Preventative Effects of *Platycodon grandiflorum* Treatment on Hepatic Steatosis in High Fat Diet-Fed C57BL/6 Mice

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**Platycodon grandiflorum** (PG) (Korean name, *Doraji*; Chinese name, *Jiegeng*; and Japanese name, *Kikyo*) is a perennial plant in the Campanulaceae family that contains triterpenoid saponins, carbohydrates, and fibers. This study was carried out to investigate effects of root of PG on fatty liver inhibition in high fat diet (HFD)-fed C57BL/6 mice. C57BL/6 mice were divided into control, total extract of PG (T-PG, 500 mg/kg) and saponin fraction (S-PG, 50 mg/kg)-treated groups. Significant decreases in body weight, associated with fat mass reduction, were observed in PG-treated groups (*p* < 0.05). Hepatic lipid content and score index calculated from morphometric observations on fatty liver were significantly decreased in the PG-treated groups (*p* < 0.05). Moreover, activities of fatty acid synthase (FAS) and carnitine palmitoyl-transferase (CPT) were significantly suppressed and increased as compared with the control group, respectively (*p* < 0.05). mRNA expressions of the sterol regulatory element binding protein (SREBP1c) and stearoyl-CoA desaturase (SCD1) gene were suppressed in the T-PG and S-PG groups (*p* < 0.05). From these findings, we speculate that fatty liver inhibition effects of PG extract and its saponins appear to be conferred by hepatic lipogenesis and acceleration of energy expenditure, along with modulation of liver FAS and CPT activities in HFD-fed C57BL/6 mice.

Key words: *Platycodon grandiflorum*; fatty liver; lipid regulating enzyme activity.

Non-alcoholic hepatic steatosis (NASH) is closely associated with obesity, diabetes, hyperlipidemia, and insulin resistance. Generally, NASH results from the accumulation of fat in the liver, primarily through excessive transport of free fatty acids from visceral adipose tissue into the liver, and from an imbalance of *de novo* lipid synthesis and catabolism in hepatocytes. Lipid-lowering drugs have been used to inhibit hepatic steatosis; however, these drugs can cause serious side effects including vomiting, headache, stomachache, and heart attack. Some oriental medicinal herb extracts have been reported to be useful for the control of obesity, hyperlipidemia, and glucose concentrations, with less significant side effects. Recently, various saponin-containing natural products have emerged as potential lead compounds, drug alternatives, and/or nutritional supplements.

*Platycodon grandiflorum* (PG) (Korean name, *Doraji*; Chinese name, *Jiegeng*; and Japanese name, *Kikyo*) is a perennial plant in the Campanulaceae family that contains triterpenoid saponins, carbohydrates, and fibers. This plant grows wild in East Asian countries and is widely used in traditional herbal medicine as an expectorant for pulmonary disease and a remedy for respiratory disorders. *Platycodon radix* is the root of PG and contains bioactive components such as platycodin saponins. These components have attracted renewed interest due to their novel pharmacological potentials for treating metabolic diseases related to obesity. Previous studies have shown anti-obesity and anti-hyperlipidemic effects of platycodin saponins in animal models due to the inhibition of intestinal fat absorption via pancreatic lipase activity suppression. Moreover, PG saponins have been reported to show preventative effects on carbon tetrachloride (CCl4)- and acute ethanol-induced hepatotoxicity by blocking cytochrome P450 2E1 (CYP2E1)-mediated CCl4 bioactivation and increasing free radical scavenging activity. In relation to high fat diet (HFD)-induced fatty liver, aqueous extract feeding of PG was found to significantly reduce hepatic lipid concentrations; however, the underlying mechanism of the anti-fatty liver effects of PG are not well elucidated. Therefore, in the present study we investigated the effects of PG and its saponins on fatty liver and the underlying mechanism leading to fatty liver in HFD-fed C57BL/6 mice.

