shown in Fig. 5C-F, these vacuolization, fatty changes of hepatocytes were effectively alleviated after the treatment of APP and AFP, showing slight scattered droplets of fat, and hepatoprotective effects of the apple peel extracts were more notable than flesh extracts. As a result, histopathological observations suggested that APP and AFP could protect the liver from acute CCl_4-induced oxidative damage and steatosis, which were in agreement with the biochemical

354 results of serum hepatotoxic markers and hepatic oxidative stress systems.

355

356 **4. Discussion**

357 The flesh of many fruits is a known source of polyphenols. To determine whether there is
358 bioactive difference between the peel and flesh, we assessed *in vitro* antioxidant activities and *in*
359 *vivo* hepatoprotective effects of APP and AFP as polyphenols-enriched extracts from Red Fuji
360 Apple peel and flesh. APP was shown to exhibit greater ferric-reducing antioxidant power and
361 scavenging activities against $DPPH^{\bullet}$, $HO^{\bullet}$, and $O_2^{\bullet-}$ *in vitro* than AFP. This might associate to
362 the quantities of polyphenols available in the peel compared to the flesh. Previous studies have
363 demonstrated that apples contain a broad spectrum of phenolic antioxidants, such as flavonol
364 glycosides, flavanones and chalcones, catechins, and phenolic acids.[23] It has also been
365 confirmed that apple polyphenols possess antioxidant activities and may attenuate
366 atherosclerosis.[24] In our research, the antioxidant effects of APP and AFP were verified in
367 CCl_4-induced liver oxidative stress injury in mice by evaluating serum enzymic activities of
368 AST, ALT and ALP, and hepatic biomarker levels of MDA, SOD, GSH-P*x*, as well as the
369 histopathological observations, and the outcomes demonstrated that the hepatoprotective effect
370 of APP was more potent than AFP. Our study presents substantial evidence illustrating that
371 apple peel is a rich and diverse source of polyphenols from the flesh, and may potentially serve
372 as a novel option for the treatment of oxidative stress-related liver diseases.

373 CCl_4-induced hepatic injury is a commonly used experimental model for screening the
374 therapeutic potential of drugs and dietary antioxidants.[25-26] The main cause of acute liver injury
375 by CCl_4 is free radicals, which are generated in its metabolism by the cytochrome P450 (CYP)
376 system.[2] The content of CYP in the liver is more abundant than that in any other organs, such as

lung, kidney, and intestine.[26] By the activation of liver CYP, CCl_4 produces the hepatotoxic metabolites trichloromethyl free radicals ($\cdot CCl_3$ or $CCl_3OO\cdot$), which immediately propagate a chain of lipid peroxidation events and finally lead to the breakdown of membrane structure and the consequent leakage of hepatic cell marker enzymes into the bloodstream.[27] In our hands, significant increases in the activities of serum ALT, AST were observed after administration of CCl_4 as reported previously.[4,25] However, the increased activities of these enzymes were decreased by the administration of APP and AFP at 250 and 500 mg/kg·bw, and APP showed more protective effects than AFP. In addition, the hepatic damage induced by CCl_4 administration was also observed by evaluating serum ALP activity as it was employed in the evaluation of hepatic disorders.[28] ALP is cytoplasmic in nature, and upon liver injury, it enters into the circulatory system due to altered permeability of membrane.[28] Therefore, an increase in ALP activity reflects acute liver damage and inflammatory hepatocellular disorders. Our results showed that CCl_4 administration caused severe acute liver damage in mice, reflected by significant elevation of serum ALP activity, which was consistent with the findings of other investigators.[4,27] Pretreatment with APP and AFP markedly reduced the enzyme activities under conditions of CCl_4 exposure, clearly implying that APP and AFP may effectively protect the hepatocytes against the toxic effects of CCl_4. It was particularly noteworthy that the inhibitory effect of APP was higher than that of AFP ($p<0.05$), and this differential hepatoprotective effect between APP and AFP may be mediated through differences in either the amounts or types of polyphenols in apple peels and fleshes. Besides, further hepatotoxic biomarkers should be investigated like the hepatic levels of CYP2e1 and other cellular stress markers in the future according to many reports.[29-30]

