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Eriobotrya japonica Improves Hyperlipidemia and Reverses Insulin Resistance in High-fat-fed Mice

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The effect of *Eriobotrya japonica* Lindl. (loquat) on insulin resistance was examined in mice fed a high-fat (HF) diet. First, the mice were divided randomly into two groups: the control (CON) group was fed a low-fat diet, whereas the experimental group was fed with a 45% HF diet for 10 weeks. After 6 weeks of induction, the HF group was subdivided into five groups and was given orally loquat or not for 4 weeks afterward. It was demonstrated that loquat was effective in ameliorating the HF diet-induced hyperglycemia, hyperleptinemia, hyperinsulinemia and hypertriglyceridemia, as well as in decreasing the levels of free fatty acid (FFA), but increasing the adipose PPARγ (peroxisomal proliferator-activated receptor γ) and hepatic PPARα mRNA levels. Loquat significantly decreased the body weight gain, weights of white adipose tissue and visceral fat accompanying the suppressed leptin mRNA levels. Loquat not only suppressed the hepatic mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis and lowered the sterol regulatory element binding protein-1c (SREBP-1c) mRNA level, but also affected fatty acid oxidation enzyme levels. These regulations may contribute to triacylglycerol accumulation in white adipose tissue. The findings provide a nutritional basis for the use of loquat as a functional food factor that may have benefits for the prevention of hyperlipidemia and diabetes. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: Eriobotrya japonica; hyperglycemia; leptin; body fat.

INTRODUCTION

The dried leaves of loquat, *Eriobotrya japonica* Lindl. (Rosaceae), is a well known Traditional Chinese medicine for relieving cough and regulating the stomach to restrain vomiting (A Group of Editors for Chinese Materia Medica, 1996). The leaves of loquat are used in the treatment of skin diseases and diabetes mellitus (Tomassi *et al*., 1992). Loquat, which consists of a large amount of pentacyclic triterpenes, would exert many biological activities. However, previous studies demonstrated that loquat had been used for the treatment of diabetes (Noreen *et al*., 1988). Triterpenoid constituents have been isolated from the leaves of this plant, and several of them were reported to have antitumor, antiviral and antiinflammatory properties (Shimizu *et al*., 1986, 1996; Liang *et al*., 1990; Tommasi *et al*., 1992; Nozato *et al*., 1994). The occurrence of polyphenolic constituents in the leaves and their cytotoxicity against human oral tumor cell lines were also reported (Ito *et al*., 2000). Current other studies have shown that the sesquiterpene glycoside, nerolidol-3-*O*-α-l-rhamnopyranosyl(1→4)-α-l-rhamnopyranosyl(1→2)-[α-l-rhamnopyranosyl(1→6)]-β-D-glucopyranoside from the leaves of *E. japonica* produces a significant antihyperglycemic effect when administered orally to alloxan-diabetic mice (Chen *et al*., 2008). The reported bioactive components of loquat include flavonoids (Louati *et al*., 2003), phenolics (Ding *et al*., 2001), amygdalin (Zhuang, 2002), triterpenic acids (Liang *et al*., 1990) and carotenoids (Godoy and Amaya, 1995). The isomeric pentacyclic oleanolic acid, ursolic acid, amygdalin and maslinic acid are predominant triterpenoids found in loquat leaves (Ju *et al*., 2003; Park *et al*., 2005; Wen *et al*., 2005, 2007). Corosolic acid (Fig. 1) is one of the primary active principles of the plant extract, which has been marketed in Japan and the United States (Judy *et al*., 2003).

High-fat (HF) diet-induced obesity can lead to insulin resistance. Obesity is defined as the accumulation of excess adipose tissue resulting from various metabolic disorders. It is a strong risk factor for hypertension, hyperlipidemia, heart disease and Type 2 diabetes (Olefsky *et al*., 1982). It is controlled by both genetic and environmental factors. Among the environmental factors, the chronic consumption of a HF diet is strongly associated with the development of obesity. Obesity is associated with a decreased capacity of insulin to regulate glucose and lipid metabolism in the peripheral tissues. The increase in adipose tissue is accompanied by elevations of circulating free fatty acids (Boden, 1997).