**MATERIALS AND METHODS**

**Extraction and Saponin Fraction Preparation** The root of PG (21 years old) was supplied by Jangsang Doraji Co., Ltd., Jinju, South Korea. Dried and powered PG roots (100 g) were refluxed with ethanol. The obtained filtrate was evaporated under reduced pressure to give a brown residue (42.2 g; total fraction). The extract (1.0 g) were fractionated by SPE cartridge CEREX® C18 HEAVY LOAD (350 mg; particle size 10 μm). The cartridges were conditioned with 20 ml of methanol followed by 20 ml water. The total fraction (1 g/5 ml in water) was passed through the cartridges, which were then rinsed using 30 ml of H2O. The retained analytes were eluted with 20 ml of 80% aqueous methanol. The eluate was concentrated at reduced pressure to give a crude saponin fraction (135 mg).

**HPLC Analysis of the *P. grandiflorum* Root Extract** Twenty microliters of the saponin fraction, which was prepared from the root extracts of PG as described above, was injected onto an Optimapak column (4.6×250 mm, 5 μm, RSTech). The Optimapak column was maintained at 40°C on a Futesc NS-300i system equipped with an ELSD detector (Softa, U.S.A.). A mixture of 50 mM ammonium acetate}
solution (NH₄Ac), acetonitrile, and methanol was used as the mobile phase as follows: eluent A = 85:10:5 NH₄Ac–acetonitrile–methanol; eluent B = 55:40:5 NH₄Ac–acetonitrile–methanol; flow rate: 0.8 ml/min; gradient: 0–5 min (0–15% B), 5–28 min (15–38% B), 28–33 min (38–40% B), 33–53 min (40–43% B), 53–63 min (43–60% B), 63–81 min (60–100% B) (Fig. 1).¹⁴

Animal Experiments Male, 8 week-old C57BL/6 mice were used in these studies. All animals were maintained at the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, Korea). The mice were housed in plastic cages in a room with controlled temperature (22±1 °C) and maintained on a reverse 12 h light/dark cycle. The mice were randomly divided into three groups: High-fat diet (Dyets Inc., Cat. #100244, U.S.A.) plus daily vehicle [0.5% carboxymethylcellulose (CMC)] as a control group, HFD plus the total extract of PG (T-PG, 500 mg/kg), and HFD plus the saponin fraction of PG (S-PG, 50 mg/kg). T-PG and S-PG were administered daily by oral gavage for 12 weeks. Body weight gain was measured once a week. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the institutional guidelines at KRIBB.

Fat Mass Weights and Plasma Analysis At the end of the experimental period, blood samples were taken from the orbital venous congestion to determine the concentrations of plasma biomarkers. Plasma was prepared by centrifugation of blood at 10000 g for 5 min at 4 °C. Plasma was stored at −70 °C until analysis. At necropsy, both sides of the inguinal, retroperitoneal, and abdominal adipose tissue were removed and weighed. The ratios of adipose tissue weights to body weights were then calculated. The tissues were rinsed in liquid nitrogen and stored at −70 °C until used for enzyme-based assay. Plasma leptin levels were determined using a radioimmunoassay kit (DY498 Mouse Leptin, R&D system, Inc., U.S.A.). Total cholesterol (TC) and triglyceride (TG) levels were measured with an automatic chemistry analyzer (Hitachi 7150, Japan).

Measurement of Hepatic Triglycerides and Total Cholesterol Hepatic lipids were extracted using the procedures developed by Bligh and Dyer.¹⁵ Briefly, frozen liver tissue was homogenized in a 0.9% NaCl solution and the homogenate was added to a CM solution (chloroform: methanol=1:2, v/v). The solution was vortexed and centrifuged at 2000 g for 20 min. The upper phase was aspirated and filtered. The filtered chloroform phase was used in further analyses. Hepatic cholesterol and triglyceride concentrations were analyzed using an enzymatic kit (Asan, Korea) at 540 nm.

