Characterization, antioxidant activities and hepatoprotective effects of polysaccharides from pre-pressing separation Fuji apple peel

Lijun Sun, Yonghong Meng, Jiaojiao Sun and Yurong Guo

College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi’an, P.R. China

ABSTRACT
Characterization, antioxidant activities and hepatoprotective effects of polysaccharides from pre-pressing separation apple (Fuji) peel pomace were investigated in this study. Gel permeation chromatography indicated that apple peel polysaccharides (APPs) were a kind of heteropolysaccharides composed of three fractions with homogenous polarity. Both GC–MS and HPLC analysis showed that arabinose, galactose and galacturonic acid were the main monosaccharides in APP. In vitro antioxidant activities of APP were characterized. Furthermore, administration of APP (450 mg/kg bw) in mice significantly decreased the CCl₄-induced elevations of serum alanine aminotransferase, aspartate aminotransferase and lactic dehydrogenase activities, and hepatic malondialdehyde level and inhibited the decreases in superoxide dismutase and glutathione peroxidase activities caused by CCl₄. Histopathological observation further confirmed that APP could protect the liver tissues from CCl₄-induced histological alternation. These findings indicate that APP has the antioxidant and hepatoprotective potentials and provide a scientific basis for comprehensive utilization of pre-pressing separation apple peel pomace.

KEYWORDS
Characterization; structural characterization; antioxidant activities; hepatoprotective effects

Palabras clave
Separación con previo prensado; polisacáridos de la pela de manzana; caracterización estructural; actividad antioxidante; efectos hepatoprotectores

Introduction
Apple is the pomaceous fruit of apple tree and known to be an important source of bioactive nutrition in human’s diets (Sun, Guo, Fu, Li, & Li, 2013). It has been revealed that apple extracts, such as polyphenols and polysaccharides, are beneficial to human health and have a potential of preventing cardiovascular diseases (Chai et al., 2013). Apple juice is one of the most popular soft drinks in the world made by maceration and pressing of apple fruits. According to the manufacturers and end products, apple fruits may be processed in different ways before pressing. Commonly, apples are directly pressed for juice after broken, so some parts of the fruits (peel, stalk and seed) are mixed and pressed together with flesh, which may cause adverse effects on juice quality, such as pesticide residues, colour burn and off-flavour. Besides, after the traditional pressing process, all the waste residues, including peel, flesh and stalk pomace, are mixed together, which decreases the recycling values of pomace as fodders, food additives and research materials. In order to solve these issues and improve juice quality, ‘pre-pressing separation technology’ is being introduced to juice industry with the design of ‘pre-pressing separator’. This separator is designed to scrape apples to remove peel, seed and stalk on the base of the differences in toughness of different fruit parts, followed by breaking and pulping flesh. Under the interception of screen mesh, the flesh pulp can be separated from other parts before pressing process (Figure 1(a)), and the juice is only from flesh part. Therefore, through this separation technique, the potential issues mentioned above can be avoided as much as possible. After the complete juicing process, apple juice with lighter colour than the traditional juice can be...
can be used to in vitro...D was purchased from Tianjin apple peel polysaccharides (APPs), especially for the apple peel obtained from pre-pressing separation. To our knowledge, there are few literatures available to address the compositional features and hepatoprotective activity of Fuji apple peel polysaccharides (APPs), especially for the apple peel obtained from pre-pressing separation.

Therefore, the objectives of this study were to identify the monosaccharide composition and primary molecular structure of polysaccharides from Fuji apple peel. Then, in vitro antioxidant activities and in vivo hepatoprotective effects against CCl$_4$-induced hepatic damage in mice were further investigated. Besides, we considered this study addressing an interdisciplinary between food engineering and food nutrition, as well as a comprehensive application of juicing manufacturing wastes.

Materials and methods

Materials and reagents

The apples (Fuji) were harvested in October 2015 in Liquan, Shaanxi province of China. Monosaccharides, including mannose, l-rhamnose, d-glucuronic acid, d-galacturonic acid, d-glucose, d-xylose, d-galactose, L-arabinose, d-fucose, mannitol and ascorbic acid (Vc), were obtained from Sigma Co. (St. Louis, US). Biphenyldicarboxylate bill (BP) was obtained from Zhejiang Wanbang Pharmaceutical Co., Ltd. (Shanghai, China). Test kits for glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trifluoroacetic acid, lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).
acid, triethylamine and 1-phenyl-3-methyl-5-pyrazolone (PMP) were obtained from Merck Co. (Darmstadt, Germany). HPLC-grade acetonitrile, methanol, potassium ferrocyanide \( [K_2Fe(CN)_6] \) and trichloroacetic acid were purchased from Sigma Co. (Steinheim, Germany). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH\(^*\)), nitroblue tetrazolium, nicotinamide adenine dinucleotide and phenazine methosulphate were products of Applichem Co. (Darmstadt, Germany). All other reagents were of analytical grade.

