Effect of Vaccinium ashei reade Leaf Extracts on Lipid Metabolism in Obese OLETF Rats

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The effects of a hot water extract and fractional extracts from rabbiteye blueberry (Vaccinium ashei reade) leaves (BBL) on lipid metabolism were studied in obese Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Feeding the hot water extract and fractional extracts from BBL alleviated hepatic triglyceride accumulation in the rats. Additionally, feeding with the flavonol glycoside (FG) and proanthocyanidin (PA) fractions lowered serum cholesterol levels in the obese rats. The results from measurements of the hepatic enzyme activity indicate that the hypolipidemic effects of the hot water extract and the PA fraction might be attributable to enhanced lipolysis in the liver. The reduced serum levels of C-reactive protein, an inflammatory cytokine, by the chlorogenic acid fraction and FG fraction might be associated with alleviating the metabolic abnormalities in obese rats. These results indicate that the BBL extracts, and especially FG and PA, exerted hypolipidemic effects on obese OLETF rats and suggest that an infusion of BBL can be useful as a dietary hypolipidemic component.

Key words: blueberry leaf; flavonol glycoside; proanthocyanidin; lipid metabolism; Otsuka Long-Evans Tokushima Fatty rat

Such lifestyle-related diseases as hyperlipidemia, arteriosclerosis, diabetes mellitus, and hypertension are widespread and increasingly prevalent in industrialized countries, thus contributing to increased cardiovascular morbidity and mortality. Accompanied by the rapid increase in the number of elderly people, this problem has become important both medically and socioeconomically. A clustering of metabolic disorders in an individual, defined as the metabolic syndrome, is known to increase cardiovascular risks. Although the pathogenesis of metabolic syndrome is complicated and the precise details of its underlying mechanisms are not known, lipid abnormality has been proposed as a feature of metabolic syndrome together with insulin resistance. Otsuka Long-Evans Tokushima Fatty (OLETF) rats develop a syndrome with multiple metabolic and hormonal disorders that shares many features with human obesity. OLETF rats have hyperphagia because they lack receptors for cholecystokinin, and become obese, developing hyperlipidemia, fatty liver, and type-2 diabetes.

Many studies have suggested that such natural compounds as phytochemicals in fruits and vegetables can be important modulators in terms of the risks associated with metabolic syndrome. Vaccinium ashei reade (blueberry) belongs to the Ericaceae plant group, and infusions of its leaf are used as a folk medicine treatment for lifestyle-related diseases in Europe. Martineau et al. have demonstrated the anti-diabetic properties of the Canadian lowbush blueberry in vitro. We have recently reported that the blueberry leaf (BBL) exerted strong inhibitory effects on the angiotensin-converting enzyme activity in vitro, and that feeding with BBL suppressed the development of essential hypertension in spontaneously hypertensive rats in vivo. We have also reported that freeze-dried BBL powder could alleviate the lipid abnormalities in OLETF rats, and that BBL used in that study contained a considerable amount of tannin. Hot water extraction and the fractional extracts from BBL were used in the present study to identify the physiologically active substances responsible for alleviating the lipid abnormalities in obese OLETF rats.

Materials and Methods

Materials. Fresh rabbiteye blueberry leaves were cultivated and collected by Unkai Shuzo Co. (Miyazaki, Japan). The leaves were freeze-dried, pulverized, and stored at −20℃ until being used.

General procedures. Column chromatography was performed in columns of Sephadex LH-20 (25–100μm; Pharmacia Fine Chemical Co.) and Diaion HP20SS, MCI-gel CHP 20P (75–150μm; Mitsubishi Chemical Co., Japan). Thin-layer chromatography (TLC) was performed on 0.2-mm pre-coated Kieselgel 60 F254 plates (Merck).

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Abbreviations: BBL, blueberry leaf; BW, body weight; CPT, carnitine palmitoyltransferase; CRP, C-reactive protein; FAS, fatty acid synthase; MCP-1, monocyte chemoattractant protein-1; NEFA, non-esterified fatty acids; OLETF, Otsuka Long-Evans Tokushima Fatty; WAT, white adipose tissue.
eluting with benzene-ethyl formate-formic acid (1:7:1, v/v) or chloroform-methanol-water (14:6:1, v/v). Spots were detected by UV illumination and by spraying with a 2% ethanolic FeCl₃ or 10% sulfuric acid reagent and then heating. Analytical high-performance liquid chromatography (HPLC) was performed in a 250 × 4.6-mm i.d. Cosmosil SC-18-AR II column (Nacalai Tesque), using gradient elution by CH₃CN in 50 mM H₃PO₄ from 10–30% for 30 min and 30–75% for 15 min at a flow rate of 0.8 mL/min, and detected with a Jasco MD-910 photodiode array detector.