Measurement of Hepatic Lipid Regulating Enzyme Activity The hepatic enzyme source was prepared according to the methods developed by Hulcher and Oleson with a slight modification.¹⁶ A homogenate was prepared in a buffer containing 0.1 mol/l of triethanolamine, 0.02 mol/l of ethylenediaminetetraacetate (EDTA), and 2 mmol/l of dithiothreitol, pH 7.0, and then centrifuged at 600 g for 10 min to remove any cellular debris. The supernatant was centrifuged at 10000 g followed by 12000 g for 20 min at 4 °C to remove the mitochondrial pellet. Thereafter, the supernatant was ultracentrifuged twice at 100000 g for 60 min at 4 °C to obtain the cytosolic supernatant. The mitochondrial and microsomal pellets were then redissolved in 800 μl of a homogenization buffer and protein content was determined by the Bradford method using bovine serum albumin as the standard. Fatty acid synthase (FAS) activity was measured according to the methods of Carl et al.¹⁷ by monitoring the malonyl-CoA-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH). Then 100 μl of the cytosolic enzyme was mixed with 125 mmol/l potassium phosphate buffer (pH 7.0), 165 μmol/l acetyl-CoA, 50 μmol/l malonyl-CoA, 50 μmol/l NADPH, 1 mmol/l β-mercaptoethanol, and 1 mmol/l EDTA. This mixture was then analyzed for 2 min at 340 nm on a spectrophotometer. The measured activity represents the oxidized NADPH nmol/min/mg protein. Carnitine palmitoyltransferase (CPT) was assayed following the release of coenzyme A (CoA)-SH from palmitoyl-CoA using the general thiol reagent (5,5'-dithiobis-2-nitrobenzoate, DTNB) as described by Markwell et al.,¹⁸ with a slight modification. The reaction mixture contained 232 mmol/l Tris–HCl (pH 8.0), 1.1 mmol/l EDTA, 220 mmol/l carnitine, 24 μmol/l DTNB, 7 μmol/l palmitoyl-CoA, and 0.09% Triton X-100. The reaction was initiated by the addition of an enzyme at 25 °C and the rate was determined at 412 nm.

Quantitative Real-Time Polymerase Chain Reaction (PCR) for Hepatic Lipogenic Genes Total RNA from homogenates of frozen liver was isolated using Trizol reagents, quantified by Nano drop, and stored at −70 °C prior to use. Relative quantification of the expression of selected genes was performed using the Exicycler 96 Real-Time PCR System (Bioneer, Korea) and SYBR (Takara, Japan) as DNA binding dye for the detection of PCR products. The primer pairs for PCR were as follows: for SREBP1c, forward 5′-CCA CCG CTA CTT CCA GCT AGA C-3′, reverse 5′-GTC GGC ATG GTC CTG ATT G-3′; for SCD1, forward 5′-GCC TCT GGA GCC ACA GAA CT-3′, reverse 5′-GCC CAT TCG TAC ACG TCA TTC-3′. The cycling conditions were 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, and 60 °C for 1 min. To detect and eliminate possible primer-dimer artifacts, the dissociation curve was generated by adding a cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. All primer sets produced amplicons of the expected size, and their identity was also verified by sequencing. Results were normalized using the reference gene 18 s.
RNA and represented as fold changes for the reference gene.

Liver Histology and Steatosis Scoring  Liver was removed from the mice and fixed in a buffer solution of 10% formalin. Fixed tissues were processed routinely for paraffin embedding, and 5-μm sections were prepared and stained with haematoxylin and eosin (H&E); stained areas were viewed using an optical microscope with a magnifying power of ×200. The stained sections were evaluated to grade the degree of hepatic steatosis and classified into four grades according to the distribution pattern of the fat accumulation as follows: 1=no or few fat droplets in the lobules (1—25% of whole specimens), 2=few fat droplets in the lobules (26—50% of whole specimens), 3=moderate fat droplets in the lobules (51—75% of whole specimens), 4=numerous fat droplets (75—100% of whole specimens).