Besides hepatic marker enzymes, the hepatic MDA level is also commonly used as an

indicator of liver tissue damage involving a series of oxidative chain reactions.[23] MDA has been well known to be the most abundant individual aldehyde resulting from lipid peroxidation, and therefore, measurement of MDA level is the most commonly used method for the evaluation of lipid peroxidation.[31] In our work, mice treated with CCl_4 showed a striking increase in MDA level as compared to untreated normal mice ($p<0.01$). However, APP at 250 and 500 mg/kg·bw could markedly prevent the increase in MDA formation (Fig. 3D), which clearly demonstrated the ability of APP to relieve lipid peroxidation. Similarly, the protective effect of APP was more potent than AFP, which is most likely to be related with the varied quantities and mixtures of different phenolic phytochemicals in apple peels and fleshes. In addition, the present results also showed a considerable difference in enhancing antioxidant constituents with treatment of APP and AFP in mice. SOD and GSH-P*x* are the major enzymes that play an important role in the elimination of the toxic metabolites, which are the major cause of liver pathology caused by CCl_4.[1,3] Here, administration of CCl_4 to mice sharply decreased antioxidant capacity of mouse liver as evidenced by inhibiting the enzymic activities of SOD and GSH-P*x*, which is in agreement with earlier results.[4] However, the decrease of these enzymic activities was significantly elevated by the pretreatment with APP and AFP, especially at the high dosage of 500 mg/kg·bw, suggesting that they could protect the two antioxidant enzymes in CCl_4-damaged liver tissue. In agreement with the results of biochemical parameters assay in serum and liver tissues, histopathological examination of both H&E and oil red O staining also showed that the CCl_4-induced severe histological alteration and steatosis of mouse liver were markedly reduced by the administration of APP and AFP. Similarly, BP as a clinical hepatoprotective drug, which has been clinically used for more than 20 years in East Asia (e.g. China & Korea),[32] also showed significant protective effects against CCl_4-induced liver oxidative injury in mice. The

observations together provide substantial evidence for the protective effect of APP and AFP against CCl_4-induced hepatic damage, and APP has more significant effects than AFP.

In this study, phenolic compounds derived from apple peels and fleshes were identified and quantified by chromatographic analysis to gain an insight into the major active compounds responsible for their hepatoprotective effect. Red Fuji apple peels were found to contain higher amount of polyphenols than the flesh parts, among which rutin (65.3 mg/g), quercitrin (23.7 mg/g) and hyperoside (8.7 mg/g) were the main constitutes in APP (Fig. 1B). It was also found that at least eight kinds of polyphenols were contained in apple peels, whereas six polyphenols were identified in apple fleshes. This difference may be most likely responsible for the remarkable antioxidant and oxidative stress-mediated hepatoprotective activities of APP and AFP. The phenolic composition of apples and their protective activity is deeply influenced by their varieties, and can be modified by postharvest factors, including storage and processing.[33] However, it is difficult to compare the bioactivities obtained from different studies performed in different conditions because the polyphenol concentration also depends on the fruit ripening degree, vegetation season, cultivation methods, soil and climatic conditions, and isolation degree.[34,35] Previous studies have demonstrated that quercitrin, quercetin, hyperoside, and rutin are potential therapeutic agents as they reduce oxidative DNA damage and lipid peroxidation via quenching free radicals.[27] Quercetin and hyperoside were shown to possess a protective effect against CCl_4-induced acute liver injury, which is likely due to the enhancement of the antioxidative defense system and suppression of the inflammatory response.[36-38] Recent work has also shed light on detailed mechanisms underlying bioactive actions of polyphenols.[39] It was reported that pretreatment with quercetin decreased mitochondrial oxidative stress in isoproterenol-induced myocardial infarct in rats via anti-lipid peroxidation and mitochondrial

membrane stabilizing effects.[39] Similar study also suggested that epigallocatechin-gallate derived from Green tea was involved in interaction with plasma membrane proteins, activation of second messengers and signal transduction pathways, modulation of metabolic enzymes and autophagy.[40] Moreover, a recent study also reported that a phenolic fraction from the mango peels contained more polyphenols and flavonoids than fleshes, and exhibited good antioxidant and antiproliferative activities, and the effects of mango peels might be due to the synergistic actions of bioactive compounds present in them.[41]