**Isolation of APP**

The fresh apples picked up in the field were transferred to Dinghe machinery manufacturing Co., Ltd. (Xi’an, China). Then, the washed apples were uploaded into the pre-pressing separator according to the operating instruction. As mentioned above, the flesh pulp was separated from the mixtures of peel, seed and stalk pomace. Then, the mixtures were collected for further separation of peel pomace. To do this, water (1:1, v/w) was added to a small amount of mixtures, followed by centrifugation at 4000 × g for 15 min. The peel pomace was easily obtained from the upper layer of the centrifuge tube because of the lower density for peel pomace than other parts (stalk and seed). Finally, the peel pomace was oven-dried for further study.

The obtained dry peel was grated and bleached to suppress the activities of polyphenol oxidases for lighter colour of polysaccharides. Then, the tissue homogenate was lyophilized. The polysaccharides of apple peel were extracted by the reported method with some modifications (Lv et al., 2009). Briefly, the pomace was extracted with 95% ethanol (1:10, w/v) refluxing at 80°C for 4 h to remove the phenolic and lipophilic components. Next, the pre-processed pomace was dried and then extracted with hot water (1:30, v/w) at 80°C for 2 h, followed by filtration. The residues were extracted again for two more cycles, and the combined extraction solution was condensed to approximately quarter of the original volume by use of a rotary vacuum evaporator under reduced pressure, followed by centrifugation at 4000 × g for 15 min. Then, the supernatant was collected and precipitated with triple volumes of 95% (v/v) ethanol solution at 4°C. The polysaccharides were obtained by centrifuging at 4000 × g for 15 min, followed by dissolved in distilled water and dialyzed intensively (cut-off Mw 8 kDa) for 3 days against distilled water to remove impurities, such as soluble monosaccharides, inorganic salts and polyphenols. Subsequently, the residual portion in the dialysis bag was deproteinized by the process of freeze-thaw, repeated 10 times, followed by filtration. Finally, the supernatant was condensed and lyophilized to obtain total APPs.

**Characterization of APP**

**Determination of contents of carbohydrates, polyphenols, proteins and nucleic acid**

The content of total carbohydrates was determined using a phenol–sulphuric acid colorimetric method with glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of total polyphenols was determined using spectrophotometry as reported before with gallic acid as a standard (Singleton, Orthofer, & Lamuela-Raventos, 1999). UV spectrum was recorded in the range of 190–400 nm to measure the potential presences of proteins and nucleic acid as the maximum absorption wavelengths for the two compounds were 280 and 260 nm, respectively (Jia et al., 2013).

**Determination of monosaccharide composition by HPLC**

The monosaccharide composition of APP was analysed using HPLC method established by Yang et al. (2013) with some modifications. Specifically, 20 mg APP was hydrolysed in a 5-mL sealed ampoule by the addition of 2.0 mL of 3 M trifluoroacetic acid (TFA) at 95°C for 8 h to fully release the constitutive monosaccharides. The hydrolysate mixture was centrifuged at 4000 × g for 5 min at room temperature, and the supernatant was evaporated to dry under reduced pressure followed by dissolved with 1.0 mL distilled water. Subsequently, 100 µL monosaccharides solution obtained was mixed with 200 µL of 0.5 M methanol solution of PMP and 300 µL of 0.3 M NaOH solution for derivatization to obtain a strengthen UV absorption. The mixture was then incubated at 70°C for 1 h before neutralization with 300 µL of 0.3 M HCl. The resulting solution was extracted with chloroform and repeated two more cycles. Finally, the aqueous layer was filtered through a 0.45-µm membrane for HPLC analysis.

The analysis of PMP-derived monosaccharides was performed on a Shimadzu® LC-2010A HPLC system. The analytical column was a Venusil® RP C18 column (4.6 mm × 250 mm, I.D., 5 µm). The mobile phases consisted of acetonitrile (A) and 3.3 mM KH\(_2\)PO\(_4\)/3.9 mM TEA buffer containing 10% acetonitrile (B) using a gradient elution: 0–4 min, 94% B; 4–9 min, 94–88% B; 9–30 min, 88% B at a flow rate of 1.0 mL/min. The column was maintained at 37°C.

