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***Citrullus lanatus* 'Sentinel' (Watermelon) Extract Reduces Atherosclerosis in LDL Receptor Deficient Mice**

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Abstract

Watermelon (*Citrullus lanatus* or *C. lanatus*) has many potentially bioactive compounds including citrulline, which may influence atherosclerosis. In this study, we determined the effects of *C. lanatus*, provided as an extract of the cultivar 'sentinel', on hypercholesterolemia-induced atherosclerosis in mice. Male LDL receptor deficient mice at 8 weeks old were given either *C. lanatus* 'sentinel' extract (2% vol/vol; n=10) or a mixture of matching carbohydrates (2% vol/vol; n=8) as the control in drinking water, while fed a saturated fat-enriched diet for 12 weeks ad libitum. Mice consuming *C. lanatus* 'sentinel' extract had significantly increased plasma citrulline concentrations. Systolic blood pressure was comparable between the two groups. Consumption of *C. lanatus* 'sentinel' extract led to lower body weight and fat mass without influencing lean mass. There were no differences in food and water intake, and urine output between the two groups. *C. lanatus* 'sentinel' extract administration decreased plasma cholesterol concentrations that were attributed to reductions of intermediate/low density lipoprotein cholesterol. Plasma concentrations of MCP-1 and IFN- γ were decreased and IL-10 increased in mice consuming *C. lanatus* 'sentinel' extract. Intake of *C. lanatus* 'sentinel' extract resulted in reductions of atherosclerosis in both aortic arch and thoracic regions. In conclusion, consumption of *C. lanatus* 'sentinel' extract led to reduced body weight gain, decreased plasma cholesterol concentrations, improved homeostasis of pro- and anti-inflammatory cytokines, and attenuated development of atherosclerosis without affecting systolic blood pressure in hypercholesterolemic mice.

Keywords

Citrullus lanatus; atherosclerosis; LDL receptor deficient mice; cholesterol

1. Introduction

Atherosclerosis is a major cause of cardiovascular diseases in the industrialized nations. Many risk factors influencing atherosclerosis involve lifestyle such as smoking and diet.

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Epidemiological and clinical studies have demonstrated that a healthy life style reduces risks of predisposition to atherosclerosis and improves overall health [1,2]. There is growing evidence from both human studies and animal models that fruit juice consumption has beneficial effects on cardiovascular diseases [3–5].

Watermelon (*Citrullus lanatus* or *C. lanatus*) belongs to the Cucurbitaceae family and is a source of multiple minerals, vitamins, and proteins that are present in skin, pulp, and seeds [6,7]. Beneficial effects of *C. lanatus* on cardiovascular diseases may be due to several mechanisms including anti-oxidant, anti-inflammatory, and vasodilatory properties [8–10]. *C. lanatus* is a rich source of citrulline that is responsible for the effects of this fruit on nitric oxide release [8,10,11]. There is literature evidence that enhanced nitric oxide release promoted by *C. lanatus* intake improves endothelial dysfunction, reduces aortic blood pressure, and decreases lipid peroxidation in liver [9,10]. However, direct effects of *C. lanatus* consumption on atherosclerosis have not been determined.

Hypercholesterolemia is a major risk factor for atherosclerosis. Among hypercholesterolemic animal models, LDL receptor deficient mice are a commonly used model for atherosclerosis studies [12]. LDL receptor deficient mice fed a saturated fat-enriched diet for 12 weeks have elevated plasma cholesterol concentrations greater than 1,000 mg/dl and develop profound aortic atherosclerotic lesions [13–15]. In the present study, we hypothesized that oral administration of *C. lanatus* 'sentinel' extract would attenuate development of atherosclerosis. To test this hypothesis, we gave *C. lanatus* 'sentinel' extract (2% vol/vol) or a carbohydrate-matched mixture (2% vol/vol) in drinking water to male LDL receptor deficient mice, while feeding these mice a saturated fat-enriched diet.

2. Materials and Methods

2.1. Mice, solutions, and diet

LDL receptor $-/-$ mice (B6.129S7-Ldlr^{tm1her}; Stock #002207; 6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in a barrier animal facility at the University of Kentucky.

One group of mice was provided with *C. lanatus* 'sentinel' extract in drinking water (2% vol/vol). The extract was obtained by pressing and filtering the skin, pulp, and seeds of *C. lanatus*. The extract contained 20–30 mg/g of citrulline (Cat# FG10812, Milne Fruit Products Inc., Prosser, WA, USA). Control mice were provided with a solution (2% vol/vol) that matched the carbohydrate content of the *C. lanatus* 'sentinel' extract. The carbohydrate solution was composed of fructose, glucose, sucrose, maltodextrin (Cat #s F3510, G6152, S1888, 419680, Sigma, St Louis, MO, USA; respectively), and silicon dioxide (Cat # 110330, Natural Flavors Inc., Orange, CA, USA). Both watermelon and carbohydrate solutions were dissolved in reverse osmosis water. Concentrations of individual components contained in the two study groups are given in Online Table I.

