Blueberry Leaf Polyphenols Prevent Body Fat Accumulation in Mice Fed High-fat, High-sucrose Diet

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Abstract: Blueberry leaf is currently a popular dietary supplement. Effects of dietary blueberry leaf and its active components on body fat accumulation were examined. C57BL/6J mice were fed high-fat, high-sucrose diet with or without 3% blueberry leaf extract (BLEx) or 3% concentrated-polyphenolic BLEx (CP BLEx) for 8 weeks. Compared to mice fed a high-fat, high-sucrose diet without blueberry leaf, BLEx and CP BLEx significantly reduced body weight and adipose tissue weight gain. Adipocytes were also smaller and liver lipid accumulation was significantly inhibited in mice fed either BLEx or CP BLEx. These effects tended to be more pronounced in mice fed CP BLEx compared to in mice fed BLEx. Together, results suggest that blueberry leaf inhibits body fat accumulation typically observed in mice fed a high-fat, high-sucrose diet, and that inhibition is attributable to polyphenolic components in leaf extracts.

1 INTRODUCTION

Obesity is a serious health problem world-wide. Accumulation of excess fat increases risk of diseases such as type 2 diabetes, non-alcoholic fatty liver disease, and atherosclerosis. An effective means to reduce or prevent obesity and obesity-related disease is therefore an important public health goal. Recently, dietary supplements have been studied as possible anti-obesity agents. Some dietary supplements do possess anti-obesity properties. Blueberry leaf extract (BLEx) supplement for 4 weeks also decreased body fat, reduced liver lipid accumulation, and prevented elevation of blood glucose in diet-induced obesity mice. Further, blueberry leaf reduces the incidence of obesity-related diseases. We examined the effect of BLEx supplement on diet-induced obesity mice compared to positive control mice not administered BLEx in the present study. In addition, we compared the effects induced by BLEx and the effects induced by concentrated polyphenolic BLEx (CP BLEx). CP BLEx has approximately twice the polyphenolic content of BLEx and was used to help identify biologically active components in BLEx. The aim of this study is to reveal the effect of chronic BLEx supplement and to investigate the active component of BLEx on diet-induced obesity.

2 Experimental Procedures

2.1 Materials

BLEx was prepared as a hot water extract by Bizen Chemical Co. Ltd (Okayama, Japan). Briefly, blueberry powder was extracted twice in 16 parts of hot water.

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100°C) for 30 min. Extracts were then combined, filtered, and heat sterilized. Finally, a powder was produced from the sterile filtrate using a spray dryer. CP BLEx was prepared by Bizen Chemical Co. Ltd (Okayama, Japan). BLEx contained 86.5 mg/g chlorogenic acid, 715 µmol/g eq. cyanidin proanthocyanidin, and a total polyphenol content of 36.3 ˚C. CP BLEx contained 219 mg/g chlorogenic acid, 1092 µmol/g eq. cyanidin proanthocyanidin, and a total polyphenol content of 72.9 ˚C.

2.2 Experimental animals and diets
C57BL/6J mice (4-week-old, males) were purchased from Japan SLC (Hamamatsu, Japan) and acclimatized for 1 week. Room temperature was maintained at 22-24°C with a 12-h light/dark cycle. Mice were divided into 4 groups: normal diet group (ND), high-fat, high-sucrose diet group (HFHS), high-fat, high-sucrose diet with 3% BLEx (BLEx) and high-fat, high-sucrose diet with 3% CP BLEx (CP BLEx). Mice remained on these diets for 8 weeks. Diets were prepared according to AIN-93G with slight modifications. Detailed diet compositions are provided in Table 1. After an overnight fast at the end of the feeding period, mice were sacrificed under Somnopentyl anesthesia. Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Miyazaki and in compliance with the Law Concerning the Protection and Control of Animals (Japan Law No.105, approval No. 2013-024); Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No.88 of the Ministry of the Environment, Japan); and Guidelines for Animal Experimentation (the Japanese Association for Laboratory Animal Science).

