Inhibitory Effect of Blueberry Polyphenolic Compounds on Oleic Acid-Induced Hepatic Steatosis in Vitro

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ABSTRACT: Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide and is closely associated with metabolic syndromes, such as obesity, diabetes, and insulin resistance. Nonalcoholic fatty liver (NAFL), also called simple steatosis, is the initial phase of NAFLD, which is accompanied the characteristic pathological overaccumulation of lipids without inflammation. To prevent NAFLD from reaching the NAFL stage through dietary therapy, in the present work, wild Chinese blueberries (*Vacciniun* spp.) were selected for their well-known benefits in inhibiting metabolic syndrome. After being purified from wild Chinese blueberries, polyphenol-rich extracts were subsequently separated into three fractions, namely, anthocyanin-rich fraction, phenolic acid-rich fraction, and ethyl acetate extract. The inhibition of oleic acid (OA)-induced triglyceride (TG) deposition in HepG 2 cells was referred to as the potential activity of preventing NAFL. Biochemical indicators, such as cytotoxicity, TG level, levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and intracellular reactive oxygen species, were used to evaluate the analogous pathological stage of NAFLD. The results show that OA $\leq$ 1.0 mM exhibits a dose-dependent induction of TG accumulation, and no inflammation was observed based on the changes in ALT and AST levels. Therefore, 1.0 mM OA was used to simulate an in vitro fatty liver. Blueberry polyphenol-rich extract efficiently inhibited OA-induced TG accumulation in HepG2 cells, and the phenolic acid-rich fraction performed efficiently. Seven phenolic acids were subsequently identified using a high-performance liquid chromatography assay, and the main types were caffeic, chlorogenic, ferulic, *p*-coumaric, and cinnamic acids. These phenolic acid standards also displayed good efficiency in inhibiting TG accumulation in HepG2 cells. These results imply that wild Chinese blueberries have a potential preventive effect on NAFLD in its early stage, and phenolic acids are the most efficient component.

KEYWORDS: Blueberries, polyphenols, nonalcoholic fatty liver, triglyceride, oleic acid, phenolic acid

INTRODUCTION

The worldwide prevalence of nonalcoholic fatty liver disease (NAFLD) continues to increase and corresponds to the frequency of metabolic syndrome.[1,2] NAFLD is becoming a serious threat to people's health globally.[3,4] A recent study estimated that in North America, Europe, Australia, and Asia, over 30% afflicted with obesity, 50% with type 2 diabetes, and nearly 100% with morbid obesity suffer from NAFLD.[5] This disease is characterized by a spectrum of disorders, ranging from simple steatosis or nonalcoholic fatty liver (NAFL), to nonalcoholic steatohepatitis (NASH), to ultimately, fibrosis or cirrhosis.[4,6,7] Studies have also confirmed that NAFLD is not only a hepatic component of metabolic syndrome, but it is also associated with an increased risk of all-cause mortality and predicts future cardiovascular disease (CVD) events.[8]

Over the years, the development and progression of NAFLD have been explained according to a "two-hit" hypothesis. Briefly, the "first hit," also called steatosis, is the deposition of triglyceride (TG) in hepatocytes.[9] This stage is generally considered to represent a risk signal for more serious forms of liver damage, such as NASH and cirrhosis.[6] Once steatosis has been established, the "second hit" is required for hepatocyte injury, which is accompanied by mitochondrial dysfunction and production of inflammatory cytokines in hepatic cells.[10,11]

Epidemiological studies reveal that diets rich in fruits and vegetables can reduce the incidence of metabolic syndrome. Therefore, bioactive compounds derived from plant foods are gaining more interest in ameliorating adverse health risks because of their low toxicity and few side effects. The World Health Organization has emphasized that phenolic components, especially from colorful fruits, play an important role in preventing diseases that seriously threaten people's health, including CVDs, diabetes, cancer, and obesity.[12] For food scientists and nutritionists, finding effective functional factors and developing a dietary therapy are important approaches in preventing NAFLD. According to the two-hit theory, NASH is closely associated with lipid peroxidation. Dietary antioxidants have been proposed as therapeutic agents to counteract liver damage. According to previous studies, oat extracts containing oxidants exhibit an inhibitory effect against hepatic steatosis.[13] Melon juice extracts and gallic acid also have implications in preventing NASH.[14,15]

Blueberries are highly recommended because of their potential health benefits. Many of the health-promoting properties of

blueberries are attributed to their phenolic compounds, which are well-known for their good antioxidant activity. Anthocyanins are one of the main constituents of blueberry polyphenols. According to the authors' previous report, the total polyphenol content and the total anthocyanin content in fresh wild Chinese blueberries (*Vacciniun* spp.) are 602.9 ± 9.1 and 177.1 ± 8.3 mg/100 g, respectively.[16] Anthocyanins are beneficial in reducing the risk of metabolic syndrome conditions, such as obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia.[17,18] Aside from being rich in anthocyanins, blueberries also contain abundant phenolic acids,[19] which are reported to have antioxidant, antimutagenic, and antiviral properties, among others.[20]

Although the activities of blueberries on metabolic syndrome have been reported, information on its preventive effect on NAFLD is still rare. Given that the prevention of a disease at an early stage is the principle of dietary or nutritional therapy, the objective of the present work is to evaluate whether blueberries have the efficacy in preventing NAFLD in its initial phase, namely, hepatic steatosis. The present paper also aims to determine which polyphenolic constituents are involved in this bioactivity and to identify the efficiencies of these components.

■ MATERIALS AND METHODS

Plant Materials. Fresh blueberries (*Vacciniun* spp.), grown from the Greater Hinggan Mountains in Northeast China, were supplied by the Science and Technology Bureau of Greater Hinggan Mountains district and were stored at −20 °C until use.

Chemicals and Reagents. Amberlite XAD-7 used for purifying blueberry polyphenols was obtained from Sigma (Sydney, Australia). The Sephadex LH20 and Oasis HLB cartridges used for isolating and purifying anthocyanins were purchased from Amersham Biosciences AB (Uppsala, Sweden) and Waters (Milford, MA), respectively. Deionized water was produced using a Milli-Q unit (Millipore, Bedford, MA). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO). Acetonitrile from Mallinckrodt Baker (Phillipsburg, NJ) was of high-performance liquid chromatography (HPLC) grade. Ethanol and hydrochloric acid were purchased from China National Pharmaceutical Industry Corporation Ltd. (Shanghai, China). Analytical reagent-grade solvents were used during extraction.