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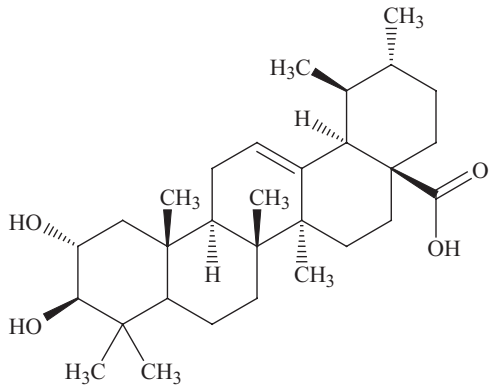
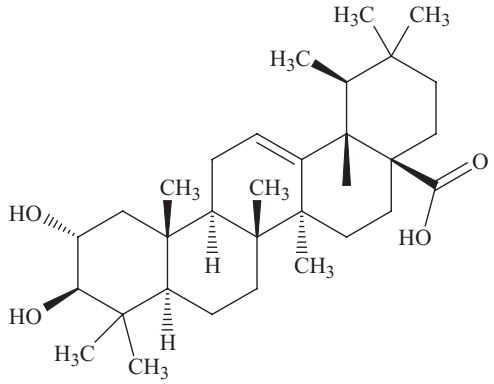


corosolic acid



maslinic acid

Figure 1. Structure of corosolic acid and maslinic acid.

Thiazolidinediones (TZDs), as peroxisome proliferator-activated receptor (PPARs) ligands are heralded as a breakthrough in the pharmacological treatment of Type 2 diabetes. PPARγ represses the gene expression of leptin, which has been implicated in insulin resistance (Rangwala and Lazar, 2004). Collectively, activation of PPARγ mitigates insulin resistance. Fibrates are hypolipidemic drugs whose effects are mediated by the activation of PPARα. The administration of the drug also increases fatty acid oxidation and the change may be involved in the reduction of fat deposits (Cabrero *et al*., 2001).

Recently, much attention has been focused on some herb and food factors that may be beneficial in preventing HF diet-induced body fat accumulation and possibly reduce the risk of diabetes and heart disease. Ide *et al*. (2000) showed that sesamin, found in sesame seed, decreased fatty acid synthesis and enhanced β-oxidation in rat liver. Murase *et al*. (2001) showed that dietary diacylglycerol suppresses HF diet-induced body fat accumulation in mice. However, there is little evidence that food factors themselves are beneficial for the prevention of obesity and the amelioration of insulin resistance.

The present study was designed to examine the preventive effect of loquat extract on the development of obesity and hyperglycemia induced by feeding a HF diet. No studies exist on a possible insulin sensitizing and hypolipidemic property of loquat extract. Loquat extract is made from loquat leaves. Loquat extract consists of corosolic acid and contains a large amount of pentacyclic triterpenes. The C57BL/6J mice used in this study develop severe obesity, hyperglycemia and hyperinsulinemia when fed a HF diet (Surwit *et al*., 1988). This study also investigated the effects of loquat on AMP-activated protein kinase (AMPK) activity in HF mouse liver and adipose tissue. Phosphorylation of Thr 172 of the α subunits is essential for AMPK activity (Crute *et al*., 1998; Stein *et al*., 2000). As one of the possible mechanisms of action, the study also examined its effect on the expression of genes involved in lipogenesis in the liver and white adipose tissue (WAT).

MATERIALS AND METHODS

Preparation of extract and determination of triterpene compounds. The leaves of *E. japonica* were purchased from the local markets in Taiwan in September 2007. All materials were sorted and identified by Associate Professor Chao-Lin Kuo with voucher specimens (CMU-EJ080001-CMU-EJ08010) deposited in the China Medical University, Taichung, Taiwan.

The fresh leaves of *E. japonica* (50 kg) were extracted twice with 95% ethanol (EtOH). After extraction, the mixture was concentrated and yielded an EtOH extract (6030 g). The EtOH extract was suspended in water and partitioned with *n*-butanol to obtain a deep green syrup (1550 g). The butanol extract was subjected to an active carbon column with 30% $CHCl_3$ in MeOH an eluate to remove the chlorophylls liquid. The grey liquid was concentrated under reduced pressure to yield a drab powder (507 g). The powder consisted of five compounds identified as tormentic acid, maslinic acid, corosolic acid, oleanolic acid and ursolic acid. The isolation procedure and analysis condition of the compounds has been previously published by our team partner (Ho *et al*., 2008). In briefly, HPLC was performed on a Shimadzu 10A system equipped with one pump (LC-10AT Shimadzu Japan) and a RI spectrophotometric detector (RID-10A, Shimadzu, Japan). The HyPURITY C-18 column (5 μm, 4.6 × 250 mm) was eluted at a rate of 0.5 L/min with a solvent of A-B (A, methanol; B, 0.15% acetic acid aqueous; A: B = 85: 15, v/v). The total contents of five triterpenes were 25.9% (tormentic acid 8.2%, maslinic acid 2.4%, corosolic acid 5%, oleanolic acid 1.6% and ursolic acid 8.7%, respectively).

