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Isolation, Characterization, and Hepatoprotective Effects of the Raffinose Family Oligosaccharides from *Rehmannia glutinosa* Libosch

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ABSTRACT: This study was aimed to isolate and characterize the raffinose family oligosaccharides (RGOs) from a novel plant source of *Rehmannia glutinosa* Libosch, and further evaluate whether RGOs can attenuate CCl_4-induced oxidative stress and hepatopathy in mice. HPLC analysis showed that RGOs were mainly composed of stachyose (61.7%, w/w), followed by 23.7% raffinose and 7.1% sucrose. Administration of RGOs orally daily in mice for 21 days significantly reduced the impact of CCl_4 toxicity on the serum markers of liver damage, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total-cholesterol (TC), and triglycerides (TG). RGOs also increased antioxidant levels of hepatic glutathione (GSH), glutathione peroxidase (GSH-P*x*), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC), and ameliorated the elevated hepatic formation of malonaldehyde (MDA) induced by CCl_4 in mice, which coincided with the histological alteration. These findings exhibited the potential prospect of RGOs as functional ingredients to prevent ROS-related liver damage.

KEYWORDS: *Rehmannia glutinosa Libosch, raffinose family oligosaccharides, HPLC, carbon tetrachloride, hepatoprotective effect*

INTRODUCTION

Nondigestible carbohydrates are natural occurring constituents of many plants or foods, and are often referred to as dietary fibers. In recent years, a great deal of attention has been paid to oligosaccharides, a major source of nondigestible carbohydrates, for their beneficial physiological effect on the microflora of gastrointestinal tract.[1−5] Meanwhile, raffinose family oligosaccharides (e.g., raffinose and stachyose) as the family of α-galactooligosaccharides are the best known and most commonly applied functional oligosaccharides because they are excellent sources of dietary fiber and prebiotics, as well as having a good potential to improve food quality, flavor, and physicochemical characteristics.[6−8] Interestingly, recent advance shows that orally administered chitosan oligosaccharides can suppress hepatic cytochrome P450 enzymes and induce phase II detoxifying reactions in the liver and kidneys of rats.[9] Agaro-oligosaccharides prepared with acid hydrolysis on agar have also been shown to exhibit their in vitro and in vivo hepatoprotective effects through antagonizing oxidative damage induced by reactive oxygen species (ROS).[10,11] These reports suggest the potential prospects of various oligosaccharides in preventing the oxidative stress-related liver diseases.

Rehmannia glutinosa Libosch (also known as Di-Huang in China) is the family of Scrophulariaceae and has been used for centuries as an oriental traditional dietary herb or functional food. Recently, the raffinose family oligosaccharides isolated from *R. glutinosa* were shown to promote antibody production of B lymphocytes in immuno-suppressed mice, and enhance the splenocyte proliferation and cellular immunity in tumor bearing mice.[12] More recently, our previous study also found that the stachyose-enriched raffinose family oligosaccharides from the roots of *Lycopus lucidus* Turcz exerted significantly cellular and humoral immune responses.[3] *R. glutinosa* is traditionally considered to be very effective for treating diabetes and liver disease.[13−15] Interestingly, *R. glutinosa* oligosaccharides have been approved as a useful element to promote the proliferation of human adipose-derived mesenchymal stem cells,[16] and have also been shown to be responsible for hypoglycemic effects of *R. glutinosa* in glucose-induced hyperglycemic and alloxan-induced diabetic rats.[13] It was also found that the iridoid glycosides from *R. glutinosa* exhibited in vitro hepatoprotective effects.[15] However, to the best of our knowledge, the hepatoprotective effects of the oligosaccharides from *R. glutinosa* have not been demonstrated in vivo.

Liver is the main organ involved in the metabolism of biological toxins and medicinal agents. Such metabolism is always associated with the disturbance of hepatocyte biochemistry and generation of ROS.[17] Lots of liver damages ranging from subclinical icteric hepatitis to necroinflammatory hepatitis, cirrhosis, and carcinoma have been proved to be associated with the redox imbalance and oxidative stress.[18] Many antioxidants and plant extracts have been evaluated for their hepatoprotective and antioxidant effects against chemicals-induced liver damage. One of such candidates is the raffinose family oligosaccharide prepared from *R. glutinosa*, which was chosen in the present study. Here, the raffinose family oligosaccharides RGOs were prepared from a novel *R. glutinosa* Libosch plant, and were first used to examine in vivo antioxidant effects depending on experimental mouse model of CCl_4-induced oxidative injury.

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MATERIALS AND METHODS

Materials and Chemicals. The roots of *R. glutinosa* Libosch were harvested in October, 2011 from the Qinyang countryside region of Henan province, China, and identified according to the standard of Pharmacopeia of the People's Republic of China. Voucher specimens of the materials were deposited at the key laboratory of ministry of education for medicinal resource and natural pharmaceutical chemistry, Shaanxi Normal University, China. Diatomaceous earth, charcoal, carbon tetrachloride (CCl_4), and naphthoresorcinol were purchased from Tianjin Tianli Chemical Reagent Co. (Tianjin, China). Sucrose, raffinose, and stachyose (>99%) were obtained from Merck (Darmstadt, Germany). Acetonitrile was purchased from Acros-Organic (Geel, Belgium). Bifendate pills (BP) were obtained from Zhengjiang Wanbang Pharmaceutical Co. Ltd. (Wenling, China). Haematoxylin and eosin (H&E) were the products of Shanghai Lanji Technological Development Co. Ltd. (Shanghai, China). Assay kits to determine serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total-cholesterol (TC), and triglycerides (TG) were the products of Huili Biotechnology (Changchun, China). The commercially diagnostic kits of glutathione (GSH), glutathione peroxidase (GSH-P*x*), superoxide dismutase (SOD), malonaldehyde (MDA), and total antioxidant capacity (T-AOC, the assay is based on reduction of Fe^{3+} to Fe^{2+}, whereas the latter forms complexes with phenanthroline substances, which can be measured using a spectrophotometer) were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China).