Phenolic acid standards were purchased from Chengdu Mansite Pharmaceutical Co., Ltd. (Chengdu, Sichuan, China). Oleic acid (OA), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Oil Red O, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemicals Co. Fetal bovine serum (FBS), penicillin, streptomycin, Hanks' balanced salt solution (HBSS), and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco Life Technologies (Grand Island, NY). Bovine serum albumin (BSA) was purchased from EMD Biosciences (La Jolla, CA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits (linearity ranges, 0.78−50 U/L) were purchased from the Beijing Zhongsheng Hightech Bioengineering Co. (Beijing, China). The TG kit (linearity ranges, 1.13−11.29 mmol/L) was purchased from Biosind Biotechnology and Science Inc. (Beijing, China). The lactic dehydrogenase (LDH) kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Extraction and Fractionation. Polyphenol separation and anthocyanin purification from blueberries were performed as previously described but with several modifications.[21,22] Fifty grams of fresh blueberries and 100 mL of absolute methanol were mixed in a 250 mL round-bottomed flask and were then homogenized using a homogenizer (XHF-D; Ningbo Science & Biotechnology Co., Ningbo, Zhejiang, China) set at 5000 rpm for 1 min. The homogenized sample

was further centrifuged for 10 min at 4000*g*, and the supernate was filtered using a moderate-speed 102 qualitative filter paper (Hangzhou Special Paper Industry Co. Ltd., Hangzhou, China). The aforementioned procedure was repeated once to reextract the residue. The two filtrates were evaporated using a rotary evaporator at 40 °C. A portion of the concentrated solution was loaded on an Amberlite XAD-7 column, whereas the remaining portion was lyophilized (crude extract) for further analysis. After 1 h, the XAD was washed with 1% aqueous formic acid solution to remove the nonpolyphenolic compounds, and then, the polyphenolics were eluted with methanol. The eluent was concentrated at 40 °C and was lyophilized in vacuum using a freeze dryer (Four-Ring Science Instrument Plant, Beijing Co., Ltd., Beijing). Friable dark-red powder was obtained 48 h later. A 50 mg lyophilized sample (polyphenol mixture) was resolubilized in a pH 7 phosphate buffer and was applied to a Sephadex LH20 column. The column was initially washed with a pH 7 phosphate buffer to remove phenolic acids, which is called the phenolic acid-rich fraction, after being collected and lyophilized, and then washed with 70% methanol acidified with 10% formic acid to elute anthocyanins and flavonols. After the anthocyanin and flavonol fraction was freeze-dried, the fraction was resolubilized with 5% formic acid in water and was applied to an Oasis HLB cartridge. The cartridge was washed with 5% formic acid, followed by ethyl acetate, and then 10% formic acid in methanol. The ethyl acetate eluted the flavonols, and this fraction was called the ethyl acetate fraction after being dried in vacuum at 40 °C using a DZF-6021 vacuum drying oven (Hangzhou Lihui Environmental Testing Equipment Co. Ltd., Hangzhou, China). Then, the anthocyanins were eluted with acidified methanol, and the eluent was freeze-dried and called the anthocyanin-rich fraction. These extracts or fractions were kept at −20 °C until use.

Analysis of Phenolic Acids. An Agilent 1100 Series high-performance liquid chromatograph (HPLC) equipped with a diode array detector was used as the analytical HPLC system. The analytical column used had a 250 mm × 4.6 mm i.d. An Agilent Zorbax Eclipse SB-C18 column (Agilent, Santa Clara, CA) was maintained at 40 °C. The injection volume was 20 μL, and the elution solvents were (A) H_2O with 0.1% TFA and (B) acetonitrile with 0.1% TFA. They were applied as follows: flow rate of 1 mL/min, isocratic 7.5% B for 5 min, 7.5−22.5% B for 20 min, 22.5−30% B for 15 min, 30−45% B for 5 min, 45−70% B for 2 min, isocratic 70% B for 20 min, 70−7.5% B for 2 min, and isocratic 7.5% B for 10 min. Phenolic acids were examined with a diode array detector at 280 nm. The phenolic acids in the phenolic acid-rich fraction were identified according to the consistency of retention time with the phenolic acid standards (caffeic, chlorogenic, cinnamic, ferulic, gallic, *p*-coumaric, and protocatechuric acids).

Cell Culture. HepG2 cells were purchased from Peking Union Medical College Hospital (Beijing, China) and were cultured in DMEM supplemented with 10% FBS containing 1% antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C in a humidified 5% CO_2 atmosphere.

