ORIGINAL ARTICLE

High hydrostatic pressure extract of mulberry leaves ameliorates hypercholesterolemia via modulating hepatic microRNA-33 expression and AMPK activity in high cholesterol diet fed rats

Eunyoung Lee†, Mak-Soon Lee†, Eugene Chang‡, Chong-Tai Kim§, Ae-Jin Choi*, In-Hwan Kim# and Yangha Kim†,‡

1Department of Nutritional Science and Food Management, Ewha Womans University, Seoul, South Korea; 2Department of Food and Nutrition, Gangneung-Wonju National University, Gangneung-si, Gangwon-do, South Korea; 3R&D Center, EastHill Corporation, Gwonseon-gu, Suwon-si, Gyeonggi-do, South Korea; 4Functional Food & Nutrition Division, National Institute of Agricultural Science (NIRAS), Rural Development Administration (RDA), Wanju, Jeonbuk-do, South Korea; 5Department of Integrated Biomedical and Life Sciences, Korea University, Seoul, South Korea; 6Graduate Program in System Health Science and Engineering, Ewha Womans University, Seoul, South Korea

Abstract

Background: Mulberry leaf (Morus alba L.) contains multiple bioactive ingredients and has been used in the treatment of obesity, diabetes, inflammation, and atherosclerosis. High hydrostatic pressure (HHP) processing has been developed for the extraction of bioactive compounds from plants. However, the hypocholesterolemic effect of the HHP extract from mulberry leaves and its underlying mechanism have never been investigated.

Objective: The specific aim of the present study was to investigate the hypocholesterolemic property of a novel extract obtained from mulberry leaves under HHP in rats.

Design: Sprague–Dawley rats were divided into four groups and fed either a normal diet (NOR), a high cholesterol diet containing 1% cholesterol and 0.5% cholic acid (HC), an HC diet containing 0.5% mulberry leaf extract (ML), or a 1% mulberry leaf extract (MH) for 4 weeks.

Results: High hydrostatic pressure extract of mulberry leaves significantly reduced the HC-increased serum levels of triglyceride (TG), cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), and hepatic contents of TG and TC. The HHP extraction from mulberry leaves also increased the HC-decreased fecal TC and bile acid levels without changing body weight, food intake, liver weight, and serum activities of alanine transaminase (ALT) and aspartate transaminase (AST) (P < 0.05). The mulberry leaf extract significantly enhanced the expression of hepatic genes such as cholesterol 7 alpha-hydroxylase (CYP7A1), liver X receptor alpha (LXRα), and ATP-binding cassette transporters, ABCG5/ABCG8, involved in hepatic bile acid synthesis and cholesterol efflux (P < 0.05). In addition, the HHP extraction of mulberry leaves significantly suppressed hepatic microRNA(miR)-33 expression and increased adenosine monophosphate-activated protein kinase (AMPK) activity.

Conclusion: These results suggest that the HHP extract of mulberry leaves lowers serum cholesterol levels by partially increasing hepatic bile acid synthesis and fecal cholesterol excretion through the modulation of miR-33 expression and AMPK activation in the liver.

†These authors contributed equally to this work and should be considered co-first authors
Cardiovascular disease (CVD) has been the leading cause of mortality across the world (1). Hyperlipidemia, which is characterized by increased blood levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and their associated lipoproteins, and decreased high-density lipoprotein cholesterol (HDL-C) is considered as a risk factor for CVDs (2). Therefore, it is critical to improve lipid abnormalities to prevent and/or treat CVDs.

Cholesterol metabolism and bile acid synthesis are associated with several transcriptional regulators and enzyme activities. Liver X receptors (LXRs) regulate ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8 expressed in the liver and intestine, thereby controlling cholesterol homeostasis through cholesterol uptake, transport, efflux, and excretion (3, 4). Liver X receptor alpha (LXRα)-deficient mice lack the expression of the rate-limiting enzyme for bile acid synthesis, cholesterol 7 alpha-hydroxylase (CYP7A1), resulting in accumulation of cholesterol in the liver, which leads to CVDs (5). LXR agonists have been shown to decrease atherosclerosis by mobilizing cholesterol from the periphery, promoting hepatic excretion, and limiting absorption (3, 6). Therefore, cholesterol metabolism and bile acid synthesis might be potential therapeutic targets for dyslipidemia and CVDs.

Mulberry leaf (Morus alba L.) is widely used to feed silk worms and as an alternative medicine with anti-obesity, anti-diabetic, anti-inflammation, and anti-atherosclerosis effects (7–11). Growing evidence demonstrates that multiple bioactive components such as alkaloids including 1-doxynojirimycin (1-DNJ), flavonoids, polyphenols, and polysaccharides are found in mulberry leaves which have physiologically beneficial effects (12–14). An aqueous extract of mulberry leaves inhibits the oxidation and lipid peroxidation of LDL in murine macrophage cells (9) and decreases the serum lipid profiles by regulating fatty acid oxidation, lipogenesis, and cholesterol clearance in high fat diet-fed rats or hamsters (15, 16). These studies demonstrate the protective effect of mulberry leaf extract on CVDs. A novel technology, HHP has been developed as an alternative to conventional high-heat processing (17). This non-thermal food processing technology at lower temperatures between −20 and 60°C and pressures above 100 MPa retains sensory and nutritional qualities by disrupting the cell membranes and releasing the intracellular products without thermally degrading the activity and structure of bioactive components (18, 19). In addition, the effectiveness of the mulberry juice’s microbiological quality, bioactive compounds, anti-oxidant activity and volatile profiles was evaluated using HHP technology suggesting that HHP processing might be an alternative to conventional heat treatment for the production of high-quality mulberry juice (20).