2.3 Isolation of Stromal Vascular Cell (SVC) in adipose tissue and flow cytometry analysis
Adipose tissue piece was excised, transferred to Hank’s buffer containing collagenase (1 mg/mL), and incubated at 37°C for 1 hour. Digested tissue was filtered using a 300 µm mesh and the filtrate centrifuged at 300 g for 5 min. Supernatant was removed and suspended in lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 9.8 mM EDTA-2Na) to remove erythrocytes, and then centrifuged again at 300 g for 5 min. Pellets containing SVCs were incubated in Fc Block (anti-CD16/32; Affymetrix Inc, California, USA) on ice for 10 min and stained with anti-mouse F4/80-PE (Affymetrix Inc, California, USA) at 4°C for 30 min. Stained cells were washed twice in PBS containing 2 mM EDTA and 2% FBS and stained with propidium iodide solution. Finally, cells were washed again with the same PBS solution and fixed in 0.1% paraformaldehyde. Analysis of stained and washed cells was performed using a flow cytometer (Cell Lab Quanta SC MPL, Beckman Coulter Inc, California, USA).

2.4 Histological analysis
Adipose and liver tissues were excised immediately after sacrifice and fixed in 4% paraformaldehyde. Subsequent

<table>
<thead>
<tr>
<th>Component (g/kg)</th>
<th>ND</th>
<th>HFHS</th>
<th>BLEx</th>
<th>CP BLEx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Choline bitartate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cystine</td>
<td>3.0</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.01</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>200</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Corn starch</td>
<td>397.5</td>
<td>148.7</td>
<td>148.7</td>
<td>148.7</td>
</tr>
<tr>
<td>Pegelatinized corn starch</td>
<td>132</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Tallow</td>
<td>0</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>Lard</td>
<td>0</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>BLEx</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>CP BLEx</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

Diets were prepared as described in AIN-93G with slight modification. ND, normal diet; HFHS, high-fat, high-sucrose; BLEx, blueberry leaf extract; CP BLEx, concentrated-polyphenolic BLEx.
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Table 2  Effect of dietary BLEx and CP BLEx on growth parameters.

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>HFHS</th>
<th>BLEx</th>
<th>CP BLEx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>17.8 ± 2.3</td>
<td>17.6 ± 1.9</td>
<td>17.6 ± 1.6</td>
<td>17.7 ± 1.8</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>26.7 ± 1.4a</td>
<td>28.9 ± 1.8b</td>
<td>26.0 ± 1.4a</td>
<td>26.0 ± 1.9b</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>2.91 ± 0.08</td>
<td>2.67 ± 0.27</td>
<td>2.57 ± 0.26</td>
<td>2.50 ± 0.21</td>
</tr>
<tr>
<td>Water intake (g/day)</td>
<td>2.99 ± 0.15</td>
<td>2.70 ± 0.20</td>
<td>3.08 ± 0.23</td>
<td>3.13 ± 0.58</td>
</tr>
<tr>
<td>Feeding efficiency (mg body weight gain/g food intake)</td>
<td>58.3 ± 11.4</td>
<td>81.3 ± 14.9</td>
<td>63.4 ± 19.8</td>
<td>63 ± 6.9</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 8). Different superscript letters identify values that differ significantly (p < 0.05).

ND, normal diet; HFHS, high-fat, high-sucrose; BLEx, blueberry leaf extract; CP BLEx, concentrated-polyphenolic BLEx.

embedding, section preparation, and hematoxylin-eosin staining were outsourced to Sapporo General Pathology Laboratory (Sapporo, Japan).

2.5 Analysis of liver lipids
A liver homogenate was prepared using Micro Smash MS-100R (Tomy Seiko, Tokyo, Japan) and total lipids extracted using a chloroform and methanol (2:1). Solvents were resolubilized in 5% Tween-20 isopropanol. Triglyceride and total cholesterol were measured by Triglyceride E test and Cholesterol E test, respectively (Wako, Osaka, Japan).

