A mixture of blackberry leaf and fruit extracts decreases fat deposition in HepG2 cells, modifying the gut microbiome

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Abstract More effective treatments are needed for non-alcoholic fatty liver disease (NAFLD). We hypothesized that water extracts of blackberry fruits (BF) and leaves (BL) and their combinations (BFL) reduce fat deposition in HepG2 cells and modulate short-chain fatty acids (SCFA) and fecal bacteria in vitro. HepG2 cells were treated with BF, BL, BFL1:2, and BFL1:3 for 1 h, and 0.5 mM palmitate was added to the cells. Moreover, low (30 µg/mL) and high doses (90 µg/mL) of BL and BF were applied to fecal bacteria in vitro, and SCFA was measured by GC. BL, BF, BFL1:2, and BFL1:3 reduced triglyceride deposition in the cells in a dose-dependent manner, and BFL1:2 and BFL1:3 had a stronger effect than BF. The content of malondialdehyde, an index of oxidative stress, was also reduced in BL, BF, and BFL1:2 with increasing superoxide dismutase and glutathione peroxidase activities. The mRNA expression of acetyl CoA carboxylase, fatty acid synthase, and sterol regulatory element-binding protein-1c was reduced in BL, BF, BFL1:2, and BFL1:3 compared to the control, and BFL1:2 had the strongest effect. By contrast, the carnitine palmitoyltransferase-1 expression, a regulator of fatty acid oxidation, increased mostly in BFL1:2 and BFL1:3. Tumor necrosis factor-α and interleukin-1β expression was reduced in BL compared to that in BF and BFL1:2 in HepG2 cells. Interestingly, BL increased propionate production, and BF increased butyrate and propionate production and increased total SCFA content in fecal incubation. BF increased the contents of Bifidobacteriales and Lactobacillales and decreased those of Clostridiales, whereas BL elevated the contents of Bacteroidales and decreased those of Enterobacteriales. In conclusion, BFL1:2 and BFL1:3 may be potential therapeutic candidates for NAFLD.

Keywords Fatty acid synthesis · Gut microbiome · Non-alcoholic fatty liver disease · Palmitate · Short-chain fatty acids

Introduction Fatty liver disease is characterized by an increase in the accumulation of lipid droplets in liver cells due to various conditions such as alcohol intake, high fat diet, and drug administration. It is categorized into alcoholic and non-alcoholic fatty liver disease (NAFLD). Although this condition is reversible, it can progress into hepatitis with inflammation, liver cirrhosis, and liver cancer [1]. The prevalence of NAFLD is rising worldwide (approximately 20-30% in the USA and 29.6% in Asia) [1,2]. NAFLD is caused by increasing free fatty acids from adipose tissues in the circulation that enter into the liver and damage the hepatic cells by accumulating lipids [3]. The elevation of serum free fatty acid concentrations may be involved in increased insulin resistance. Obesity, dyslipidemia, and hyperglycemia are accompanied by non-alcoholic liver diseases, and obesity with high visceral fat is the major cause of the disease [4,5]. Thus, the reduction of insulin resistance is necessary to prevent and treat NAFLD.

Oxidative stress and inflammation increase with elevated insulin resistance, leading to the progression of NAFLD into severe liver diseases. Inflammation is associated with gut microbiomes [6]. Bacteria produce lipopolysaccharide (LPS), metabolites, and short-chain fatty acids (SCFAs). LPS causes low-grade inflammation mediated by the induction of inflammatory cytokines by immune
cells and adipocytes, and SCFA such as acetate or butyrate can modulate immune cell function [7]. LPS and some SCFAs, especially acetate, can affect adipogenesis and/or insulin resistance [8,9]. Thus, the gut microbiota can produce SCFAs that can modulate systemic inflammation and adipogenesis, especially in the gut-liver axis, as the molecules produced in the intestines go to the liver through the portal vein [10].