In conclusion, this was the first investigation with unequivocal evidence that polyphenolic extract from Red Fuji apple peels presented more effective hepatoprotective effects than the fleshes. This differential hepatoprotective effect was most likely related to the different classes of polyphenolic constituents found in peels and fleshes. This finding provides the interesting information for further utilizing apple products and pericarps, and also serves as a useful reference to allow the future exploitation of apple polyphenols as a novel preventive and therapeutic ingredient for the treatment of oxidative stress-related liver diseases.

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584 **Figure Captions**

585 **Fig. 1.** The HPLC chromatograms of the authentic polyphenol standards (A) and polyphenolic
586 components in methanolic extracts APP (B) and AFP (C). Peaks: (1) Chlorogenic acid, (2)
587 Caffeic acid, (3) Epicatechin, (4) Rutin, (5) Hyperoside, (6) Quercitrin, (7) Quercetin, (8)
588 Phloretin.

589

590 **Fig. 2.** *In vitro* antioxidant activities of APP or AFP determined using $DPPH^{\bullet}$, $HO^{\bullet}$ and $O_2^{\bullet-}$
591 scavenging assays as well as ferric-reducing antioxidant power. (A) $DPPH^{\bullet}$-scavenging effects,
592 (B) $HO^{\bullet}$-scavenging assay, (C) $O_2^{\bullet-}$-scavenging activities, (D) Ferric-reducing antioxidant power
593 assay. Vit. C was used as positive control. Results of three separated experiments are presented
594 as means ± SD (n = 3). *p<0.05, **p<0.01, compared with AFP.

595

596 **Fig. 3.** Effects of APP and AFP on enzymic activities of serum AST (A), ALT (B), ALP (C) and
597 hepatic levels of MDA (D), SOD (E) and GSH-Px (F) in CCl_4-intoxicated mice. All values are
598 expressed as means ± SD (n=8). Mice were treated intragastrically (ig.) with APP or AFP (250
599 and 500 mg/kg·bw) or BP (250 mg/kg·bw) once daily for 14 consecutive days, respectively, and
600 subsequently intraperitoneally (ip.) single injection of CCl_4 (0.8%, v/v). $^{\#\#}p$<0.01, compared to
601 the normal group. *p<0.05, **p<0.01, compared with the CCl_4-intoxicated group.

602

603 **Fig. 4.** Effects of APP or AFP on the liver histological changes after CCl_4 treatment in mice
604 (original magnification of 400×). The liver section of each mouse from different groups was
605 stained by hematoxylin and eosin (H & E) staining, and the images were examined under a
606 microscope. (A) Normal group, (B) CCl_4 alone-treated mice, (C) Mice treated with APP (250

607 mg/kg·bw) + CCl_4, (D) Mice treated with APP (500 mg/kg·bw) + CCl_4, (E) Mice treated with
608 AFP (250 mg/kg·bw) + CCl_4, (F) Mice treated with AFP (500 mg/kg·bw) + CCl_4.
609
610 **Fig. 5.** Lipid staining of mouse liver sections (oil red O, 400×). The images showed
611 representative sections of the liver stained with oil red O. (A) Normal group, (B)
612 CCl_4-intoxicated group, (C) 250 mg/kg·bw APP + CCl_4, (D) 500 mg/kg·bw APP + CCl_4, (E)
613 250 mg/kg·bw AFP + CCl_4, (F) 500 mg/kg·bw AFP + CCl_4.
614
615

616 **Table 1**

617 Calibration curves and the contents of the identified polyphenols in the extracts APP and AFP

618 from Red Fuji apples by HPLC.