2.6 Serum parameters
Serum GOT and GPT, triglyceride, free fatty acid, and total cholesterol were measured by using the following assays -- Transaminase C II (serum enzymes), Triglyceride E, Lab Assay™ NEFA (free fatty acids), Cholesterol E (Wako). Serum glucose was measured by LAB Gluco (Foracare Japan Co Ltd, Tokyo, Japan). Serum insulin was measured using an ELISA-based commercial kit (Morinaga, Yokohama, Japan). Serum adiponectin was measured by Duo Set® Mouse Adiponectin/Acrp30 (R&D Systems Inc, Minneapolis, USA). All chemical analyses were performed according to manufacturer’s protocols.

2.7 Statistical analysis
All data were expressed as the mean ± SD. Statistical analysis was performed by one-way ANOVA, followed by Tukey-Kramer post hoc test. Differences were considered significant at p < 0.05.

3 Results
3.1 Growth parameters
No significant differences in initial body weights were observed among animals in the 4 test groups. Final body weights of animals in BLEx and CP BLEx groups were significantly lower than body weights of animals in the HFHS group and about the same as body weights of animals in the ND group (Table 2). Further, no significant differences in food and water intake were observed among animals in any of the four groups. Feeding efficiency in the HFHS group did tend to be higher than in the other three groups: these differences were not statistically significant.

3.2 Body fat accumulation and macrophage infiltration
Perirenal, epididymal, and subcutaneous fat weight was significantly less in animals in BLEx and CP BLEx groups than fat weights observed in animals in the HFHS group (Fig. 1A-C). Brown fat weight significantly decreased in animals in CP BLEx group than HFHS group (Fig. 1D). Differences between fat weights in animals in CP BLEx and HFHS groups tended to be greater than differences in fat weights between animals in BLEx and HFHS groups. Size of adipocytes collected from animals in the HFHS group was greater than the size of adipocytes collected from animals in the ND group. In contrast, adipocytes collected from animals in BLEx and CP BLEx groups were smaller than adipocytes from animals in the HFHS group (Fig. 1E-H). In epididymal fat, the fraction of adipocytes displaying a macrophage marker tended to be higher in cells from animals in the HFHS group compared to cells from animals in the ND group, but lower in cells from animals in the CP BLEx group compared to cells from animals in the HFHS group (Fig. 1I).

3.3 Liver lipid accumulation
Liver weight was significantly higher in animals in the ND group compared with that in animals in either HFHS or BLEx groups (Fig. 2A). Serum GOT levels in animals in the ND group were significantly higher than levels in animals from BLEx and CP BLEx groups (Fig. 2B). No significant differences in serum GPT levels were observed in mice among the four groups (Fig. 2C). Triglyceride and total cholesterol levels in serum from animals in BLEx and CP BLEx groups were significantly lower than in animals from the HFHS group (Fig. 2D, E). Histologically, lipid droplets in liver cells from animals in BLEx and CP BLEx groups were
Fig. 1  Effect of BLEx and CP BLEx on body fat and macrophage infiltration
(A-D) Adipose tissue weight. (E-H) Histology of adipose tissue. (I) Macrophage infiltration in adipose tissue. Data are means ± SE (n = 4-8). Different superscript letters identify values that differ significantly (p < 0.05). ND, normal diet; HFHS, high-fat, high-sucrose; BLEx, blueberry leaf extract; CP BLEx, concentrated-polyphenolic BLEx.
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**Fig. 2** Effect of BLEx and CP BLEx on liver lipid.
Liver weight. (B, C) Serum GOT and GPT level. (D, E) Liver triglyceride and total cholesterol level. (F-I) Histology of liver. Data are means ± SE (n = 4-8). Different superscript letters identify values that differ significantly (p < 0.05). ND, normal diet; HFHS, high-fat, high-sucrose; BLEx, blueberry leaf extract; CP BLEx, concentrated-polyphenolic BLEx.
smaller than lipid droplets in liver cells from animals in the HFHS group (Fig. 2E-I). These differences in effects on liver were more pronounced in animals in the CP BLEx group than in animals from the BLEx group.