The medicine market for the treatment of NAFLD is growing and the need for novel treatments for NAFLD is increasing. The ideal treatment agent should enhance lipid utilization in the liver and induce liver cell regeneration. Ursodeoxycholic acid, diphenyl dimethyl dicarboxylate, glutathione, silymarin, and a mixture of flavonolignans extracted from milk thistle (Silybum marianum L. Gaertneri) are used for the treatment for NAFLD [11]. However, more effective therapeutic agents are needed. Blackberry (Rubus alceaeifolius) leaves and fruits are reported to decrease insulin resistance, oxidative stress, and inflammation. Anthocyanins, including cianidin-3-glucoside, and total alkaoids in blackberry fruits have a protective effect against non-alcoholic hepatic steatosis in hepatic cells and rats [12,13]. Blackberry extracts also have anti-inflammatory activity by modulation of the NF-κB signaling induced non-alcoholic hepatic steatosis in rats [14]. The leaves of blackberry also contain flavonoids, and their water extracts may have an anti-non-alcoholic hepatic steatosis effect.

In the present study, we hypothesized that water extracts of blackberry fruit (BF) and leaves (BL) and their combinations reduce fat deposition in HepG2 cells. Their effects on fecal bacteria were also measured.

Materials and Methods

Water extract of blackberry leaves and fruits
Dried Korean blackberry fruits and blackberry leaves (Korea Prime Co., Ltd., Suwon, Korea) were separately powdered and extracted in 20-fold volumes of 50% ethanol at 92 °C for 4 h, and the extracts were concentrated at 40 °C using a rotary evaporator. The concentrated extracts were lyophilized in a freeze-dryer, and the yield of BF and fruit extracts was 30.8 and 39.0%, respectively.

HPLC was performed using an Ultra-Fast Liquid Chromatography instrument (Shimadzu, Kyoto, Japan) equipped with an autoinjector and a UV detector (Shimadzu SPD-M20A). The extract was analyzed using the Phenomenex Gemini-NX C18 (250 mm × 4.6 mm I.D., 5 µm). The mobile phase was 0.5% phosphoric acid in water (A) and acetonitrile (B). The gradients were used as follows: 0 min, A:B 95:5 (v/v); 5 min, A:B 85:15; 25 min, A:B 25:80; 35 min, A:B 0:100; 40 min, A:B 0:100; 41 min, A:B 95:5; and 50 min, 95:5. The mobile phase flow rate was 1.0 mL/min under the following conditions: column temperature, 35 °C; injection volume, 20 µL; and UV detection at 254 nm. The indicator compounds were cyanidin-3-glucoside for BF and ellagic acid for BL. Each indicator compound was purchased from Sigma-Aldrich (St. Louis, MO, USA) or ChromaDex (Irvine, CA, USA).

Effects of HepG2 cell line on cell damage
Human hepatocellular carcinoma HepG2 cells were obtained from American Type Culture Collection (HB-8065; Manassas, VA, USA) and were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum [15]. Cells were transferred into 96-well plates at 4×10^3 cells/well in high-glucose DMEM containing 0.3% bovine serum albumin and allowed to grow to 70% confluence. The cells were incubated with vehicle, 30 or 90 µg/mL BF and BL or their mixtures (ratio of BF and BL=1:2 (BFL1:2), 1:3 (BFL1:3), and 1:5 (BFL1:5)). After 1 h of pretreatment with blackberry extracts, 0.5 mM palmitate emulsified with fatty acid free albumin was added to the HepG2 cells, and they were incubated for another 24 h. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using an Aureon plate reader (Aureon Biosystems, Vienna, Austria).

HepG2 cells were grown in 24-well plates at 6×10^4 cells/well until the cells were 70% confluent, then, they were treated with vehicle, 30 or 90 µg/mL BF, BL, or their mixtures (BFL1:2, BFL1:3, and BFL1:5) for 1 h. The cells were subsequently added with 0.5 mM palmitate and incubated for an additional 24 h, and the cells were lysed with lysis buffer at 4 °C. The cells were used to measure antioxidant and inflammation status and to investigate the mRNA expression of targeted genes.