Cytotoxicity Assay. The MTT assay is based on the protocol established by Mossmann (1983). Briefly, at the end of the incubation period, the culture fluid in the wells was removed, and the cells were carefully washed once with phosphate-buffered saline (PBS) and incubated with 200 μL of a serum-free DMEM medium containing 0.05% MTT in each well for 4 h. Afterward, the culture medium was removed, and 150 μL of dimethyl sulfoxide was added to dissolve the formed formazan. The absorbance of each well was measured at 570 nm using a microplate spectrophotometer system (Molecular Devices Co., Sunnyvale, CA). The cellular release of LDH was used as a measure of cellular damage/integrity. The enzymatic activity was determined using the LDH kit according to the manufacturer's instructions. To ensure that the absorbance of the reaction solution matched the linearity ranges (absorbance, 0.05−0.5) recommended by the manufacturer, a reasonable dilution multiple of the cellular supernate must initially be chosen.
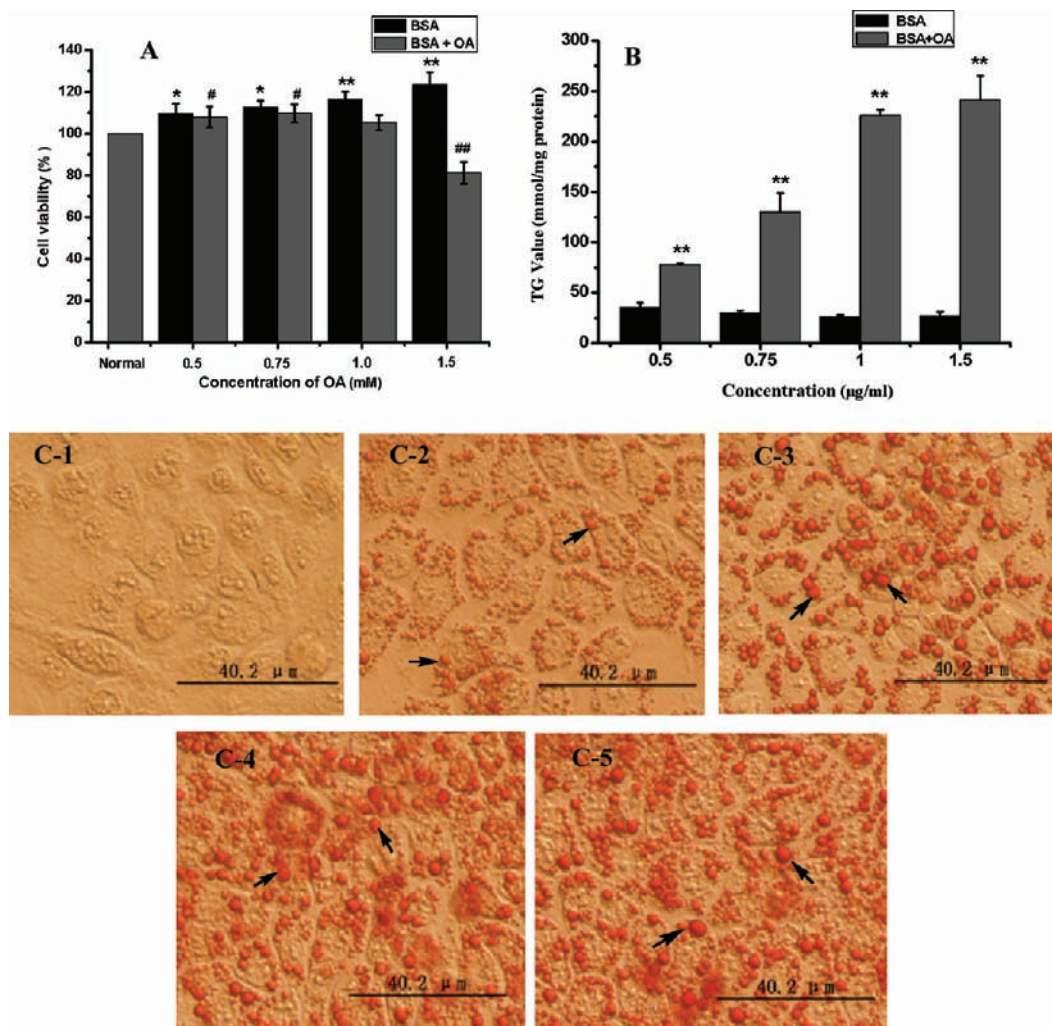
Figure 1. Effect of OA on cell viability and TG accumulation in HepG2 cells. (A) Cell viability, (B) TG value, and (C1−C5) oil red O staining: (C-1) normal cells, (C-2) 0.5 mM OA, (C-3) 0.75 mM OA, (C-4) 1.0 mM OA, and (C-5) 1.5 mM OA (**, $P < 0.01$).

TG Analysis and Oil Red O Staining. Cells were cultured into a 24-well plate and were washed twice with PBS after 24 h. Then, the cells were fixed with 75% ethanol for 15 min. After they were rewashed with PBS, the cells were stained using 0.5% Oil Red O in isopropanol for 30 min. For the optical microscopy observation, the cells were washed with 70% ethanol containing 0.5% HCl for 1−2 min and were then washed with distilled water. Intracellular TG content was evaluated using a commercial TG kit after the cells were lysed with cell lysis solution.

ALT and AST Analyses. The ALT and AST levels in coculture supernates were investigated using commercial kits according to the manufacturer's instructions.

Cellular Reactive Oxygen Species (ROS) Measurement. The ROS level in HepG2 cells was monitored using the fluorescent dye DCFH-DA. HepG2 cells were cultured on the bottom of a transparent black 96-well plate in a 200 μL growth medium at a density of 1.0×10^5 cells/mL. After 24 h of incubation, the medium was replaced with a serum-free medium containing 0.5, 0.75, 1.0, and 1.5 mM of OA, and the cells were cultivated for another 24 h. Then, the new medium with 25 μM DCFH-DA was added into the 96-well plate, and the cells were incubated sequentially for another 1 h at 37 °C. After the supernate containing DCFH-DA was removed, the cells were carefully washed twice with HBSS. The fluorescence of the cells from each well was recorded using a multifunctional microplate reader (Molecular Devices Co.) at 530 nm emission and 485 nm excitation.

Statistical Analysis. The data were analyzed using one-way analysis of variance (ANOVA) with Origin version 8.0. Results are expressed as means ± standard deviations for each measurement. The significance of the difference (* denotes $p < 0.05$; ** denotes $p < 0.01$) between two groups was assessed using a one-way ANOVA followed by Tukey's procedure.

■ RESULTS

Effect of OA on Cell Viability and Steatosis in HepG2 Cells. To simulate the pathological features of steatosis in vitro, the lipotoxicity of OA on HepG2 cells was determined (Figure 1A). OA at 0.5 and 0.75 mM showed a proliferative effect on HepG2 cells ($p < 0.05$). The corresponding BSA concentrations used to dissolve OA also exhibited a dose-dependent proliferative effect on HepG2 cells. This phenomenon indicates that BSA can alleviate the cytotoxicity of OA on HepG2 cells. However, the cells exhibited significant ($p < 0.01$) apoptosis when OA concentration was 1.5 mM, and nearly 81% of the living cells remained viable as compared with the normal group (no OA treatment).