**Thiol degradation of fraction 4.** A solution of 0.1% (w/v) of fraction 4 (Fr. 4) in 70% EtOH (0.2 mL) was mixed with 5% mercaptoethanol in 60% EtOH containing 0.1% HCl (0.8 mL), and then heated at 70°C for 7 h. The HPLC analysis revealed 3 major peaks. The retention time and UV absorption of each of these peaks coincided with those of catechin (tR, 19.8 min), epicatechin (tR, 24.2 min), and epicatechin-4-hydroxyethylthioether (tR, 28.8 min).**

**Animals and experimental diets.** All aspects of the experiment were conducted according to the guidelines provided by the ethical committee on experimental animal care at Saga University. Four-week-old male OLETF rats were provided by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). The rats were individually housed in metal cages in a temperature-controlled room (24°C) under a 12-h light/dark cycle. After a 1-week adaptation period on a powder diet (CE-2; Clea Japan, Tokyo, Japan), they were assigned to 5 groups of 5 rats each, each group being fed with one of 5 diets: (i) a semi-synthetic diet containing (by weight) casein, 20; corn oil, 7; cornstarch, 15; vitamin mixture (AIN-76); 1; mineral mixture (AIN-76); 3.5; and 5% sucrose, 45 (control group); (ii) a semi-synthetic diet supplemented with the 2% hot water extract from BBL at the expense of sucrose (water extract group, WE); (iii) a semi-synthetic diet supplemented with 0.3% of the chlorogenic acid + rutin fraction (Fr. 2) at the expense of sucrose (chlorogenic acid + rutin fraction group, CR); (iv) a semi-synthetic diet supplemented with 0.15% of the flavonol glycoside fraction (Fr. 3) at the expense of sucrose (flavonol glycoside fraction group, FG); and (v) a semi-synthetic diet supplemented with 0.4% of the proanthocyanidin fraction (Fr. 4) at the expense of sucrose (proanthocyanidin fraction group, PA). The respective amounts of the polyphenols in BBL were first separated (Fig. 1). Freeze-dried leaf powder (300 g) was extracted for 10 min with boiling water (15 L) and then cooled in an ice bath. After removing the plant debris by centrifugation (3,000 rpm for 10 min), the supernatant (11.9 L) was concentrated under reduced pressure (at below 40°C) to give an extract as an aqueous suspension (1.3 L) containing an insoluble precipitate. This extract was directly subjected to Sephadex LH-20 column chromatography (10 cm i.d. × 33 cm), eluting with water containing increasing proportions of MeOH (3 L of water and then 20% (1 L), 30% (1 L), 50% (1 L), 70% (1 L), 80% (1 L) and 100% (1 L) MeOH), and finally eluted with 60% acetone (3.0 L). The eluate was collected in test tubes (150 mL in each), and the constituents were examined by TLC and HPLC. Fr. 1

### Table 1. Composition of the Experimental Diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>WE</th>
<th>CR</th>
<th>FG</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>β-Cornstarch</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
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<tr>
<td>Cellulose</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mixture (AIN-76)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mixture (AIN-76)</td>
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<td>1.0</td>
<td>1.0</td>
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<td>1.0</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Corn oil</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Hot water extract</td>
<td>—</td>
<td>2.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chlorogenic acid + rutin fraction</td>
<td>—</td>
<td>—</td>
<td>0.3</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Flavonol glycoside fraction</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.15</td>
<td>—</td>
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<tr>
<td>Proanthocyanidin fraction</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>48.0</td>
<td>46.0</td>
<td>47.7</td>
<td>47.85</td>
<td>47.6</td>
</tr>
</tbody>
</table>

WE, water extract group; CR, chlorogenic acid + rutin fraction group; FG, flavonol glycoside fraction group; PA, proanthocyanidin fraction group.