Fecal bacterial cultivation
Fecal samples from the large intestine of healthy rats were collected and immediately dissolved in saline containing 1% L-cysteine to make 30% fecal solution. Thioglycolate 0.75% (BD Medical Technology, 225650, Franklin Lakes, NJ, USA) in phosphate buffer containing 1% L-cysteine was prepared, autoclaved at 121 °C for 15 min, and cooled down. The fecal sample (300 µL) was added to the 0.75% thioglycolate medium (30 mL) for control and supplemented 0.5% BL or BF. These processes were conducted in an anaerobic chamber, and the samples were put into the anaerobic jar with an anaerobic pack. The jar was incubated in a shaking bath at 38 °C at 200 rpm for 48 h. After incubation, the medium was centrifuged, and the bacteria were separated. The medium was used for measuring SCFA, and the bacterial composition was determined by Next Generation Sequencing (NGS).

Oxidative stress and inflammation status
Lipid peroxide levels in the HepG2 cells treated with 0.5 mM palmitate and BF and BL extracts were measured using a thiobarbituric acid reactive substance assay kit (Cayman Chemical, Ann Arbor, MI, USA). The activities of antioxidant enzymes such as Cu/Zn superoxide dismutase (SOD) and glutathione (GSH)-peroxidase were measured from the lysates of the HepG2 cells by using colorimetry kits (Cayman Chemical, Ann Arbor, MI, USA).
and Biovision, Milpitas, CA, USA, respectively). One unit of each enzyme activity was defined as the amount of SOD needed to inhibit the reaction by 50%, and the enzyme activity was normalized by milligrams of protein in the lysate. GSH level in the liver was also determined using a GSH assay kit (Sigma-Aldrich, St. Louis, MO, USA).

Isolation of total RNA in HepG2 cells and real-time polymerase chain reaction (PCR) of target genes
Treated HepG2 cells were mixed with a monophasic solution of phenol and guanidine isothiocyanate (TRizol reagent; Gibco-BRL, Rockville, MD, USA) for total RNA extraction, according to the manufacturer’s instructions. cDNA was synthesized from equal amounts of total RNA using SuperScript III reverse transcriptase, and PCR was performed with high fidelity Taq DNA polymerase. Equal amounts of cDNA were added to SYBR Green mix (Bio-Rad, Richmond, CA, USA) and amplified using a real-time PCR instrument (Bio-Rad). The expression levels of the genes of interest were normalized to that of the housekeeping gene β-actin. To assess changes in the expression of genes related to fatty acid synthesis and oxidation in the liver, the following primers were used: sterol regulatory element-binding protein (SREBP)-1c forward 5'-GGAGCCATGGATTGCACAT-3', reverse 5'-AGGAAGGCTTCCAGAGGAA-3'; acetyl CoA carboxylase (ACC) forward 5'-GGAAGATGGGTCGCTGGGTAC-3', reverse 5'-GGGGAGATGTGCTGGGTCAT-3'; fatty acid synthase (FAS) forward 5'-AGTGCTAGAGGCCCTGCTA-3', reverse 5'-GTGC3'-GGGGAGATGTGCTGGGTCAT-3'; carnitine palmitoyl transferase I forward 5'-AGGAAGGCTTCCAGAGGAA-3'; TNF-α forward, 5'-AC CCCCACACTTATGAAGAAA-3', reverse 5'-TCCACGCAAACG GGAATGAA-3'; interleukin (IL)-1β forward, 5'-TTGTTGGCTGT GGAAGAAGCTG-3', reverse 5'-GCCGCTTCTTACACAGG-3'; β-actin forward 5'-AAGTCCCTCACCCTCCAAAAG-3', reverse 5'-AAGCAATGCTGTCACCTTCCC-3'. The primers were designed to sandwich at least one intron in order to distinguish between the products derived from mRNA and genomic DNA. At least four PCR reactions per group were performed as previously described [16].