Extraction and Isolation of RGOs. The roots of *R. glutinosa* were thoroughly air-dried and pulverized into powder, and screened through a 60-mesh sieve. The oligosaccharides were extracted as previously described with some modifications.[19] Briefly, the powder (100 g) was soaked in anhydrous ethanol for refluxing at 60 °C for 4 h. After the extraction mixture was filtered, the defatted residues were dried in air and further extracted with 10-fold volume of 50% ethanol (1:10, w/v) at 80 °C for 4 h, and this extraction was repeated three times. The combined extract was centrifuged for 15 min at 3000*g*, and the supernatants were concentrated under reduced pressure at 65 °C and then were precipitated three times by adding 7-fold volume of 100% (v/v) ethanol at 4 °C overnight. The precipitate was completely dissolved in a certain amount of water and was deproteinized by the freeze−thaw process 10 times, followed by centrifugation at 3000*g* for 15 min. The resulting supernatants were lyophilized as crude extract of water-soluble oligosaccharide RGOs.

RGOs Purification. The RGOs were purified as previously described with some modifications.[19] 5 mL of 200 mg/mL crude RGOs was loaded onto a column (5.0 i.d. × 100 cm) packed with diatomaceous earth and charcoal (1:1 w/w), followed by an elution with 6% aqueous ethanol to remove the *R. glutinosa* monosaccharides (RGMs, Figure 1A). Concerning the effect of water or lower concentrations of ethanol on oligosaccharide elution, crude RGOs were eluted by 20%, 40%, 60%, and 80% aqueous alcoholic solution, respectively. The flow rate was 1.0 mL/min, and fractions were collected using a Redifrac fraction collector (BSZ-100, Qingpu Huxi Instruments Factory, Shanghai, China). Each fraction (8 mL) was collected and then analyzed with phenol-sulfuric acid method at 490 nm with a spectrophotometer (UV-723, Guangpu Co Ltd., Shanghai, China). Fractions corresponding to the main peak were collected, respectively, and concentrated at 65 °C with a rotary evaporator (RE-52AA, Shanghai Yarong Biochemical Equipment Co., Shanghai, China) under vacuum, and finally lyophilized as a fine white powder. The highest content of oligosaccharides part was designated as RGOs (Figure 1A), and this part was further applied in the following experiments for the determination of structural analysis and in vivo antioxidant activity.

HPLC Analysis of RGOs. The quantification of the component oligosaccharides in RGOs was carried out using a Shimadzu LC-2010A high performance liquid chromatography (HPLC) system (Kyoto, Japan) with a refraction index (RI) detector (RID-10A) and Shimadzu Class-VP 6.1 workstation (SHIMADZU, Kyoto, Japan).[3] HPLC-RI analysis was performed on a Huapu XAmide column (4.6 i.d. × 150 mm, 5 μm) using acetonitrile/water (65:35, v/v) as the mobile phase at a flow rate of 1.0 mL/min. Stock standard solutions of each carbohydrate at a concentration of 5.0 mg/mL were prepared in deionized water. Working standard solutions were prepared as needed by appropriate dilution of the stock solutions in 65% aqueous acetonitrile and stored at 4 °C. Quantification of each carbohydrate was performed by comparing the peak areas with those of the standard solutions. Before injection, all samples were filtered through a 0.22 μm Millipore membrane.

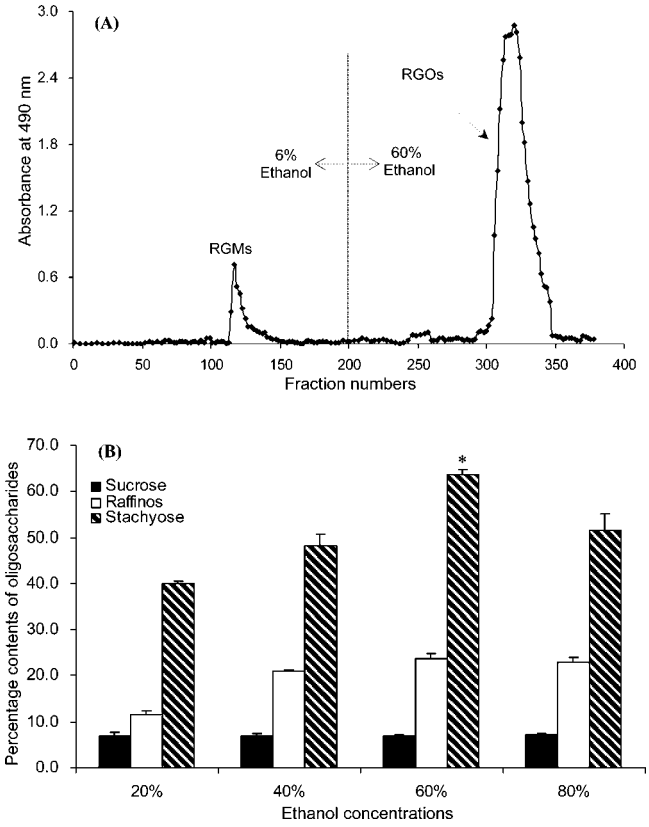

Figure 1. Elution profiles (A) of *R. glutinosa* oligosaccharides (RGOs) on diatomaceous earth and charcoal (1:1 w/w) column (5.0 i.d. × 100 cm), and percentage contents of sucrose, raffinose, and stachyose (B) in each elution by HPLC analysis, $*p < 0.05$, as compared to 20%, 40%, and 80% ethanol elution, respectively. The crude RGOs were dissolved in deionized water and applied to the column. The eluted solution was collected, and the total carbohydrate content of collected fractions was monitored by phenol−sulfuric acid method.