3.4 Serum parameters

No significant differences in serum triglyceride or free fatty acid levels were observed in animals among the four groups (Fig. 3A, B). In contrast, serum total cholesterol levels in animals from the CP BLEx group were significantly lower than levels in animals from the HFHS group (Fig. 3C). Serum glucose levels in animals in BLEx and CP BLEx groups tended to be lower than levels in animals from the HFHS group (Fig. 3D). No significant differences in serum insulin levels were observed in animals among the four groups (Fig. 3E). Serum adiponectin levels in BLEx and CP BLEx groups were significantly lower than levels in animals from the HFHS group (Fig. 3F).

4 Discussion

Obesity is characterized by accumulation of excessive visceral fat and increased size and lipid content of adipocytes. Our previous study reported that BLEx decreased white adipose tissue weight gain in mice fed a high-fat, high-sucrose diet. The experimental diet in that report was replaced cellulose with BLEx, but the experimental diet in the present study was replaced sucrose with BLEx or CP BLEx to avoid the influence on diet-induced obesity.
by the different quantity of dietary fiber among each group according to several reports (the composition of BLEX and CP BLEX are not shown)\textsuperscript{14, 10}. And it was known that the difference of the amount of cystine brought the promotion of body fat accumulation\textsuperscript{161}. Therefore we lowered the amount of cystine in ND group compared to the other group to induce obesity by high-fat diet according to previous report\textsuperscript{171}. The results indicate that mice fed BLEX or CP BLEX along with a high-fat, high-sucrose diet gained less white adipose tissue weight. These results demonstrate that BLEX can significantly inhibited the accumulation of body fat. Further, lower brown adipose tissue weight and serum adiponectin level was observed in BLEX and CP BLEX groups in this study. Although, brown adipose tissue has anti-obesity effect by its thermogenesis property, previous study reported that brown adipose tissue weight was increased in high-fat diet induced obesity mice due to accumulation of lipid droplets in brown adipose tissue cells\textsuperscript{8, 19}. The result in this study suggest that BLEX might also inhibit accumulation of lipid droplets in brown adipose tissue. Adiponectin exerts anti-diabetic property and is secreted from mature adipocytes\textsuperscript{20}. Several studies showed anti-obesity materials increased serum adiponectin level in diet induced obesity animal model and the mechanism of this effect was considered as reduction of adipocyte size because abnormally enlarged adipocytes decrease the adiponectin production. On the other hand, dietary BLEX and CP BLEX failed to increase adiponectin despite the fact they reduced the weight body fat. Adiponectin is exclusively produced from mature adipocytes, therefore, it is considered that BLEX and CP BLEX may prevented the differentiation and maturation of adipocytes. Actually, our preliminary study revealed that BLEX prevented the differentiation of 3T3-L1 cells to the adipocytes.

Obesity can also lead to chronic inflammation in adipose tissue. Macrophages accumulated in intraperitoneal adipose tissue of obese mice fed a high-fat diet; similar results have been reported in subcutaneous adipose tissue of obese human subjects\textsuperscript{21, 22}. Present results show that the fraction of M1-type macrophages in epidydimal adipose tissue in mice from the CP BLEX group tended to be less than the fraction in mice from the HFHS group. This observation suggests that polyphenols in blueberry leaf are active constituents in preventing obesity-induced inflammation in adipose tissue.