Peak no.	t_R (min)	Identified polyphenols	Regression equation $Y = aX + b$	R^2	Content (mg/g)[a]	
					APP	AFP
1	14.0 ± 0.02	Chlorogenic acid	$Y = 2642.3X + 13390$	0.9985	4.1	1.8
2	19.2 ± 0.09	Caffeic acid	$Y = 8761.6X + 143609$	0.9989	0.4	0.03
3	20.4 ± 0.07	Epicatechin	$Y = 1216.8X + 176001$	0.9984	0.7	0.1
4	29.8 ± 0.09	Rutin	$Y = 11331X + 8826.5$	0.9999	65.3	1.0
5	30.9 ± 0.08	Hyperoside	$Y = 1785.4X - 3493$	0.9997	8.7	1.2
6	35.2 ± 0.08	Quercitrin	$Y = 1008.6X + 4271$	0.9999	23.7	1.8
7	47.0 ± 0.07	Quercitin	$Y = 1537.6X + 18656$	0.9998	0.5	
8	52.5 ± 0.07	Phloretin	$Y = 39411X + 49916$	0.9999	0.1	

619

620 **Table 2**

621 Effects of APP and AFP on body weight, liver weight, and hepatosomatic index (HI) of mice
622 subjected to CCl_4 treatment. Date are shown as means ± SD (n=10). $^{\#}p<0.05$,$^{\#\#}p<0.01$, as
623 compared with the normal group. $*p < 0.05$,$**p < 0.01$, compared with the CCl_4 group.

Parameters	Body wt (g)	Liver wt (g)	HI (%)
Normal	31.27±2.33	1.53±0.26	4.89±0.88
CCl_4 alone	29.91±2.51	1.78±0.20$^{\#}$	5.83±0.35$^{\#\#}$
CCl_4+APP 250 mg/kg·bw	30.23±1.35	1.71±0.29	5.66±0.35*
CCl_4+APP 500 mg/kg·bw	30.13±2.15	1.62±0.35*	5.51±0.67*
CCl_4+AFP 250 mg/kg·bw	29.97±2.31	1.73±0.19	5.77±0.42
CCl_4+AFP 500 mg/kg·bw	29.45±4.23	1.68±0.23*	5.67±0.54*
CCl_4+BP 250 mg/kg·bw	29.60±2.59	1.52±0.19*	5.12±0.39*

