



# Cranberry anthocyanin as an herbal medicine lowers plasma cholesterol by increasing excretion of fecal sterols

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## ABSTRACT

**Background:** Interest in using herbal medicines to treat the hypercholesterolemia is increasing. Cranberry extract could decrease plasma cholesterol, however, the active ingredients and the underlying mechanisms remain largely unknown.

**Hypothesis:** The present study was to test the hypothesis that cranberry anthocyanins (CrA) were at least one of the active ingredients responsible for the cholesterol-lowering activity of cranberry fruits via a mechanism of increasing fecal sterol excretion.

**Methods:** Forty-four hamsters were randomly divided into five groups and fed one of the five diets, namely a non-cholesterol control diet (NCD), a high-cholesterol control diet (HCD), a HCD diet supplemented with a low dose of 1% CrA (CL), a HCD diet supplemented with a high dose of 2% CrA (CH), and a HCD diet supplemented with 0.5% cholestyramine as a positive control drug (P-CTL), respectively, for six weeks. Plasma lipoprotein cholesterol was quantified using the enzymatic kits, while the gene expressions of transporters, enzymes and receptors involved in cholesterol absorption and metabolism were quantified using the quantitative RT-PCR. Fecal sterols were quantified using gas chromatography (GC).

**Results:** Plasma total cholesterol and aorta atherosclerotic plaque decreased dose-dependently with the increasing amounts of CrA added into diets. This was accompanied by a dose-dependent increase in excretion of both neutral and acidic sterols. CrA had no effect on the mRNA levels of intestinal Niemann-Pick C1 like 1 protein (NPC1L1), acyl CoA:cholesterol acyltransferase2 (ACAT2), microsomal triacylglycerol transport protein (MTP), and ATP binding cassette transporter 5 (ABCG5) as well as hepatic cholesterol-7 $\alpha$ -hydroxylase (CYP7A1), 3-Hydroxy-3-methylglutaryl reductase (HMG-CoA-R), sterol regulatory element binding protein 2 (SREBP2), LDL receptor (LDL-R), and Liver X receptor alpha (LXR $\alpha$ ).

**Conclusion:** CrA as an herbal medicine could favorably modify the lipoprotein profile in hamsters fed a high cholesterol diet by enhancing excretion of fecal neutral and acidic sterols, most likely not mediated by interaction with genes of transporters, enzymes and proteins involved in cholesterol absorption and metabolism.

## Introduction

Coronary heart disease (CHD) is the number one killer globally. Cumulative Evidences suggest that elevated concentration of plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) as well as reduced concentration of high-density lipoprotein cholesterol

(HDL-C) are the major risk factors for CHD (Benderly et al., 2009; Sharrett et al., 2001). There is a long history of consuming cranberry fruit as a phytomedicine (McKay and Blumberg, 2007; Howell, 2007; Hurta et al., 2013; Hurta et al., 2012; Liberty et al., 2012; Okla et al., 2013; Shukitt-Hale et al., 2005). Cranberry has been demonstrated to be able to lower TC and LDL-C in ovariectomized rats and

**Abbreviations:** ABCG5 and 8, ATP binding cassette transporter 5 and 8; ACAT2, acyl CoA:cholesterol acyltransferase2; CYP7A1, cholesterol-7 $\alpha$ -hydroxylase; CrA, cranberry anthocyanins; HMG-CoA-R, 3-Hydroxy-3-methylglutaryl coenzyme reductase; LDL-R, LDL receptor; LXR $\alpha$ , Liver X receptor alpha; NPC1L1, Niemann-Pick C1 like 1 protein; MTP, microsomal triacylglycerol transport protein (MTP); SREBP2, sterol regulatory element binding protein 2

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hypercholesterolemic swine (Reed, 2002; Yung et al., 2013). Clinical studies suggest that chronic cranberry consumption is able to reduce the atherosclerotic cholesterol in patients with coronary artery disease and type II diabetics (Lee et al., 2008). However, the active ingredients in cranberry fruits responsible for the cholesterol-lowering activity remain largely unknown. Cranberry fruit is rich in anthocyanins. We hypothesize that cranberry anthocyanins (CrA) are the active phytochemicals or at least one of the active ingredients accountable for the cholesterol-lowering activity of cranberry fruits.

Inhibition on cholesterol absorption is effective in reducing plasma TC. Cholesterol absorption is a complex process involving several proteins, enzyme and transporters, namely intestinal Niemann-Pick C1 like 1 protein (NPC1L1), acyl-CoA: cholesterol acyltransferase 2 (ACAT2), microsomal triacylglycerol transport protein (MTP) and ATP-binding cassette transporters sub-family G member 5 and 8 (ABCG5 and 8). NPC1L1 is a sterol transporter, which transfers the cholesterol from intestinal lumen to enterocytes where ACAT2 converts cholesterol to cholesteryl ester (CE). MTP places CE into chylomicron, which is then diffused into blood via lymphatic system. ABCG5 and 8 transfer the unabsorbed cholesterol from enterocyte back to the lumen of intestine for elimination. Although some studies have reported the cholesterol-lowering activity of cranberry extract (Reed, 2002; Yung et al., 2013), no study to date has examined the interaction of CrA with intestinal NPC1L1, ACAT2, MTP, ABCG5 and 8.