In the present study, we investigated the hypocholesterolemic effect of HHP-treated mulberry leaf extract in rats fed a high-cholesterol diet. We evaluated several parameters such as lipid levels in serum, liver, and feces, hepatic gene expression related to cholesterol uptake and excretion, microRNA-33 (miR-33) expression, and adenosine monophosphate-activated protein kinase (AMPK) activity.

Materials and methods

Preparation of high hydrostatic pressure-mulberry leaf extract
The mulberry leaf extract prepared by using the HHP method was kindly supplied from Korea Food Research Institute (Wanju, Jeolabuk-do, Korea). Mulberry leaves were harvested locally (Sangju, Geongsangbuk-do, Korea) in May 2017 and 500 g of the leaves were homogenized in a Waring blender for 5 min. A total of 40,000 units of enzymes of Pectinex ultra color (Daejong Trade Co., Korea) and Pectinex BE XXL (Daejong Trade Co., Korea) were added to the ground mulberry leaves. The mixture was then poured into plastic bags and transferred to a high-pressure apparatus (TFS-50L, Innoway Co. Ltd., Korea), where it was subjected to a pressure of 100 MPa for 4 h at 50 °C. The enzymes were inactivated by boiling at 100 °C for 10 min. The extracts were then cooled and centrifuged at 11,000 g for 5 min and filtered through a Whatman No. 5 filter paper. These extracts were then lyophilized and stored at −20 °C until further use.

Ultra performance liquid chromatography-photodiode detector-quadrupole/time of flight-mass spectrometry (UPLC-PDA-Q/TOF-MS) analysis
Polyphenols in HHP extract of mulberry leaves were identified and quantified using an ACQUITY Ultra Performance LC system. This system was equipped with a...
photodiode array detector with a binary solvent manager (Waters Corporation, USA) series and a mass detector G2 Q/TOF micro mass spectrometer (Waters, UK) equipped with an electrospray ionization (ESI) source as previously described (21). Individual polyphenols were separated using a Kinetex column (1.7 μm XB-C18 100 A, 150 × 2.1 mm; Phenomenex, USA) at 30 °C. The samples (2 μL) were injected and eluted within 40 min with a sequence of linear gradients. The sample flow rate was set at 0.3 mL/min and the wavelength at 350 nm. The mobile phase comprised solvent A (0.5% formic acid in water, v/v) and solvent B (0.5% formic acid in acetonitrile). The amount of quercetin was calculated by using the following formula:

\[
\text{Content (mg/100 g)} = \left( \frac{P_1}{P_2} \times C \times \text{dilution factor} \right) / 1,000 \times 100,
\]

where \(P_1\) is the peak area of the sample, \(P_2\) is the peak area of the internal standard, and \(C\) is the concentration of the internal standard.

**Animals and diets**

Six-week old male Sprague-Dawley rats weighing 180–200 g were purchased (Doo Yeol Biotech, Korea). Each rat was housed individually by caging in a controlled environment maintaining a constant temperature (22 ± 2°C), humidity (55 ± 5%), and a 12-h light and dark cycle. After 1 week of acclimatization with free access to water and a normal chow diet (Harlan 2018S rodent diet, United States), 40 Sprague-Dawley rats were randomly divided into four groups (\(n = 10\)/group) as follows: a normal diet (NOR), a high-cholesterol diet containing 1% cholesterol and 0.5% cholic acid (HC), an HC diet containing 0.5% HHP extract of mulberry leaves (ML), and a 1% HHP extract of mulberry leaves (MH). A commercial chow diet of Harlan 2018S contains 44.2% carbohydrate, 18.6% crude protein, 6.2% fat, 18.2% fiber, and 5.3% ash. The diet compositions for NOR, HC, ML, and MH groups are shown in Table 1. The body weights and food intake amounts were measured twice a week during the 4-week experimental period. Feces were collected on the last three consecutive days of the experiment and stored at −40°C. After 12 h of overnight fasting, the rats were anesthetized with a mixture of Zoletil 50 (Virbac Laboratories, France) and Rompun (Bayer Korea, Seoul Korea). Blood samples were collected by cardiac puncture, separated by centrifugation (2,800 rpm, 20 min, 4°C), and stored at −40°C until further use. Liver and epididymal white adipose tissue (eWAT) were excised, immediately frozen in liquid nitrogen, and stored at −70°C for further analysis. All the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ewha Womans University (IACUC No. 17-057).

**Serum biochemical markers**

Based on the enzymatic colorimetric methods, serum activities of alanine transaminase (ALT) and aspartate transaminase (AST) and serum levels of HDL-C, TC, and TG were measured using commercial kits (Asan pharmaceutical, Korea) in accordance with the manufacturer’s instructions. LDL-C was calculated by using the Friedewald’s formula (22).

\[
\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TC} / 5)
\]

**Determination of hepatic and fecal lipids**

Lipids from the liver and feces were extracted using the method of Bligh and Dyer with a slight modification as previously described (23). About 0.1 g wet liver tissues were briefly homogenized in 1.5 mL of 0.9% saline and 7.5 mL of methanol:chloroform (2:1, v:v). A total of 2.5 mL of chloroform was added and centrifuged at 3,000 rpm for 20 min. The lower phase was collected by using a Pasteur pipette, moved to a fresh tube, filtered through Whatman No. 6 filter paper, and subsequently dried and weighed. A solution of n-hexane/isopropanol (3/2, v/v) was employed to dissolve the lipid extract. Collected feces were dried in a dry oven (65°C) for 1 day, ground, and weighed. Fecal lipid extraction was carried out in a way similar to the liver lipid extraction. Hepatic and fecal TG and TC levels were analyzed by using the enzymatic colorimetric method using commercial kits as described here.