**Measurement of the lipid levels and enzyme activities in the liver.** Liver lipids were extracted and purified according to the method of Folch et al.[13] The concentrations of triglyceride and cholesterol were measured according to the methods of Fletcher[40] and Sperry and Webb.[55] The activities of fatty acid synthase (FAS)[66] and the malic enzyme[75] in the cytosol fraction, and of carniitine palmitoyltransferease (CPT)[18] in the mitochondrial fraction were determined as described. The protein concentration of each fraction was determined according to the method of Lowry et al.[79] with bovine serum albumin used as the standard.

**Statistical analysis.** All values are expressed as the mean ± SE. Data were analyzed by 1-way ANOVA, and all differences were analyzed by the Tukey-Kramer post-hoc test (KaleidaGraph; Synergy Software, Reading, PA, USA). Differences were considered significant at p < 0.05.

**Results and Discussion.**

The polyphenols in BBL were first separated (Fig. 1). Freeze-dried leaf powder (300 g) was extracted for 10 min with boiling water (15 L) and then cooled in an ice bath. After removing the plant debris by centrifugation (3,000 rpm for 10 min), the supernatant (11.9 L) was concentrated under reduced pressure (at below 40°C) to give an extract as an aqueous suspension (1.3 L) containing an insoluble precipitate. This extract was directly subjected to Sephadex LH-20 column chromatography (10 cm i.d. × 33 cm), eluting with water containing increasing proportions of MeOH (3 L of water and then 20% (1 L), 30% (1 L), 50% (1 L), 70% (1 L), 80% (1 L) and 100% (1 L) MeOH), and finally eluted with 60% acetone (3.0 L). The eluate was collected in test tubes (150 mL in each), and the constituents were examined by TLC and HPLC. Fr. 1
contained sugars and glycosides and was further separated by Diaion HP20SS column chromatography (5.0 cm i.d. × 33 cm), eluting with water and increasing proportions of MeOH (0–100% MeOH at 20% stepwise, each 300 mL) to give Fr. 1-1 (59.9 g, mainly containing sugars) and Fr. 1-2 (6.21 g, containing glycosides and non-polar constituents). An HPLC analysis of Fr. 2 (17.9 g) from the initial Sephadex LH-20 column chromatography revealed chlorogenic acid and rutin to be the major constituents of this fraction. TLC and HPLC analyses indicated that Fr. 4 (8.23 g) contained chlorogenic acid and rutin fraction group; BW, body weight; WAT, white adipose tissue; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein. Data are expressed as mean ± SE. Different superscript letters show significant differences at p < 0.05. had been responsible for the activities. Infusions of BBL have been used as a folk medicine treatment for lifestyle-related diseases, and recent reports have revealed that the polyphenolic content and anthocyanidin from BBL had several physiological functions. Taken together, these findings led us to explore the effects of hot water extraction and the polyphenolic extracts from BBL on the lipid metabolism in OLETF rats.

After the 4-week feeding period, the intake amount of food, final body weight, liver weight and total WAT weight were not significantly altered among the groups of food, final body weight, liver weight and total WAT weight were not significantly altered among the groups of OLETF rats (Table 2). The effects on serum contained sugars and glycosides and was further separated by Diaion HP20SS column chromatography (5.0 cm i.d. × 33 cm), eluting with water and increasing proportions of MeOH (0–100% MeOH at 20% stepwise, each 300 mL) to give Fr. 1-1 (59.9 g, mainly containing sugars) and Fr. 1-2 (6.21 g, containing glycosides and non-polar constituents). An HPLC analysis of Fr. 2 (17.9 g) from the initial Sephadex LH-20 column chromatography revealed chlorogenic acid and rutin to be the major constituents of this fraction. TLC and HPLC analyses indicated that Fr. 4 (8.23 g) contained chlorogenic acid and rutin fraction group; BW, body weight; WAT, white adipose tissue; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein. Data are expressed as mean ± SE. Different superscript letters show significant differences at p < 0.05. had been responsible for the activities. Infusions of BBL have been used as a folk medicine treatment for lifestyle-related diseases, and recent reports have revealed that the polyphenolic content and anthocyanidin from BBL had several physiological functions. Taken together, these findings led us to explore the effects of hot water extraction and the polyphenolic extracts from BBL on the lipid metabolism in OLETF rats.