Animals and Treatment. Kunming male mice (weight 18−22 g) were purchased from the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). They were allowed free access to tap 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, Qianmin Feed Factory). All of the animals were housed under standard conditions with 12/12 h light−dark cycle at a temperature of 22 ± 2 °C and a humidity of 60 ± 5%. All of the experiments were approved by the Fourth Military Medical University Committee on Animal Care and Use (SYXK-007-2007).

After environmental adaptation for 3 days, mice were divided into six groups with 10 mice selected at random in each group. In both normal group and CCl_4-intoxicated group, mice were given a single dose of physiological saline (0.5 mL, intragastrically, ig) once daily. In the BP group, animals received reference drug BP at 400 mg/kg·bw (0.5 mL, ig) once daily. In low-, medium-, and high-dosage of RGOs-treated group, animals received RGOs at 200, 400, and 800 mg/kg·bw once daily (0.5 mL, ig), respectively. All administrations were conducted for 21 consecutive days. On the 22nd day, all mice except those in the normal group were given simultaneously a 1% CCl_4/

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peanut oil mixture (v/v, 0.3 mL) by intraperitoneal injection, whereas the normal group received peanut oil alone. After 2 h, all of the animals were fasted but given enough water to drink for 12 h. At the end of the experimental period, 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.[20] The blood samples were centrifuged and serum was stored at 4 °C until use, and the isolated livers were refrigerated at −80 °C for further analysis.

Measurement of Serum ALT, AST, TC, and TG. Liver damage and protection were assessed by estimating serum enzyme activities of ALT and AST, and the serum levels of TC and TG, using commercially available diagnostic kits, and the results were expressed in U/L, U/L, mmol/L, and mmol/L, respectively.

Assay of Hepatic MDA, GSH, SOD, GSH-P*x*, and T-AOC Levels. The liver tissue was homogenated by an automatic homogenate machine (F6/10-10G, FLUKO Equipment Shanghai Co. Ltd., Shanghai, China). During the preparation, 0.5 g of each hepatic tissue was homogenated in 9-fold frozen normal saline in volume, and centrifuged at 1500*g* for 10 min. The supernatant was used for the assay of MDA, GSH, SOD, GSH-P*x*, and T-AOC, reflected as common indexes of antioxidant status of hepatic tissues. The protein concentration in homogenates was measured by the method of Coomassie brilliant blue.[21] The assay for hepatic MDA and GSH levels was performed with commercially available diagnostic kits, and the results were expressed as nmol/mg protein and mg GSH/g protein, respectively. SOD, GSH-P*x*, and T-AOC were assessed using common commercial kits, and the results were expressed as U/mg protein.

Histopathological Studies in Liver Tissue. The tested mouse livers were fixed in 4% paraformaldehyde, and then the fixed tissues were embedded in paraffin, sectioned (6 μm), and stained with H&E. The slides were detected under an olympus light microscope for observations and photograph.[22]

Statistical Analysis. All of the experiments were performed at least in triplicate. The data were expressed as means of ± SD (standard deviation), and subjected to an analysis of variance (ANOVA, $p < 0.05$) and Duncan's multiple range test. *P*-Values of <0.05 were considered to be statistically significant.

RESULTS

Chemical Characterization of RGOs. The extraction procedure of crude oligosaccharides from the roots of *R. glutinosa* was optimized, and sufficient extraction was achieved with 50% aqueous ethanol. As depicted in Figure 1A, pure RGOs were first obtained from crude oligosaccharide extract by a separation on a diatomaceous earth and charcoal (1:1 w/w) column. The effects of various concentrations of aqueous ethanol as eluate on oligosaccharide recovery are shown in Figure 1B. As a result, 60% aqueous ethanol was found to be the most effective of the various elution systems investigated for the recovery of stachyose ($p < 0.05$) and raffinose. The yield of RGOs was 31.3% (w/w) of the dried *R. glutinosa* roots.

It is very important to known the components of RGOs that contribute to their bioactivity, and a routine HPLC-RI chromatographic procedure was performed to further measure the component digestible sucrose and nondigestible oligosaccharides in the RGOs preparation. As shown in Figure 2A, the tested standard oligosaccharides could be completely baseline separated within 13 min, and the peaks in the chromatogram were identified in the order of sucrose, raffinose, and stachyose. As shown in Table 1, this assay had excellent linearity from 50 to 1000 μg/mL with the correlation coefficients (r) in the range of 0.9997−0.9999. The limit of detection (LOD) calculated as 3 times the baseline noise was below 47 ng (10 μL injection).