**Fecal bile acid analysis**

Fecal bile acid was extracted following the fecal lipid extraction method as described earlier. Total bile acids (TBAs) in the feces were analyzed using the TBA-test kit (Wako, Japan) according to the manufacturer’s instructions. In these assays, the reacted substrates create colored end products considered to be directly proportional to the concentration of TBA.

**Histological analysis**

Dissected liver tissues from the rats were fixed in 10% formalin solution for 24 h, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E). The histology sections were visualized by using a microscope (Olympus, Japan) at 400× magnification.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from the liver tissues using Ribo Ex (Geneall Biotechnology Co., Ltd., Korea). Complementary DNA (cDNA) was synthesized from 4 μg of isolated RNA using a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Bioneer Co., Korea) in accordance with the manufacturer’s instructions. LDL-C was calculated by using the Friedewald’s formula (22).

\[
\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TC} / 5)
\]

**Determination of hepatic and fecal lipids**

Lipids from the liver and feces were extracted using the method of Bligh and Dyer with a slight modification as previously described (23). About 0.1 g wet liver tissues were briefly homogenized in 1.5 mL of 0.9% saline and 7.5 mL of methanol:chloroform (2:1, v:v). A total of 2.5 mL of chloroform was added and centrifuged at 3,000 rpm for 20 min. The lower phase was collected by using a Pasteur pipette, moved to a fresh tube, filtered through Whatman No. 6 filter paper, and subsequently dried and weighed. A solution of n-hexane/isopropanol (3/2, v/v) was employed to dissolve the lipid extract. Collected feces were dried in a dry oven (65°C) for 1 day, ground, and weighed. Fecal lipid extraction was carried out in a way similar to the liver lipid extraction. Hepatic and fecal TG and TC levels were analyzed by using the enzymatic colorimetric method using commercial kits as described here.

**Fecal bile acid analysis**

Fecal bile acid was extracted following the fecal lipid extraction method as described earlier. Total bile acids (TBAs) in the feces were analyzed using the TBA-test kit (Wako, Japan) according to the manufacturer’s instructions. In these assays, the reacted substrates create colored end products considered to be directly proportional to the concentration of TBA.

**Histological analysis**

Dissected liver tissues from the rats were fixed in 10% formalin solution for 24 h, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E). The histology sections were visualized by using a microscope (Olympus, Japan) at 400× magnification.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from the liver tissues using Ribo Ex (Geneall Biotechnology Co., Ltd., Korea). Complementary DNA (cDNA) was synthesized from 4 μg of isolated RNA using a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Bioneer Co., Korea). An AccuPower 2X Greenstar qPCR MasterMix (-ROX Dye) (Bioneer Co., Korea) and a fluorometric thermal cycler (Corbett Research, Australia) were used for quantitative
real-time polymerase chain reaction (qRT-PCR). Primers used for qRT-PCR are described in the supplementary Table 1. β-actin was used as a reference gene for normalization and the results were relatively quantified using the ΔΔCt method (24) and expressed as a fold-difference compared with the HC group.

The miR-33 expression was measured as demonstrated before (25). cDNA was synthesized by using a miRNA cDNA Synthesis Kit with Poly (A) Polymerase Tailing (ABM Inc., Canada) and amplified using the EvaGreen miRNA qPCR Master Mix (ABM Inc.). The miR-33 expression was normalized to U6 snRNA using the 2−ΔΔCt method.

Adenosine monophosphate-activated protein kinase activity

An AMPK kinase assay kit (MBL Life Science, USA) was used to measure the AMPK activity. The protein levels were determined using a bicinechonic acid (BCA) protein assay kit (Thermo Scientific, USA). AMPK activity was normalized to the protein concentration and expressed as a fold change compared with the HC group.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were conducted using the SPSS software (version 23; IBM Corporation, USA). Significant differences among the different groups were determined by a one-way analysis of variance (ANOVA) following Tukey’s multiple comparison tests. A p-value less than 0.05 was considered as statistically significant.

Table 1. The composition of experimental diets (g/kg diet)

| Component            | NOR | HC   | ML   | MH   |
|----------------------|-----|------|------|------|
| Corn starch          | 150.0 | 150.0 | 150.0 | 150.0 |
| Casein               | 200.0 | 200.0 | 200.0 | 200.0 |
| Sucrose              | 500.0 | 485.0 | 484.5 | 484.0 |
| Corn oil             | 50.0  | 50.0  | 50.0  | 50.0  |
| Cellulose            | 50.0  | 50.0  | 50.0  | 50.0  |
| Mineral mix†         | 35.0  | 35.0  | 35.0  | 35.0  |
| Vitamin mix‡         | 10.0  | 10.0  | 10.0  | 10.0  |
| DL-Methionine        | 3.0   | 3.0   | 3.0   | 3.0   |
| Choline bitartrate   | 2.0   | 2.0   | 2.0   | 2.0   |
| Cholesterol          | 0.0   | 10.0  | 10.0  | 10.0  |
| Cholic acid          | 0.0   | 5.0   | 5.0   | 5.0   |
| Mulberry leaves extract | 0.0 | 0.0  | 5.0   | 10.0  |
| Total                | 1,000 | 1,000 | 1,000 | 1,000 |
| Total Calorie (kcal) | 3,774 | 3,714 | 3,712 | 3,710 |
| Carbohydrates (% as kcal) | 67.3 | 66.8 | 66.8 | 66.7 |
| Protein (% as kcal)  | 19.6  | 19.6  | 19.6  | 19.6  |
| Fat (% as kcal)      | 11.6  | 12.1  | 12.1  | 12.1  |