Statistical Analysis  All data are presented as the mean±standard error of the mean (SEM). Statistically significant differences among the groups were determined by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. A p-value of less than 0.05 was considered statistically significant. Weekly body weight changes were compared with the control group, and significant differences between the groups were analyzed by Student’s t-test.

RESULTS AND DISCUSSION

The root of *Platycodon grandiflorum* (PG), which is used as a traditional medicine in Korea, has a wide range of beneficial health effects, including anti-inflammatory actions and the prevention of hyperlipidemia and diabetes. To yield a fraction of PG, saponin from root of PG were fractionated via open-column chromatography on silica gel and subjected to semi-preparative HPLC: Deapio-Platycoside E (1), Platycoside E (2), Deapio-platycosid D₃ (3), platycodin D₃ (4), polygalacin D₂ (5), platyconic acid A (6), platycodin D₂ (7), platycodin D (8), 2′-O-acetyl-polygalacin D₂ (9).

This study was carried out to investigate effects of total extract and saponin from PG on fatty liver inhibition in high fat diet (HFD)-fed C57BL/6 mice. The present study demonstrates that HFD-induced hepatic steatosis is effectively inhibited by daily oral administration of total extract and saponin from PG, concomitantly accompanied with anti-obesity effects through fat mass reduction.

The body weights appeared to increase with time in all groups (Fig. 2A). However, the rate of body weight change in the T-PG and S-PG groups showed gradually decreasing patterns from 4 weeks, and the final body weights of the T-PG group (34.4±0.9 g) and S-PG group (34.1±1.0 g) were significantly decreased, as compared with the control group (37.9±1.2 g) (p<0.05). Also relative fat mass to body weight ratios of the T-PG and S-PG groups were significantly decreased as compared with the control group (p<0.05) (Fig. 2B), which indicated that the body weight decreases in the T-PG and S-PG treated mice were due to fat mass reductions rather than other factor changes such as muscle and bone mass changes.

Plasma leptin levels of the mice in the T-PG (5.76±2.8 ng/ml) and S-PG (7.88±4.5 ng/ml) groups were significantly decreased, as compared with the control group (13.41±2.6 ng/ml), (p<0.05) (Fig. 3A). These results support that reductions in body weight were due to the T-PG and S-PG treat-
ments. Moreover, although no significant changes were found among groups in the present study, plasma triglycerides and total cholesterol levels were reduced in T-PG- and S-PG-treated mice (Figs. 3B, C). Previous reports demonstrated that platycodon saponin and PG aqueous extract have anti-obesity and anti-hyperlipidemic effects in Sprague-Dawley rats \(^{19,20}\) and ICR mice \(^{21}\) with HFD-induced obesity. The present study reconfirms these effects. We found that the T-PG and S-PG treatments inhibited body weight increases via fat mass reduction and decreased plasma leptin levels, which were predominantly expressed in adipocytes.

High fat diet caused fatty liver with increased accumulation of total cholesterol and triglyceride. Fatty liver generally results from hepatic lipid accumulation due to increases in the supply of circulating free fatty acids to the liver. In addition, increased endogenous hepatic fatty acid synthase, a key enzyme that catalyzes the synthesis of saturated long-chain fatty acids, plays a significant role. \(^{21}\) In this study, hepatic TC levels showed a significant reduction with PG treatment (\(p<0.05\)) (Fig. 4A); TC levels in the T-PG and S-PG groups were lowered by up to 34.97 and 47.46%, as compared with the control group, respectively. The PG groups tended to have decreased hepatic TG levels as compared with the control group at week 12 (Fig. 4B). Moreover, the activities of FAS and CPT, as representative enzymes mediating lipid biosynthesis and energy expenditure, were assessed to investigate PG effects on lipid metabolism in the liver. The activity of hepatic cytosolic FAS was significantly lower in the T-PG and S-PG groups as compared with the control group (Fig. 4C). In contrast, hepatic mitochondrial CPT activity in the T-PG and S-PG groups was significantly higher than that of the control group (\(p<0.05\)) (Fig. 4D). CPT is the rate-limiting enzyme that facilitates the entry of fatty acids into the mitochondria for oxidation. \(^{22}\) These results suggest that the reducing effects of PG on hepatic triglycerides and total cholesterol levels are likely mediated by decreases in hepatic fatty acid formation and increases in fatty acid oxidation.