624

Fig. 1

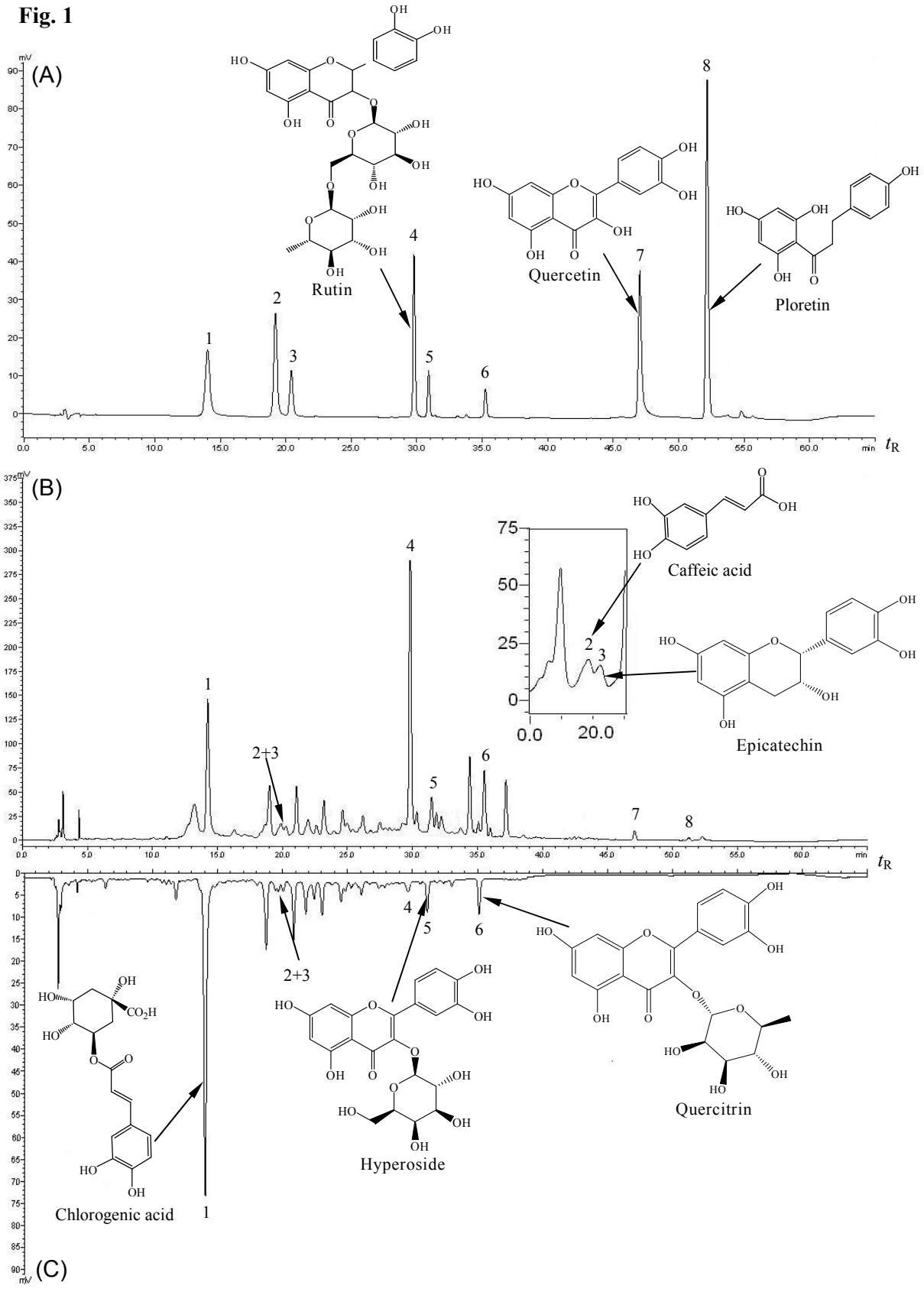

Fig. 2

