A Mixture of Ethanol Extracts of Persimmon Leaf and *Citrus junos* Sieb Improves Blood Coagulation Parameters and Ameliorates Lipid Metabolism Disturbances Caused by Diet-Induced Obesity in C57BL/6J Mice

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This study investigated the effects of a flavonoid-rich ethanol extract of persimmon leaf (PL), an ethanol extract of *Citrus junos* Sieb (CJS), and a PL–CJS mixture (MPC) on mice fed a high-fat diet (HFD). We sought to elucidate the mechanisms of biological activity of these substances using measurements of blood coagulation indices and lipid metabolism parameters. C57BL/6J mice were fed a HFD with PL (0.5% (w/w)), CJS (0.1% (w/w)), or MPC (PL 0.5%, CJS 0.1% (w/w)) for 10 weeks. In comparison with data obtained for mice in the untreated HFD group, consumption of MPC remarkably prolonged the activated partial thromboplastin time (aPTT) and prothrombin time (PT), whereas exposure to PL prolonged aPTT only. Lower levels of plasma total cholesterol, hepatic cholesterol, and erythrocyte thiobarbituric acid-reactive substances, hepatic HMG-CoA reductase, and decreased SREBP-1c gene expression were observed in mice that received PL and MPC supplements compared with the respective values detected in the untreated HFD animals. Our results indicate that PL and MPC may have beneficial effects on blood circulation and lipid metabolism in obese mice.

Keywords: Obesity, persimmon leaf, *Citrus junos* Sieb, blood circulation, lipid metabolism

Introduction

Hemostasis maintains the integrity of the blood vessel system by regulating blood coagulation and platelet activation [6, 9, 29]. During hemostasis, three stages of the response to a blood vessel damage can be recognized. The first stage is the vascular spasm when blood vessels constrict to reduce blood loss. During the second stage, platelets adhere together to form a temporary seal, a platelet plug, to cover the break in the vessel wall. The third and final stage is coagulation or blood clotting [32, 50], which involves formation of a thrombus that prevents any further loss of blood from damaged tissues, blood vessels, or organs [32].

In general, regulation of blood coagulation occurs via extrinsic and intrinsic pathways and involves the fibrinolytic system [9]. Activation of the extrinsic pathway takes place upon extravascular plasma factor VIIa binding to the tissue factor [9]. Activation of the intrinsic pathway involves plasma coagulation factors XIIa, XIa, IXa, and VIIIa [6]. Finally, the two pathways jointly induce formation of factor Xa that catalyzes cleavage of prothrombin yielding active thrombin (FIIa), which in turn facilitates transformation of soluble fibrinogen to insoluble fibrin [6, 9].
When an endothelial cell is damaged, activated platelets are aggregated at the site of the vascular injury [34]. Initially, the inner vascular wall promotes the release of α-granules containing protein mediators such as von Willebrand factor and glycoprotein Ib/V/IX in response to collagen [8]. Then, various agents, including adenosine diphosphate (ADP), thromboxane A2 (TXA2), and serotonin are released from storage granules in the platelets [8, 34]. In addition, soluble P-selectin (sP-selectin), an adhesion molecule that is upregulated upon platelet activation, is also secreted to regulate platelet-to-platelet cohesion [2, 37].

However, obesity and excessive adipose tissue can result in multiple thrombi due to elevated concentrations of the plasma coagulation factor and upregulated platelet adhesion and activation [35, 45]. Furthermore, obesity can induce reactive oxygen species (ROS) generation as a consequence of oxidative stress and effects of inflammatory mediators, such as macrophage chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1), and thereby recruit monocytes/macrophages to move toward the damaged endothelial cells [10, 45]. In particular, LDL, oxidized and accumulated by migrated macrophages in the intima of the blood vessel, turns these cells into foam cells, which in turn contribute to the arteriosclerosis process, exacerbate cytotoxicity, and generally manifest pro-inflammatory and pro-coagulant properties [14, 16]. These processes, which take place in narrow arterial walls, disrupt blood flow and cause blood circulation disorders [11, 14, 16].

Persimmon (Diospyros kaki) leaves and Citrus junos Sieb are widely cultivated in East Asia and used in herbal teas and traditional medicine in South Korea, Japan, and China. The persimmon leaf is rich in polyphenols, tannins, phenols, organic acids, chlorophyll, vitamin C, and caffeine. It is commonly used for brewing tea in Asia and is colloquially praised for its alleged beneficial effects on constipation, hypertension, apoplexy, and atherosclerosis [28, 47]. In particular, tannins with a gallate group have a number of beneficial physiological properties, such as free radical scavenging, antibacterial, and anti-allergic properties. They are also known to lower blood pressure, decrease serum and hepatic cholesterol concentrations, and augment fecal sterol excretion in rats with hypercholesterolemia [25, 41].

C. junos Sieb contains various flavonoids, including naringenin and naringin, which have been pharmacologically evaluated as potential anticancer and hypolipidemic agents [27]. In addition, Citrus junos Sieb constituents possibly regulate collagen-induced platelet aggregation and arachidonic acid metabolism in platelets [52]. Naringin has been demonstrated to have antioxidant effects, such as the inhibition of lipid peroxidation in blood cell membranes and free radical scavenging. Recently, several studies have reported that hesperidin, a substance chemically similar to naringin, can lower blood cholesterol levels [18, 42].

Epidemiologic studies suggest that a higher intake of flavonoids from fruit and vegetables is associated with lower incidence of metabolic syndromes, such as obesity, hyperglycemia, dyslipidemia, and hypertension. Numerous in vivo and in vitro studies demonstrated that flavonoids improved endothelial and platelet function and had a beneficial effect on antioxidant biomarkers [36, 43, 47]. We previously reported that the ethanol extract of persimmon leaf (PL) improved anticoagulation systems and lipid profiles in rats fed with a high-fat diet (HFD) [40]. However, to date, the effects of the ethanol extract of Citrus junos Sieb (CJS) alone or as part of a PL–CJS mixture (MPC) on the anticoagulation system and lipid profiles have not been reported. In this study, we investigated the potential mechanisms of action of PL and CJS administered separately and in combination in the form of MPC. We particularly focused on gene expression and the action of enzymes involved in lipid metabolism and oxidative stress in the liver as well as on the lipid content in feces.