Gas chromatography for measuring SCFA
After incubation of rat fecal samples, the supernatants of the media were mixed with ethanol (1:2), and 5 M HCl was added to reach pH 2. The mixture was centrifuged at 4 °C for 2,000× g for 15 min, and the supernatant was filtered with a 0.45-µm syringe filter. The filtrate was injected into a gas chromatograph (Perkin-Elmer, Waltham, MA, USA) using a fused-silica capillary column (30 m×0.25 mm) coated with a 0.25-im-thick free fatty acid phase film (DB FFAP 122-3232, J&K Scientific, Agilent Technologies Inc., Santa Clara, CA, USA). Gas chromatography was conducted. Helium was supplied as the carrier gas using a linear velocity mode at 22.2 cm sec⁻¹. Glass wool was inserted in the liner of the splitless injection port at 230 °C. The initial oven temperature was set at 100 °C, then gradually increased at 10 min⁻¹ to 180 °C, then to 220°C, and maintained at 220 °C for 8 min. The FID detector was used, and the temperature was 240°C. Flow rates were 40, 400, and 80 mL min⁻¹ for hydrogen, air, and helium, respectively.

Gut microbiome measured by NGS
The gut microbiome composition was measured from culture rat feces by analyzing metagenome sequencing using NGS. Bacterial DNA was extracted from the samples of each rat using a Power Fecal DNA Isolation kit (MoBio, Carlsbad, CA, USA) according to the manufacturer’s instructions. Each library was prepared using PCR products according to the GS FLX plus library prep guide. The emPCR, corresponding to clonal amplification of the purified library, was carried out using the GS FLX plus emPCR kit (454 Life Sciences, Branford, CT, USA). Libraries were immobilized onto DNA capture beads. The library beads were added to the amplification mix and the oil, and the mixture was vigorously shaken on a Tissue Lyser II (Qiagen, Valencia, CA, USA) to create micro-reactors containing both amplification mix and a single bead. The emulsion was dispensed into a 96-well plate, and the PCR amplification program was run with 16S universal primers in the Fast Start High Fidelity PCR System (Roche, Basel, Switzerland) according to the manufacturer’s recommendations. Sequencing of bacterial DNA in the feces was performed by the Macrogen Ltd. (Seoul, Korea) using a Genome Sequencer GS FLX plus (454 Life Sciences) as previously reported [17]. The 16S amplicon sequences were processed using Mothur v.1.36. We followed the Miseq SOP to identify the taxonomy and count the bacteria in each fecal sample. The selection of operational taxonomic units was delimited at 98% identity, which was taxonomically classified by consensus using Greengenes 13.8.99.

Statistical analysis
Statistical analysis was performed using SAS, version 7.0. Results are expressed as means ± standard deviations. The differently treated groups were compared by one-way analysis of variance. Significant differences between groups were identified by Tukey’s tests at p <0.05.

Results
The contents of cyanidin-3-glucoside and ellagic acid, index compounds
The 50% ethanol extracts of BF and BL contained 12±0.4 mg cyanidin-3-glucoside and 28.3±1.2 mg ellagic acid, respectively (n =3; Fig. 1).
Improvement of cell viability by BFL1:2 and BFL1:3
Palmitate administration (0.5 mM) inhibited cell viability in HepG2 cells, and BL and BF dose-dependently protected against cell death by palmitate intoxication (Fig. 2A). High dosage of BL (30 and 90 µg/mL) showed greater protective activity than that of BF (Fig. 2A). BFL1:2, BFL1:3, BFL1:4, and BFL1:5 improved cell viability dose-dependently, and BFL1:2 and BFL1:3 were the most effective in protecting against cell damage (Fig. 2B). However, the improvement was not as much as the normal control group in HepG2 cells (Fig. 2B).