OA can induce a significant ($p < 0.01$) increase in TG deposition in HepG2 cells in a dose-dependent manner
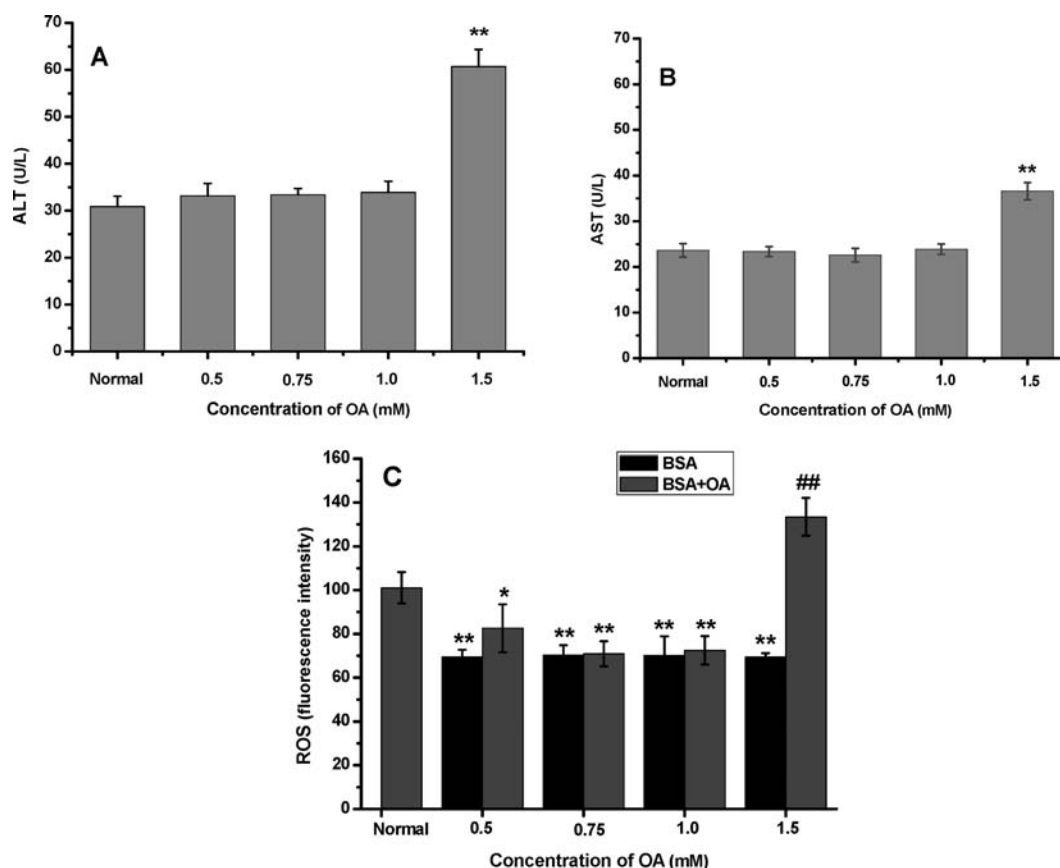
Figure 2. Effect of OA on ALT and AST levels, as well as intracellular ROS level, expressed by HepG2 cells. (A) ALT level, (B) AST level, and (C) intracellular ROS level (*, $p < 0.05$; ** and ##, $P < 0.01$).

(Figure 1B). Simultaneously, lipid staining was used in morphological observations. The cells in the normal group exhibited irregular polygons, clear edges, and no red lipid droplets (Figures 1C1—5). At 0.5 mM OA, lipid droplets began to appear in the HepG2 cells. With an increase in OA concentration, intracellular lipid droplets became larger with a deeper red color.

Effect of OA-Induced TG Accumulation on the Production of ALT and AST as Well as Intracellular ROS. Significantly high ALT and AST levels in the blood are indicators of hepatitis. In other words, ALT and AST can detect hepatic cell inflammation. Therefore, ALT and AST release assays were used to judge the progress of NAFLD in the cell model. The ALT and AST levels did not increase significantly until the OA concentration is 1.5 mM (Figure 2A,B). The intracellular ROS level was investigated further (Figure 2C). Correspondingly, the ROS level increased significantly ($p < 0.01$) when the OA concentration was 1.5 mM. However, at OA concentrations less than 1.5 mM, the intracellular ROS levels were lower than that in the normal cells. This condition may be attributed to the protective effect of BSA on the HepG2 cells because a much lower ($p < 0.01$) intracellular ROS level was also observed in the HepG2 cells treated with the corresponding BSA concentration used to dissolved OA (Figure 2C).

According to the NAFLD processes in vivo, TG deposition is the main pathological feature of hepatocyte steatosis, and apoptosis, as well as inflammatory response, is not involved during this stage. Therefore, 1.0 mM OA was chosen to induce hepatocyte steatosis in vitro in the following experiments.

Cytotoxicity of Blueberry Polyphenol-Rich Extract and Its Fractions on HepG2 Cells. The dose—response cytotoxicity of the blueberry polyphenol-rich extract and its fractions on HepG2 cells is shown in Figure 3. As compared with the normal cells, at the range of 20—300 μg/mL, both the blueberry polyphenol-rich and the ethyl acetate extracts showed no significant effect on cell viability over a 24 h period (Figure 3A). The anthocyanidin-rich and the phenolic acid-rich fractions did not inhibit cell growth at concentrations below 250 and 120 μg/mL, respectively (Figure 3A). However, the LDH results show that all of the experimental samples began to display cytotoxicity at 150 μg/mL (Figure 3B). According to this analysis, the safe doses of these samples are below 120 μg/mL in the following experiments.

Inhibitory Effect of Blueberry Polyphenol-Rich Extract and Its Fractions on TG Accumulation in HepG2 Cells. To evaluate the inhibitory effect of blueberry polyphenols on OA-induced lipid accumulation and to identify the effective constituents, HepG2 cells were treated with safe doses of the blueberry polyphenol-rich extract and its fractions in the presence of OA for 24 h. Then, the TG level was analyzed quantitatively, and the morphology was observed by oil red O staining under an optical microscope (Figure 4). As compared with the model group, the blueberry polyphenol-rich extract could effectively suppress TG deposition in HepG2 cells in a dose-dependent manner. When the concentration was 80 μg/mL, the inhibitory rate of the TG synthesis reached a maximum value of approximately 60%.