† AIN-76 Mineral mix (mg/kg diet), ‡ AIN-76 Vitamin mix (mg/kg diet). NOR, normal diet; HC, high-cholesterol diet containing 1% cholesterol and 0.5% cholic acid; ML, HC + 0.5% high hydrostatic pressure mulberry leaf extract; MH, HC + 1% high hydrostatic pressure mulberry leaf extract. This experimental diet was formulated based on the AIN-76 diet composition.

Results

Composition of high hydrostatic pressure extract of mulberry leaves

Figure 1 and Table 2 show that the mulberry leaves contain quercetin 3-O-rutinoside (rutin), quercetin 3-O-glucoside (isoquercitrin), quercetin 3-O-(6''-O-malonyl)glucoside, kaempferol 3-O-rutinoside (nicotiflorin), quercetin 3-O-(2''-O-malonyl)glucoside (morkotin), kaempferol 3-O-glucoside (astragalin) and kaempferol 3-O-(6''-O-malonyl)glucoside. Mulberry leaves extracted by HHP contained 14.25 mg phenolic compounds per 100 g of the dried sample.

Effects of mulberry leaf extract on body weight, food intake, and adipose tissue mass

After supplementing mulberry leaf extract in the HC diet for 4 weeks, no statistical differences in the final body weight, body weight gain, food intake, energy intake, and energy efficiency ratio were noted for any of the experimental groups. In addition, HHP-treated mulberry leaf extract did not change eWAT mass (Table 3).

Influence of high hydrostatic pressure-treated mulberry leaf extract on the liver weight and serum alanine transaminase (ALT) and aspartate transaminase (AST) activities

To investigate whether supplementing the HC diet with the HHP extract from mulberry leaves results in liver toxicity and the HC-induced hepatomegaly, the liver tissue weights, and activities of serum ALT and AST were
Preventive effects of mulberry leaves on hypercholesterolemia measured. The HC diet significantly increased the liver tissue weight by about 1.5 fold as compared with the NOR diet, indicating that the HC leads to liver hypertrophy. However, supplementing the HC diet with HHP-treated mulberry leaf extract (ML or MH diet) did not change the liver weight, compared with the HC diet (Table 3). Next, we investigated whether using mulberry leaf extract is safe in rats. Serum ALT and AST activities were not statistically different among the experimental groups.

Effects of high hydrostatic pressure-treated mulberry leaf extract on serum and hepatic lipid profiles
Rats fed on a HC diet had significantly increased serum concentrations of TC and LDL-C and reduced serum concentrations of HDL-C, as compared with rats fed on a NOR diet (P < 0.05) (Fig. 2a). The HC-increased serum TC and LDL-C concentrations were significantly reduced by 22.5 and 31.5%, respectively, in the MH group (Fig. 2a). A total of 0.5 and 1% mulberry leaf extract supplementation in the HC diet significantly increased fecal TG levels by at least 1.43 times compared with the HC group (P < 0.05) (Fig. 2d). Fecal TC concentrations were significantly increased in a dose-dependent fashion when mulberry leaves were used (P < 0.05) (Fig. 2d). In addition, HHP extract of mulberry leaves dose-dependently increased fecal bile acids as measured by TBA levels, reaching statistical significance at the 0.5% dose (ML) (P < 0.05) (Fig. 2d). Therefore, it is possible that mulberry leaf extract-reduced serum and hepatic lipid profiles is associated with its-increased fecal cholesterol excretion.

Effects of high hydrostatic pressure-treated mulberry leaf extract on hepatic gene expression related to cholesterol efflux and bile acid synthesis
In the present study, hepatic mRNA levels involved in cholesterol efflux and bile acid synthesis were measured by qRT-PCR. The high hydrostatic pressure extract of mulberry leaves significantly upregulated the HC-decreased hepatic mRNA levels of LXRα, ABCG5, and ABCG8.
Eunyoung Lee et al.

which are the key transcriptional regulators of cholesterol efflux ($P < 0.05$) (Fig. 3a). Thus, we suggest that the hypocholesterolemic effect of mulberry leaves helps to promote cholesterol efflux through LXR$\alpha$ and ABCG5/8 expression. To investigate the influence of HHP-treated mulberry leaf extract on bile acid synthesis, we measured the hepatic