Materials and Methods

Preparation of PL

Persimmon (Diospyros kaki Thunb.) leaves were harvested in Cheongdo County in Korea. The leaf constituents were extracted with 50% ethanol and then concentrated under reduced pressure. The PL yield was 19.6% (3.92 kg/20.0 kg) after drying. The concentrations of three major flavonoids (catechin, epicatechin, and gallic acid) in the dried ethanol extract of the leaf were determined by high-performance liquid chromatography (HPLC) using an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA, USA) with an Xbridge C18 column (4.6 × 150 mm, film thickness 5 µm; Waters Corp., Millford, MA, USA) and a two-mobile-phase gradient elution system. Mobile phase A contained water with 2% acetic acid (v/v) and mobile phase B contained acetonitrile. The flow rate was 0.8 ml/min with an injection volume of 10 µl, while ultraviolet detection was performed at 280 nm. According to these measurements, the dried ethanol extract of the leaf contained 1.5 mg/g of gallic acid, 1.1 mg/g of catechin, and 119 mg/g of total polyphenols.

Preparation of CJS

Citrus junos Sieb. ex Tanaka was harvested in Goheung County in Korea and the seeds were removed. The fruit flesh and peel were treated with 50% ethanol and then concentrated under reduced pressure. The yield of the CJS was 40% (8.0 kg/20.0 kg)
after the drying process. The concentrations of two major flavonoids (naringin and hesperidin) present in the dried CJS were determined by HPLC using the Agilent 1100 series system with a Luna C18 column (4.6 × 150 mm, film thickness 5 µm; Phenomenex, Torrance, CA, USA) and a two-mobile-phase gradient elution system. Mobile phase A contained water with 2% acetic acid (v/v) and mobile phase B contained acetonitrile. The flow rate was 1.0 ml/min with an injection volume of 10 µl, while ultraviolet detection was performed at 280 nm. According to our measurements, the dried ethanol extract of the fresh CJS contained 3.1 mg/g of naringin and 6.7 mg/g of hesperidin.

Animals and Diet

Male C57BL/6j mice (4 weeks old) were purchased from Orient Inc. (Seoul, Republic of Korea). The mice were individually housed in polycarbonate cages at 22 ± 2°C with a 12-h light–dark cycle. All mice were fed a pelleted commercial chow diet for 2 weeks after arrival. The mice were then randomly divided into five groups, which received the control or one of several experimental diets for 10 weeks: the normal diet (ND), HFD, HFD containing 0.5% PL, HFD containing 0.1% CJS, and HFD containing a mixture of 0.5% PL and 0.1% CJS (MPC). The ND was prepared in accordance with a purified AIN-76 rodent diet. The HFD was modified from the AIN-76 rodent diet to include 20% (w/w) fat, 1% (w/w) cholesterol, and 0.25% (w/w) cholate. The detailed compositions of the experimental diets fed to each group are shown in Table 1. The diets were administered in the pellet form for 10 weeks. The mice had free access to food and distilled water during the experimental period. The food intake was measured daily and the body weight was measured once a week. Feces were collected during the last 5 days and, upon drying, fecal material was used for analysis of fecal lipids.

On the 10th week, the mice were anesthetized with diethyl ether and sacrificed after 12 h of fasting. Blood was taken from the inferior vena cava and then centrifuged at 1,000 ×g for 15 min at 4°C. The plasma was separated for biomarker analysis. After blood collection, the liver and adipose tissue were promptly removed, rinsed, weighed, frozen in liquid nitrogen, and stored at −70°C. All procedures were approved by the animal ethics committee of the Kyungpook National University (Approval No. KNU-2013-18).

Anticoagulation Assay

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined using a thrombotimer (Behnk Elektronik, Norderstedt, Germany) according to the manufacturer’s instructions. In brief, platelet-rich plasma (PRP, 50 µl) was incubated with PL, CJS, or MPC for 3 min at 37°C, and then the aPTT reagent (50 µl) was added to the mixture, which was then re-incubated under the same conditions. Subsequently, 20 µM CaCl₂ (50 µl) was added and the clotting time was recorded. For the PT assay, PRP (30 µl) was incubated with PL, CJS, or MPC for 3 min at 37°C. The PT reagent (100 µl) was then added and the clotting time was recorded.

Platelet Activation Marker Levels

Concentrations of serotonin, thromboxane B₂ (TXB₂), and sP-selectin, markers of platelet activation, were measured using commercial serotonin, TXB₂, and sP-selectin ELISA kits (MyBioSource, San

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**Table 1.** Composition of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>ND</th>
<th>HFD</th>
<th>PL</th>
<th>CJS</th>
<th>MPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.00</td>
<td>200.00</td>
<td>200.00</td>
<td>200.00</td>
<td>200.00</td>
</tr>
<tr>
<td>D,L-Methionine</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>150</td>
<td>108.5</td>
<td>108.5</td>
<td>108.5</td>
<td>108.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500</td>
<td>370</td>
<td>365</td>
<td>369</td>
<td>364</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50.00</td>
<td>30.00</td>
<td>30.00</td>
<td>30.00</td>
<td>30.00</td>
</tr>
<tr>
<td>Lard</td>
<td>-</td>
<td>170.00</td>
<td>170.00</td>
<td>170.00</td>
<td>170.00</td>
</tr>
<tr>
<td>Mineral mixture (AIN-76)</td>
<td>35.00</td>
<td>42.00</td>
<td>42.00</td>
<td>42.00</td>
<td>42.00</td>
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<td>Vitamin mixture (AIN-76)</td>
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<td>12.00</td>
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<td>12.00</td>
<td>12.00</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Cholate</td>
<td>-</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>tert-Butylhydroquinone¹</td>
<td>0.01</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Persimmon leaf ethanol extract</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td><em>Citrus junos</em> Sieb ethanol extract</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>1,000.0</td>
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<td>1,000.0</td>
<td>1,000.0</td>
<td>1,000.0</td>
</tr>
</tbody>
</table>

**Notes:** ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of *Citrus junos* Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of *Citrus junos* Sieb.