**Determination of monosaccharide composition by GC–MS**

Monosaccharide composition of APP was also analysed using GC–MS method. Specifically, APP (~10 mg) was hydrolysed in 1 mL of 2 M TFA at 120°C for 2 h. Then, the solution was dried under a stream of nitrogen gas, followed by a derivatization procedure according to one previously reported method (Sassaki et al., 2008). Briefly, 1 mL of 0.5 M NH\(_4\)OH was added and mixed thoroughly for 10 min. Then, 1 mg of NaBH\(_4\) was added and stirred for 5 h. The solution was dried under nitrogen gas. The residue was dissolved in 1 mL of 0.5 M HCl–MeOH and heated at 100°C for 15 min and then dried under nitrogen gas. 1 mL of pyridine–acetic anhydride was added and heated at 95°C for 30 min. The solution was then dried under nitrogen gas and dissolved in 1 mL MeOH. The procedure of GC–MS was conducted on an Agilent® GC system (7890A, California, USA) coupled with an Agilent® MS system (5975C, California, USA). The separation was performed according to one previously reported method (Cheong et al., 2016) on a DB-5 column (30 m × 0.25 mm×0.25 µm). The column temperature was held at 165°C for 7 min, programmed at 5°C/min to 185°C and held for 5 min, then at 4°C/min to 200°C, finally at 20°C/ min to 280°C and held for 2 min. The injection volume was 2 µL and the split ratio was 1:50. A flow rate of 1.0 mL/min helium gas was used as the carrier gas. The mass spectrometer was operated in electron-impact mode (70 eV) at 2.89 s per scan from the range of 10–500 amu. The temperatures of the interface and ionization source were 250 and 280°C, respectively. Monosaccharide standards mix including rhamnose, fucose, arabinose, xylose, galacturonic acid,
glucuronic acid, inositol (internal standard), mannose, glucose and galactose was prepared and analysed in the same way.

**Fourier transform infrared analysis**

The infrared spectrum of APP was performed using a Bruker® Fourier transform infrared (FT-IR) spectrophotometer (EQUINX 55, US). A volume of 4 mg APP was mixed with KBr powder and ground together, followed by pressed into a 1-mm pellet for measurement. The FT-IR of APP was recorded in the range of 4000–500 cm⁻¹ (You et al., 2013).

**Separation of APP by gel permeation chromatography**

To characterize the homogeneity of APP, gel permeation columns were applied to separate it according to the molecular size and polarity. The purified APP was separated sequentially by Cellulose DEAE-52 and Sephadex G-150 chromatography columns based on the reported method with some modifications (Jia et al., 2015; Ye, Wang, Zhou, Liu, & Zeng, 2008). Briefly, 0.5 g purified polysaccharides were dissolved in 10 mL distilled water and then centrifuged at 4000 × g for 15 min. The supernatant was subjected onto a Cellulose DEAE-52 column (2.5 cm×60 cm). Subsequently, the column was eluted gradiently with distilled water and NaCl solution (0.1–0.5 M) at a flow rate of 0.8 mL/min. The elutions were collected automatically tube by tube, and the carbohydrate contents were determined using a phenol–sulphuric acid method (Dubois et al., 1956). Then, the separated fractions were collected, condensed, dialyzed before further purified on a Sephadex G-150 column (1.6 cm×60 cm) eluted with distilled water at a flow rate of 0.5 mL/min. The elutions through the Sephadex G-150 column were collected automatically tube by tube, and the carbohydrate contents were determined using the phenol–sulphuric acid method above. Finally, the tubes with high contents of respective fractions were collected, condensed, dialyzed and lyophilized.

**Determination of average molecular distributions of three APP fractions**

The molecular distributions of three APP fractions were measured with high performance size exclusion chromatography by use of a Shimadzu® LC-2010A HPLC system equipped with a Shodex® size exclusion chromatography column (SB-804 HQ). A volume of 20 µL of 2 mg/mL respective APP fraction was injected into the column and separated using distilled water at a flow rate of 0.8 mL/min. The calibration curve was made with the Dextran standards with known molecular weights (Shao, Chen, & Sun, 2014).

**In vitro antioxidant activities of APP**

The antioxidant activities of APP were demonstrated by reducing powder (Yang et al., 2013) and three radicals scavenging methods reported previously, including DPPH*, O₂− and HO’ (Chun, Kim, & Lee, 2003; Ye et al., 2008).

**Animal and treatment**

Kunming male mice (weight 18–22 g, around 1 month of age) were allowed free access to distilled water and rodent chow (40% corn flour, 26% wheat flour, 10% bran, 10% fish meal, 10% bean cake, 2% mineral, 1% coarse meal and 1% vitamin complex). All the mice were housed together in a plastic cage (20 × 40 cm) in a specific pathogen-free animal room with a 12-h light/dark cycle at a temperature of 22 ± 2°C and a humidity of 60 ± 5%. The mice were allowed to acclimatize to the environment for 3 days before the experiments, and all the related studies were conducted according to the Regulation of Experimental Animal Administration issued by the State Committee of Science and Technology of People’s Republic of China.