### Table 3. Effect of high hydrostatic pressure mulberry leaf extract on physiological variables

| Group          | NOR   | HC    | ML    | MH    |
|----------------|-------|-------|-------|-------|
| Initial body weight (g) | 216.36 ± 1.28 | 217.08 ± 1.31 | 215.59 ± 1.70 | 215.91 ± 1.40 |
| Final body weight (g)     | 378.58 ± 5.20  | 388.87 ± 3.76  | 380.95 ± 3.35  | 373.55 ± 5.53  |
| Body weight gain (g / 4 weeks) | 162.22 ± 4.59 | 171.79 ± 3.24 | 165.37 ± 3.47 | 157.64 ± 5.33 |
| Food intake (g/day)       | 23.15 ± 0.47   | 23.56 ± 0.59   | 23.25 ± 0.32   | 23.05 ± 0.65   |
| Energy intake (kcal/day)  | 87.37 ± 7.18   | 87.50 ± 2.18   | 85.87 ± 1.20   | 84.67 ± 2.38   |
| Energy efficiency ratio (EER)$^a$ | 0.064 ± 0.001 | 0.068 ± 0.001 | 0.066 ± 0.001 | 0.064 ± 0.001 |
| Epididymal fat weight (g / 100 g body weight) | 1.52 ± 0.06 | 1.33 ± 0.04 | 1.27 ± 0.04 | 1.36 ± 0.09 |
| Liver weight (g / 100 g body weight) | 3.21 ± 0.05$^a$ | 4.75 ± 0.06$^a$ | 4.77 ± 0.07$^a$ | 4.94 ± 0.11$^a$ |
| Serum alanine transaminase (IU/L) | 13.08 ± 1.98 | 24.18 ± 7.32 | 22.32 ± 4.68 | 20.73 ± 2.93 |
| Serum aspartate transaminase (IU/L) | 61.21 ± 7.17 | 60.84 ± 8.18 | 63.64 ± 7.93 | 59.93 ± 6.14 |

$^a$Energy efficiency = body weight gain (g/day)/energy intake (kcal/day); Values are expressed as mean ± SEM ($n = 10$). Different letters (a, b) show significant difference ($P < 0.05$). NOR, normal diet; HC, high-cholesterol diet containing 1% cholesterol and 0.5% cholic acid; ML, HC + 0.5% high hydrostatic pressure mulberry leaf extract; MH, HC + 1% high hydrostatic pressure mulberry leaf extract.

Fig. 2. Effects of the high hydrostatic pressure mulberry leaf extract on serum, hepatic and fecal lipid profiles. (a) Serum lipid profiles, LDL-C = TC – HDL-C – (TG/5). (b) Representative hematoxylin and eosin (H&E)-stained liver sections (scale bars, 50 $\mu$m; magnification, 400X). (c) Hepatic lipid profiles. (d) Fecal lipid profiles together with bile acid levels. Values are expressed as mean ± SEM ($n = 10$). Bars with different letters (a, b, c) show significant differences ($P < 0.05$). HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TBA, total bile acid; TC, total cholesterol; TG, triglyceride. NOR, normal diet; HC, high-cholesterol diet containing 1% cholesterol and 0.5% cholic acid; ML, HC + 0.5% high hydrostatic pressure mulberry leaf extract; MH, HC + 1% high hydrostatic pressure mulberry leaf extract.
Preventive effects of mulberry leaves on hypercholesterolemia

mRNA expression of CYP7A1, a rate-limiting enzyme in bile acid synthesis. Consistent with the increased fecal TBA concentration as indicated by the fecal bile acids, HC-decreased hepatic CYP7A1 gene expression was significantly enhanced by the HHP extraction of mulberry leaves in a dose-dependent manner with increments of 1.56 fold when using ML and 1.89 fold when using MH (P < 0.05) (Fig. 3b). Therefore, we suggest that the beneficial effects of mulberry leaves on HC-diet induced hypercholesterolemia may be partially mediated through the regulation of hepatic CYP7A1 expression.

Effects of high hydrostatic pressure-treated mulberry leaf extract on hepatic miR-33 expression and adenosine monophosphate-activated protein kinase (AMPK) activity

To investigate the molecular mechanisms by which HHP extract of mulberry leaves regulate cholesterol homeostasis, we measured the hepatic expression of miR-33, a
potent post-transcriptional gene regulator in cholesterol efflux, high-density lipoprotein biogenesis, and fatty acid oxidation. In the present study, miR-33 expression in rats fed with HC diet including 0.5 or 1% mulberry leaf extract was significantly downregulated by 41.3 and 47.1%, respectively, compared with HC-fed rats ($P < 0.05$) (Fig. 4a). Next, we analyzed AMPK activity, a cellular energy metabolic sensor involved in the hepatic lipid metabolism at the transcriptional and post-transcriptional levels by regulating fatty acid oxidation and cholesterol and TG synthesis. Supplementing the HC-diet with HHP-treated mulberry leaf extract significantly increased the hepatic AMPK activity in a dose-dependent manner, with a 1.11 fold-increment by 0.5% mulberry leaf-contained HC diet, as compared with the HC diet ($P < 0.05$) (Fig. 4b).

**Discussion**

Based on numerous epidemiological studies, hypercholesterolemia has been considered as an important risk factor for CVDs (1, 2, 26). Cholesterol homeostasis is fairly well-regulated in the body, which involves the synthesis of bile acid, the conversion of cholesterol to bile acids in the liver and cholesterol efflux, and biliary cholesterol excretion to feces (27). Natural foods and their bioactive functional ingredients have gained attention due to their multiple pharmacological effects. High hydrostatic pressure has been introduced as a non-thermal food processing technique to keep the activity and structure of bioactive ingredients in foods (18). In the present study, we investigated the favorable effect of the HHP-treated mulberry leaf extract on cholesterol metabolism in rats fed a high-cholesterol diet.