Mice were provided either solution for 13 weeks ad libitum. After 1 week of drinking solutions, mice were fed a diet supplemented with saturated fat (milk fat 21% wt/wt) and cholesterol (0.2 % wt/wt; Cat # TD.88137, Harlan Teklad, Madison, WI, USA) for 12 weeks to induce hypercholesterolemia. This study was performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

2.2. Systolic blood pressure measurement

Systolic blood pressure was measured using a non-invasive tail-cuff system (Coda 8, Kent Scientific Corporation, Torrington, CT, USA) as described previously [16].

2.3. Echo magnetic resonance imaging

Fat mass and lean mass were measured during the last week of the study by an echo-magnetic resonance imaging system (Echo-MRI; Echo Medical Systems, Houston, TX, USA). This system uses the distinct resonance frequency of protons in lipid and water to determine body mass [17].

2.4. Measurements of food and water intake and urine output

Food and water intake, and urine output were measured over a 24-hour interval during week 12 of the study using a metabolic cage system (Techniplast, Inc., Philadelphia, PA, USA).

2.5. Measurement of plasma citrulline concentrations

Plasma was collected via cardiac puncture at termination. Plasma citrulline concentration was measured using HPLC, and on the basis of the authentic standards (Sigma, St Louis, MO, USA) using MillenniumTM-32 software (Waters Inc., Milford, MA, USA) as described previously [18].

2.6. Measurement of plasma cholesterol concentrations and lipoprotein distribution

Plasma cholesterol concentrations were measured using an enzymatic kit (Cat # 439-17501, Wako Chemicals USA, Richmond, VA, USA). Plasma lipoprotein distribution (n=4) was resolved by fast performance liquid chromatography. Eluted fractions of samples from individual mice were collected and subsequently measured to determine lipoprotein-cholesterol distribution. Lipoprotein cholesterol distribution of very low density (VLDL), intermediate/low density (I/LDL) and high density lipoproteins (HDL) were analyzed using Peak-Fit software 4.1 version (Seasolve Software Inc., San Jose, CA, USA). The software analyzed chromatographic data using nonlinear peak separation function [19].

2.7. Measurement of plasma cytokine concentrations

Plasma cytokine concentrations were measured using multiplex ELISA kits (Cat # M60000C909, Richmond, CA, USA) and were detected with a Bio-Plex 200 instrument (Bio-Rad, Richmond, CA, USA). The following cytokines were measured: monocyte chemoattractant protein-1 (MCP-1), interferon-gamma (IFN- γ), tumor necrosis factor (TNF- α), interleukin-1 β (IL-1 β), and interleukin-10 (IL-10). Plasma SAA concentrations were measured using ELISA kits (Cat # TP802-M, Tridelata Development Ltd, Maynooth, County Kildare, Ireland) and detected using a Bio-Rad microplate reader (Bio-Rad). Assays were performed according to manufacturers' protocols.

2.8. Quantification of atherosclerosis

Atherosclerosis was quantified on the intima of both arch and thoracic aortic regions as described previously [20].

2.9. Statistics

SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) was used for all statistical analyses. Data are summarized as mean $\pm$ standard error of mean (SEM). Student's *t* test or Rank Sum test were performed as appropriate. Weekly body weight and systolic blood pressure data were analyzed by one way repeated measure ANOVA. A *P* value < 0.05 was considered statistically significant.

3. Results

3.1. *C. lanatus* 'sentinel' extract increased plasma citrulline concentration but had no effect on blood pressure

Since *C. lanatus* is enriched in citrulline, we measured its concentration in plasma as a marker of consumption. Plasma citrulline concentrations were significantly increased in mice provided with *C. lanatus* 'sentinel' extract compared to control mice ($P<0.05$, Figure 1). However, there was no difference in systolic blood pressure between the two groups (Table 1).

3.2. *C. lanatus* 'sentinel' extract reduced body weight gain and fat mass

All mice were weighed weekly throughout the study. Both groups steadily gained ~1.8 g of body weight per week during first 7 weeks. However, at week 8, mice provided with *C. lanatus* 'sentinel' extract significantly gained less body weight compared to those provided with control (Control: 1.6 g/week vs *C. lanatus*: 1.1 g/week; $P<0.05$, Figure 2A). Body composition was measured using an Echo MRI during week 12 of the fat-enriched diet feeding. Although there was no difference in lean mass, a significant reduction of fat mass was found in mice supplied with *C. lanatus* 'sentinel' extract compared to the control group ($P<0.05$, Figure 2B). Food and water intake, and urine output were measured. There were no significant differences in any of these parameters between the two groups (Table 1).