To verify which constituent is effective in attenuating lipid accumulation in HepG2 cells, the blueberry polyphenol-rich
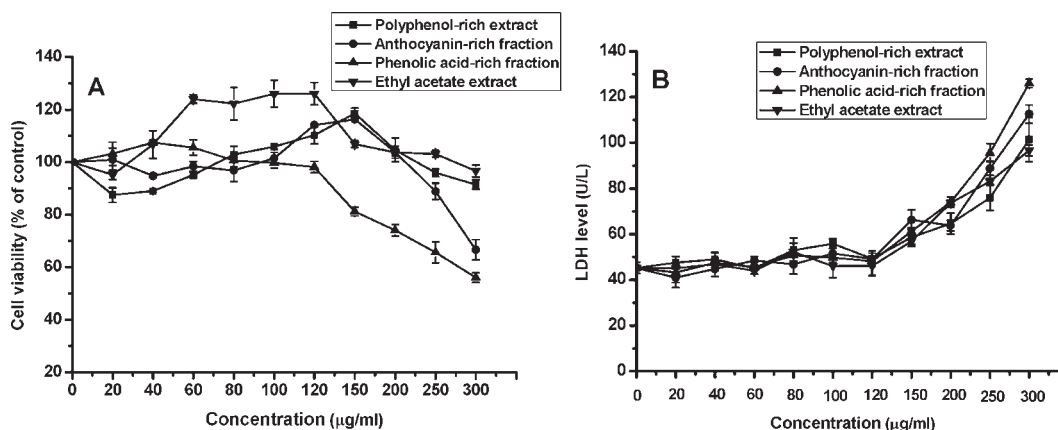
Figure 3. Inhibitory effect of blueberry polyphenol extracts and different fractions on TG accumulation in HepG2 cells. (A) Cell viability and (B) LDH.

extract was separated into three fractions (anthocyanin-rich fraction, phenolic acid-rich fraction, and ethyl acetate extract). After the fractionation by Sephadex HL20 and the extraction by ethyl acetate, approximately 20.5 mg of the anthocyanin fraction, 24.1 mg of the phenolic acid fraction, and 2.2 mg of the ethyl acetate extract were derived from the 50.0 mg blueberry polyphenol-rich extract. Among these fractions, the phenolic acid-rich fraction exhibited the best effect on TG clearance. The maximal clearance was $58.6 \pm 4.7\%$ when the concentration was 100 μg/mL. As for the anthocyanin-rich fraction, the maximal TG clearance could reach 30% at 100 μg/mL. However, at low concentrations ranging from 20 to 60 μg/mL, the anthocyanin-rich fraction could promote OA-induced TG accumulation in HepG2 cells. The ethyl acetate extract showed the lowest bioactivity of inhibiting TG accumulation in HepG2 cells. Intracellular lipid drops in cultures with different treatments at 80 μg/mL were also simultaneously observed through oil red O staining under an optical microscope. As compared with the control group, the lipid drops in cells treated with the blueberry polyphenol-rich extract and the phenolic acid-rich fraction had a light color and a smaller volume. These results imply that blueberry polyphenols are beneficial in inhibiting OA-induced lipid accumulation in hepatic cells and that the most effective constituents are phenolic acids, which is about 48.1% of the total amount of blueberry polyphenols.

Qualitative Analysis of Phenolic Acid Compounds in Phenolic Acid-Rich Fraction. Considering the high bioactivity of the phenolic acid-rich fraction in inhibiting lipid deposition in HepG2 cells, the composition of phenolic acids in this fraction was analyzed by HPLC. According to the related reports on the composition of phenolic acids in different blueberries, the seven most common phenolic acids in blueberries are caffeic, chlorogenic, cinnamic, ferulic, gallic, p-coumaric, and protocatechuric acids.[22−26] These phenolic acid standards were chosen to identify the phenolic acid composition in Chinese wild blueberries. The HPLC chromatogram of the phenolic acid-rich fraction identified all of these phenolic acids according to the identical retention times with the phenolic acid standards. On the basis of the response values of the peaks, the identified main phenolic acids in Chinese wild blueberries are caffeic, chlorogenic, cinnamic, ferulic, and p-coumaric acids.
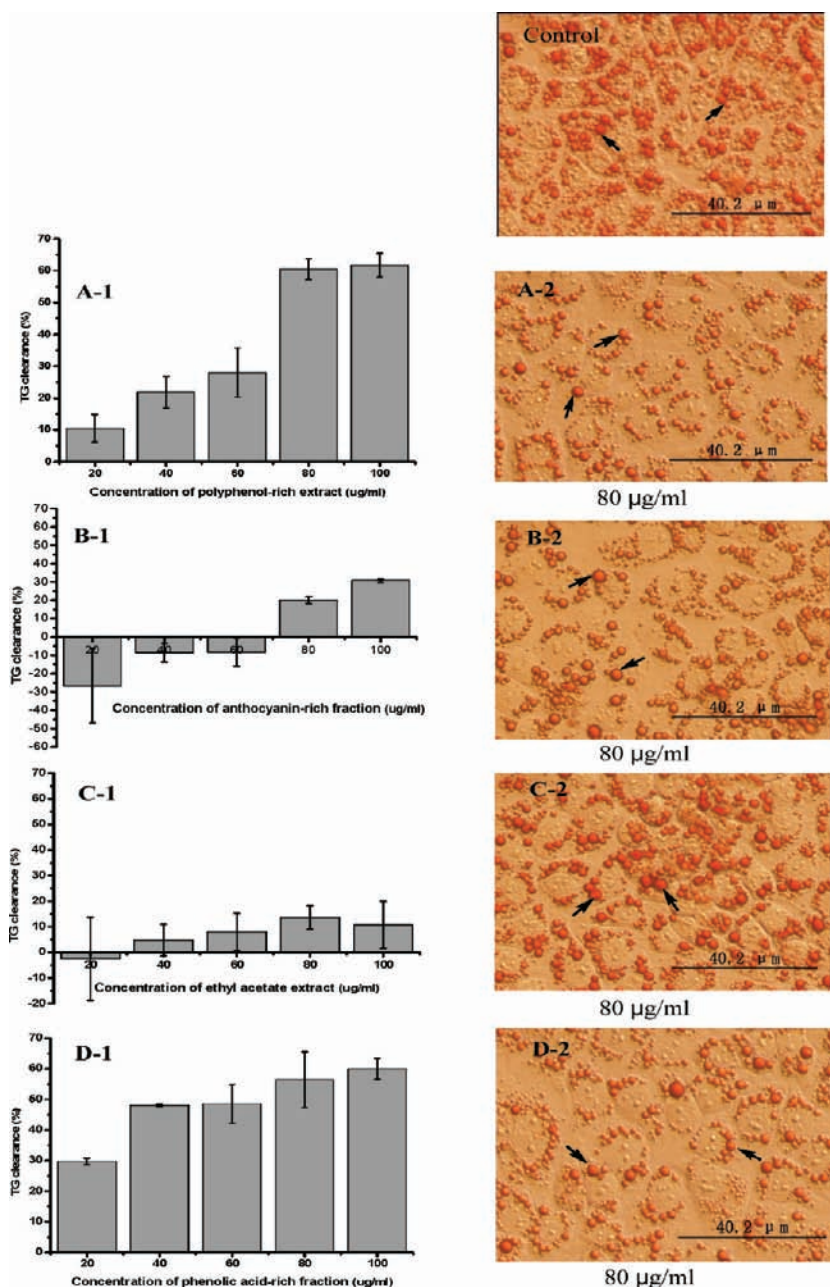
Inhibitory Effect of Standard Phenolic Acids on TG Accumulation in HepG2 Cells. To confirm further which component in the phenolic acid-rich fraction is the most effective in