Among multiple bioactive components including 1-DNJ, flavonols, polyphenols, and polysaccharides (12, 14), a previous study detected quercetin 3-glucoside (isoquercitrin) and kaempferol 3-glucoside (astragalin) as flavonols in mulberry leaves (28). High hydrostatic pressure extraction of mulberry leaves contained 14.25 mg total flavonols including isoquercitrin and astragalin in 100 g of the dried sample. In the present study, we evaluated the influence of HHP-treated dietary mulberry leaves on hypercholesterolemia in rats fed with a HC diet. With regard to used two doses determined as previously described (8, 15), no significant differences in serum ALT and AST activities and liver tissue weight were observed. It suggests that both doses of mulberry leaves used in the present study at least did not induce liver toxicity. Consistent with previous studies using hot water extract (15, 16), the HHP extract of mulberry leaves significantly reduced HC-increased serum TC, TG, and LDL-levels. In addition, a dose-dependent increase in serum HDL-C level was observed in rats fed on the HC diet supplemented with the HHP extract from mulberry leaves. Several randomized clinical trials have shown the beneficial effects of mulberry leaves on serum TC, TG, and LDL concentrations in subjects with dyslipidemia without severe adverse reactions (29–31). It suggests that mulberry leaves might be a potential therapeutic resource for modulating hypercholesterolemia and CVD risk.

To investigate the mechanism underlying the cholesterol lowering effect of HHP-treated mulberry leaf extract, we measured the expression of hepatic genes related to cholesterol homeostasis. Cholesterol homeostasis is sustained by the balance between hepatic cholesterol biosynthesis and hepatic cholesterol catabolism (32). In the maintenance of cholesterol homeostasis, bile acids are the end products of cholesterol catabolism, and their biosynthesis and excretion to feces are involved in a reduction of excess hepatic cholesterol (33). In this study, supplementation with HHP-treated mulberry leaves in the HC diet significantly decreased hepatic TG and TC levels and increased fecal TG, TC, and bile acid contents, when compared with the HC diet. In addition, rats that were fed a HC diet with the HHP extract of mulberry leaf extract had higher hepatic LXR$\alpha$ and ABCG5/8 gene expression involved in cholesterol efflux and CYP7A1 mRNA levels related to bile acid synthesis. LXR$\alpha$ regulates its target genes such as ABCA1, ABCA1, ABCG5, ABCG8, and CYP7A1, thereby coordinating the balance between cholesterol biosynthesis and catabolism (3, 4). When cholesterol levels are high, LXR$\alpha$ activation upregulates hepatic and intestinal ABCG5/8 expression (3). Genetic modifications of ABCG5 and ABCG8 in a rodent model depict their role in cholesterol absorption and biliary cholesterol secretion (34, 35). In addition, LXR$\alpha$ upregulates CYP7A1 expression, the rate-limiting enzyme in bile acid synthesis and cholesterol excretion (5). It suggests that the hypcholesterolemic effect of HHP-treated mulberry leaf extract might at least, in part, interact with LXR$\alpha$ nuclear receptor and its-target genes, ABCG5/8 and CYP7A1 in the liver.

Apart from the classical transcriptional regulators, miRNAs, members of a class of non-coding RNAs have been identified as critical post-transcriptional regulators of cholesterol homeostasis by binding to complementary target sites in the 3’ untranslated regions of mRNAs (36). The miR-33 expression has been shown to post-transcriptionally inhibit key target genes such as ABCA1 and ABCG1 involved in cholesterol efflux and high-density lipoprotein biogenesis, and fatty acid oxidation-related genes including carnitine palmitoyltransferase 1z (CPT1z), carnitine O-octanoyltransferase (CROT), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase beta subunit (HADHB), and AMPK (37). During increasing fatty acid oxidation, AMPK, a critical energy sensor and metabolic master switch, inhibits hepatic cholesterol and TG synthesis (38). Previous studies show that mulberry leaf extract
or mulberry leaf polyphenol extract activates AMPK phosphorylation and inhibits hepatic lipogenesis (39, 40). In addition, AMPK activation suppresses endogenous LXR ligand production leading to the decrease in LXR expression and its transcriptional regulation (41). In this study, the HHP extract of mulberry leaves significantly decreased HC-induced miR-33 expression and increased HC-decreased AMPK activity in the liver. Taken together, mulberry leaf extract-mediated modulation of miR-33 expression and AMPK activity might be essential in regulating cholesterol homeostasis by increasing bile acid synthesis and cholesterol efflux.

**Conclusion**
This study has demonstrated for the first time that a 4-week supplementation with HHP extraction of mulberry leaves improves the HC diet-induced serum and hepatic lipid abnormalities and increases fecal lipid excretion in rats fed a high cholesterol diet. The mulberry leaf extract also increases fecal bile acid content, which is accompanied by an increase in the expression of hepatic genes involved in cholesterol metabolism, miR-33 expression, and AMPK activity. These findings suggest that HHP-treated mulberry leaf extract has the potential to prevent/treat hypercholesterolemia and CVDs associated with it.