After environment adaption for 3 days, mice were divided into 6 groups with 10 mice selected at random in each group. For the normal and CCl₄-intoxicated groups, mice were provided 0.5 mL of physiological saline once a day. For the BP (positive) group, mice were given 0.5 mL of 450 mg/kg bw BP once a day. For the polysaccharide-treated group, mice were received 0.5 mL of respective 150, 300 and 450 mg/kg bw APP once a day. All the administrations were conducted for seven consecutive days. After that, all the mice except the normal group were given a dose of 0.3 mL of 0.8% CCl₄/peanut oil mixture, while the normal group was given 0.3 mL peanut oil alone. Then, all the mice were fasted with only water provided for 48 h. The mice were weighted and blood samples were collected from the orbit. After the animals were decapitated and eviscerated, the livers were immediately taken out and weighted after rinsing by physiological saline (ice-cold) and then stored at −80°C before use. The blood samples were centrifuged and the serum was stored at 4°C before use. Hepatosomatic index (HI) was calculated as the ratio of body weight to the corresponding liver weight.

**Assay for biochemical index in serum and liver**

**Determination of the activities of ALT, AST and LDH in serum**

The serum was separated after clot by centrifugation at 4°C. The activities of ALT, AST and LDH were measured according to the method of Reitman–Frankel (1957) by commercially available assay kits. All the experiments were conducted in triplicates, and the average counts were obtained from each individual sample.

**Determination of hepatic MDA, GSH-Px and SOD levels**

A volume of 1.0 g each liver sample was mixed with 9 mL cold physiological saline and then homogenized, followed by centrifugation at 1400 × g for 10 min. The content of MDA in the supernatant was measured by the thiobarbituric acid reaction method, and the activities of GSH-Px and SOD were assayed as described by the manufacturer’s instruction of commercially available test kits. Total protein contents were determined by Coomassie Brilliant Blue with bovine serum albumin as a standard. All the experiments were conducted in triplicates, and the average counts were obtained from each individual sample.

**Histopathological observation**

Some parts of liver tissues were mixed with a 4% paraformaldehyde solution at 4°C for 24 h and then embedded in paraffin wax (~5 µm) and stained with H & E solution. The slides were observed under an Olympus® light microscope and photographed.
Statistical analysis

The data were presented as the mean ± standard deviations of triplicate measurements and evaluated by one-way analysis of variance using SPSS 18.0 Statistics (SPSS Inc., Chicago, US). The mean values were compared using Dunnett’s t-test at 95% or 99% significant level (p < 0.05 or p < 0.01).

Results and discussion

Chemical composition of APP

The crude APP was obtained from pre-pressing separation apple peel using traditional hot water extraction and ethanol precipitation. After deproteinization and dialysis, the yield of APP was 9.53% (w/w) based on the weight of dried peel pomace. The content of total carbohydrate in APP was determined as 83.75% by use of the phenol–H$_2$SO$_4$ method. Reaction was hardly observed between Folin–Ciocalteu reagent and APP, indicating that there existed no phenolic components. Besides, little absorbance at 280 and 260 nm in the UV spectrum indicated the absence of proteins and nucleic acid in APP, whereas the absorbance at ~190 nm suggested that the main component in APP was polysaccharides (You et al., 2011). In addition, no starch was detected in APP because no reaction was observed after the addition of I$_2$/KI solution.

Monosaccharide composition of APP

Monosaccharide composition of APP by HPLC

The monosaccharide composition and contents in APP were determined using HPLC method and are shown in Figure 2. Nine kinds of standard monosaccharides derived with PMP could be separated well through the column within 25 min. As shown, APP was composed of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose, and their corresponding mole percentages were 10.5%, 9.2%, 5.3%, 18.5%, 6.5%, 7.2%, 20.3% and 21.5% of all the quantitative monosaccharides, respectively, which was similar to the results obtained by Yang et al. (2013), even though the polysaccharides tested were from two different varieties (Fuji and Pink Lady, respectively). Both kinds of polysaccharides contained some parts of pectin. The HPLC method established in our study could simultaneously separate a number of monosaccharides both for neutral and uronic sugars with a short analytical time.

Monosaccharide composition of APP by GC–MS

To encourage the monosaccharide composition of APP, GC–MS was performed and the chromatography is presented in Figure 3. As shown, the monosaccharide composition of APP determined by GC–MS was consistent with that determined by HPLC (Figure 2), with the mole percentages of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose in total monosaccharides of 12.4%, 8.1%, 2.0%, 13.2%, 8.2%, 10.1%, 21.0% and 24.3%, respectively.

Figure 2. The HPLC chromatograms of PMP derivatives of 9 standard monosaccharides (a) and component monosaccharides released from APP (b). Peaks: 1 mannose, 2 rhamnose, 3 glucuronic acid, 4 galacturonic acid, 5 glucose, 6 xylose, 7 galactose, 8 arabinose, 9 fucose (internal standard).