inhibiting TG accumulation in HepG2 cells, five identified phenolic acid standards were evaluated. Under toxicologic evaluation, these phenolic acid standards exhibited cytotoxicity to HepG2 cells at 120 μg/mL (Figure 5). Therefore, safe doses ranging from 20 to 100 μg/mL were chosen. Caffeic acid was most efficient on suppressing TG accumulation in HepG2 cells (Figure 6), and its maximal clearance was over 50%. Chlorogenic acid, ferulic acid, and p-coumaric acid shared a maximal TG clearance of approximately 40%. Cinnamic acid exhibited the lowest maximal TG clearance, which was about 22%. However, at a lower concentration, such as 60 μg/mL, the order of the five phenolic acids in inhibiting TG deposition in HepG2 cells is caffeic acid > chlorogenic acid > ferulic acid = p-coumaric acid > cinnamic acid. These results confirm that phenolic acids are blueberry polyphenolic compounds effective in inhibiting TG deposition in HepG2 cells, and the most active one is caffeic acid.

■ DISCUSSION

The liver is an important organ that maintains whole-body energy homeostasis by metabolizing fatty acids and glucose.[10] NAFLD is one of the most common liver diseases worldwide and is the result of an imbalance between the hepatic uptake of free fatty acids (FFAs), TG synthesis, and excretion. Overaccumulation of TG in hepatocytes can impair liver function and increase the risk of other diseases. To date, there is a growing body of evidence suggesting that NAFLD is associated with an increased risk of incident CVD.[27] When hepatic steatosis occurs, TG can be transported to the blood in the form of very-low density lipoprotein TGs,[28] which is closely related to CVD. Therefore, NAFLD has been the subject of much attention and research because of the serious health risks of this disease.

Intracellular lipid accumulation is the main pathological characteristic of a human liver with NAFLD.[29] FFAs are the main substrates of synthetic TG in hepatocytes. Excessive ingested fat and metabolic syndrome conditions, such as obesity, insulin resistance, and type 2 diabetes, can result in increased FFA levels in the blood. In metabolic syndrome, the lipolysis of lipids in adipose tissue increases. As a result, the delivery of FFA to the liver is amplified.[6] Related research has shown that the influx of plasma FFA derived from the lipolysis of adipose tissue to the liver accounts for about 60−80% of the source of intrahepatic fat and approximately 15% of liver fat derived from dietary FFA.[30] OA, a monounsaturated fatty acid, and palmitic acid (PA), a saturated fatty acid, are the main dietary FFAs.

Figure 4. Inhibitory effect of blueberry polyphenol-rich extract and different fractions on TG accumulation in HepG2 cells. Left, A-1 to D-1 are the polyphenol-rich extract, anthocyanin-rich fraction, ethyl acetate extract, and phenolic acid-rich fractions, respectively; right, A-2 to D-2 are oil red O staining of corresponding extracts or fractions at 80 μg/mL.

The most important enzyme associated with TG synthesis is acyl-coenzyme A:diacylglycerol acyltransferase (DGAT), which catalyzes the final step in TG synthesis in hepatocytes and others.[10] Before being synthesized as TG, PA must be directly desaturated to palmitoleic acid or elongated to stearic acid, which is then desaturated to form OA.[31] Previous research has illustrated that OA is more steatogenic but less apoptotic than PA in hepatic cell cultures.[32] Therefore, OA was chosen in the current work. Dietary sugars, such as fructose and glucose, can also result in FFA overload in hepatocytes through de novo synthesis.[33]

In the liver, the formation of lipid droplets may be a protective response of preventing lipotoxicity from fatty acid-induced oxidative stress.[6] However, the storage of TG in the liver is only

a temporary strategy. If hepatic cells are unable to handle them appropriately through metabolic pathways, the stored TG could render the liver more susceptible to NASH.[33] Therefore, regulation of the hepatic TG synthesis may be critical in producing new treatments for NAFLD. The inhibitory effect on TG synthesis in vitro was determined to evaluate the potential bioactivity of blueberries on NAFLD.

