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Wild *Lonicera caerulea* berry polyphenol extract reduces cholesterol accumulation and enhances antioxidant capacity in vitro and in vivo

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ARTICLE INFO

Chemical compounds studied in this article:
Cyanidin-3-glucoside (PubChem CID: 441667)
Cyanidin-3,5-diglucoside (PubChem CID: 441688)
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Pelargonidin-3-glucoside (PubChem CID: 443648)
Cyanidin-3-rutinoside (PubChem CID: 44256716)
Peonidin-3-rutinoside (PubChem CID: 44256842)
Delphinidin-3-rutinoside (PubChem CID: 44256887)
Catechin (PubChem CID: 9064)
Chlorogenic acid (PubChem CID: 1794427)
Neochlorogenic acid (PubChem CID: 5280633)
Vanillic acid (PubChem CID: 8468)
Quercetin-3-O-rutinoside (PubChem CID: 5280805)
Hyperoside (PubChem CID: 5281643)
Quercetin-3-O-glucoside (PubChem CID: 25203368)
3,5-Dicaffeoylquinic acid (PubChem CID: 6474310)
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ABSTRACT

The hypocholesterolemic effect of *Lonicera caerulea* berry extract rich in polyphenols (LCBP) on high cholesterol-induced hypercholesterolemia and lipoprotein metabolite changes was examined in Caco-2 cells and rats. Cyanidin-3-glucoside, catechin, and chlorogenic acid are the major phenolic components of LCBP. The cholesterol-reducing effect and antioxidant capacity of these components were compared in Caco-2 cells. LCBP (80 μg/mL) and cyanidin-3-glucoside, catechin, and chlorogenic acid (50 μM) were found to be effective ($p < 0.05$). Rats were fed a high cholesterol diet (HCD) with or without LCBP supplementation (75, 150, and 300 mg/kg body weight intragastrically once daily) for 12 weeks. Compared with the HCD control group, LCBP supplementation at 150 and 300 mg/kg decreased the levels of TC, TG, and LDL-C, but increased that of HDL-C. LCBP treatment promoted greater neutral and acidic sterol excretion ($p < 0.05$) and improved the antioxidant capacity of the colon tissue, colon contents, and blood. Moreover, trimethylamine N-oxide (TMAO) levels were decreased in serum ($p < 0.05$). NPC1L1, ACAT2, and MTP mRNA and protein expression were reduced and ABCG5/8 expression was increased ($p < 0.05$) after LCBP treatment. Our results suggest that LCBP could be used as a functional food for the prevention and treatment of diseases related to excessive cholesterol accumulation.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death globally (Godard et al., 2009). Hypercholesterolemia is a key risk factor for cardiovascular disease and is positively correlated with the incidence and mortality of coronary heart disease (O'Connor, Taylor, Campbell, Epstein, & Libby, 2001). As a reduction of cholesterol levels in blood by 1% can reduce the risk of coronary heart disease by 3% (Lora, Morse, Gonzalez-Krtiger, & Driskell, 2007), the maintenance of the cholesterol balance is very important. Numerous studies have suggested that polyphenols are able to lower blood total cholesterol (TC) through the restriction of cholesterol absorption (Kim, Park, Wegner, Bolling, & Lee, 2013; Lam et al., 2008; Sanz-Buenhombre et al., 2016; Zou et al., 2014).

Cholesterol absorption in the small intestine is mainly affected by

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the regulation of two types of transporters, Niemann-Pick C1-Like 1 (NPC1L1) and ATP-binding cassette transporter subfamily G members 5/8 (ABCG5/8). NPC1L1 can actively transport cholesterol from the lumen to the intestinal epithelial cells. Acyl-coenzyme A (CoA):cholesterol acyltransferase 2 (ACAT2) converts cholesterol to cholesterol ester. Subsequently, microsomal triacylglycerol transport protein (MTP) packs cholesterol ester into chylomicrons, which are transferred into the blood through the lymphatic system. The unabsorbed cholesterol in the intestinal epithelial cells is then moved into the lumen by ABCG5/8, where it is subjected to gut microbe reactions and excreted (Chen, Ma, Liang, Peng, & Zuo, 2011; Jiao et al., 2013; Yang et al., 2014).

Recently, research showed that the gut microbe-dependent metabolite trimethylamine, N-oxide (TMAO), directly increased platelet hyperreactivity and thrombosis potential in vivo (Zhu et al., 2016). Resveratrol inhibits TMAO synthesis and eliminates atherosclerosis (AS) (Chen et al., 2016). Reactive oxygen species (ROS), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) were significantly correlated with gut microbiota (Qiao, Sun, Ding, Le, & Shi, 2013). These studies have revealed that a diet with high cholesterol and fat induces gut microbiota changes that increase gut and blood oxidation products, which subsequently aggravate the atherosclerotic process. Therefore, the reduction in oxidative stress in the gut will improve body lipid levels and reduce the risk of cardiovascular disease.