After the 4-week feeding period, the intake amount of food, final body weight, liver weight and total WAT weight were not significantly altered among the groups of OLETF rats (Table 2). The effects on serum

Table 2. Effect of BBL Extracts on Growth and Serum Parameter Levels in OLETF Rats

<table>
<thead>
<tr>
<th>Growth parameters</th>
<th>Control</th>
<th>WE</th>
<th>CR</th>
<th>FG</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (g)</td>
<td>112 ± 3</td>
<td>112 ± 3</td>
<td>112 ± 3</td>
<td>112 ± 3</td>
<td>112 ± 2</td>
</tr>
<tr>
<td>Final BW (g)</td>
<td>310 ± 7</td>
<td>314 ± 5</td>
<td>314 ± 3</td>
<td>313 ± 2</td>
<td>310 ± 5</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>571 ± 16</td>
<td>574 ± 11</td>
<td>574 ± 4</td>
<td>574 ± 5</td>
<td>574 ± 4</td>
</tr>
<tr>
<td>Liver weight (g/100 g BW)</td>
<td>3.67 ± 0.14</td>
<td>3.61 ± 0.10</td>
<td>3.83 ± 0.07</td>
<td>3.79 ± 0.12</td>
<td>3.75 ± 0.16</td>
</tr>
<tr>
<td>WAT weight (g/100 g BW)</td>
<td>10.1 ± 0.9</td>
<td>10.2 ± 0.3</td>
<td>10.2 ± 0.2</td>
<td>9.84 ± 0.37</td>
<td>10.2 ± 0.5</td>
</tr>
</tbody>
</table>

Serum parameters

| Triglyceride (mg/dL) | 114 ± 23 | 90.4 ± 8.8 | 85.4 ± 12.5 | 71.0 ± 17.7 | 96.5 ± 11.3 |
| Cholesterol (mg/dL) | 154 ± 7a | 148 ± 4ab | 138 ± 4ab | 133 ± 6ab | 120 ± 6b |
| Glucose (mg/dL) | 184 ± 15 | 187 ± 7 | 208 ± 6 | 199 ± 5 | 189 ± 9 |
| Leptin (ng/mL) | 2.51 ± 0.47 | 2.42 ± 0.15 | 2.47 ± 0.19 | 2.25 ± 0.26 | 2.41 ± 0.17 |
| Insulin (ng/mL) | 5.77 ± 2.34 | 4.03 ± 0.48 | 4.44 ± 0.20 | 4.85 ± 0.36 | 3.93 ± 0.55 |
| MCP-1 (pg/mL) | 379 ± 10 | 357 ± 24 | 382 ± 15 | 368 ± 12 | 297 ± 44 |
| CRP (µg/mL) | 425 ± 16a | 421 ± 7ab | 317 ± 26b | 339 ± 10c | 390 ± 13a |

WE, water extract group; CR, chlorogenic acid + rutin fraction group; FG, flavonol glycoside fraction group; PA, proanthocyanidin fraction group; BW, body weight; WAT, white adipose tissue; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein. Data are expressed as mean ± SE. Different superscript letters show significant differences at p < 0.05.

Many reports have suggested that blueberry fruit had physiologically beneficial activities, including anti-cancer and anti-vascular disease effects, and such polyphenols as flavonols and proanthocyanidins are believed to

![Fig. 2. Effects of Blueberry Leaf Extracts on the Hepatic Lipid Levels in OLETF Rats.](image)

The rats were fed for 4 weeks with a control (Con) diet, or with diets containing a 2% hot water extract (WE), 0.3% chlorogenic acid + rutin fraction (CR), 0.15% flavonol glycoside fraction (FG), or 0.4% proanthocyanidin fraction (PA). Each value is expressed as the mean ± SE (n = 5). Different letters show significant differences at p < 0.05.

![Fig. 3. Effects of Blueberry Leaf Extracts on the Activity of an Enzyme Related to Lipid Metabolism in the Liver of OLETF Rats.](image)