¹Weight (g) of tert-Butylhydroquinone was not included the total weight 1000 g.
Diego, CA, USA) according to the manufacturer’s instructions. Because TXA₂ is unstable and quickly converts to TXB₂, generation of TXA₁ in platelets was measured by determining the TXB₂ concentration using a commercial TXB₂ ELISA kit (MyBioSource).

**Plasma Lipid Measurements**

Plasma concentrations of triglycerides (TG), total-cholesterol (Total-C), and high-density lipoprotein (HDL-C) were determined using a commercial kit (Asan, Seoul, Republic of Korea). Plasma concentrations of free fatty acids (FFA) and phospholipids were measured using a commercial kit from Wako Chemicals (Richmond, VA, USA). Apolipoprotein (Apo) A-I and B levels were determined by immunonephelometry using a Cobas Integra Cassette (Germany) and a Cobas Integra 800 (Germany). The HTR (%) and atherogenic index (AI) were calculated using the following formulae: HTR (%) = (HDL-C/Total-C) × 100 and AI = ([Total-C] - [HDL-C])/[HDL-C].

**Hepatic and Fecal Lipid Measurements**

Hepatic lipids were extracted and then dried lipid residues were dissolved in 1 ml of ethanol for TG, cholesterol, and fatty acids assays. Triton X-100 and a sodium cholate solution in distilled water were added to 200 µl of the dissolved lipid solution for emulsification. Fecal lipids were extracted using the procedure developed by Folch et al. [12]. Cholesterol, TG, and fatty acid levels in the liver and feces were analyzed using the same commercial kit (Asan) as in the plasma analysis.

**Hepatic Morphology**

The liver was dissected and fixed in a buffer solution of 10% formalin. Fixed tissues were processed routinely for paraffin embedding and 4-µm-thick sections were prepared and dyed with hematoxylin and eosin (H&E). The stained areas were viewed using an optical microscope (Zeiss Axioscope, Germany) with a magnifying power of ×200.

**Measurements of Plasma Levels of Glucose, Insulin, and Inflammatory Cytokines**

The plasma glucose concentration was determined using a commercial kit (Asan). For detection of plasma adiponectin levels, a quantitative sandwich enzyme immunoassay kit (ELISA kit; R&D Systems, MN, USA) was used. The same approach was used to measure plasma insulin and adipocytokine levels. Plasma levels of the adipocytokines MCP-1, interferon gamma, leptin, adiponectin, PAI-1, and insulin were determined with a multiplex detection kit from Bio-Rad (Hercules, CA, USA). All samples were assayed in duplicate and analyzed with a Luminex 200 LabMap system (Luminex, Austin, TX, USA). Data analysis was performed by Bio-Plex Manager software ver. 4.1.1 (Bio-Rad).

**Measurement of the Fatty Acid Synthase and β-Oxidation Activity**

Hepatic cytosolic, mitochondrial, and microsomal samples were prepared by a previously reported method of Hulcher and Oleson [17] with a slight modification. Protein concentrations were determined using Bradford’s method [4]. To measure the activity of lipid-regulating enzymes in the liver, samples were prepared and analyzed according to the methods described previously by Kim et al. [24]. Briefly, fatty acid synthase (FAS) activity was determined with a spectrophotometric assay according to a previously described method [39]. Enzyme activity was measured in nmol NADPH oxidized/min/mg protein. Fatty acid β-oxidation was determined by monitoring the reduction of NAD to NADH at 37°C and 340 nm for 5 min, as mentioned previously [26]. Enzyme activity was measured in nmol NADPH oxidized/min/mg protein.

**Hydrogen Peroxide and Lipid Peroxidation Measurements**

Hydrogen peroxide (H₂O₂) levels in the liver were measured using Wolff’s method [51]. The FOX-1 (ferrous oxidation with xylene orange) reagent contained the following constituents: 100 µM xylene orange, 250 µM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H₂SO₄. The test sample (50 µl) was added to 950 µl of the FOX-1 reagent, vortexed, and then incubated at room temperature for a minimum of 30 min, by which time color development was nearly complete. The absorbance was read at 560 nm and the data for a standard were linear in the 0–5 µM concentration range. Concentration of the erythrocyte thiobarbituric acid-reactive substances (TBARS), a marker of lipid peroxide production, was measured spectrophotometrically using the method of Tarladgis et al. [48].

**Paraoxonase Activity Measurements**

The plasma paraoxonase (PON) activity was determined using the method of Mackness et al. [33] with a slight modification. The hydrolysis rate of paraoxon was assessed by measuring the release of p-nitrophenol at 405 nm and 25°C for 90 sec. The basal reaction mixture included 5.5 mM paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma-Aldrich Chemical Co.) and 2 mM CaCl₂ in 0.1 M Tris-HCl buffer (pH 8.0). A molar extinction coefficient of 17,000 M⁻¹ cm⁻¹ was used to determine the activity, which was expressed in nmol p-nitrophenol/min/mg protein.

**RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction Analysis**

Total RNA was isolated from the liver and aorta using the TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. DNase digestion was used to remove any DNA contamination, and RNA was precipitated in ethanol to ensure no phenol contamination. RNA purity and integrity were evaluated by using an Agilent 2100 Bioanalyzer (Agilent Technologies). Equal amounts of RNA from each experimental group were pooled to normalize individual differences. Total RNA (1 µg) was reverse transcribed using a commercial kit (Qiagen, Germany). Expression of mRNA was then quantified by real-time quantitative PCR using a Quantitect SYBR green PCR kit (Qiagen) on a CFX96TM real-time PCR system (Bio-
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Rad, UK). The sequences of the primers were as follows: 3-hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, forward 5'-GAGGCCTTTGATAGCACCAG-3', reverse 5'-AGCAGTGCTTTC TCTCCGTACC-3'; sterol regulatory element binding protein-1c (SREBP-1c), forward 5'-ACAGATGTGTCTATGGAGGG-3', reverse 5'-AAAAGACAAGGGGCTACTCT-3'; GAPDH, forward 5'-ACCACA GTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

### Statistical Analyses

All data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS, ver. 18; SPSS Inc., Chicago, IL, USA). Significance of differences between the means was determined by one-way ANOVA. Duncan’s multiple-range test was performed if a difference was identified between HFD groups at \( p < 0.05 \). The differences between the ND group and HFD groups were compared by the Student’s t-test using SPSS.

### Results

#### Body Weight, Body Weight Gain, Food Intake, and Food Efficiency Ratio

The body weight gain (BWG) of the HFD group was significantly higher than that of the ND group. The final body weights of mice that received PL, CJS, or MPC were however significantly lower than of mice from the HFD group. Supplementation with PL markedly decreased the BWG compared with its level in the HFD group. Although the food intake of the HFD group was significantly lower than that of the ND group, the food efficiency ratio (FER) in the HFD group was significantly higher. Mice that consumed PL or MPC had a significantly lower FER than animals from the HFD group (Table 2).

#### Liver and Adipose Tissue Weights

The relative weights (per body weight) of the liver and all white adipose tissue (WAT) were significantly higher in the HFD group than in the ND group. Consumption of PL-containing diet was associated with significantly lower liver and all WAT weights compared with parameters measured in the HFD group. In addition, supplementation with MPC led to lower weights of the liver, interscapular WAT, epididymal WAT, mesenteric WAT, and retroperitoneal WAT. Furthermore, consumption of either PL or MPC was associated with lower weights of the visceral WAT (including epididymal WAT, perirenal WAT, mesenteric WAT, and retroperitoneal WAT) and total WAT compared with the values observed in the HFD group (Table 3).

#### PT and aPTT Measurements

The relative PT and aPTT were significantly lower in the HFD group than in the ND group. In mice that received MPC, the PT was significantly longer than in the HFD animals. Furthermore, in the PL and MPC groups, aPTT values were also significantly longer than in the HFD mice (Table 4).

#### Concentrations of sP-Selectin, TXB\(_2\), and Serotonin

The plasma level of sP-selectin was significantly higher in the HFD group than in the ND group (Fig. 1). Animals that were exposed to PL had significantly lower levels of sP-selectin. The relative plasma TXB\(_2\), concentration was significantly higher in the HFD group than in the ND group. However, there were no significant differences in TXB\(_2\) levels between any of the HFD-fed groups. Likewise, concentrations of plasma serotonin did not significantly differ between the experimental groups (Fig. 1).

#### Plasma Lipid Profiles

TG, total-C, non-HDL-C, Apo B, and Apo A-I levels were
Table 3. Effects of PL, CJS, and MPC on the weight of the liver and adipose tissue in HFD-fed mice

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>HFD</th>
<th>PL</th>
<th>CJS</th>
<th>MPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g/100 g body weight)</td>
<td>3.49 ± 0.08</td>
<td>5.11 ± 0.25***</td>
<td>3.97 ± 0.08ab</td>
<td>4.71 ± 0.14c</td>
<td>3.86 ± 0.07b</td>
</tr>
<tr>
<td>Interscapular BAT</td>
<td>0.31 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>Interscapular WAT</td>
<td>1.30 ± 0.08</td>
<td>2.29 ± 0.18**</td>
<td>1.57 ± 0.19b</td>
<td>1.82 ± 0.14b</td>
<td>1.50 ± 0.17b</td>
</tr>
<tr>
<td>Epididymal WAT</td>
<td>3.04 ± 0.29</td>
<td>5.04 ± 0.40***</td>
<td>3.23 ± 0.25b</td>
<td>4.24 ± 0.43ab</td>
<td>3.69 ± 0.28b</td>
</tr>
<tr>
<td>Perirenal WAT</td>
<td>3.03 ± 0.02</td>
<td>0.48 ± 0.03***</td>
<td>0.32 ± 0.03b</td>
<td>0.39 ± 0.04ab</td>
<td>0.46 ± 0.03c</td>
</tr>
<tr>
<td>Mesenteric WAT</td>
<td>1.11 ± 0.09</td>
<td>1.47 ± 0.13**</td>
<td>0.98 ± 0.05b</td>
<td>1.27 ± 0.11ab</td>
<td>1.21 ± 0.12b</td>
</tr>
<tr>
<td>Retroperitoneum WAT</td>
<td>0.88 ± 0.10</td>
<td>1.47 ± 0.010***</td>
<td>0.88 ± 0.07c</td>
<td>1.12 ± 0.11bc</td>
<td>1.20 ± 0.10b</td>
</tr>
<tr>
<td>Subcutaneous WAT</td>
<td>1.33 ± 0.12</td>
<td>2.32 ± 0.17***</td>
<td>1.61 ± 0.15b</td>
<td>2.22 ± 0.23b</td>
<td>1.80 ± 0.17b</td>
</tr>
<tr>
<td>Visceral WAT</td>
<td>5.36 ± 0.48</td>
<td>8.45 ± 0.59***</td>
<td>5.39 ± 0.36c</td>
<td>7.02 ± 0.60ab</td>
<td>6.31 ± 0.46c</td>
</tr>
<tr>
<td>Total WAT</td>
<td>7.98 ± 0.63</td>
<td>13.05 ± 0.92***</td>
<td>8.58 ± 0.64bc</td>
<td>11.06 ± 0.91bc</td>
<td>9.22 ± 0.79b</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Means from the HFD-fed groups in the same row not sharing a common superscript letter are significantly different at p < 0.05.

ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of Citrus junos Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of Citrus junos Sieb; BAT, brown adipose tissue; WAT, white adipose tissue.

Table 4. Effects of PL, CJS, and MPC on blood clotting parameters in HFD-fed mice.

<table>
<thead>
<tr>
<th></th>
<th>PT (seconds)</th>
<th>aPTT (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>10.72 ± 0.36</td>
<td>53.77 ± 1.51</td>
</tr>
<tr>
<td>HFD</td>
<td>8.37 ± 0.58bc</td>
<td>40.90 ± 2.90bc</td>
</tr>
<tr>
<td>PL</td>
<td>9.17 ± 0.33bc</td>
<td>51.15 ± 1.52</td>
</tr>
<tr>
<td>CJS</td>
<td>9.18 ± 0.27bc</td>
<td>41.27 ± 0.47bc</td>
</tr>
<tr>
<td>MPC</td>
<td>10.43 ± 0.65</td>
<td>49.75 ± 3.15</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: *p < 0.05.

Means from the HFD-fed groups in the same column not sharing a common superscript letter are significantly different at p < 0.05.

ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of Citrus junos Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of Citrus junos Sieb; PT, prothrombin time; aPTT, activated partial thromboplastin time.

Table 5. Hepatic and Fecal Lipid Profiles

<table>
<thead>
<tr>
<th></th>
<th>Hepatic Cholesterol</th>
<th>Hepatic TG</th>
<th>Fecal Cholesterol</th>
<th>Fecal TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>2.22 ± 0.17</td>
<td>0.98 ± 0.05</td>
<td>0.39 ± 0.04</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>HFD</td>
<td>1.80 ± 0.17</td>
<td>1.27 ± 0.11</td>
<td>1.12 ± 0.11</td>
<td>1.20 ± 0.10</td>
</tr>
<tr>
<td>PL</td>
<td>1.82 ± 0.23</td>
<td>1.61 ± 0.15</td>
<td>2.22 ± 0.23</td>
<td>1.80 ± 0.17</td>
</tr>
<tr>
<td>CJS</td>
<td>1.22 ± 0.03</td>
<td>0.98 ± 0.05</td>
<td>0.39 ± 0.04</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>MPC</td>
<td>1.21 ± 0.12</td>
<td>1.27 ± 0.11</td>
<td>1.12 ± 0.11</td>
<td>1.20 ± 0.10</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Means from the HFD-fed groups in the same row not sharing a common superscript letter are significantly different at p < 0.05.

ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of Citrus junos Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of Citrus junos Sieb; PT, prothrombin time; aPTT, activated partial thromboplastin time.

Significantly higher in the HFD group than in the ND group. At the same time, the Apo A-I/Apo B ratio was significantly lower in the HFD group compared with the ND group. There were no significant differences in the plasma TG, FFA, phospholipid, HDL-C, and Apo B concentrations, as well as in HTR, between any HFD-fed groups. However, plasma total-C and non-HDL-C concentrations were significantly lower in the PL and MPC groups than in the HFD group. The Apo A-I plasma concentration was significantly reduced in the CJS group compared with that in the HFD group. However, there were no significant differences in the Apo B concentrations and Apo A-I/Apo B ratios between any HFD-fed groups. Moreover, mice that consumed MPC had significantly lower Apo A-I compared with the value observed in the HFD group (Table 5).

Hepatic Morphology

Representative micrographs of liver samples stained with H&ﬁ are shown in Fig. 2. There were more lipid
droplets in samples from the HFD group than in those from the ND group and the droplets in the former were larger. In addition, HFD resulted in the accumulation of hepatic lipid droplets in the center of the portal vein of the liver tissue, whereas they were relatively rare in samples from the PL and MPC groups.