The extract was diluted and adjusted, then it was administered orally to mice in a volume of 0.2, 0.5, 1.0 g/kg body weight, respectively. Distilled water was administered in a similar volume to the control mice.

Experimental animals and protocol. Male C57BL/6J mice, 5 weeks of age, were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council. Mice were housed in an air-conditioned room at 22 ± 3°C with 12 h of light and tap water *ad libitum*.

After a 1-week acclimation period, the mice were divided randomly into two groups. The control (CON) group was fed a low-fat diet (Diet 12450B, Research Diets), whereas the experimental group was fed a 45% high-fat diet (Diet 12451, Research Diets) for 10 weeks. The low-fat diet was composed of protein 20%, carbohydrate 70% and fat 10%, whereas the high-fat diet was composed of protein 20%, carbohydrate 35% and fat

Table 1. Composition of the high- and low- fat diets (g/kg diet)

Ingredient	Low-fat	High-fat
Casein	800	800
L-Cystine	12	12
Corn starch	1260	291
Maltodextrin 10	140	400
Sucrose	1400	691
Cellulose, BW200	0	0
Soybean oil	225	225
Lard	180	1598
Mineral mix S10026	0	0
Dicalcium carbonate	0	0
Calcium carbonate	0	0
Potassium citrate, $1H_2O$	0	0
Vitamin mix V10001	40	40
Choline bitartrate	0	0
FD&C yellow dye #5	0	
FD&C red dye #40		0
FD&C blue dye #1		
Total	4057	4057

Composition of the low-fat and high-fat diet is expressed as shown and as a percentage of total calories.

45% (of total energy, % kcal). After the first 6 weeks, the high-fat treated mice were further subdivided into five groups and were administered by gavage with or without *Eriobotrya japonica* or rosiglitazone (ROS) for 4 weeks, while the mice were still on the high-fat diet. During the last 4 weeks, the CON and high-fat control (HF) mice were treated with vehicle only. The other groups received loquat extract (including 0.2, 0.5, 1.0 g/kg/day extracts), or ROS 10 mg/kg, respectively.

The body weight was measured weekly throughout the study. These dietary periods lasted for 10 weeks, and the mice were maintained in accordance with the Animal Experiment Committee guidelines. The compositions of the experimental diets are shown in Table 1.

At the end of the feeding period, the mice were killed by exsanguination under diethyl ether anesthesia. The liver and white adipose tissues (WATs) (including epididymal, mesenteric and retroperitoneal WAT) and interscapular brown adipose tissue (BAT) were dissected according to defined anatomical landmarks. The weights of the tissues were measured. Visceral fat was defined as the sum of epididymal and retroperitoneal WAT. They were then immediately frozen using liquid nitrogen and kept at −80°C until use. The collected blood was kept at room temperature for 5 min for coagulation. Then, the plasma was obtained from the coagulated blood by centrifugation at 1600 × **g** for 15 min at 4°C. The separation of the plasma was finished within 30 min. The plasma was immediately frozen at −80°C until use.

Food intake and body weight. Food intake and body weight were monitored daily. First, the pelleted food was weighed then placed in a cage food container. After 24 h, the remaining food was weighed. The difference represented the daily food intake. The animal weight and food weight were measured using an electronic scale. Unconsumed pelleted HF food was discarded each day and fresh pelleted high-fat diet was provided to ensure consistent food quality to the mice throughout the study. The HF food was stored at 4°C.

Measurement of blood parameters. The concentrations of triglyceride (TG), total cholesterol (TC) and FFA were measured using commercial assay kits according to the manufacturer's directions (Triglycerides-E test, Cholesterol-E test and FFA-C test, Wako Pure Chemical, Osaka, Japan). The level of glucose was measured by the glucose oxidase method (Model 1500; Sidekick Glucose Analyzer; YSI Incorporated, Yellow Springs, USA).

Measurement of adipocytokine levels. The levels of insulin and leptin were measured by ELISA using a commercial assay kit according to the manufacturer's directions (mouse insulin ELISA kit, Sibayagi, Gunma, Japan and mouse leptin ELISA kit, Morinaga, Yokohama, Japan).

Histology analysis of epididymal WAT. Small pieces of epididymal WAT were fixed with formalin (200 g/kg) neutral buffered solution and embedded in paraffin. Sections (8 μm) were cut and stained with hematoxylin and eosin. For microscopic examination, a microscope (Nikon, ECLIPSE, 80C) was used, and the images were taken using a Nikon Digital Sight camera (DS-5MC-U2) at 10 (ocular) × 10 (object lens) magnification.