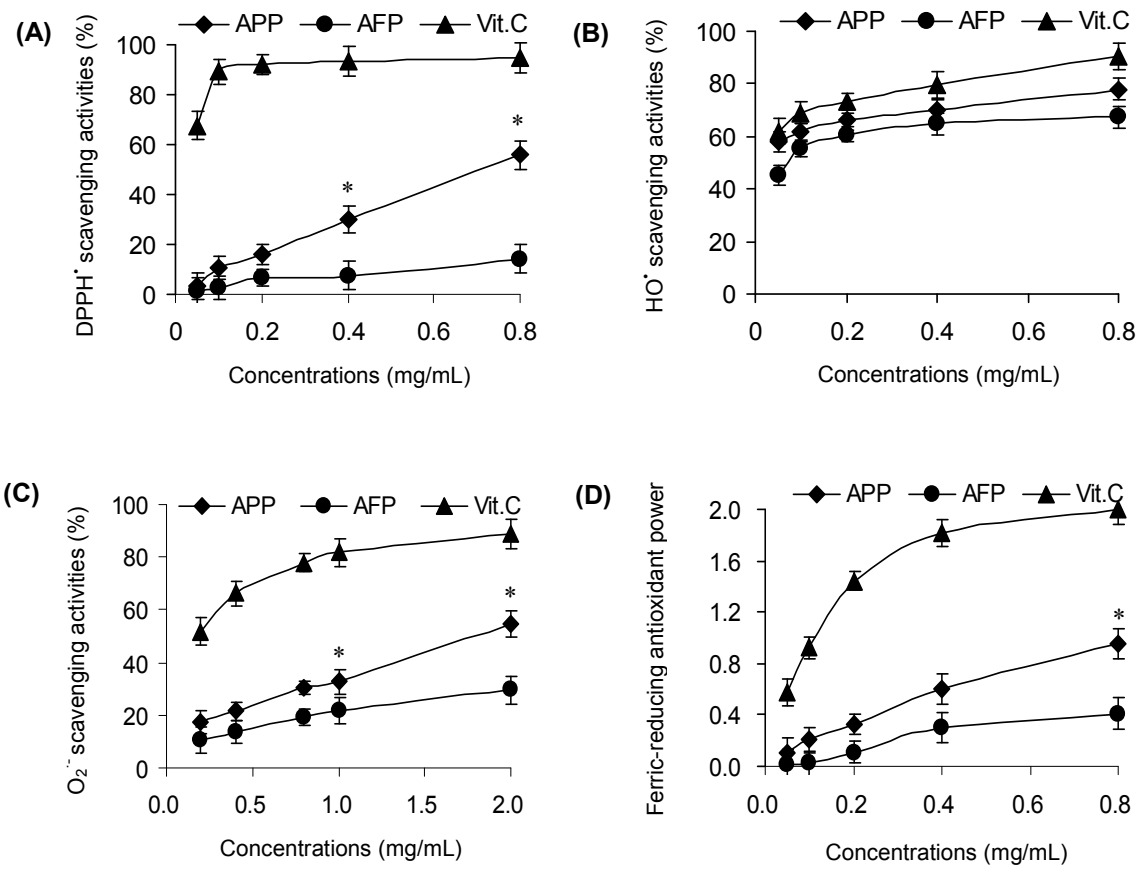

Fig. 3

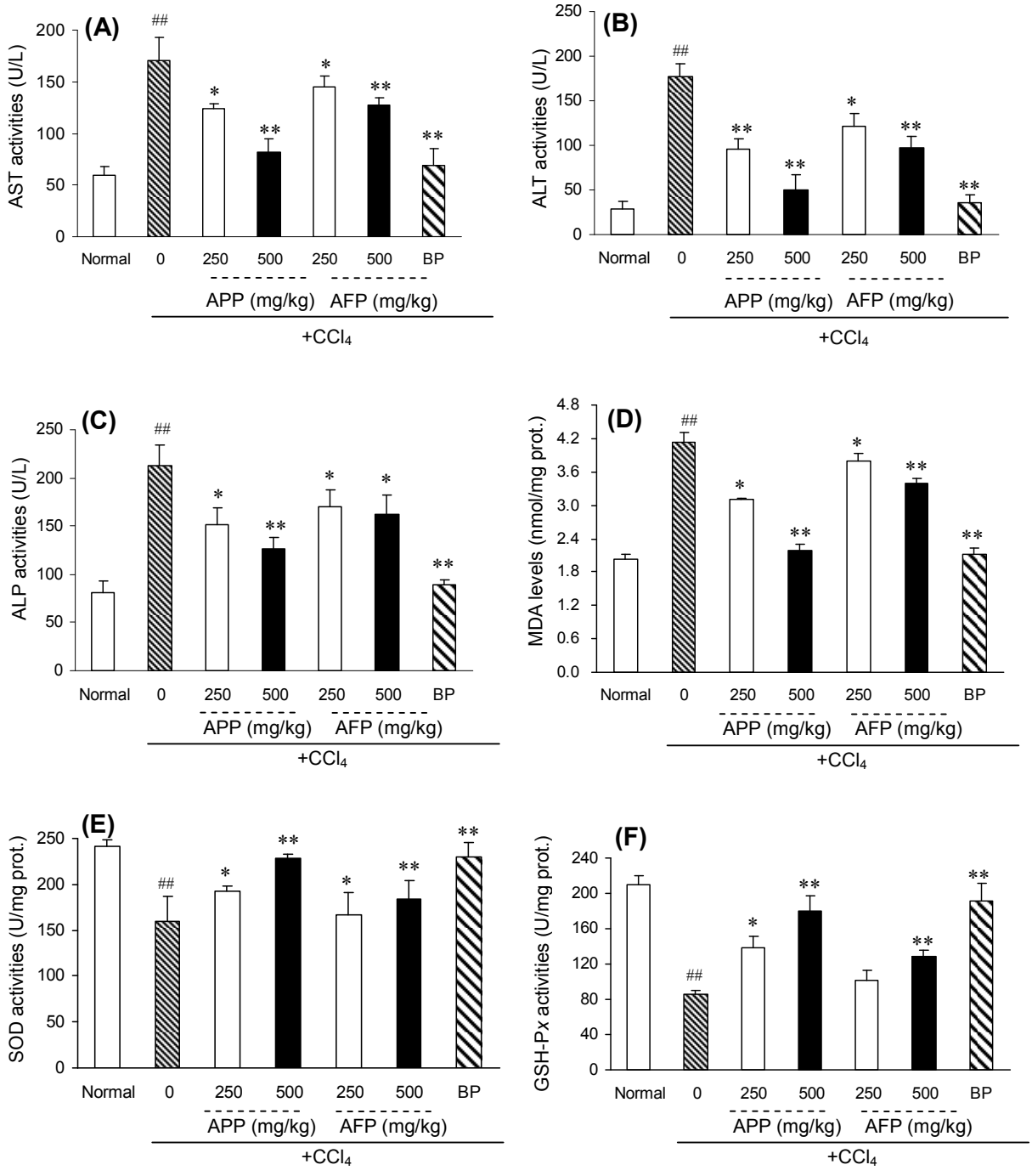