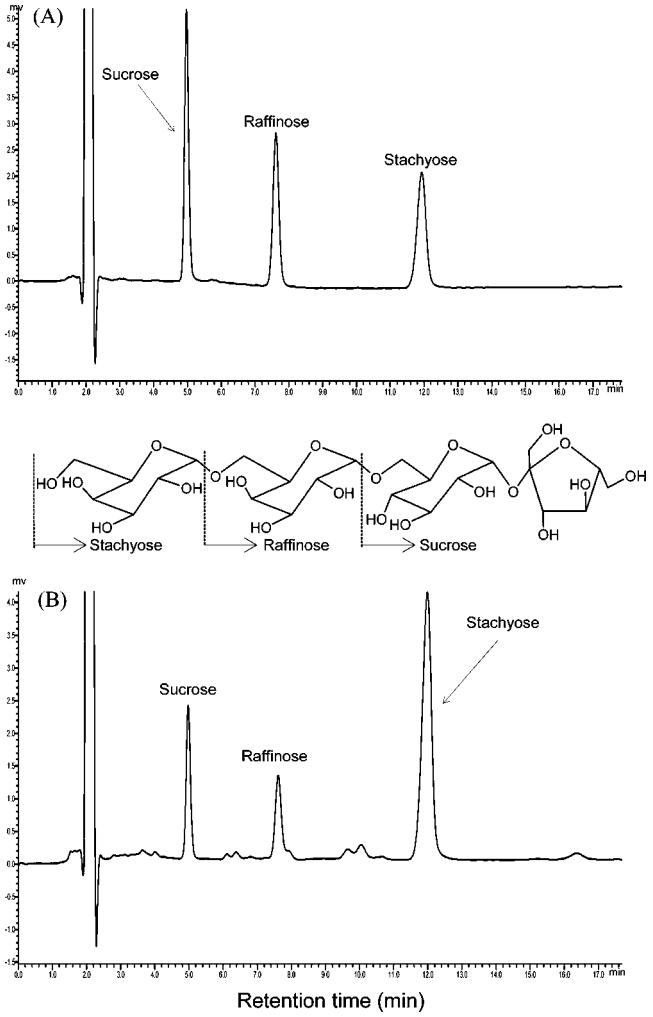

Figure 2. The HPLC-RI chromatographic profiles of the digestible sucrose and nondigestible oligosaccharide standards (A) and the contents of compositional sucrose, raffinose, and stachyose of RGOs sample (B). HPLC-RI conditions: Column, Huapu XAmide column (4.6 i.d. × 150 mm, 5 μm). Mobile phase, acetonitrile−water (65:35, v/v). Flow rate, 1.0 mL/min. The analytical procedure was performed as described in the Materials and Methods.

In addition, a typical HPLC-RI chromatogram for oligosaccharide composition in RGOs samples is shown in Figure 2B, and the quantified constituents are listed in Table 1. As can be seen, the component oligosaccharides could be identified by comparison with the standard oligosaccharide mixture (Figure 2A). The results indicate that RGOs are of a typical raffinose family oligosaccharide mixture and are composed of sucrose, raffinose (trisaccharide), and stachyose (tetrasaccharide) in the amounts of 7.1%, 23.7%, and 61.7% (grams per 100 g of RGOs), respectively. The HPLC-RI identification results clearly showed that the major oligosaccharides present in RGOs were stachyose and raffinose, accounting for up to 85.4% of the RGOs preparation, and only a trace amount of sucrose (7.1%) was detected.

Effects of RGOs on Enzymatic Activities of Serum ALT and AST in Mice. Serum enzymes, such as ALT and AST, are considered as effective biochemical markers for early hepatic damage.[21] As shown in Figure 3A and B, a single acute application of CCl_4 caused hepatotoxicity in mice, as indicated by a significant increase in serum ALT and AST activities as

Table 1. Performance Characteristics of the Proposed HPLC-RI Method for the Assay of Sucrose, Raffinose, and Stachyose from *R. glutinosa* Libosch

component	content (mg/100 mg)	t_R (min)	equation of regression ($Y = aX + b$)	R^2	linearity range (μg/mL)	LOD[a] (μg/mL)
sucrose	7.1	5.01 ± 0.008	$Y = 47551X - 288.60$	0.9999	50−800	2.8
raffinose	23.7	7.69 ± 0.039	$Y = 40090X - 573.48$	0.9998	100−800	3.2
stachyose	61.7	12.08 ± 0.084	$Y = 41809X - 965.37$	0.9997	100−1000	4.7

[a]Limit of detection (LOD): Correspond to concentrations given at a signal-to-noise ratio of 3 (S/N).