Figura 2. Cromatogramas HPLC de los derivados de PMP de 9 monosacáridos estándar (a) y componentes de monosacáridos liberados de APP (b). Picos: 1 manosa, 2 ramnosa, 3 ácido glucurónico, 4 ácido galacturónico, 5 glucosa, 6 xilosa, 7 galactosa, 8 arabinosa, 9 fucosa (estándar interno).
Obviously, the order of individual mole percentages of mono-saccharides in APP by GC–MS method was essentially same as that by HPLC, in which galactose and arabinose accounted for the higher mole percentages than other monosaccharides. This indicates that the analysis of monosaccharide composition in APP by GC–MS and HPLC methods was shown comparable results; therefore, the two methods may be combined together in determining the mole percentages of monosaccharides in some certain plant polysaccharides. Furthermore, the contents (mass percentage) of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose in APP were determined to be 11.0%, 6.5%, 1.9%, 12.5%, 7.2%, 7.5%, 18.5% and 17.8%, respectively, by GC–MS. It has been reported that the bioactivity of polysaccharides is highly related to the monosaccharide composition and contents (Yang et al., 2013). Besides, polysaccharides with more uronic acids have higher bioactivities due to the modified properties of polysaccharides, such as solubility (Tian, Zhao, Guo, & Yang, 2011). The total content of uronic acids (including both galacturonic acid and glucuronic acid) in APP was 14.4%, suggesting that APP may have a potential to be developed as one important plant extracts with biological responses.

**FT-IR spectrum of APP**

To characterize APP composition more precisely, the characteristic FT-IR spectrum was performed in the wavelength range of 4000–500 cm$^{-1}$ (Figure 4). Specifically, the intense broad peak at 3432.12 cm$^{-1}$ was characteristic stretching vibration of hydroxyl groups (Zhao, Yang, Yang, Jiang, & Zhang, 2007), and the weak absorption peak at 2917.55 cm$^{-1}$ was caused by the stretching vibration of the C–H bonds (You et al., 2013). As reported, the characteristic absorption peak at 1342.42 cm$^{-1}$ was ascribed to the bending vibration of C–H (Ma et al., 2013; Zheng et al., 2014). In addition, the stretching absorption peak at 1613.42 cm$^{-1}$ along with the peak at 1419.59 cm$^{-1}$ indicated the presence of COO$^{-}$ (Lee et al., 2013; Tian, Zhao, Guo, & Yang, 2011).

![Figure 4](image_url)
which conforms to the presence of uronic acids detected by HPLC method. Each particular polysaccharide has specific absorptions in the region of 1200–1000 cm\(^{-1}\); therefore, this is often considered as the fingerprints of polysaccharides because it allows the characterization of the position and intensity of special bonds in each polysaccharide molecules (Zhang et al., 2010). The characteristic peaks at 1038.34 and 1079.94 cm\(^{-1}\) were conformed to the stretching vibration of C–OH side groups and C–O–C glycosidic bond, indicating that there existed pyranose unit in APP (Zhao et al., 2014).

Separation of APP by gel permeation chromatography

The purified APP was initially separated through a Cellulose DEAE-52 anion-exchange column. As a result, three main elution peaks were obtained with distilled water, 0.1 and 0.2 M NaCl solutions, respectively (Figure S5(a)), indicating that APP was a kind of heteropolysaccharides. The three eluted fractions were collected and concentrated for further purification by gel-filtration chromatography using a Sephadex G-150 column. The column was eluted with distilled water and the resulting elutions were collected. It is shown in Figure S5(b–d) that each eluted fraction obtained through DEAE-52 column generated one single elution peak through Sephadex G-150 column, indicating that three polysaccharides components with homogeneous polarity were obtained through the two columns. The respective molecular weights for the three fractions (APP-1, APP-2 and APP-3) were 201, 393 and 301 kDa (Figure S5(e–g)), using a regression equation \( \lg M_w = 9.0356 - 0.5729 t_R \) \((R^2 = 0.9968)\).