Fig. 4

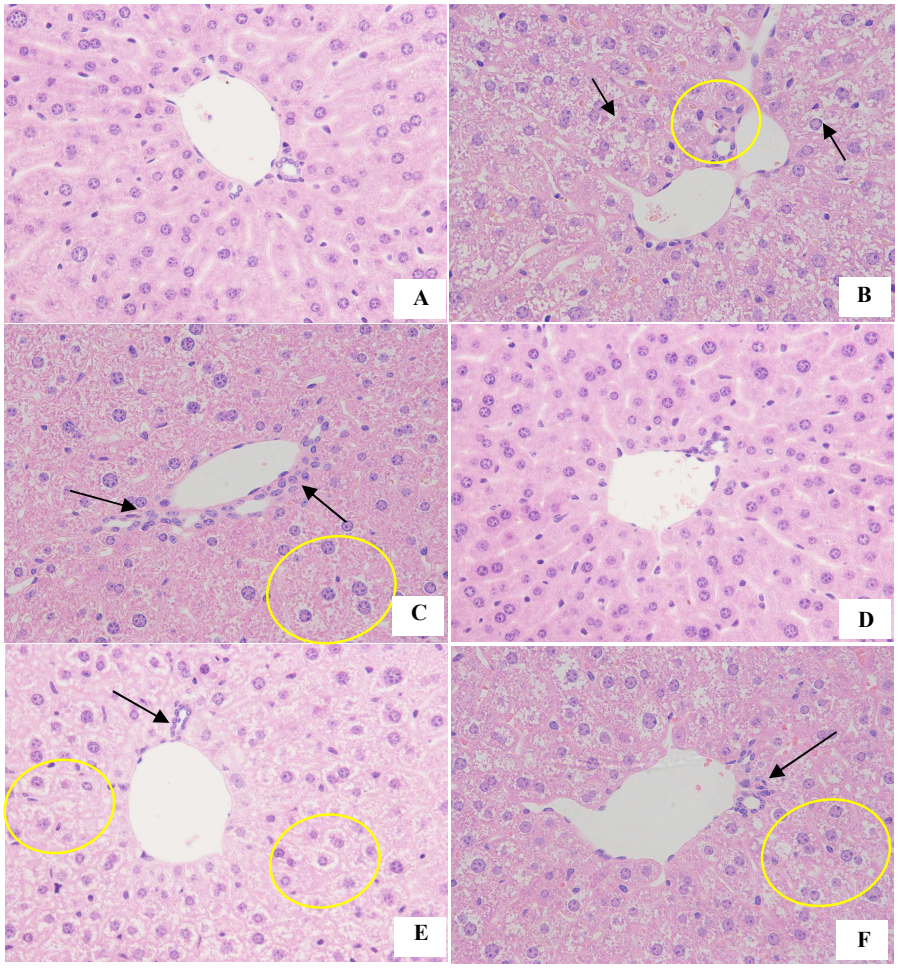

Fig. 5