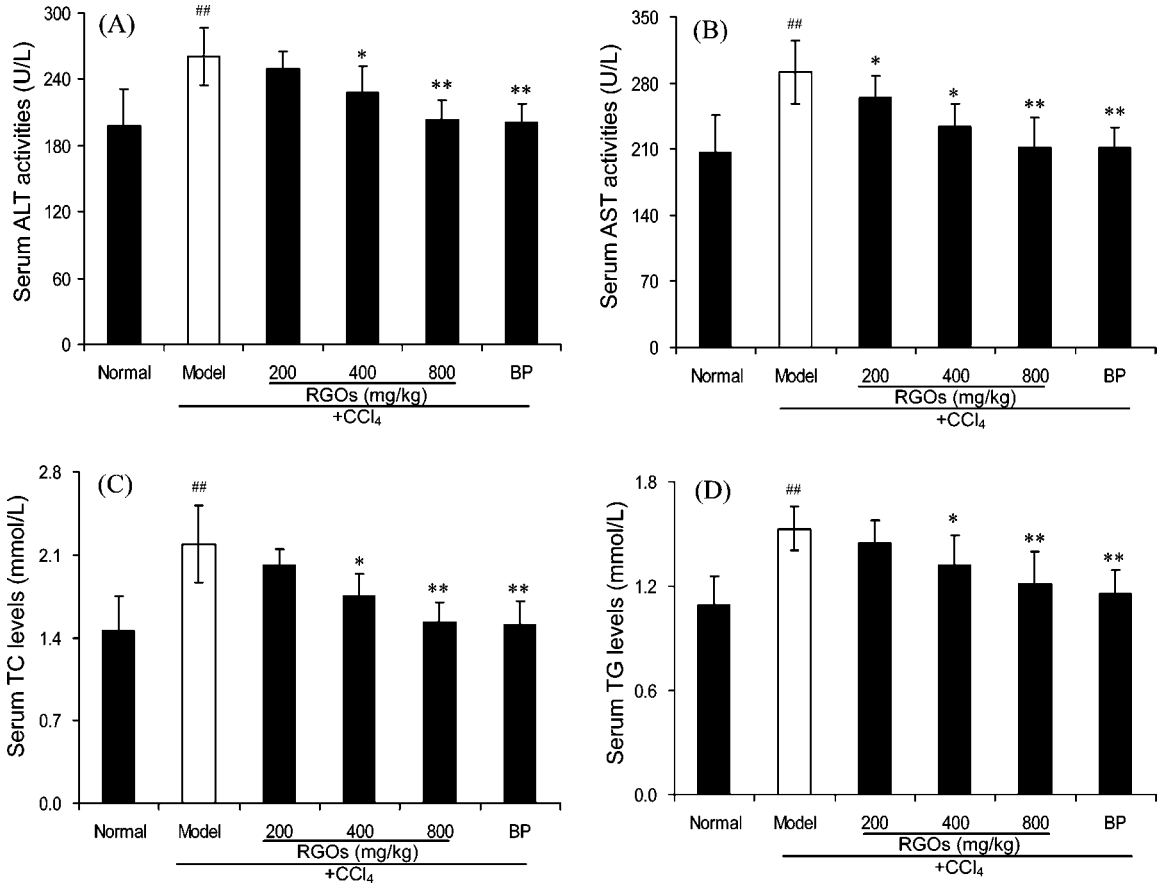

Figure 3. Effects of RGOs on serum ALT (A), AST (B), TC (C), and TG (D). Mice were administrated intragastrically with RGOs at 200, 400, and 800 mg/kg·bw or BP at 400 mg/kg·bw once daily for 21 consecutive days prior to the single administration of CCl_4 (1%). Values are expressed as means ± SD of 10 mice in each group. $^{\#\#}p < 0.01$, versus the normal group. $^{*}p < 0.05$ and $^{**}p < 0.01$, as compared to the CCl_4-intoxicated group.

compared to the normal group ($p < 0.01$), respectively. The protective administration of RGOs along with a single injury of CCl_4 decreased the level of these functional markers relative to the CCl_4 group. RGOs at 400 and 800 mg/kg·bw were found to be more effective ($p < 0.05$) when compared to the low dose (200 mg/kg·bw). At a dosage of 400 mg/kg·bw, ALT and AST activities decreased to 227.7 ± 23.5 and 234.6 ± 24.1 U/L from 260.5 ± 25.9 and 291.3 ± 33.6 U/L of CCl_4-intoxicated mice ($p < 0.05$), and at 800 mg/kg·bw, the corresponding value was 203.7 ± 17.4 and 212.2 ± 30.9 U/L, respectively ($p < 0.01$). Similarly, ALT and AST activities of mice treated with positive control BP at 400 mg/kg·bw also maintained remarkably low levels of 201.2 ± 16.3 and 211.0 ± 22.1 U/L, relative to the CCl_4-intoxicated control group, respectively ($p < 0.01$).

Effects of RGOs on Serum TC and TG Levels. The levels of serum TC and TG were estimated, and the results were given in Figure 3C and D, respectively. Serum TC and TG levels in CCl_4-intoxicated mice were sharply increased to 2.19 ± 0.32 mmol/L ($p < 0.01$) and 1.53 ± 0.12 mmol/L from 1.46 ± 0.29 and 1.09 ± 0.16 mmol/L of the normal mice, respectively ($p < 0.01$). However, the pretreatment of RGOs at all of the tested concentrations dose-dependently reduced the serum TC and TG levels elevated by CCl_4, as compared to the CCl_4-intoxicated group, especially when the dosage increased to 800 mg/kg·bw, where TC and TG activities decreased to 1.53 ± 0.16 and 1.21 ± 0.18 mmol/L, respectively ($p < 0.01$). Similarly, BP as positive control also exhibited a good protective effect (Figure 3). However, the pretreatment with RGOs at low dose of 200 mg/kg·bw led to a slight decrease in the levels of serum TC and TG, but there was not the statistical significance ($p > 0.05$).

Assay for Hepatic MDA and GSH Levels in Mice. The change of hepatic MDA and GSH levels in the experimental mice is shown in Figure 4A and B. CCl_4 administration caused a severe increase in liver metabolic concentrations of MDA in comparison with the normal group ($p < 0.01$), whereas GSH store was markedly depleted in the mice treated with CCl_4 as compared to the normal group ($p < 0.01$). However, the acute