**Conflicts of interest and funding**
The authors report no conflict of interest. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2019R1A2C1002861)

**References**

1. Barquera S, Pedroza-Tobias A, Medina C, Hernandez-Barrera L, Bibbins-Domingo K, Lozano R, et al. Global overview of the epidemiology of atherosclerotic cardiovascular disease. Arch Med Res 2015; 46(5): 328–38. doi: 10.1016/j.arcmed.2015.06.006

2. Stone NJ, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, et al. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2014; 63(25 Pt B): 2889–934. doi: 10.1016/j.jacc.2013.11.002

3. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. J Biol Chem 2002; 277(21): 18793–800. doi: 10.1074/jbc.M109927200

4. Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science 2009; 325(5936): 100–4. doi: 10.1126/science.118059299

5. Peti D, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell 1998; 93(5): 693–704. doi: 10.1016/s0092-8674(00)81432-4

6. Joseph SB, McKilligan E, Pei L, Watson MA, Collins AR, Laffitte BA, et al. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. Proc Natl Acad Sci U S A 2002; 99(11): 7604–9. doi: 10.1073/pnas.112059299

7. Katsube T, Yasamaki M, Shiwaku K, Ishijima T, Matsumoto I, Abe K, et al. Effect of flavonol glycoside in mulberry (Morus alba L.) leaf on glucose metabolism and oxidative stress in liver in diet-induced obese mice. J Sci Food Agric 2010; 90(14): 2386–92. doi: 10.1002/jsfa.4096

8. Ann Y, Eo H, Lim Y. Mulberry leaves (Morus alba L.) ameliorate obesity-induced hepatic lipogenesis, fibrosis, and oxidative stress in high-fat diet-fed mice. Genes Nutr 2015; 10(6): 46. doi: 10.1007/s12263-015-0495-x

9. Yang MY, Huang CN, Chan KC, Yang YS, Peng CH, Wang CJ. Mulberry leaf polyphenols possess antiatherosclerosis effect via inhibiting LDL oxidation and foam cell formation. J Agric Food Chem 2011; 59(5): 1985–95. doi: 10.1021/jf103661v

10. Peng CH, Lin HT, Chung DJ, Huang CN, Wang CJ. Mulberry leaf extracts prevent obesity-induced NAFLD with regulating adipocytokines, inflammation and oxidative stress. J Food Drug Anal 2018; 26(2): 778–87. doi: 10.1016/j.jfda.2017.10.008

11. Sheng Y, Liu J, Zheng S, Liang F, Luo Y, Huang K, et al. Mulberry leaves ameliorate obesity through enhancing brown adipose tissue activity and modulating gut microbiota. Food Funct 2019; 10(8): 4771–81. doi: 10.1039/c9cf00883g

12. Kim GN, Jang HD. Flavonol content in the water extract of the mulberry (Morus alba L.) and their anti-oxidant capacities. J Food Sci 2011; 76(6): C869–73. doi: 10.1111/j.1750-3841.2011.02262.x

13. Park E, Lee S-M, Lee EJ, Kim J-H. Anti-inflammatory activity of mulberry leaf extract through inhibition of NF-κB. J Funct Foods 2013; 5(1): 178–86. doi: 10.1016/j.jff.2012.10.002

14. Ren C, Zhang Y, Cui W, Lu G, Wang Y, Gao H, et al. A polysaccharide extract of mulberry leaf ameliorates hepatic glucose metabolism and insulin signaling in rats with type 2 diabetes induced by high fat diet and streptozotocin. Int J Biol Macromol 2015; 72: 951–9. doi: 10.1016/j.ijbiomac.2014.09.060

15. Kobayashi Y, Miyazawa M, Kamei A, Abe K, Kojima T. Ameliorative effects of mulberry (Morus alba L.) leaves on hyperlipidemia in rats fed a high-fat diet: inhibition of fatty acid oxidation, inhibition of lipogenesis, and suppression of oxidative stress. Biosci Biotechnol Biochem 2010; 74(12): 2385–95. doi: 10.1271/bbb.100392

16. Huang J, Wang Y, Ying C, Liu L, Lou Z. Effects of mulberry leaf extract on experimental hyperlipidemia rats induced by high-fat diet. Exp Ther Med 2018; 16(2): 547–56. doi: 10.3892/etm.2018.6254

17. Lopes ML, Valente Mesquita VL, Chiarradia AC, Fernandes AA, Fernandes PM. High hydrostatic pressure processing of tropical fruits. Ann N Y Acad Sci 2010; 1189: 6–15. doi: 10.1111/j.1749-6632.2009.05177.x

18. Yamamoto K. Food processing by high hydrostatic pressure. Biosci Biotechnol Biochem 2010; 74(12): 2385–95. doi: 10.1271/bbb.100392

19. Butz P, Garcia AF, Lindauer R, Dieterich S, Bognar A, Tauscher B. Influence of ultra high pressure processing on fruit and vegetable products. J Food Eng 2003; 56(2–3): 233–6. doi: 10.1016/S0260-8774(02)00258-3

20. Wang F, Du BL, Cui ZW, Xu LP, Li CY. Effects of high hydrostatic pressure and thermal processing on bioactive compounds, antioxidant activity, and volatile profile of mulberry juice. Food Sci Technol Int 2017; 23(2): 119–27. doi: 10.1021/jf103661v

21. Jung S, Lee MS, Choi AJ, Kim CT, Kim Y. Anti-inflammatory effects of high hydrostatic pressure extract of mulberry (Morus alba L.) leaves on hypercholesterolemia in rats. Food Chem 2011; 125(4): 1390–7. doi: 10.1016/j.foodchem.2010.09.060

22. Palmieri BV, Yoon KJ, Park J, Lee S, Kim Y, Kim SY, et al. Antioxidant, anti-inflammatory and anti-atherogenic effects of high hydrostatic pressure extract from mulberry leaves. Food Sci Biotechnol 2019; 28(4): 1445–55. doi: 10.1007/s10068-019-00729-w