3.3. *C. lanatus* 'sentinel' extract decreased plasma cholesterol concentrations

Since hypercholesterolemia has a direct association with atherosclerosis, plasma cholesterol concentrations were measured at 12 weeks. *C. lanatus* 'sentinel' extract consumption significantly reduced plasma total cholesterol concentrations ($P<0.05$, Figure 3A). Resolution of lipoproteins through size exclusion chromatography (Figure 3B) followed by nonlinear curve fitting analysis determined that the *C. lanatus* 'sentinel' extract had significantly decreased LDL-cholesterol concentrations ($P<0.05$, Figure 3C). However, there was no difference in VLDL-cholesterol or HDL-cholesterol concentrations between the study groups (Figure 3C).

3.4. *C. lanatus* 'sentinel' extract reduced atherosclerosis

Atherosclerotic lesion area was measured in both arch and thoracic regions of the aorta. Mice ingesting *C. lanatus* 'sentinel' extract had significantly reduced percent lesion area in both aortic arches ($P<0.05$, Figure 4A) and thoracic aortas ($P<0.05$, Figure 4B).

3.5. *C. lanatus* 'sentinel' extract decreased plasma concentrations of pro-inflammatory cytokines while increasing anti-inflammatory cytokines

To determine the effect of *C. lanatus* 'sentinel' extract on inflammation, we measured an array of plasma cytokine concentrations. Significant decreases in MCP-1 and IFN- γ concentrations, but a profound increase of IL-10, were detected in plasma from mice consuming *C. lanatus* 'sentinel' extract compared to control mice (Figure 5). There were no significant differences in plasma TNF- α , IL-1 β , and SAA concentrations were found between the two study groups (Online Table II).

4. Discussion

This study demonstrates that oral administration of *C. lanatus* 'sentinel' extract to LDL receptor deficient mice reduced hypercholesterolemia-induced atherosclerosis. In addition, we also found that mice administered *C. lanatus* 'sentinel' extract had less body weight gain and reduced plasma cholesterol concentrations.

One of the main bioactive compounds of *C. lanatus* is citrulline. Mice administered with *C. lanatus* 'sentinel' extract exhibited increased plasma citrulline concentrations compared to mice administered a control solution. This finding is consistent with a previous report that dietary watermelon pomace juice supplementation increased plasma citrulline concentrations in Zucker diabetic rats [8]. Citrulline is known to affect blood pressure. However, there are inconsistent reports on the effects of citrulline on systolic blood pressure with either a reduction [21,22] or no change [23] in humans and spontaneously hypertensive rats. In this present study, administration of *C. lanatus* 'sentinel' extract did not change systolic blood pressure in mice. Consistently, a randomized clinical trial study demonstrated that oral intake of *C. lanatus* juice did not change branchial systolic blood pressure in humans [10]. However, the explanation for this inconsistency is not clarified in the reported findings.

In the present study, dietary supplementation of *C. lanatus* 'sentinel' extract reduced body weight gain that was induced by a saturated fat-enriched diet. The decrease in body weight was not due to any detectable difference in food consumption or water intake. There is a similar observation that showed less body weight gain in Zucker diabetic rats fed *C. lanatus* watermelon pomace juice [9]. In that study, they found an increased mass of brown adipose tissue and reduced mass of subcutaneous and retroperitoneal fat in rats after watermelon pomace juice supplementation. In the present study, we measured fat and lean mass weight and found significantly decreased fat mass in mice administered *C. lanatus* 'sentinel' extract. Increased fat mass contributes to enhanced risk for developing atherosclerosis [24,25]. Despite lack of direct evidence, it is possible that supplementation of *C. lanatus* 'sentinel' extract may be beneficial for protecting against atherosclerosis by reducing fat mass.

In this study, while both groups of mice were hypercholesterolemic, we found that administration of *C. lanatus* 'sentinel' extract led to a reduction of plasma cholesterol concentrations that were attributable to reduced plasma I/LDL-cholesterol concentrations. It has been reported that dietary supplementation of a watermelon extract, *C. vulgaris*, reduced LDL-cholesterol concentrations in Wistar rats [26]. Plasma cholesterol concentrations have a direct effect on development of experimental atherosclerosis. Clinical and epidemiological studies have demonstrated that elevated LDL-cholesterol concentrations are a primary risk factor for development of atherosclerosis [27–29]. Decreased plasma I/LDL-cholesterol concentrations are a potential mechanism that contributed to reducing atherosclerosis in mice administered with *C. lanatus* 'sentinel' extract.