In vitro antioxidant activity of APP

The antioxidant activities of plant extracts are highly related to their reducing powers (Chen, Ma, Liu, Liao, & Zhao, 2012). It is shown in Figure 6(a) that the reducing power of APP increased with the concentration increasing, indicating that the reducing effect was concentration dependant in the range of 0.25–2.0 mg/mL. Even though, the reducing...
power of APP was weaker than that of Vc, a well-recognized reducing agent. DPPH\(^{-}\) is a stable free-radical compound that is widely applied to evaluate the ability of an antioxidant to scavenge-free radicals. The DPPH\(^{-}\) scavenging rate of APP is presented in Figure 6(b). APP showed a weaker scavenging effect than Vc, which was also correlated positively with the concentration in the range of 0.25–5.0 mg/mL. Hydroxyl radical (HO\(^{-}\)) is one of the most ROS and mainly responsible for the oxidative injury of biomolecules such as lipids, proteins and DNA (Spencer et al., 1994). The HO\(^{-}\) scavenging activity of APP is shown in Figure 6(c). Although the scavenging activity was around 50% at 0.5 mg/mL, it remained lower than that of Vc in the concentration range measured. Hence, APP seemed to be a moderate HO\(^{-}\) scavenger. Furthermore, superoxide anion radical (O\(_2\)\(^{-}\)) is less reactive but has a longer half-life than some other radicals. Numerous biological and photochemical reactions can generate this free radical that may subsequently form secondary radicals including hydrogen peroxide and hydroxyl radical through dismutation reaction and lead to tissue damages and even various cancers (Liu, Wang, Pang, Yao, & Gao, 2010). Therefore, the determination of scavenging effect on O\(_2\)\(^{-}\) is a necessary way to elucidate the mechanism of antioxidant activity (Wade, Jackson, Highton, & Vanrij, 1987). As shown in Figure 6(d), the O\(_2\)\(^{-}\) scavenging activity of APP was concentration dependent as well, and the activity may not be developed as potentially as Vc. Generally, the weaker antioxidant activity of APP than both Vc (Figure 6) and apple peel polyphenols (reported by Wolfe, Wu, and Liu, 2003) indicated that APP might provide hydrogen protons more hardly than both the compounds when contacting with free radicals (Schulz, 1991).

**Effects of APP on body weight and HI**

It has been reported that in vivo CCl\(_4\)-induced liver damage is the mechanism of lipid peroxidation (Altas, Kizil, Kizil, Ketani, & Haris, 2011; Hsiao et al., 2003). In this study, the hepatoprotective effects of APP were further measured in mice model. The effects of APP on the body weight, liver weight and for experimental mice were summarized in Table 1. For the CCl\(_4\)-intoxicated mice group, significant increases in liver weight and HI were observed compared to the normal mice (\(p < 0.05\) and \(p < 0.01\), respectively). Although there was no significant difference in the changes of body weights, the administration of APP at the high doses of 300 and 450 mg/kg bw resulted in significant decreases in liver weights and HIs, relative to the CCl\(_4\) group, respectively.

### Table 1. Effects of APP on body weight, liver weight and hepatosomatic index (HI) of mice after treatment with CCl\(_4\)

| Treatments          | Body weight (g) | Liver weight (g) | HI (%)         |
|---------------------|-----------------|------------------|----------------|
| Normal              | 31.36 ± 1.64    | 1.49 ± 0.25      | 4.93 ± 0.62    |
| CCl\(_4\)           | 30.39 ± 1.25    | 1.78 ± 0.16      | 5.92 ± 0.62**  |
| CCl\(_4\) + BP 450 mg/kg bw | 29.66 ± 2.31    | 1.55 ± 0.19**    | 5.04 ± 0.39**  |
| CCl\(_4\) + APP 150 mg/kg bw | 29.72 ± 2.56    | 1.62 ± 0.21*     | 5.72 ± 0.45*   |
| CCl\(_4\) + APP 300 mg/kg bw | 30.33 ± 3.14    | 1.60 ± 0.35*     | 5.53 ± 0.56*   |
| CCl\(_4\) + APP 450 mg/kg bw | 29.98 ± 1.52    | 1.59 ± 0.15**    | 5.16 ± 0.41**  |

All the values are expressed as mean ± SD (\(n = 10\)). Mice were treated intragastrically with APP (150, 300 and 450 mg/kg bw) and BP (450 mg/kg bw) once a day for 7 consecutive days before a single administration of 0.8% CCl\(_4\) \(p < 0.05\) and \(**p < 0.01\), significantly different compared to the normal group. \(*p < 0.05\) and \(**p < 0.01\), significantly different compared to the CCl\(_4\)-intoxicated group.

Todos los valores se expresan como promedio ± SD (\(n = 10\)). Los ratones se trataron intragástricamente con APP (150, 300 y 450 mg/kg bw) y BP (450 mg/kg bw) una vez al día durante 7 días consecutivos antes de una única administración de 0.8% de CCl\(_4\) \(p < 0.05\) y \(**p < 0.01\) significativamente diferente en comparación con el grupo normal. \(*p < 0.05\) y \(**p < 0.01\) significativamente diferente en comparación con el grupo intoxicado con CCl\(_4\).
(p < 0.05 and p < 0.01, respectively). Pre-treatment with positive 450 mg/kg bw of BP showed similar decreasing effects on both the indexes (Table 1), indicating that the administration of APP to mice may generate a comparable preventive effect against CCl₄-induced damage as BP. Our results present consist with the reported observations (Wang et al., 2012; Yang et al., 2013).