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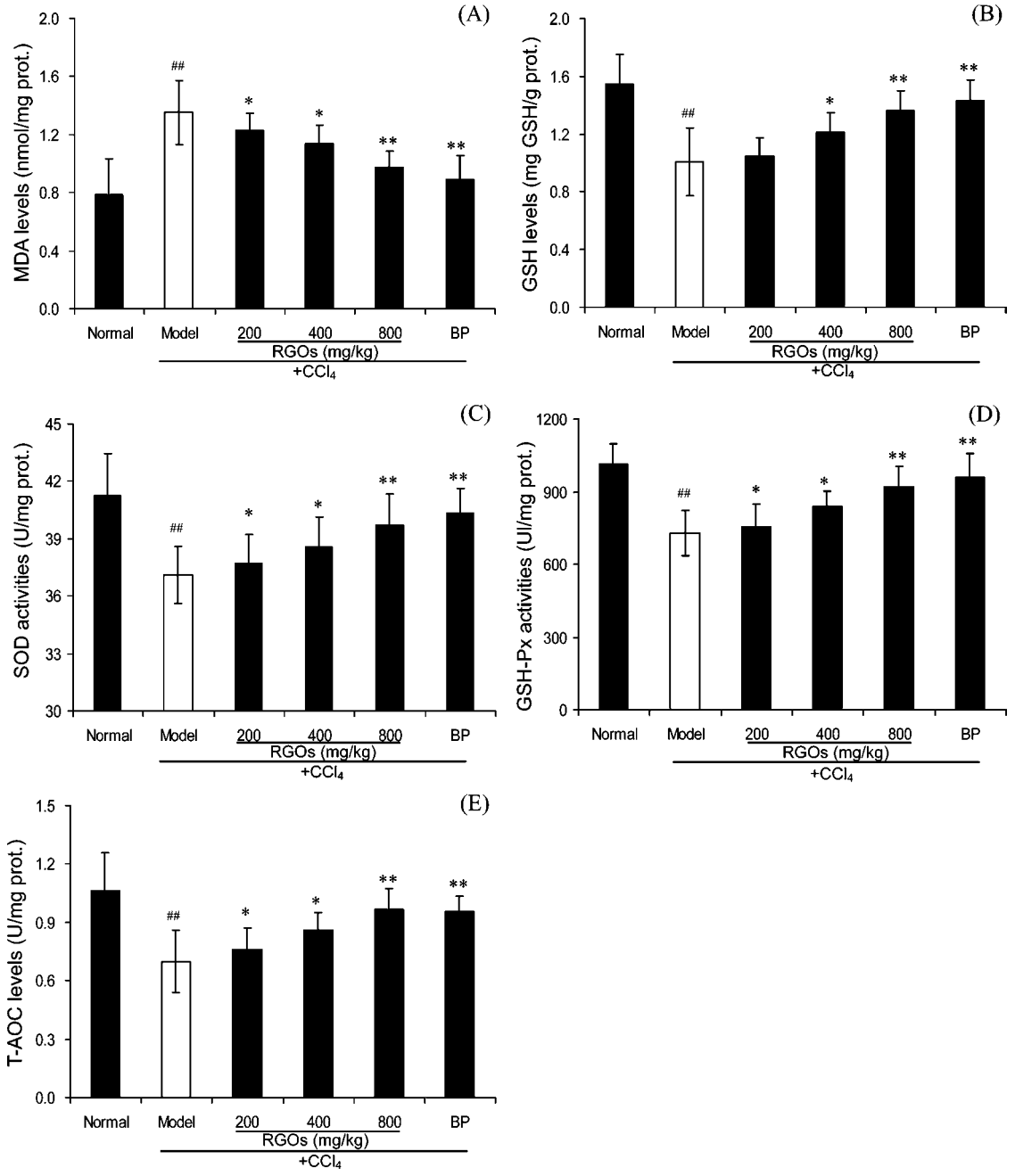

Figure 4. Effects of RGOs on hepatic MDA (A), GSH (B), SOD (C), GSH-P*x* (D), and T-AOC (E) levels against acute CCl_4-induced damage in mice. All values are expressed as means ± SD (n = 10). $^{\#\#}p < 0.01$, as compared to the normal group. $^{*}p < 0.05$ and $^{**}p < 0.01$, as compared to the CCl_4-intoxicated group.

CCl_4-induced elevation in MDA concentration was significantly lowered to 1.14 ± 0.05 and 0.97 ± 0.11 nmol/mg protein after the tested mice were subjected to the pretreatment of medial-dose and high-dose of RGOs for 21 days, as compared to CCl_4-intoxicated mice (1.35 ± 0.21 nmol/mg protein, $p < 0.05$, $p < 0.01$, Figure 4A). The pretreatment with RGOs at 400 and 800 mg/kg·bw also significantly elevated GSH level ($p < 0.05$, $p < 0.01$, Figure 4B), as indicated by an increase of up to 1.21 ± 0.13 and 1.36 ± 0.13 mg GSH/g protein from 1.01 ± 0.23 mg GSH/g protein of CCl_4-intoxicated mice, respectively. BP as a clinical hepatoprotective drug also significantly decreased the MDA level and increased the GSH concentration ($p < 0.01$ vs CCl_4-treated mice).

Effects of RGOs on Hepatic Levels of SOD, GSH-P*x*, and T-AOC. The hepatic antioxidant enzyme activities of SOD and GSH-P*x*, considered as an index of antioxidant status of tissues, were depicted in Figure 4C and D. A single acute application of CCl_4 in mice caused characteristic hepatotoxicity in antioxidant parameters of liver tissue, as indicated by a significant decrease in SOD and GSH-P*x* from 41.25 ± 2.19 and 1014.85 ± 84.40 U/mg protein in untreated normal group to 37.10 ± 1.50 U/mg protein ($p < 0.01$) and 728.42 ± 94.32 U/mg protein ($p < 0.01$, Figure 4E), as well as a remarkable decrease in the level of T-AOC, an index of total enzymatic and nonenzymatic antioxidant capacity, from 1.06 ± 0.19 U/mg protein of normal group to 0.69 ± 0.15 U/mg protein, respectively ($p < 0.01$). Administration of RGOs at the dose of