**Table 5.** Effects of PL, CJS, and MPC on plasma lipid profiles in HFD-fed mice.

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>HFD</th>
<th>PL</th>
<th>CJS</th>
<th>MPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mmol/l)</td>
<td>0.85 ± 0.07</td>
<td>1.06 ± 0.07*</td>
<td>1.26 ± 0.15</td>
<td>1.03 ± 0.07</td>
<td>1.27 ± 0.14</td>
</tr>
<tr>
<td>Total-C (mmol/l)</td>
<td>2.76 ± 0.14</td>
<td>3.30 ± 0.10**</td>
<td>2.51 ± 0.13b</td>
<td>2.89 ± 0.09ab</td>
<td>2.76 ± 0.14b</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.49 ± 0.25</td>
<td>0.93 ± 0.11</td>
<td>1.03 ± 0.07</td>
<td>1.00 ± 0.12</td>
<td>1.14 ± 0.10</td>
</tr>
<tr>
<td>Non HDL-C (mmol/l)</td>
<td>1.69 ± 0.11</td>
<td>2.44 ± 0.08***</td>
<td>1.66 ± 0.21b</td>
<td>2.54 ± 0.43a</td>
<td>1.65 ± 0.12b</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>0.47 ± 0.12</td>
<td>0.62 ± 0.05</td>
<td>0.66 ± 0.06</td>
<td>0.51 ± 0.03</td>
<td>0.56 ± 0.11</td>
</tr>
<tr>
<td>Phospholipids (mmol/l)</td>
<td>0.56 ± 0.03</td>
<td>0.53 ± 0.07</td>
<td>0.74 ± 0.04</td>
<td>0.68 ± 0.10</td>
<td>0.55 ± 0.11</td>
</tr>
<tr>
<td>Apo A-I (mg/dl)</td>
<td>35.78 ± 2.04</td>
<td>32.47 ± 0.65a</td>
<td>32.02 ± 1.09b</td>
<td>28.57 ± 0.85b</td>
<td>31.10 ± 0.73ab</td>
</tr>
<tr>
<td>Apo B (mg/dl)</td>
<td>8.32 ± 0.31</td>
<td>10.23 ± 0.28**</td>
<td>9.88 ± 0.57</td>
<td>10.31 ± 0.83</td>
<td>9.57 ± 0.31</td>
</tr>
<tr>
<td>Apo A-I/Apo B</td>
<td>4.16 ± 0.23</td>
<td>3.30 ± 0.14*</td>
<td>3.30 ± 0.21</td>
<td>2.86 ± 0.29</td>
<td>3.16 ± 0.18</td>
</tr>
<tr>
<td>HTR (%)</td>
<td>36.41 ± 3.55</td>
<td>30.89 ± 2.92</td>
<td>35.83 ± 2.79</td>
<td>29.56 ± 4.93</td>
<td>33.78 ± 1.95</td>
</tr>
<tr>
<td>AI</td>
<td>1.14 ± 0.12</td>
<td>2.22 ± 0.20**</td>
<td>1.57 ± 0.30ab</td>
<td>1.99 ± 0.18ab</td>
<td>1.34 ± 0.14b</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Means from the HFD-fed groups in the same row not sharing a common superscript letter are significantly different at p < 0.05.

ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of Citrus junos Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of Citrus junos Sieb; non HDL-C = (Total-C) - (HDL-C); HTR = (HDL-C/Totall-C) × 100; AI, atherogenic index = (Total-C) - (HDL-C)/(HDL-C).
Plasma Levels of Glucose, Insulin, Leptin, Ghrelin, and Adiponectin and the Leptin:Adiponectin Ratio

Plasma levels of glucose, insulin, leptin, adiponectin, and ghrelin, as well as the leptin:adiponectin (L:A) ratio, are shown in Table 7. Plasma levels of fasting glucose at the end of the experiment were significantly higher in the HFD group than in the ND group. The plasma level of glucose was indicative of the improved glucose clearance in the PL, CJS, and MPC groups compared with the glucose clearance in the HFD group. The plasma insulin level was significantly lower in the PL, CJS, and MPC groups than in the HFD group. There were no differences in plasma leptin concentrations between the groups. The plasma level of adiponectin was significantly lower in the HFD group than in the ND group. CJS supplementation was associated with a significantly elevated plasma adiponectin level compared with that of the HFD group. The L:A ratio was significantly higher in the HFD group than in the ND group; however, this ratio was significantly lower in the PL group than in the HFD group. The plasma level of ghrelin was significantly lower in the HFD group than in the ND group. Remarkably, PL supplementation significantly elevated the plasma ghrelin.

### Table 6. Effects of PL, CJS, and MPC on hepatic and fecal lipid profiles in HFD-fed mice.

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>HFD</th>
<th>PL</th>
<th>CJS</th>
<th>MPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFA</td>
<td>0.08 ± 0.008</td>
<td>0.09 ± 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.06 ± 0.01</td>
<td>0.29 ± 0.02&lt;sup&gt;****&lt;/sup&gt;</td>
<td>0.19 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG</td>
<td>0.61 ± 0.05</td>
<td>0.73 ± 0.05</td>
<td>0.74 ± 0.03</td>
<td>0.72 ± 0.07</td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td>Fecal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acid</td>
<td>0.36 ± 0.04</td>
<td>0.34 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.36 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.42 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.08 ± 0.01</td>
<td>0.89 ± 0.04&lt;sup&gt;****&lt;/sup&gt;</td>
<td>1.06 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.99 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.97 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG</td>
<td>0.15 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: *<i>p</i> < 0.05, ***<i>p</i> < 0.001.

<sup>a</sup>Means from the HFD-fed groups in the same row not sharing a common superscript letter are significantly different at <i>p</i> < 0.05.

ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of *Citrus junos* Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of *Citrus junos* Sieb; FFA, free fatty acid; TG, triglyceride.

**Fig. 2.** Effects of PL, CJS, and MPC on hepatic morphology in HFD-fed mice (×200).
ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of *Citrus junos* Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of *Citrus junos* Sieb. Representative photomicrographs of the liver are shown at ×200 magnification.
Thereapeutic Effects of Persimmon Leaf and Citrus junos Sieb

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Levels of Inflammatory Cytokines

Fig. 3 shows the levels of PAI-1, interferon gamma, and MCP-1. The level of plasma PAI-1 was significantly higher in the HFD group than in the ND group. Furthermore, supplementation with PL or MPC was associated with a significantly reduced plasma PAI-1 level compared with that of the HFD group. PL supplementation significantly lowered levels of the plasma interferon gamma and MCP-1 compared with their concentrations detected in the HFD group.

Activity of Hepatic Lipogenic Enzymes

Activity levels of hepatic lipogenic enzymes are shown in Table 8. FAS activity was significantly higher in the HFD group than in the ND group, whereas supplementation with PL, CJS, or MPC significantly reduced the FAS activity compared with its level in the HFD group. In addition, FAS activity in mice from either PL or MPC groups was statistically lower than that in the CJS group animals. Hepatic β-oxidation activity was significantly lower in the HFD group than in the ND group. Furthermore, consumption of PL or MPC significantly increased the hepatic β-oxidation activity compared with that in the HFD group.