NAFLD has two stages, namely, simple steatosis (also called NAFL) and NASH.[29] Steatosis is a benign noninflammatory condition.[31] NASH is the more severe end of NAFLD and is associated with progressive liver disease, fibrosis, and cirrhosis.[29] In the present study, the pathological characteristics of NAFLD were characterized in an in vitro cell model. HepG2 cells, a
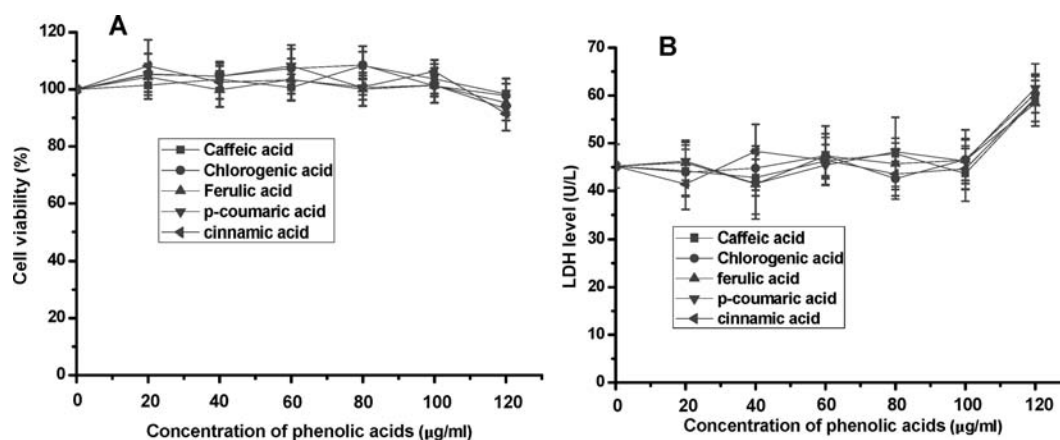
Figure 5. Cytotoxic effect of phenolic acid standards on HepG2 cells. (A) Cell viability and (B) LDH.

well-characterized and widely used human hepatoma cell line expressing a variety of liver functions,[31,34,35] have been used. To identify the NAFLD stage in this cellular model, aside from TG accumulation, cytotoxicity, ALT and AST levels, and intracellular ROS were examined. The current data demonstrate that the incubation of HepG2 cells with OA results in dose-dependent TG accumulation. Consistent with the previous studies of Okamoto et al.[36] and Gomez-Lechon et al.,[31] when the OA concentration is ≤ 1.0 mM, no cytotoxicity was observed, and the ALT and AST levels, as well as the intracellular ROS, did not exhibit abnormalities. These results demonstrate that these phenomena correspond with the pathological characteristic of NAFL. However, 1.5 mM OA produced significant toxicity on HepG2 cells ($p < 0.01$). The ALT and AST levels in the supernate and intracellular ROS also increased significantly ($p < 0.01$), indicating that inflammation occurs in HepG2 cells, which is the pathological characteristic of NASH.

According to the previous research and the current data, TG overaccumulation in hepatocytes can subsequently result in inflammation. To reduce TG synthesis in the liver and to bring this condition under a comparatively lower level are efficient means to prevent the progression of NASH. In the present work, at the primary stage of NAFLD, namely, hepatic steatosis, the potential mediatory role of blueberries on TG overaccumulation in the liver was evaluated. The present study shows that blueberry polyphenols are efficient against OA-induced TG accumulation in HepG2 cells, and the maximal percentage of inhibition is about 60%. This bioactivity of blueberry polyphenols may be closely associated with the phenolic acids. Among the three fractions of blueberry polyphenol-rich extract, the phenolic acid-rich fraction shows the best inhibitory effect on TG synthesis, and its maximal percentage of suppression is 59.2% at 100 μg/mL. Subsequently, the composition of the phenolic acid-rich fraction was identified through HPLC analysis, and then, the inhibitory effect of five phenolic acid standards on TG accumulation was evaluated further. The results indicate that the selected phenolic acids can significantly reduce TG synthesis in HepG2 cells and that the most active ones are caffeic and chlorogenic acids.

The benefits of polyphenols in suppressing TG accumulation in hepatocytes have also been reported in previous studies. Rutin, a common dietary flavonoid in fruits and vegetables, can significantly reduce TG synthesis and cholesterol level in an OA-induced in vitro model of fatty liver, and under a safe dosage, their maximal percentages of inhibition are 17.85 and 30.5%, respectively.[37] The

ethyl acetate fraction of the oat ethanol extract, rich in polyphenol compounds, also shows an inhibitory effect on OA-induced TG accumulation in HepG2 cells, and the percentage of inhibition is 27.2% at 50 μg/mL.[13] Under this model, phenolic acids show better efficacy in inhibiting TG accumulation compared with those from the previous reports.

Considering the effects of FFA, the mechanisms of functional compounds inhibiting TG synthesis in hepatocytes are still not completely clear due to complications. One approach may be through mediating related enzymes or proteins that are involved in the lipid synthesis in the liver. The inhibition of DGAT2 expression is consistent with the marked efficacy in reducing the TG content in the liver in the NALFD model.[6] Moreover, Wu et al.[37] observed that aside from reducing OA-induced TG accumulation, rutin simultaneously suppresses the expression of sterol regulatory element binding protein-1. This endoplasmic reticulum-resident transcription factor is in charge of upregulating most enzymes in the fatty acid synthesis as well as enzymes that supply acyl-coenzyme A units.[6] Rutin can also inhibit the transcriptions of 3-hydroxy-3-methylglutarylcoenzyme A reductase, glycerol-3-phosphate acyltransferase, fatty acid synthase, and acetyl-coenzyme carboxylase.[37] These results further confirm that OA-induced hepatic steatosis is closely associated with endoplasmic reticulum stress, which is characterized by the accumulation of unfolded proteins in the endoplasmic reticulum lumen and disrupted intracellular Ca^{2+} homeostasis.[38] Therefore, endoplasmic reticulum stress might serve as a novel target in the pathogenesis and therapy of NAFLD.