23. Palmieri BV, Yoon KJ, Park J, Lee S, Kim Y, Kim SY, et al. Antioxidant, anti-inflammatory and anti-atherogenic effects of high hydrostatic pressure extract from mulberry leaves. Food Sci Biotechnol 2019; 28(4): 1445–55. doi: 10.1007/s10068-019-00729-w
alba) fruit on LPS-stimulated RAW264.7 Cells. Molecules 2019; 24(7): 1425. doi: 10.3390/molecules24071425

22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18(6): 499–502. doi: 10.1093/clinchem/18.6.499

23. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37(8): 911–17. doi: 10.1139/e59-099

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25(4): 402–8. doi: 10.1006/meth.2001.1262

25. Kim S, Lee MS, Jung S, Son HY, Park S, Kang B, et al. Ginger extract ameliorates obesity and inflammation via regulating MicroRNA-21/132 expression and AMPK activation in white adipose tissue. Nutrients 2018; 10(11): 1567. doi: 10.3390/nu10111567

26. Sharrett AR, Ballantyne CM, Coady SA, Heiss G, Sorlie PD, Catellier D, et al. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: the Atherosclerosis Risk in Communities (ARIC) study. Circulation 2001; 104(10): 1108–13. doi: 10.1172/jci16001

27. Tontonoz P, Mangelsdorf DJ. Liver X receptor signaling pathways in cardiovascular disease. Mol Endocrinol 2003; 17(6): 985–93. doi: 10.1210/me.2003-0061

28. Doi K, Kojima T, Makino M, Kimura Y, Fujimoto Y. Studies on the constituents of the leaves of Morus alba L. Chem Pharm Bull (Tokyo) 2001; 49(2): 151–3. doi: 10.1248/cpb.49.151

29. Kojima Y, Kimura T, Nakagawa K, Asai A, Hasumi K, Oikawa S, et al. Effects of mulberry leaf extract rich in 1-deoxynojirimycin on blood lipid profiles in humans. J Clin Biochem Nutr 2010; 47(2): 55–61. doi: 10.3164/jcn.10-53

30. Aramwit P, Petcharot K, Supasyndh O. Efficacy of mulberry leaf tablets in patients with mild dyslipidemia. Phytother Res 2011; 25(3): 365–9. doi: 10.1002/ptr.3270

31. Aramwit P, Supasyndh O, Sritiintongthong T, Bang N. Mulberry leaf reduces oxidation and C-reactive protein level in patients with mild dyslipidemia. Biomed Res Int 2013; 2013: 787981. doi: 10.1155/2013/787981

32. Groen AK, Bloks VW, Verkade H, Kuipers F. Cross-talk between liver and intestine in control of cholesterol and energy homeostasis. Mol Aspects Med 2014; 37: 77–88. doi: 10.1016/j. mam.2014.02.001

33. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, et al. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 2000; 6(3): 507–15. doi: 10.1016/s1097-2765(00)00050-2

34. Yu L, Hammar RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC, et al. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. Proc Natl Acad Sci U S A 2002; 99(25): 16237–42. doi: 10.1073/pnas.252582399

35. Yu L, Li-Hawkins J, Hammar RE, Berge KE, Horton JD, Cohen JC, et al. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J Clin Invest 2002; 110(5): 671–80. doi: 10.1172/jci16001

36. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 132(6): 215–33. doi: 10.1016/j.cell.2009.01.002

37. Goedeke L, Fernandez-Hernando C. Regulation of cholesterol homeostasis. Cell Mol Life Sci 2012; 69(6): 915–30. doi: 10.1007/s00018-011-0857-5

38. Hardie DG, Pan DA. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. Biochem Soc Trans 2002; 30(Pt 6): 1064–70. doi: 10.1042/bst0301064

39. Wu C-H, Chen S-C, Ou T-T, Chyau C-C, Chang Y-C, Wang C-J. Mulberry leaf polyphenol extracts reduced hepatic lipid accumulation involving regulation of adenosine monophosphate activated protein kinase and lipogenic enzymes. J Funct Foods 2013; 5(4): 1620–32. doi: 10.1016/j.jff.2013.07.004

40. Kobayashi Y, Miyazawa M, Araki M, Kamei A, Abe K, Hiroi T, et al. Effects of Morus alba L (Mulberry) leaf extract in hypercholesterolemic mice on suppression of cholesterol synthesis. J Pharmacogn Nat Prod 2015; 2: 1–9. doi: 10.4172/jff.2013.07.004

41. Yang J, Craddock L, Hong S, Liu ZM. AMP-activated protein kinase (AMPK) activation by the AMP-activated protein kinase. Biochem Soc Trans 2002; 30(Pt 6): 1064–70. doi: 10.1042/bst0301064

42. Yang J, Craddock L, Hong S, Liu ZM. AMP-activated protein kinase (AMPK) activation by the AMP-activated protein kinase. Biochem Soc Trans 2002; 30(Pt 6): 1064–70. doi: 10.1042/bst0301064

43. Yang J, Craddock L, Hong S, Liu ZM. AMP-activated protein kinase (AMPK) activation by the AMP-activated protein kinase. Biochem Soc Trans 2002; 30(Pt 6): 1064–70. doi: 10.1042/bst0301064

*Yangha Kim

Department of Nutritional Science and Food Management and Graduate Program in System Health Science and Engineering, Ewha Womans University
Seoul 03760, Korea
Email: yhmoon@ewha.ac.kr