Levels of Lipid Peroxidation, Hydrogen Peroxide, and PON Activity

The levels of erythrocyte TBARS, a marker of lipid peroxide concentration, and H$_2$O$_2$, a marker of lipid hydrogen concentrations, are shown in Fig. 4. Erythrocyte
TBARS levels were significantly lower in all groups of animals that received food supplements compared with the levels detected in the HFD group. Erythrocyte H$_2$O$_2$ concentration was significantly higher in the HFD group than in the ND group. Hepatic H$_2$O$_2$ concentration was significantly lower in the PL, CJS, and MPC groups compared with its level in the HFD group.

PON plays an important role in anti-atherogenic processes. Plasma PON activity was significantly lower in the HFD group compared with the ND group. Consumption of supplements (PL, CJS, or MPC) was associated with significantly lower SREBP-1c mRNA expression compared with that in the HFD group.

**Discussion**

The present study demonstrated that PL and MPC may beneficially affect blood coagulation parameters and lipid metabolism disturbances in C57BL/6J mice fed with a HFD. We observed that when mice consumed a HFD that included 20% fat, 1% cholesterol, and 0.25% cholate for 10 weeks, they developed obesity signs; namely, a significant increase in the body weight and body fat mass. The presence of excessive adipose tissue in overweight or obese humans is associated with a higher risk of cardiovascular disease (CVD) development, whereas weight reduction decreases CVD risk [11, 45]. Consumption of PL, CJS, or MPC was associated with significantly lower final body weights. The weights of the liver, visceral WAT, and total

<table>
<thead>
<tr>
<th>Table 8. Effects of PL, CJS, and MPC on hepatic lipid-regulating enzyme activity in HFD-fed mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FAS</strong> (nmol/min/mg protein)</td>
</tr>
<tr>
<td><strong>β-Oxidation</strong> (mmol/min/mg protein)</td>
</tr>
<tr>
<td>ND</td>
</tr>
<tr>
<td>HFD</td>
</tr>
<tr>
<td>PL</td>
</tr>
<tr>
<td>CJS</td>
</tr>
<tr>
<td>MPC</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: $^p < 0.05$.

$^a$Means from the HFD-fed groups in the same column not sharing a common superscript letter are significantly different at $p < 0.05$.

ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of *Citrus junos* Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of *Citrus junos* Sieb; FAS, fatty acid synthase.

RT-qPCR Analysis of mRNA Expression of Genes Regulating Hepatic Lipid Content

Fig. 5 illustrates levels of hepatic expression of the HMG-CoA reductase and SREBP-1c mRNA. HMG-CoA reductase is involved in cholesterol synthesis and esterification. HMG-CoA reductase mRNA expression was significantly higher in the HFD group than in the ND group. However, supplementation with PL, CJS, or MPC significantly lowered the HMG-CoA reductase mRNA expression compared with that in the HFD group. Furthermore, the level of SREBP-1c mRNA was significantly higher in the HFD group than in the ND group. Consumption of supplements (PL, CJS, or MPC) was associated with significantly lower SREBP-1c mRNA expression compared with that in the HFD group.

**Fig. 4.** Effects of PL, CJS, and MPC on erythrocyte TBARS, hepatic H$_2$O$_2$, and plasma PON in HFD-fed mice.

Data are expressed as the mean ± standard error. Significant differences between the HFD and ND groups are indicated as follows: $^p < 0.05$.

$^a$Means from the HFD-fed groups not sharing a common superscript letter are significantly different at $p < 0.05$. ND, normal diet; HFD, high-fat diet (20% fat, 1% cholesterol, 0.25% cholate); PL, HFD + 0.5% ethanol extract of persimmon leaf; CJS, HFD + 0.1% ethanol extract of *Citrus junos* Sieb; MPC, HFD + mixture of 0.5% ethanol extract of persimmon leaf and 0.1% ethanol extract of *Citrus junos* Sieb; TBARS, thiobarbituric acid reactive substance; H$_2$O$_2$, hydrogen peroxide; PON, paraoxonase.
WAT in mice from the PL and MPC groups, but not from the CJS group, were lower than corresponding parameters measured in animals that received only HFD. These results suggest that including PL, CJS, and MPC into everyday diet may help to treat obesity.

Blood coagulation and platelets are crucial for the formation of thrombi [32]. A HFD can promote hyperactive hemostatic activity and dyslipidemia, which can result in CVDs [10, 35]. The propensity to coagulation can be measured by monitoring the aPTT and PT [40]. Our results revealed that consumption of MPC was associated with a significant prolongation of the PT and aPTT, whereas PL supplementation only led to a longer aPTT, compared with the parameters measured in the HFD group. Similar to our results, a previous study reported that PL decreased the vascular thrombotic risk, suppressing hyperactivation of the coagulation factor and platelet activation [40]. TXA\textsubscript{2}, sP-selectin, and serotonin play key roles in platelet binding to adhesive ligands, as well as in the formation and release of activating mediators that initiate the aggregation process [1]. Mice that received PL had significantly lower sP-selectin levels compared with those in the HFD group. At the same time, concentrations of serotonin and TXB\textsubscript{2} were not significantly affected by food supplements.