The potential benefit of phenolic acid compounds on preventing NAFLD has also been observed in early studies in vivo. As early as 1984, Kimura et al. discovered that both caffeic and chlorogenic acids could inhibit liver lipid peroxide in rats with lipid metabolic abnormality.[39] Polyphenol compounds of coffee can reduce TG accumulation and improve inflammation in a high fat diet (HFD)-induced rat model of steatohepatitis.[40] After being administered with decaffeinated coffee and its subfractions, polyphenols and melanoidins, lipid droplets in the livers of all treatment groups are reduced significantly through histological observation, and the serum ALT level is regulated to the normal level.[40] The active compounds may be closely associated with chlorogenic acid. On the one hand, coffee is a rich source of chlorogenic acid;[41] however, given that chlorogenic acid only circulates at low amounts in vivo, the chlorogenic acid metabolites may reach the liver.[42] Considering that chlorogenic acid can
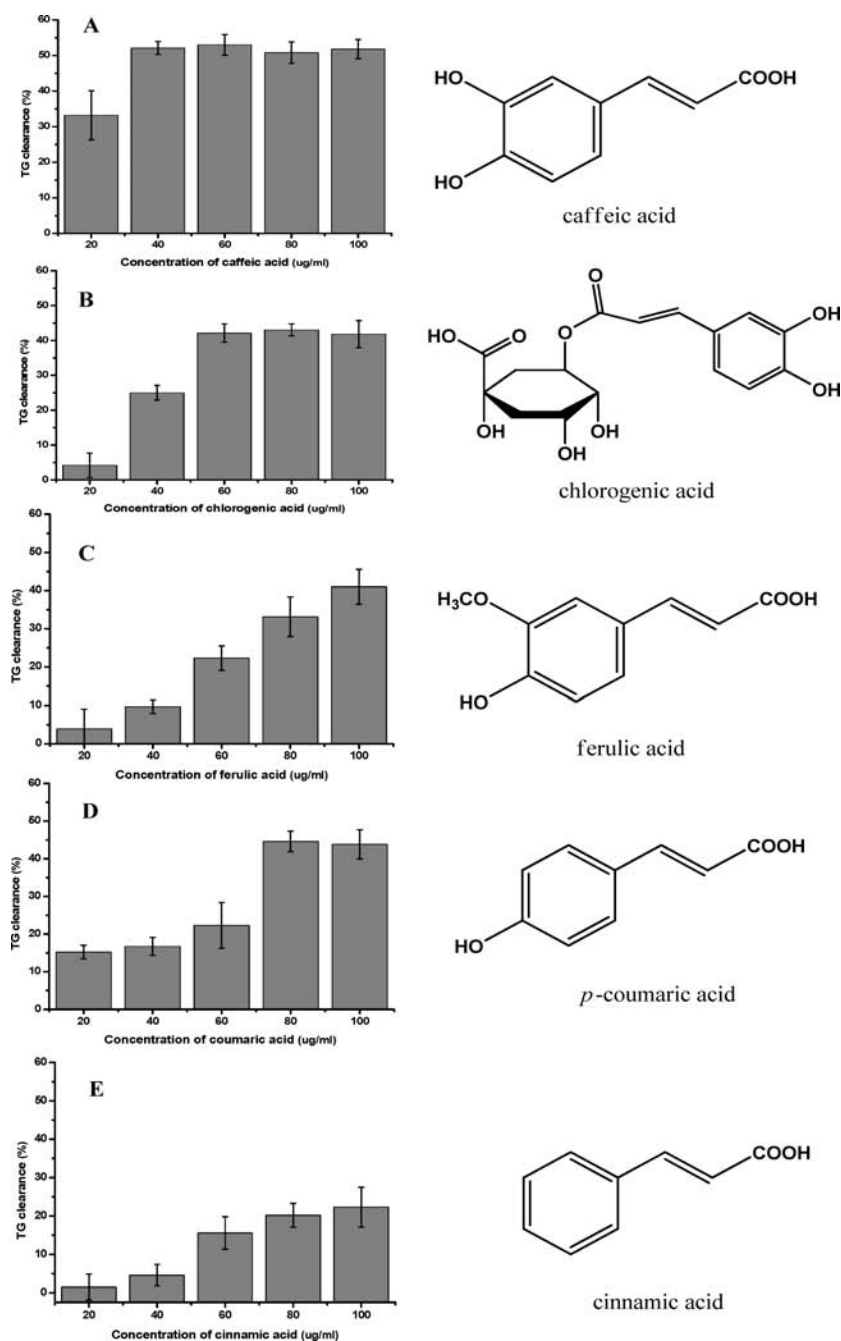
Figure 6. Inhibitory effect of the five phenolic acid standards from blueberries on TG accumulation in HepG2 cells. (A) Caffeic acid, (B) chlorogenic acid, (C) ferulic acid, (D) p-coumaric acid, and (E) cinnamic acid.

increase the β-oxidation of fatty acids through peroxisome proliferator-activating receptor (PPAR)-α in the liver,[43] which can regulate lipid metabolism by inducing catabolism of fatty acids, this acid can therefore prevent fat deposition and subsequent hepatic damage.[40,43] Gallic acid, a naturally abundant plant phenolic compound in the human diet, is also beneficial for the suppression of HFD-induced hepatosteatosis in rats. According to the previous study of Hsu and Yen,[15] as compared with the HFD group, the number of lipid droplets in the HFD + gallic acid groups is significantly decreased, and the TG level is reduced by about 38% in the liver. Furthermore, o-coumaric acid can inhibit the elevation of hepatic triacylglycerol and cholesterol levels in

rats with HFD-induced obesity, which indicates that o-coumaric acid potentially impedes the development of hepatosteatosis.[44] Antiobesity or antidiabetes is another efficient pathway to prevent and treat NAFLD. o-Coumaric acid has antiobesity potential because it can inhibit intracellular TG in 3T3-L1 preadipocytes by inhibiting the glycerol-3-phosphate dehydrogenase activity and the expression of PPAR-γ.[45]

Earlier studies showed that berry anthocyanins are beneficial in preventing metabolic syndrome. The administration of purified anthocyanins from blueberries and strawberries can prevent the development of dyslipidemia and obesity in HFD-induced mice, but feeding whole berries does not alter the development of

obesity.[46] Anthocyanins from Cornelian cherries (*Cornus mas*) can ameliorate obesity and insulin resistance in HFD-induced mice.[47] However, the present authors discovered that the anthocyanin-rich fraction at lower doses could even accelerate the progress of OA-induced TG accumulation, and the maximal percentage of inhibiting the TG synthesis was only about 30% at 100 μg/mL, which was much lower than that of the phenolic acid-rich fraction. In vivo experiments are needed to confirm the present data further because the molecular structure and bioactivities of functional compounds, such as anthocyanins and phenolic acids, are susceptible to change during digestion and absorption in the alimentary tract and upon metabolism in the liver.

The results indicate that blueberries may have a preventive effect on NAFLD. Therefore, people should daily consume some fruits or vegetables rich in polyphenols, such as phenolic acids and anthocyanins. Foods with high fat and sugar content must be avoided.

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12263

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