Lonicera caerulea (*Lonicera caerulea* L. var. edulis) belongs to the Caprifoliaceae family. It is a small shrub that yields edible wild berries. The “wild” type of *L. caerulea* berries (hereafter referred to as Wild) is common in China. The fruit of *L. caerulea* contains many anthocyanins (Liu et al., 2016), flavonoids, and phenolic acids (Oszmiański, Wojdyło, & Lachowicz, 2016), and the berries are used as an ingredient in a variety of processed food and beverages. Recent studies have suggested that Haskap berry extract, which contains 51.7% anthocyanins, decreased plasma lipids other than HDL and normalized triglyceride concentrations. Again, the effects were observed for the whole berry extract and not for individual compounds (Jurgoński, Juśkiewicz, & Zduńczyk, 2013). Polyphenols from *L. caerulea* decreased both the TG level and TC/HDL-C ratio in a mouse model of nonalcoholic steatohepatitis (Wu et al., 2016). However, it is unclear how *L. caerulea* berry extract rich in polyphenols (LCBP) can intervene in cholesterol efflux or whether it can prevent intestinal cholesterol absorption. To prove this hypothesis, we analyzed the gene and protein expression of cholesterol absorption in Caco-2 cells and the small intestine of rats. Moreover, we measured the oxidative stress levels of the colon tissue, colonic contents, and serum in rats and Caco-2 cells. The results of this study may serve as a theoretical reference for the use of Wild *L. caerulea* berry.

2. Materials and methods

2.1. Characterization of polyphenols in L. caerulea berry extract

LCBP was extracted as described previously (Liu et al., 2016). Briefly, Wild *L. caerulea* berries were obtained from Baishan City, Jilin Province, China and stored at −60 °C prior to extraction and analysis. The fruits were placed in a 1:10 solid-liquid ratio with 80% acidic ethanol (0.1% hydrochloric acid), homogenized, extracted for 1 h at 30 °C, and filtered; the extraction was repeated twice and the filtrates were merged. The merged filtrate was rotary evaporated (N1100 rotary evaporator, Tokyo, Japan) at 40 °C, until no ethanol remained, and purified by filtration through resin (D101, Sigma). This liquid was eluted with 80% acidic ethanol (0.1% hydrochloric acid), and the eluent was rotary evaporated at 40 °C. The concentrate was stored overnight at −20 °C before vacuum freeze drying. LCBP samples were stored at −20 °C in brown tubes until analysis. Ten milligrams of the LCBP sample were accurately weighed, diluted with 5 mL of methanol, passed through a 0.22 μm filter, and analyzed by HPLC-DAD-ESI-MS/MS. Anthocyanins were measured in positive ionization mode; and other phenol compounds were measured in negative ionization mode. A mass spectrometer equipped with electrospray ionization (ESI) was used to obtain MS/MS data (Ultimate 3000, Thermo LTQXL, USA). The sheath gas pressure was set 35 bar and the assist device was at 10 bar. The flow velocity of the dry gas was 12 L/min and the dry-gas temperature was 320 °C. The purge voltage was controlled at +4 kV. The scope of MS/MS scanning and data collection was 100–1500 *m/z*.

2.2. Caco-2 cell culture

Caco-2 (Huaao, Nanjing, China) cells were grown at 37 °C and 5% CO_2 in complete DMEM supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% (100 U/mL penicillin, and 100 g/mL streptomycin). When the degree of cell fusion reached 85%, the cells were digested by using 0.25% trypsin and resuspended. The resuspended cells were seeded in a 6-well Transwell training board (Corning, USA) at 2×10^5 cells/well and incubation at 37 °C and 5% CO_2 (HF-90 incubator, Lishen, Shanghai, China). The culture medium was changed every 2 days until 8 days, and every day thereafter. After approximately 21 days, close connections were formed between cells (Kosińska-Cagnazzo, Diering, Prim, & Andlauer, 2015); these cells were then used in the following experiments.

2.3. Cholesterol micellar solution preparation

The cholesterol micellar solution was prepared in accordance the method of Zou and Feng (2015), with some modifications. First, the cholesterol micellar solution [5 mM taurocholic acid sodium (dissolved in phosphate-buffered saline, PBS), 0.5 mM oleic acid, 0.16 mM lysophosphatidylcholine, 0.3 mM oleic acid monoglyceride, 5 μM cholesterol, 10 μM NBD-cholesterol (dissolved in isopropanol)] was prepared and filtered through a 0.22 μm microporous membrane. The solution was diluted 0, 1, 2, 4, and 8 times, and then incubated with cells for 16 h at 37 °C and 5% CO_2. In total, 10 μL CCK-8 solution (commercial kits, WLA074b, Wanleibio, Shenyang, China) was added to every well, followed by an incubation period of 2 h in which cell growth was observed (Brown, Hang, Dussault, & Carr, 2010).

2.4. Cell viability assay

The MTT assay after LCBP treatment was performed in accordance with the method of Lammi, Zanoni, Scigliuolo, Amato, and Arnoldi (2014), with some modifications. Caco-2 cells were incubated until a confluence of approximately 90% was reached. The cells were counted and seeded in a 96-well culture plate (3×10^3 cells/well). Each group was assigned five wells, to which 0, 20, 40, 80, or 160 μg/mL of LCBP was added, followed by incubation for 12, 24, or 48 h, respectively. Each group of cells was then treated with 5 mg/mL MTT and incubated for 4 h at 37 °C in an atmosphere of 5% CO_2. When the solution turned clear, 200 μL DMSO was added to dissolve the purple crystals, and the cell viability of each group was calculated from the absorbance of the solution measured at 490 nm by using a microplate reader.

2.5. Animals and treatments

Eight-week-old male Sprague-Dawley rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The animals were housed in a room with controlled temperature (22 ± 1 °C) and humidity (60 ± 5%) under a 12 h light-dark cycle and with free access to water and feed (a standard diet purchased from Beijing HFK Bioscience Co., Ltd.) containing 23% (w/w) protein, 4.8% fat, 3.6% fiber, 1.1% calcium, 0.78% phosphorus, 52.4% nitrogen-free extract, and 9.2% water. All experimental procedures with regard to animal care and handling were performed in accordance with the guidelines provided by the Animal Care Committee and approved by the Ethics Committee of Hebei Normal University of Science and

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