Plasma lipid profiles are well-known markers of the cardiovascular risk [5]. In the present study, plasma lipid profiles modified by the HFD indicated a high cardiovascular risk with a high AI. In a previous study [20], PL attenuated plasma lipid profiles in type 2 diabetic db/db mice. Our present results demonstrated that consumption of PL and MPC led to a significant downregulation of the plasma total-C, hepatic cholesterol, and plasma non HDL-C levels compared with those seen in HFD mice that did not receive any supplements. In addition, animals that consumed MPC had a significantly lower level of hepatic FFA and a lower AI compared with the respective values in the HFD group. Furthermore, histological analysis of the liver revealed lower numbers and smaller sizes of hepatic lipid droplets in mice that were fed the PL and MPC supplements, which supports our data on corresponding plasma and hepatic lipid profiles. Interestingly, higher excretion of fecal fatty acids and cholesterol in the groups that received the PL and MPC supplements indicates a potentially lower absorption of intestinal fatty acids and cholesterol. In contrast, plasma, hepatic, and fecal levels of TG were not altered by these supplements. Hepatic FAS is a key enzyme in the fatty acid and TG synthesis pathway [23]. Supplementation with PL, CJS, or MPC was associated with a significantly lower level of hepatic FAS activity, particularly in the PL and MPC groups, compared with activity levels in the HFD group. The improvement of hepatic steatosis in the PL and MPC groups was probably directly associated with lower hepatic FAS activity, as this key enzyme is involved in de novo fatty acids biosynthesis. Lower hepatic HMG-CoA reductase mRNA expression is associated with a lower risk of atherosclerotic lesion formation [3]. According to our findings, consumption of PL, CJS, or MPC was associated with dramatically lower expression levels of the hepatic HMG-CoA reductase mRNA. The transcription factor SREBP-1c is a critical intermediate in the pro- or anti-lipogenic action of several hormones and nutrients [22]. In particular, SREBP-1c stimulates synthesis of FAS, a critical...
lipogenic enzyme [22]. We observed that consumption of PL, CJS, or MPC led to a considerable downregulation of the hepatic SREBP-1c mRNA expression. Taken together, the action of PL and MPC on activity of lipid-regulating enzymes is consistent with lower levels of plasma total-C and hepatic cholesterol seen upon consumption of these supplements.

Effects of leptin, the first adipokine discovered, are mediated by its direct action on the central nervous system. It suppresses food intake and elevates energy expenditure by regulating glucose and fat metabolism. In the obese state, overproduction of leptin increases the levels of cytokines, such as TNF-α and interleukin (IL)-6, as well as enhances human platelet activation by potentiating the normal platelet response to ADP and thrombin [15]. In contrast, adiponectin exerts its beneficial effect on vascular endothelial cells, macrophages, and vascular smooth cells, thereby suppressing atherosclerosis [30]. When the vascular endothelial layer is damaged, adiponectin attaches to endothelial collagens V, VIII, and X [31]. The present study indicates that the plasma adiponectin level was significantly higher in the CJS group than in the HFD group. Nevertheless, the L:A ratio was markedly lower in mice that consumed PL. Ghrelin, a gastric peptide with cardiovascular action, has been shown to inhibit proatherogenesis in the endothelium [49]. We showed here that PL supplementation was associated with higher plasma ghrelin levels.

Excess adipose tissues secrete inflammatory cytokines, such as MCP-1, TNF-α, interferon gamma, IL-1, IL-6, and IL-8. In addition, vascular remodeling has been reported to promote insulin resistance and atherosclerotic vascular walls [10]. Inflammatory agents increase synthesis of the tissue factor [35]. Many studies have demonstrated that flavonoids, abundant ingredients of vegetables and fruits, prevent CVDs owing to their anti-inflammatory activity. In particular, compounds such as catechin and naringin can reduce plasma cytokine concentrations [18, 21, 36]. Elevated PAI-1 levels are associated with coagulation and fibrinolysis in obesity [19]. Plasma PAI-1 levels were significantly lower in the PL and MPC groups than in the HFD group. The levels of plasma interferon gamma and MCP-1 were also significantly lower in mice that consumed PL. Accordingly, PL, CJS, and MPC supplementation downregulated plasma levels of inflammatory adipokines.

Elevation of the blood glucose level increases lipogenesis by stimulating insulin release while inhibiting glucagon release from the pancreas [22]. The levels of fasting plasma glucose and insulin were significantly improved by consumption of PL, CJS, or MPC. Fatty liver disease is caused by oxidative stress and lipid peroxidation [13, 46]. Oxidative stress from ROS production leads to endothelial dysfunction, platelet aggregation, and thrombosis [1]. In addition, FFA, inflammatory cytokines, and oxidized LDL-C increase the production of ROS, superoxide, and H2O2 in the endothelium [17, 44, 46]. Beneficial properties of consumption of persimmon leaves and Citrus junos Sieb in relation to the antioxidant activity may be attributed to protective effects of their constituents against free radical scavengers in vivo and in vitro [18, 20, 44, 47]. In the present study, lower levels of hepatic H2O2 and erythrocyte TBARS in the PL, CJS, and MPC groups were most probably the cause of the observed reduction in hepatic lipid peroxidation, which in turn may have contributed to a general decrease of the hepatic oxidative stress in diet-induced obese mice. Moreover, MPC supplementation significantly increased the activity of plasma PON, which is another antioxidant enzyme present in the HDL fraction. PON plays an important antiatherogenic role by protecting LDL and HDL from their oxidation by ROS [38].

The present study demonstrated for the first time that supplementation with dietary MPC could potentially have an anti-thrombotic effect, as this treatment downregulated the expression of several coagulation factors of the intrinsic and extrinsic blood coagulation system in mice fed with a HFD. PL and MPC ameliorated dyslipidemia by downregulating levels of plasma lipids and hepatic FAS, as well as by increasing β-oxidation enzymatic activity and fecal cholesterol excretion. Beneficial metabolic effects of PL and CJS also included lower levels of erythrocyte and hepatic oxidative stress, as well as decreased expression of inflammatory cytokines. These effects partially contributed to a decrease in the number and size of adipocytes and lipid droplets in the PL and MPC groups. In mice that received PL, CJS, or MPC, hepatic HMG-CoA reductase and SREBP-1c gene expression was lower than in HFD mice, which did not consume any supplements. Thus, our findings suggest that PL and MPC may be useful for improving blood coagulation parameters, amelioration of dyslipidemia, and prevention of CVDs. These results reinforce the need to carry out long-term intervention studies in humans to explore cardiovascular effects of regular consumption of a mixture of ethanol extracts of persimmon leaf and Citrus junos Sieb.

Acknowledgments

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Reference


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