**Effects of APP on ALT, AST and LDH activities in serum**

The activities of enzymes in serum, such as ALT, AST and LDH, have been known as the sensitive indicators of liver injury (Zhang, Zheng, Pan, & Zheng, 2004) because the damage to hepatocytes alters the transport function and membrane permeability, resulting in the leakages of these enzymes from the injured cells (Jayakumar, Ramesh, & Geraldine, 2006). The activity levels of ALT, AST and LDH for the normal group were determined as 35.64 ± 5.69, 25.16 ± 3.87 and 160.56 ± 11.66 U/L, respectively. As shown in Figure 7(a-c), the acute hepatotoxicity induced by CCl₄ in the experimental mice was observed as the levels of ALT, AST and LDH in serum increased significantly (p < 0.01), relative to the normal group. As expected, daily administration of APP for seven consecutive days prior to the single intoxication of CCl₄ effectively lowered the CCl₄-induced elevations of serum enzymes (ALT, AST and LDH) in a dosage-dependent manner. Specifically, at a dosage of 300 mg/kg bw of APP, the activities of ALT, AST and LDH decreased to 58.45 ± 5.12, 50.24 ± 6.05 and 190.69 ± 25.36 U/L, and at 450 mg/kg bw, the corresponding data were 45.21 ± 3.52, 37.89 ± 2.55 and 172.45 ± 5.69 U/L, respectively, which were close to that of the positive control BP at 450 mg/kg bw, for which the respective values were 58.45 ± 5.12, 50.24 ± 6.05 and 190.69 ± 25.36 U/L, respectively. As shown in Figure 7(d), the acute hepatotoxicity induced by CCl₄ in the experimental mice was observed as the levels of ALT, AST and LDH in serum increased significantly (p < 0.01, respectively). Pre-treatment with CCl₄, under the reductive dehalogenation by cytochrome P450, a highly reactive free radical 'CCl₃ can be formed, which can further promote the generation of reactive peroxy radical CCl₄OO' (Mccay, Lai, Poyer, Dubose, & Janzen, 1984). The peroxy radical may cause lipid peroxidation of membranes and result in liver damage, leading to the leakages of ALT, AST and LDH from the hepatic cells. In our study, APP was shown to scavenge-free radicals in vitro (Figure 6); hence the mechanism of hepatoprotective effects of APP on CCl₄-induced liver injury may partially lie in its radical scavenging activity in vivo. In this study, treatment with CCl₄ alone resulted in significant increases in the levels of ALT, AST and LDH in the serum of mice, and these elevations could be effectively inhibited by pre-administration with APP. A similar hepatoprotective observation has been reported for polysaccharides from Pink Lady apple peel (Yang et al., 2013). The polysaccharides in this study were from Fuji apple peel obtained by pre-pressing separation technology (one kind of separation technique, through which peel pomace can be separated with little flesh attached on it). Therefore, the present results from in vivo mice experiments indicate that in spite of the differences in monosaccharide compositions and contents, APPs from both the apple cultivars can develop comparable hepatoprotective effect. Notably, both the APPs contain relatively high

![Figure 7](image-url)
mole percentage of uronic acids (23.8% and 17.7% for Fuji and Pink Lady, respectively) that have been shown to play an important role in polysaccharides bioactivities (Chen, Zhang, & Xie, 2004; Yang et al., 2013).

**Effects of APP on the levels of MDA, GSH-Px and SOD in hepatic tissue**

MDA is a well-known index of free radical chain reaction of lipid peroxidation (Yang et al., 2010). As shown in Figure 7 (d), treatment with CCl₄ caused a significant increase in liver MDA level from 1.70 ± 0.30 to 2.56 ± 0.35 nmol/mg prot, compared to the normal group (p < 0.01). However, the elevated MDA level by CCl₄ was found to be reduced by the administration of APP in a dosage-dependent manner. Especially, at 450 mg/kg bw, the MDA level in hepatic tissue was significantly reduced to 1.95 ± 0.21 nmol/mg prot in comparison with the CCl₄-intoxicated group (p < 0.01).

It is well known that SOD and GSH-Px are two important and active antioxidants in liver and that the increased lipid peroxidation is usually attributed to the reduction of free radical scavenging antioxidant system. Therefore, the levels of hepatic SOD and GSH-Px in mice liver need further determination. As shown in Figure 8, under the administration of CCl₄, the activities of GSH-Px and SOD were significantly decreased to 814.7 ± 65.6 prot and 77.4 ± 6.59 U/mg prot from 950.7 ± 40.3 and 215.3 ± 14.6 U/mg prot in comparison with the untreated normal group, respectively. Interestingly, the reduced GSH-Px and SOD levels by CCl₄ were observed to be recovered by the administration of APP in a dosage-dependent manner. Specifically, for the group pretreated with 450 mg/kg bw APP, the GSH-Px activity was significantly elevated to 907.4 ± 40.2 U/mg prot (p < 0.01), very close to that of positive 450 mg/kg bw BP group (914.8 ± 20.1 U/mg prot). In the case of SOD activity, this dosage treatment resulted in a similar increase to 186.7 ± 9.3 U/mg prot, relative to the inhibited enzymatic activity caused by CCl₄ intoxication (p < 0.01).