To confirm the effects of PG on the expression of adipogenic genes such as sterol regulatory element binding protein (SREBP1c) and stearyl-CoA desaturase (SCD1), real-time PCR analysis was performed. mRNA expressions of the SREBP transcription factor, which plays a key role in energy homeostasis, and of the SCD1 gene, which is involved in the synthesis and regulation of unsaturated fatty acids, were suppressed in the T-PG and S-PG groups (\(p<0.05\)) (Fig. 5).

In addition, histological observations of liver tissues showed that hepatic steatosis was clearly blocked by the T-PG and S-PG. As expected, the HFD-fed control group showed hepatic steatosis, characterized by irregularly shaped microvesicular fat droplets in hepatocytes consisting of hepatic lobules (Fig. 6). In contrast, the PG-treated groups did not show recognizable fatty changes in hepatocytes. On the basis of morphometric observations of liver tissue, a marked

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Fig. 4. Hepatic Lipid Concentrations and Activities of Hepatic Lipid Regulating Enzymes Determined in C57BL/6 Mice Treated with High Fat Diet Plus Total Extract (T-PG) or Saponin Fraction (S-PG) of \(P.\) grandiflorum

Values are mean±S.E.M. (n=3, each group). (A) Hepatic total cholesterol; (B) hepatic triglyceride; (C) fatty acid synthase; (D) carnitine palmitoyltransferase. \(^{ab}\) Means not sharing a common letter are significantly different between groups at \(p<0.05\).

Fig. 5. Quantitative mRNA Expressions of Liver Lipogenic Genes in C57BL/6 Mice Treated with High Fat Diet Plus Total Extract (T-PG) or Saponin Fraction (S-PG) of \(P.\) grandiflorum

Values are mean±S.E.M. (n=3, each group). SREBP1c: sterol regulatory element binding protein 1c; SCD1: stearyl coenzyme A desaturase. \(^{ab}\) Means not sharing a common letter are significantly different between groups at \(p<0.05\).

Fig. 6. Representative Photomicrographs Showing the Inhibition Effects of \(P.\) grandiflorum on Fatty Liver in High Fat Diet Fed C57BL/6 Mice

Haematoxylin and eosin (H&E) stain, original magnification: \(\times 200\).
reduction in the score index of fatty liver was noted in the livers from T-PG (0.56±0.17) and S-PG groups (0.62±0.24) as compared with the control group (2.14±0.31), suggesting that PG treatment improved microvesicular hepatic steatosis. Hepatic steatosis scores were significantly lower in the PG groups as compared with the control group (p<0.05). Thus, PG might prevent the development of hepatic steatosis by suppressing lipid-regulating enzyme actions and down-regulating the expression of lipogenic genes. With respect to the bioactive materials of PG, saponins isolated from foodstuffs have been shown to have anti-obesity actions and hypolipidemic and fatty liver inhibitory effects.7,23) The similar effect levels of the T-PG and S-PG treatments suggest that the saponin fraction of PG might be a major player in suppressing HFD-induced hepatic fatty changes.

In conclusion, the present study has shown anti-obesity effects via fat mass reduction, fatty liver prevention, and suppression of fatty acid formation through the FAS and CPT activity modulation of PG and its saponins in C57BL/6 mice fed a high fat diet. Based on these findings, PG and its saponins may be a potentially useful dietary intervention for fatty liver and metabolic syndrome.

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