In general, effects elicited by dietary CP BLEX were more pronounced than effects elicited by dietary BLEX. Preparation of CP BLEX from BLEX removed organic acids and increased polyphenolic content. Polyphenol-rich foods show anti-obesity properties. For example, grape peel extract decreased adipose tissue weight gain in mice fed a high-fat diet, and green tea, black tea, and oolong tea reduced visceral fat in mice fed a high-fat, high-sucrose diet\textsuperscript{9, 101}. BLEX contains polyphenols, mainly chlorogenic acid (86.5 mg/g dry weight) and proanthocyanidin (715 μmol/g dry weight, eq. cyanidin). These polyphenols are concentrated by factors of approximately 2.5 and 1.5, respectively in CP BLEX. In a 12-week feeding study, a high-fat diet containing green coffee bean extract (0.1-0.9%) which contains substantial amounts of chlorogenic acid inhibited body weight and decreased adipose tissue weight gains observed in mice fed the same diet without the extract\textsuperscript{195}. A similar study that used grape seed extract (250 mg/kg body weight/day) instead of green coffee bean extract produced similar results\textsuperscript{197}. Grape seed extract contains substantial amounts of proanthocyanidin. Previously, chlorogenic acid decreased body weight gain and visceral fat mass in mice fed a high-fat diet, proanthocyanidin decreased body weight gain and improved adiposity index in rats fed a high-fat, high-carbohydrate diet\textsuperscript{25, 267}. These reports show that chlorogenic acid and proanthocyanidin have anti-obesity properties. Various polyphenols have anti-obesity effect and their mechanisms can be explained at least in part by inhibition of lipid absorption. Therefore, one of the plausible mechanisms of these materials, inhibition of lipid absorption is a reasonable because the major polyphenols in BLEX have been reported to inhibit lipid absorption. Actually, chlorogenic acid and proanthocyanidin inhibited pancreas lipase activity in previous study and it is suggested that they prevent dietary fat absorption\textsuperscript{27, 28}. Total polyphenol, chlorogenic acid, and proanthocyanidin intakes in the present study were calculated from food intake. Mice in the BLEX group consumed approximately 28.0, 6.7, and 15.8 mg/day respectively, and mice in the CP BLEX group consumed approximately 54.5, 16.4, and 23.5 mg/day respectively. These intakes were higher than intakes reported in previous studies. Thus, anti-obesity effects observed in the present study may be attributable to chlorogenic acid and proanthocyanidin and be explained in part by inhibition of dietary fat absorption. More pronounced outcomes seen in mice from the CP BLEX group compared with mice in the BLEX group may be due to differences in concentrations of these polyphenols.

Mice from BLEX and CP BLEX groups demonstrated reduced liver lipid accumulation and improved (lower) serum total cholesterol levels. Notable suppression of liver lipid accumulation is consistent with BLEX suppression of liver lipid accumulation in mice fed a high-fat, high-sucrose diet\textsuperscript{122}. This effect was more pronounced in mice consuming CP BLEX instead of BLEX. No significant difference between mice in BLEX and HFHS groups was observed for total serum cholesterol, but serum levels did tend to be lower in mice fed BLEX. This tendency is consistent with the same tendency previously reported\textsuperscript{112}. Mice fed CP BLEX did have total serum cholesterol levels that were significantly lower than mice from the HFHS group. Constituents of BLEX and CP BLEX include polyphenols, particu-
larly chlorogenic acid and proanthocyanidin. Addition of chlorogenic acid (10 mg/kg body weight/day) to a high-cholesterol diet reduced liver lipid accumulation and improved plasma total cholesterol level compared to mice fed with diet without chlorogenic acid. Moreover, supplementation of diet with grape seed proanthocyanidin extract (400 mg/kg boy weight/day) reduced liver lipid accumulation in rats with carbon tetrachloride-induced fatty liver, and incursion of a fraction of blueberry leaf containing 0.4% proanthocyanidin in diet of OLETF rats lowered serum cholesterol level. Intake rates of polyphenols in previous studies were lower than intakes calculated in this study. The present results may also be attributable to chlorogenic acid and proanthocyanidin. Differences in intake rates of these two polyphenols may underlie the more pronounced effects observed in mice from the CP BLEX group.

5 Conclusion
The present study showed dietary BLEX and CP BLEX were decreased body weight and adipose tissue weight gain, and reduced liver lipid accumulation in mice fed a high-fat, high-sucrose diet. These effects tended to be more pronounced in mice fed CP BLEX compared to in mice fed BLEX. CP BLEX has approximately twice the polyphenolic content of BLEX. Thus, anti-obesity effects observed in the present study may be attributable to polyphenols in blueberry leaf.

References
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