In the aerobic metabolism, in vivo oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants. The balance of intracellular oxidative stress depends on both the cellular oxidant species and the antioxidant defence system in cells (Jia et al., 2011). Therefore, antioxidant supplementation to suppress the free radical-induced damage has become an alternative and promising therapeutic strategy for reducing the risk of liver diseases (Tian, Zhao, Guo, & Yang, 2011). In our study, APP was shown to have hepatoprotective effects against CCl₄-induced liver damage, through significantly decreasing the level of MDA and elevating the enzymatic activities of GSH-Px and SOD in liver tissues, especially at the dosage of 450 mg/kg bw.

**Histopathological observation of mice liver tissues**

To support the hepatoprotective effects of medicines and plant extracts on CCl₄-induced liver damage, histopathological observation is commonly provided additionally (Cui, Yang, Lu, Chen, & Zhao, 2014). As shown in Figure 9, for the normal group, typical hepatic cells with complete cytoplasm, prominent nucleus and visible veins could be observed (Figure 9(a)). However, the CCl₄ treatment could cause visible liver tissue damage, parenchymal disarrangement, ballooning degeneration and loss of cellular boundaries (Figure 9(b)). As demonstrated in Figure 9(c–f), the pre-administration with BP or APP brought about a marked improvement in the histopathology of liver tissues against CCl₄-induced histological degradation. Besides, the recovering effect of APP on histological alteration was dosage dependent, indicating that administration of high dosage of APP might access to almost full recovery of histological alternation of liver tissues.

It has been widely considered that one of the principal causes of liver diseases is the occurrence of lipid peroxides induced by free radicals or corresponding derivatives that are thought to cause intracellular oxidative stress (Shim et al., 2010; Zhang et al., 2004). Therefore, it may be hypothesized that the supplement of dietary antioxidants can inhibit the oxidative stress and thus reduce the risk of liver diseases. Besides apple polyphenols reported before, apple polysaccharides are also shown to be a source of antioxidants in this study. It has been widely recognized
that acidic polysaccharides from plants or fruits have antioxidant, antitumor and hepatoprotective activities (Ma et al., 2013; Xin et al., 2012; Yang et al., 2013). The present study clearly implied that the polysaccharides from Fuji apple peel pomace were also one kind of acidic saccharides (including some parts of pectin). The hepatoprotective effects of polysaccharides from Pink Lady apple peel have been reported by Yang et al. (2013). Although there are some structural differences in the two cultivar polysaccharides, the similar molar percentage of uronic acids partially explained the comparable antioxidant and hepatoprotective effects. Apple peel is usually considered as a waste of juicing industry. Besides, through the pre-pressing separation technology, large amount of apple peel pomace with little flesh attached on it can be more easily obtained, compared to peeling apples in the lab level. It has been previously found that galacturonic acid, arabinose and galactose are the main component monosaccharides of apple polysaccharides (Mehrlander, Dietrich, Sembries, Dongowski, & Will, 2002; Yang et al., 2013), and our quantitative results for the structural composition of APPs are in agreement with the previous founding, but in different amounts and ratios. This may be caused by the differences in extraction process and apple cultivars. Herein, we showed for the first time that the polysaccharides from pre-pressing separation apple (Fuji) peel pomace have antioxidant and hepatoprotective activities, suggesting that apple peel pomace, as the by-product of apple juice industry, has the potential as a source of hepatoprotective polysaccharides.

**Conclusion**

Pre-pressing separation technology is being introduced to apple juicing industry for enhancing the quality of apple juice. Through this technology, large amount of apple peel pomace with very little flesh attached can be easily obtained after juicing process. It is valuable and necessary to utilize this juicing waste; therefore, APPs were isolated from the pre-pressing separation Fuji peel pomace in this study. APP was found to be a heteropolysaccharides with arabinose, galactose and galacturonic acid as the main monosaccharides. The potential carboxylic group of uronic acids was identified by FT-IR, and the total contents of uronic acid, including both galacturonic and galacturonic acid, were determined to be 14.4% by GC–MS. APP was shown to have in vitro antioxidant activity and in vivo hepatoprotective effect, decreasing the CCl$_4$-induced elevations of enzymic activities (serum ALT, AST and LDH) and hepatic malondialdehyde level, as well as inhibiting the decreases in SOD and GSH-Px activities. Our findings here provide not only the potential for utilization of juicing industry peel wastes, but also a base for further exploitation of APPs as a novel hepatoprotective pharmaceutical ingredient.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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