Excessive cholesterol in the liver is metabolized to produce bile acids, which are eliminated via bile duct. In this regard, hepatic cholesterol-7 $\alpha$ -hydroxylase (CYP7A1) is an enzyme, which regulates the conversion of cholesterol to bile acids. In contrast, 3-hydroxy-3-methylglutaryl coenzyme reductase (HMG-CoA-R) is a regulatory enzyme responsible for cholesterol synthesis in the liver, while hepatic LDL receptor is to remove the excessive LDL cholesterol (LDL-R) from circulation into the liver. To maintain the cholesterol homeostasis, liver X receptor alpha (LXR $\alpha$ ) regulates the gene expression of CYP7A1, whereas sterol regulatory element binding protein 2 (SREBP2) regulates the gene expression of HMG-CoA-R and LDL-R. To the best of our knowledge, no study has investigated the effect of CrA on gene expression of CYP7A1, HMG-CoA-R, LDL-R, LXR $\alpha$  and SREBP2.

The present study was designed to (i) investigate if CrA could reduce plasma TC, and (ii) examine the interaction of CrA with gene expression of intestinal NPC1L1, ACAT2, MTP, ABCG5 and 8, as well as hepatic CYP7A1, HMG-CoA-R, LDL-R, and SREBP2.

## Materials and methods

### CrA extract

CrA extract was obtained from Xi'an Realin Biotechnology Co., Ltd (Xi'an, China). As we previously described (Wang et al., 2015), the individual anthocyanins were separated and quantified on an Apollo C18 column (250  $\times$  4.6 mm, 5  $\mu$ m, Grace, Chicago, Illinois, USA) and quantified on a HPLC system with a UV detector at 520 nm. The flow rate was set at 1 ml min<sup>-1</sup>, whereas the gradient mobile phase consisted of 5% acetic acid in water (Solvent A) and methanol (Solvent B). The gradient elution was programmed: 0–10 min, 40% B; 10–30 min, 40–45% B; 30–40 min, 45% B; and 40–50 min, 45–40% B. Each anthocyanin was identified according to the retention time and UV spectrum of authentic standards. HPLC analysis showed that the CrA extract used in the present study mainly contained cyanidin 3-galactoside (4.25%), cyanidin 3-araboside (4.75%), peonidin 3-galactoside (36.36%) and peonidin 3-araboside (4.72%) (Fig. 1).

### Diets

Five diets were prepared according to the method described by Lei et al. (2014) with minor changes. In brief, the non-cholesterol control diet (NCD) was prepared by mixing the list powdered

ingredients (g kg<sup>-1</sup>): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix, 40; vitamin mix, 20; gelatin, 20; DL-methionine, 1 (Table 1). The high-cholesterol control (HCD) diet was similarly prepared by adding 0.2% cholesterol in NCD. The other three experimental diets were prepared by adding a low dose of 1% CrA (CL), a high dose of 2% CrA (CH) or 0.5% cholestyramine as a positive control (P-CTL) into the HCD diet, respectively (Table 1). Cholestyramine, a bile acid sequestrant, binds and prevents the reabsorption of bile acids, leading to lower cholesterol concentration in the blood.

### Hamsters

Forty-four male Golden Syrian hamsters (aged 3 months, body weights = 100–120 g) were randomly divided into five groups (n = 10 each for HCD and CH diets; n = 8 each for NCD, CL, and P-CTL diets) and fed one of the five diets for 6 weeks. The hamsters were kept in Wire-bottomed cages in an animal room at 23 °C with 12/12-h light-dark cycle. Food consumption and body weights were recorded daily. At week 0 and 6, about 0.5 ml blood sample from each hamster was collected from the retro-orbital sinus into heparinized capillary tube under a light anesthesia using a mixture of ketamine, xylazine and saline (v/v/v; 4:1:5) after overnight fasting. At the end of week 6, all hamsters were sacrificed by carbon dioxide anaesthesia and the abdomen was cut open with blood being collected from the aorta into syringe. The liver, heart, kidney, testis, epididymal and perirenal adipose tissues were removed, washed with saline, weighted, and frozen in liquid nitrogen. The small intestine was washed 3 times in phosphate-buffered saline. All tissues were kept in a –80 °C freezer until analysis. Thoracic aortas were dissected and stored in DEPC-PBS solution until analysis. At week 6, total fecal output from each cage was separated from the bedding, freeze-dried, ground and saved for neutral and acidic sterols analyses. The entire experimental protocol was approved and performed under the guidelines of the Animal Experimental Ethical Committee, The Chinese University of Hong Kong [Ref No:(15–327) in DH/HA&P/8/2/1 Pt.49].

### Analysis of plasma lipids

The commercial enzymatic kit obtained from Infinity (Waltham, MA, USA) and Stanbio Laboratories (Boerne, TX, USA) were used to measure the TC, HDL-C, and total triacylglycerols (TG), respectively. Non-HDL-C was calculated by reducing HDL-C from TC.

### Analysis of atherosclerotic plaque

The percentage area of atherosclerotic plaque on the endothelial layer was measured as we previously described (Ng et al., 2008). In brief, the thoracic aorta was cut opened vertically after surrounding connective tissue being removed. The aortas were stained with saturated oil red (Sigma-Aldrich, St. Louis, Mo, USA) followed by three times of washing with 2-propanol and two times of washing with distilled water. Then the aortas were scanned with a table scanner (Epson 1220 perfection, Epson Co., Tokyo, Japan). The total aortic endothelial layer area and atherosclerotic plaque area were analyzed using a computer image analyzing program Sigma Scan Pro 5.0 (SPSS, Chicago, USA). The percentage area of atherosclerotic plaque was calculated according to the following equation: Aortic Plaque (%) = (atherosclerotic plaque area / total aortic endothelial layer area)  $\times$  100%.

### Analysis of liver cholesterol

Liver cholesterol was measured using a GC method as we previously described (Lei et al., 2014; Ng et al., 2008). In brief, total lipids were extracted into a solvent mixture of chloroform-methanol with addition of 1 mg of 5  $\alpha$ -cholestane as an internal standard. After saponification, cholesterol in the non-saponified fraction was converted to TMS-ether

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