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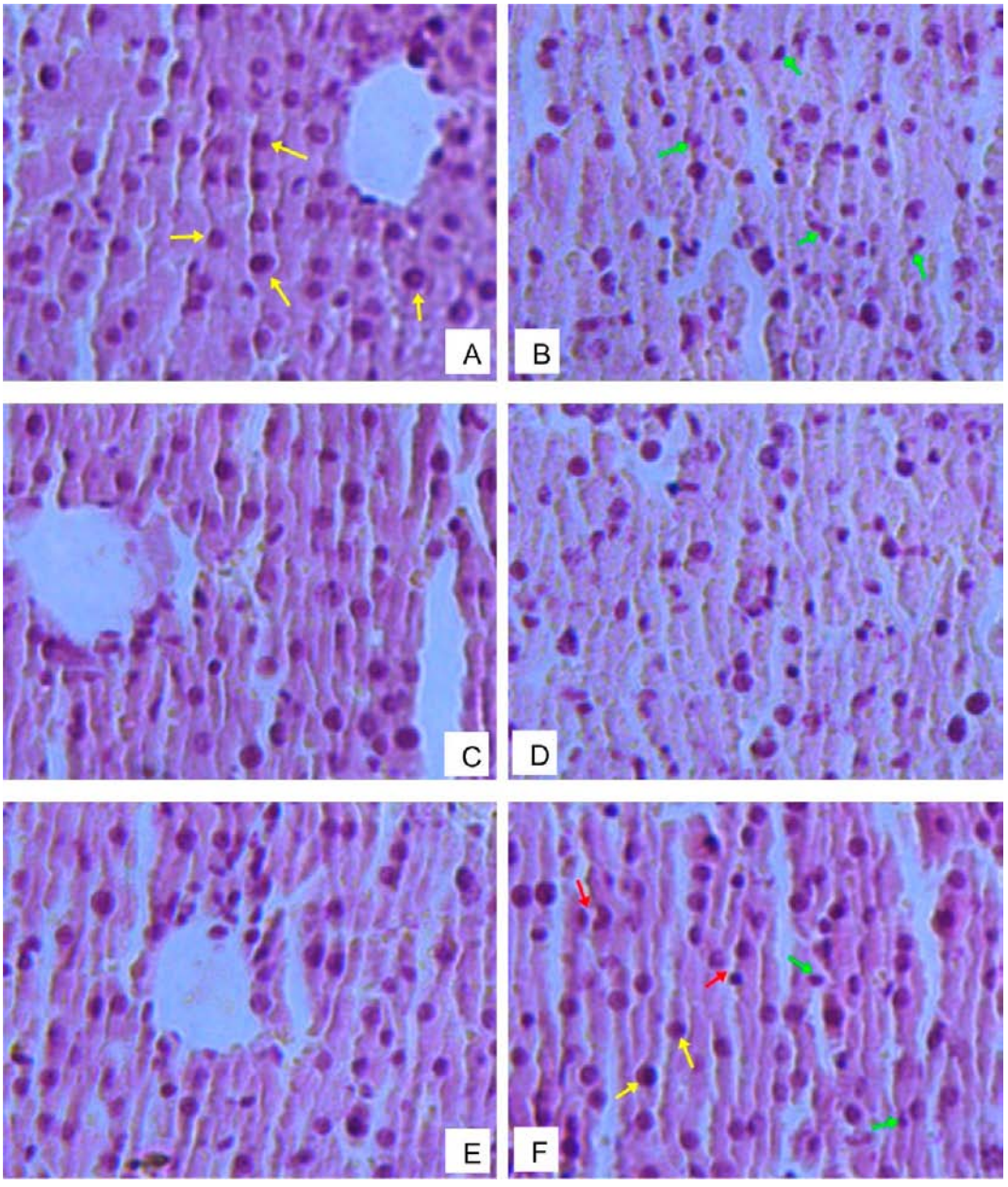

Figure 5. Preventive effects of RGOs against CCl_4-induced liver histopathological changes in mice (original magnification of 400×). (A) Normal group, (B) CCl_4-intoxicated group, (C) BP positive group (400 mg/kg·bw+CCl_4), (D) 200 mg/kg·bw RGOs (low-dose+CCl_4), (E) 400 mg/kg·bw RGOs (medium-dose+CCl_4), and (F) 800 mg/kg·bw RGOs (high-dose+CCl_4). The yellow arrows indicate normal cellular architecture with clear hepatic cell nucleus. The green arrows indicate the hepatic cell necrosis. The red arrows indicate the enlarged sinusoids between the plates of hepatocytes.

200, 400, and 800 mg/kg·bw once daily for 21 consecutive days prior to the single administration of CCl_4 (0.1%) effectively protected against a decrease in hepatic SOD, GSH-P*x*, and T-AOC ($p < 0.05$ vs CCl_4-treated mice), and BP as positive control also exhibited a good protective effect.

Histopathological Examination of Mouse Liver. To further investigate the protective effect of RGOs against acute CCl_4-induced hepatocyte morphological changes, the histopathological microscopy of liver tissue sections was examined. In the normal group, liver slices showed typical hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins (Figure 5A). In contrast, the liver sections of a single injection of CCl_4 caused strong fatty changes of mouse hepatocytes around the central vein, and a parenchymal disarrangement, such as ballooning degeneration, infiltration of inflammatory cells, and the loss of cellular boundaries (Figure 5B). However, administration of positive control BP drug showed an effective protection against CCl_4-induced liver damage (Figure 5C). As depicted in Figure 5D–F, the hepatic lesions caused by CCl_4 were markedly ameliorated by the pretreatment with RGOs, and the RGOs at 800 mg/kg·bw were more effective when compared to two other doses (200 and 400 mg/kg·bw), showing near normal appearance with well-preserved cytoplasm, prominent nuclei, and legible nucleoli (Figure 5F). This histopathological observation conformably suggests that RGOs may exert a good hepatoprotective effect.

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DISCUSSION

Naturally occurring raffinose family oligosaccharides are widely distributed in the plant kingdom and have received widespread attention due to their healthy benefits.[23,24] Recent studies have initially shown that the consumption of a prebiotic mixture and a *R. glutinosa* supplement beneficially reduces the blood indices of peroxidation status and liver diseases.[14] In fact, *R. glutinosa* has become attractive as a functional food and novel source of bioactive oligosaccharide compounds, which can proliferate beneficial bacteria.[25] However, there are no reports in linking the hepatoprotective effects to the oligosaccharide constituents of Chinese *R. glutinosa*. In the present study, this hepatoprotective effect of RGOs was first demonstrated by suppressing CCl_4-induced oxidative stress in the livers of mice, and attenuating the morphological changes caused by CCl_4. Our finding contributes to the understanding that dietary RGOs treatment reduces the occurrence of liver oxidative injury, and this nutritional strategy represents alternatives to pharmaceutical approaches for reducing hepatotoxicity.