The rats were fed for 4 weeks with a control (Con) diet, or with diets containing a 2% hot water extract (WE), 0.3% chlorogenic acid + rutin fraction (CR), 0.15% flavonol glycoside fraction (FG), or 0.4% proanthocyanidin fraction (PA). Each value is expressed as the mean ± SE (n = 5). Different letters show significant differences at p < 0.05.
parameters of feeding with the BBL extracts are shown in Table 2. The 9-week-old OLETF rats exhibited mild hyperlipidemia after the 4-week feeding period. The serum triglyceride, glucose, insulin, leptin, and MCP-1 levels were not significantly altered among the groups. Our previous report has indicated that feeding with freeze-dried BBL powder reduced the serum CRP and cholesterol levels in OLETF rats.11) The serum cholesterol levels in the present study were significantly less in both the FG and PA groups than in the control group. This result suggests that the hypocholesterolemic effect of freeze-dried BBL powder was attributable to the physiological functions of the polyphenols in both the flavonol glycoside and proanthocyanidin fractions. However, since rats differ from humans in the plasma distribution of cholesterol, evaluating the cholesterol-lowering effects of the BBL extracts in other animal models such as guinea pigs and hamsters, in which the majority of the cholesterol is carried in low-density lipoprotein,26) would be required. Moreover, the serum CRP levels were significantly less in both the CR and FG groups than in the control group. CRP is an inflammatory cytokine, and its serum concentration reflects the inflammatory condition of animals and humans. Given that obesity enhances CRP synthesis and that elevated CRP levels have been suggested as risk factors of cardiovascular diseases and type 2 diabetes in humans and animals,27,28) we presumed that the polyphenols in both the chlorogenic acid + rutin and flavonol glycoside fractions contributed to suppressing the development of obesity-induced metabolic abnormalities in the OLETF rats. At present, however, there is no report indicating that any polyphenols directly suppressed CRP expression. The physiological function of vaccinin A, a new compound found in the flavonol glycoside fraction,29) has not yet been clarified; an evaluation of its anti-inflammatory activity is of great interest for future study.

The liver is the vital organ concerned with lipid metabolism, so we next investigated the effects of feeding the BBL extracts on the distribution of lipids to the liver (Fig. 2). Although the hepatic cholesterol levels were not altered among the groups, the accumulation of hepatic triglyceride was markedly alleviated in the WE, CR, FG, and PF groups when compared with the control group. These results suggest that hot water extraction and its fractional extracts could prevent the development of obesity-induced fatty liver in OLETF rats.

We further investigated the regulation of hepatic lipid metabolism by analyzing the effect of feeding the BBL extracts on the activities of enzymes related to fatty acid synthesis and fatty acid beta-oxidation. Figure 3 shows that the activity of the malic enzyme, which provides NADPH that is required for fatty acid synthesis, was significantly lower in the PF group than in the control group. However, the activity of FAS, a key enzyme in fatty acid synthesis, was not altered among the groups. Previous reports have indicated that polyphenols from plants had FAS inhibitory activity in vitro30) and that proanthocyanidins from persimmon peel down-regulated the expression of lipogenic genes by reducing the transcription factor steroid response element binding protein 1.30) These results suggest that the polyphenols from BBL were different from those found in other plants. We noted that both the hot water extract and proanthocyanidin fraction significantly enhanced the activity of CPT, a key enzyme in mitochondrial fatty acid beta-oxidation, when compared with the control group (Fig. 3). This result suggests that the hypolipidemic effect of the hot water extract and the proanthocyanidin fraction can be attributed to enhanced lipolysis in the liver. The stimulative effects of both extracts on hepatic fatty acid oxidation should be confirmed by a more detailed examination of fatty acid metabolism, such as by a liver perfusion experiment,31) in a future study. Murase et al. have reported that a green tea extract, which contained high levels of polyphenolic compounds, increased fatty acid beta-oxidation in the liver and muscles of mice.32,33) Li et al. have also shown that a green tea leaf extract enhanced the hepatic expression of lipolytic transcriptional factor PPAR-alpha and improved lipid homeostasis in a fructose-fed insulin-resistant hamster model.34) Moreover, a grape seed procyanidin extract upregulated CPT under the regulation of the nuclear receptor small heterodimer partner in mouse liver.35) It is therefore likely that the proanthocyanidins from the BBL hot water extract were also actively involved in lipolysis. A previous study has demonstrated that BBL extracts contained a large amount of oligomeric and polymeric anthocyanidins. The MALDI-TOF MS data indicated that proanthocyanidins were at least present as dodecamers in the BBL extracts and that the A-type linkage and cinchonain units characterized the BBL proanthocyanidins.20) The extent to which BBL anthocyanidins contributed to the activation of fatty acid beta-oxidation is of great interest for a future study.

In conclusion, our results indicate that the BBL extracts, especially FG and PA, had a hypolipidemic effect on obese OLETF rats, and suggest that an infusion of BBL could be useful as a dietary hypolipidemic component. Further purification and identification of the bioactive polyphenols from each fractional extract will be necessary to clarify the physiological functions of a BBL infusion.

Acknowledgments

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References