Decrease of TG deposition and mRNA expression of genes related fatty acid synthesis by BFL1:2 and BFL1:3
Palmitate pretreatment increased TG accumulation in HepG2 cells. BL, BF, and BFL inhibited TG deposition in a dose-dependent manner, and BL decreased TG deposition more than BF. The high dosage of BFL1:2 and BFL1:3 was the most effective in suppressing TG accumulation (Fig. 3A). The mRNA expression of ACC, FAS, and SREBP-1c was involved in the fatty acid synthesis, and the mRNA expression of CPT-1 was associated with fatty acid oxidation in HepG2 cells (Fig. 3B). The mRNA expression of ACC was reduced in the normal control compared to the control, and BL, BF, BFL1:2, and BFL1:3 also decreased. The decrease associated with BF was smaller than that associated with BL. The mRNA expression of FAS and SREBP-1c exhibited similar patterns under different treatments (Fig. 3A). The mRNA expressions of FAS and SREBP-1c were reduced in a descending order of the control, BF, BL, BFL1:3, BFL1:2, and normal control. The mRNA expression of CPT-1 was opposite to that of FAS, and it was lower in the control than in the normal control. The CPT-1 mRNA expression increased in ascending order of the control, BF, BL, BFL1:2, BFL1:3 and normal control (Fig. 3B).

Reduction of oxidative stress and inflammation by BFL1:2 and BFL1:3
The cell damage by palmitate administration was associated with increased oxidative stress and inflammation. The MDA levels representing lipid peroxide deposition were higher by approximately 2.5 folds in the HepG2 cells damaged with palmitate (control) compared to those in the normal control (Fig. 4A). MDA contents in HepG2 cells increased in the control compared to the normal control, and BL and BF suppressed the MDA increase from the control. BFL1:2 (90 µg/mL) inhibited MDA contents the most. SOD and GSH-Px levels were reduced in the control compared to the normal control (Fig. 4A). BF treatment (90 µg/mL) increased SOD level more than BL, but GSH-Px activity was elevated in BL.
and BF. BFL1:2 increased SOD and GSH-Px activities the most, but the activities in BFL1:2 were lower than that in the normal control (Fig. 4A).

In contrast to the activities of antioxidant enzymes, the mRNA expression of proinflammatory cytokines was elevated in the control group compared to the normal control (Fig. 4B). The mRNA expression of TNF-α and IL-1β decreased in BL, BF, BFL1:2, and BFL1:3 groups compared to the control, and it was the lowest in BFL1:2 and BFL1:3 groups. Especially, TNF-α expression in BFL1:2 and BFL1:3 groups decreased as much as that in the normal control group (Fig. 4B).

**Increase of propionic acid and butyric acid by BF and BL, respectively, in fecal culture**

Total production of SCFA was highest in the group treated with BF, followed by BL and then control (Fig. 5A). BF increased butyric acid and propionic acid the most, whereas BL increased propionic acid more than the control (Fig. 5A).

**Increase of Bifidobacteriales by BF and Bacteroidales by BL**

As shown in Fig. 5B, BF increased Bifidobacteriales and Coriobacteriales but decreased Bacteroidales compared to the control. BL increased Bacteroidales (2.5 folds) and Fibrobacterales compared to the control. Lactobacillales did not differ between BF and control, but they decreased in BL. BL decreased Clostridiales compared to the control. Enterobacteriales, harmful bacteria, decreased in both BL and BF compared to the control, and they were lower in BL than in BF. Thus, BL and BF differently influenced gut microbiome composition, but both improved gut microbiome composition compared to the control.

**Discussion**

Although the incidence of NAFLD is increasing, preventive and therapeutic measures are not sufficient. NAFLD results in increased hepatic fat deposition, and it accompanied by inflammation that
progressed into non-alcoholic steatohepatitis. Excessive energy intake through a high fat diet is the major cause of these diseases, although this does not completely account for NAFLD induction.