It is widely recognized that the functional oligosaccharides have positive influences in reducing the risk of development of nutrition-related chronic diseases, and this fact has stimulated the search for new oligosaccharide sources.[5] Among these, legume α-galactooligosaccharides, including those from pea and lupin, have been considered as a main source of raffinose family oligosaccharides.[26] Herein, we successfully isolated the oligosaccharides RGOs, a novel raffinose family oligosaccharide preparation, from the roots of herb *R. glutinosa* with 50% aqueous ethanol extracting, and subsequently purified with column chromatography packed with simple diatomaceous earth and charcoal, and the elution was performed with 60% aqueous alcoholic solution. RGOs are shown to be the mixture of linear raffinose and stachyose oligomers, which are built of 1−6 linked α-galactosyl residue to the glucosyl residue of sucrose (Figure 2). HPLC analysis showed that RGOs presented 7.1% sucrose, 23.7% raffinose, and 61.7% stachyose, and dietary herb *R. glutinosa* was found to be a potential source rich in raffinose family oligosaccharides (raffinose and stachyose) with the high content of up to 31.3% of its dried roots. This method allows for fast preparation of raffinose family oligosaccharides for animal tests and may be a basis for raffinose family oligosaccharides production on an industrial scale. Interestingly, *R. glutinosa* was approved as a potential source of obtaining functional raffinose family oligosaccharides due to its stachyose-enriched composition and attractive oligosaccharide-high abundance.

Among therapeutics for liver diseases, protective antioxidants have attracted more attention because liver metabolism of biological toxins is always associated with the disturbance of hepatocyte biochemistry and generation of ROS.[27] In liver injury, trichloromethyl free radicals ($^{\bullet}CCl_3$ or $CCl_3OO^{\bullet}$), the hepatotoxic metabolites of CCl_4, are mainly associated with CCl_4-induced hepatic damage,[28] and, therefore, the CCl_4-induced hepatotoxicity is the most commonly used oxidative stress model system for the screening of hepatoprotective activity of plant-derived antioxidants. The increases in serum ALT and AST activities by CCl_4 have been attributed to hepatic structural damage because these enzymes are normally localized to the cytoplasm, and the injury to the hepatocytes alters their transport function and membrane permeability, leading to the leakage of these enzymes into the circulation from the cells.[14,22] In our study, administration of CCl_4 markedly raised the serum AST and ALT activities in mice. However, the pretreatment of the mice with RGOs for 21 days prior to CCl_4 administration remarkably protected liver from the elevation of serum ALT and AST activities, respectively. Besides successful protection of liver damage by efficiently inhibiting ALT and AST activities, RGOs also reduced CCl_4-elevated TC and TG levels in mice, indicating that this is involved in the maintenance of normal structure and function of cells, probably by its redox, metabolism, and detoxification reactions.[29] This finding contributes to the understanding that RGOs is one of the main active ingredients responsible for the hepatoprotective effect of *R. glutinosa*.

It is well-known that ROS is produced in cells during normal aerobic metabolism,[22] and oxidative stress occurs when there is an imbalance between cellular oxidant species production and antioxidant capability.[20] Meanwhile, an increase in hepatic MDA levels indicates the enhanced lipid peroxidation of the polyunsaturated fatty acid of biological membrane, leading to tissue damage and failure of the antioxidant-defense mechanisms to prevent the formation of excessive ROS.[18,30] In our work, mice treated with CCl_4 showed a striking increase in MDA levels as compared to the untreated normal mice ($p < 0.01$), and RGOs could significantly block the ROS-mediated lipid peroxidation. It is well-known that nonenzymatic antioxidant GSH can provide protection against oxidative stress, and several endogenous antioxidant enzymes such as SOD and GSH-P*x* can also convert ROS into less noxious compounds in living organisms.[20,30,31] For example, SOD catalyzes the dismutation of superoxide anions into hydrogen peroxide that subsequently converts to water by GSH-P*x* and converts lipid hydroperoxides to nontoxic alcohols.[30−32] In our hands, RGOs were also shown to markedly enhance the activities of antioxidant enzyme system of the host, including SOD, GSH-P*x*, and the level of nonenzymic antioxidant GSH, implying that RGOs may effectively protect the hepatocytes against the toxic effects of CCl_4, essentially by preventing the formation of superoxide anion and H_2O_2 and/or by exerting a sparing effect on the antioxidant enzymes. T-AOC consists of enzymatic and nonenzymatic antioxidant defense systems and reflects the body's ability to regulate antioxidants and scavenge free radicals.[33] In this study, CCl_4 administration greatly decreased the total antioxidant capacity in the liver, and the pretreatment with RGOs can significantly dispute the decrease of T-AOC (Figure 4E), suggesting that RGOs may increase the activities of both the nonenzymatic and the enzymatic antioxidant defense systems. In addition, histopathological examination also showed that the CCl_4-induced severe pathological damage of mouse liver was markedly reduced by the administration of RGOs (Figure 5). These findings were in line with previous studies that chitosan oligosaccharides and agaro-oligosaccharides protected oxidative liver damage in cell-based and animal studies.[9,11] Taken together, these results further support a beneficial relationship between antioxidant activity and hepatoprotective effect of RGOs.

In conclusion, this is the first study to show that RGOs exerted systematic protective effect against acute CCl_4-induced hepatotoxicity in mice. Chinese *R. glutinosa* is a potential novel source of raffinose family oligosaccharides, and the predominant individual oligosaccharide in RGOs was found to be raffinose and stachyose responsible for hepatoprotective activity of *R. glutinosa*. This finding opens the possibility of exploitation of functional oligosaccharides RGOs as the novel preventive

and therapeutic ingredients for the mitigation of oxidative stress-induced liver injury and some chronic disease.

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Notes

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