Administration of *C. lanatus* 'sentinel' extract attenuated atherosclerosis in both arch and thoracic regions of aortas in mice. Aortic arch and thoracic aorta regions represent different developmental origins of aortic smooth muscle cells [30]. Reduction of atherosclerosis in these two distinct aortic regions provides strong evidence of a role for *C. lanatus* 'sentinel' extract. There is evidence that the main component of *C. lanatus*, citrulline, has a beneficial effect on atherosclerosis and aortic vascular remodeling. Citrulline administration improved endothelium-dependent vasorelaxation and decreased aortic medial thickening [31,32]. It is possible that citrulline in *C. lanatus* 'sentinel' extract may contribute to reducing atherosclerosis as shown in this study.

Inflammation has long been recognized as a critical mechanism in the development of atherosclerosis [33]. In this study, we found significantly decreased of pro-inflammatory cytokines in plasma, such as MCP-1 and IFN- γ , in mice consuming *C. lanatus* 'sentinel' extract. In contrast, consumption of *C. lanatus* 'sentinel' extract increased plasma concentrations of IL-10, an anti-inflammatory cytokine. These findings indicate that administration of *C. lanatus* 'sentinel' reduced inflammatory responses in LDL receptor $-/-$ mice fed a saturated fat-enriched diet. In agreement with this result, a recent study has also

demonstrated anti-inflammatory activities of *C. lanatus* 'citroides' in murine macrophages cells [34]. Albeit not direct evidence, it is possible that decreased systemic inflammation, as shown in the present study, is a potential mechanism by which *C. lanatus* 'sentinel' extract reduces atherosclerosis.

In summary, the present study demonstrated that oral administration of *C. lanatus* 'sentinel' extract attenuated atherosclerosis in LDL receptor $-/-$ mice. The beneficial effects of *C. lanatus* 'sentinel' extract supplementation also included less body weight gain due to less fat mass, and reduced plasma cholesterol concentrations that was due to decreased I/LDL-cholesterol concentrations. In addition, *C. lanatus* 'sentinel' extract reduced pro-inflammatory and increased anti-inflammatory cytokines concentrations. This study demonstrates that dietary supplementation of *C. lanatus* 'sentinel' extract provides a potential health benefit against cardiovascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *C. lanatus* 'sentinel' extract increased plasma citrulline concentrations

Plasma citrulline concentrations were measured in 5 random samples from each group.

Histobars represent means and error bars are SEMs. * denotes $P < 0.05$ by Student's t test.

Figure 2. *C. lanatus* 'sentinel' extract reduced weight gain and decreased fat mass

(A) Body weights were measured weekly during the study. The diet enriched in saturated fat was started at week 0. Circles represent group means and error bars are SEMs. * denotes $P < 0.05$ by one way repeated measures ANOVA. (B) Lean and fat mass were measured during the last week of the study. Histograms represent means and error bars the SEMs. * denotes $P < 0.05$ by Student's *t* test.

Figure 3. *C. lanatus* 'sentinel' extract reduced plasma total cholesterol and intermediate/low density lipoprotein cholesterol concentrations

(A) Plasma cholesterol concentrations were measured at termination. Histograms represent means and error bars are SEMs. (B) Lipoproteins were resolved by size exclusion chromatography. Circles of each point represent mean values of each fraction from 4 individual mice of each group and bars are SEMs. (C) Plasma cholesterol concentrations of lipoprotein fractions were calculated using a non linear curve fitting approach. Histograms represent means and bars represent SEM. * denotes $P < 0.05$ by Student's t test.

Figure 4. *C. lanatus* 'sentinel' extract reduced atherosclerosis

(A) Percent of atherosclerotic lesion areas were measured on the intimal surface of aortic arches. Inverted triangles represent individual mice, circles represent means and bars are SEMs. (B) Percent of atherosclerotic lesion areas were measured on the intimal surfaces of thoracic regions. Inverted triangles represent individual mice, circles represent means and bars are SEMs. * denotes $P < 0.05$ by Student's t test.

Figure 5. *C. lanatus* 'sentinel' extract influenced plasma concentrations of MCP-1, IFN- γ , and IL-10

Plasma concentrations of (A) MCP-1, (B) IFN- γ , and (C) IL-10 were measured at termination of the study. Inverted triangles represent values from individual mice, circles represent means, and bars are SEMs. * denotes $P < 0.05$ by Rank Sum test or Student's *t* test.

Table 1

Characterization of LDL receptor $-/-$ mice fed a saturated fat-enriched diet and either a *C. lanatus* 'sentinel' extract or carbohydrate solution

Groups	Control	<i>C. lanatus</i>
N	8	10
Systolic blood pressure (mmHg)	120 $\pm$ 7	124 $\pm$ 4
Food intake (gm/24 hours)	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1
Water intake (ml/24hours)	2.5 $\pm$ 0.2	3.1 $\pm$ 0.2
Urine output (ml/24 hours)	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1

Values are represented in mean $\pm$ SEM.