It is accompanied by oxidative stress and inflammation. Anthocyanins and flavonoids are well-known to reduce fatty acid synthesis, oxidative stress, and inflammation. Silymarin, a flavonoid, is used for treating NAFLD. In the present study, we determined whether BF and BL and their combinations (BFL) could be a potential treatment for NAFLD. BL and BF reduced fat deposition in HepG2 cells compared to the control, and BL was more effective than BF. BL reduced mRNA expression of genes related to fatty acid synthesis and increased mRNA expression of genes related to fatty acid oxidation compared to BF. Interestingly, the mixture of BF and BL (1:2 and 1:3) decreased mRNA expression more than individual extracts. Lipid peroxide contents were lowered in BF and BL than the control. BFL1:2 reduced the lipid peroxide contents compared to individual extracts. BL and BF increased SCFA compared to the control and modulated the composition of fecal bacteria in vitro. Therefore, BL, BFL1:2, and BFL1:3 may have a therapeutic activity for NAFLD, and further experiments in animals and humans are needed.

NAFLD is associated with excessive triglyceride deposition, with increased oxidative stress and inflammation. In cell-based studies, palmitate administration develops excessive triglyceride deposition, showing characteristics similar to NAFLD. Increased serum concentrations of free fatty acids are the major cause of NAFLD by stimulate fat deposition in the liver in experimental animals and humans. Berry fruits and leaves have anthocyanins and flavonoids that are involved in reducing oxidative stress and inflammation in vitro and in vivo [18,19]. Berries, including cranberry, blackberry, wild blueberry, strawberry, and chokeberry have antioxidant activities in HepG2 cells in oleic acid-induced hepatic steatosis [13]. The major index compound of blackberry and blueberry is a cyanidin-3-glucoside that improves metabolic
disorders, including NAFLD, and increases the infiltration of inflammatory markers in the liver [20-22]. Ellagic acid in berries is a natural antioxidant polyphenol and alleviates NAFLD by mitigating hepatic oxidative stress and insulin resistance in type 2 diabetic female rats [23,24]. Thus, BL and BF can reduce fat deposition in the liver to attenuate NAFLD.

The gut microbiome is also involved in NAFLD [25]. High fat diet can cause intestinal dysbiosis by modulating the production of substances such as SCFA, bile acids, and bacterial components and by activating toll-like receptors [26]. Some substances including acetate increase insulin resistance and inflammation and induce intestinal permeability due to malfunction of tight junctions. However, butyrate-producing bacteria reduces NAFLD progression [27,28]. Thus, the dysbiosis of gut microbiome in a high fat diet is possible mechanism to develop NAFLD. Polyphenols, including anthocyanins and flavonoids, show low bioavailability [29]. Polyphenol biotransformation by colonic fermentation improves bioavailability to exert health benefits [29,30]. Digested and fermented phenolic compounds in blackberry by gut microbiota fermentation ameliorate palmitate-induced lipid deposition in HepG2 cells [31]. The present study showed that the composition of intestinal bacteria and SCFA was differently changed by BL and BF extracts. The modulation may affect the fat deposition in the liver by altering the gut-liver axis. Gowd et al. [31] have demonstrated that gut metabolites of blackberry significantly elevate the glucose uptake and glycogen content in HepG2 cells. Gut metabolites also ameliorate high-glucose plus palmitate-induced oxidative stress by reducing reactive oxygen species overproduction and glutathione depletion in HepG2 cells. The present study also showed that BL, BF, and the mixtures of BL and BF decreased fat deposition and oxidative stress. BFL1:2 showed the most beneficial activity in reducing fat accumulation, oxidative stress, and inflammation in HepG2 cells.

In conclusion, both BL and BF decreased triglyceride deposition, and their mixture (BFL1:2) reduced it the most in HepG2 cells. This was associated with an increase in fatty acid synthesis and a decrease in fatty acid oxidation. BF reduced oxidative stress better than BL, and BFL1:2 decreased oxidative stress the most by...
elevating the activities of antioxidant enzymes. BL and BF increased SCFA and altered the gut microbiome composition, increasing their effect on NAFLD. Thus, BFL1:2 may be beneficial to prevent or alleviate NAFLD. Further animal and human studies are needed to confirm these benefits.

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Authors’ contributions SP designed the studies, analyzed the data and wrote the paper. HJY and XW conducted the measurement of index compounds. XW and BRJ performed gut microbiome and HepG2 cell experiments.

Competing interests The authors declare that they have no competing interests.

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