Measurement of hepatic lipids. Hepatic lipids were extracted using a previously described protocol (Folch *et al.*, 1957). For the hepatic lipid extraction, the 0.375 g liver samples were homogenized with 1 mL distilled water for 5 min. A fresh 2.5 mL solvent mixture containing chloroform and methanol (2:1) was added to the homogenate and centrifuged at 3000 rpm for 10 min to separate the phases. The lower phase was removed carefully and transferred to a new tube. Additional solvent was added to the upper phase and the pellet in a final volume of 6.25 mL was vortexed, then centrifuged at 3000 rpm for 10 min. The lower phase was repeatedly collected with 1.5 mL of washing buffer containing chloroform, methanol and 0.05% $CaCl_2$ (3: 48: 47). After vortexing and centrifugation, the lower phase containing lipids was evaporated under a nitrogen stream. The dried pellet was resuspended in 0.5 mL ethanol and analysed using a triglycerides kit as used for serum lipids.

RNA extraction. Total RNA from the epididymal WAT and liver was isolated with a Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's directions. The integrity of the extracted total RNA was examined by 2% agarose gel electrophoresis, and the RNA concentration was determined by the ultraviolet (UV) light absorbancy at 260 nm and 280 nm (Spectrophotometer U-2800A, Hitachi). The quality of the RNA was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA after electrophoresis on 2% agarose gel containing 6% formaldehyde.

Relative quantitation of mRNA indicating gene expression. Total RNA (1 μg) was reverse transcribed to cDNA in a reaction mixture containing buffer, 2.5 mM dNTP (Gibco-BRL, Grand Island, NY), 1 mM of the

Table 2. Primers used in this study

Gene	Accession number	Forward primer and reverse primer	PCR product (bp)	Annealing temperature (°C)
White adipose tissue				
PPARγ	NM_013124	F: CATGCTTGTGAAGGATGCAAG	190	55
		R: TTCTGAAACCGACAGTACTGACAT		
Leptin	NM_008493	F: GGCATTTTCTTACCTCTGTG	303	55
		R: ACTTTGGATGAACCAATCAG		
aP2	NM_024406	F: TCACCTGGAAGACAGCTCCT	143	50
		R: TGCCTGCCACTTTCCTTGT		
SREBP1c	NM_011480	F: GGCTGTTGTCTACCATAAG	219	55
		R: AGGAAGAAACGTGTCAAGAA		
FAS	NM_007988	F: TGGAAAGATAACTGGGTGAC	240	55
		R: TGCTGTCGTCTGTAGTCTTG		
Liver				
PPARα	NM_011144	F: ACCTCTGTTCATGTCAGACC	352	55
		R: ATAACCACAGACCAACCAAG		
FAS	NM_007988	F: TGGAAAGATAACTGGGTGAC	240	50
		R: TGCTGTCGTCTGTAGTCTTG		
ACC	NM_198837	F: TCGCCATAACCAAGTAGAGT	313	55
		R: TCTGTTTAGCGTAGGGATGT		
CPT-1	NM_153679	F: GCAGGAAATTTACCTCTGTG	288	55
		R: ACATGAAGGGTGAAGATGAG		
SREBP1c	NM_011480	F: GGCTGTTGTCTACCATAAGC	219	50
		R: AGGAAGAAACGTGTCAAGAA		
AMPK	NM_178143	F: ATTGAAATCACCGAAAACAC	266	50
		R: CCTAACAGCTGCTGAAGTTT		
GAPDH	NM_031144	F: TGTGTCCGTCGTGGATCTGA	99	55
		R: CCTGCTTCACCACCTTCTTGA		

oligo (dT) primer, 50 mM dithiothreitol, 40 U RNase inhibitor (Gibco-BRL, Grand Island, NY) and 5 μL Moloney murine leukemia virus reverse transcriptase (Epicentre, USA) at 37°C for 1 h, and then heated at 90°C for 5 min to terminate the reaction. The polymerase chain reaction (PCR) was performed in a final 25 μL containing 1U Blend Taq™ -Plus (TOYOBO, Japan), 1 μL of the RT first-strand cDNA product, 10 μM of each forward (F) and reverse (R) primer, 75 mM Tris-HCl (pH 8.3) containing 1 mg/L Tween 20, 2.5 mM dNTP and 2 mM $MgCl_2$. Preliminary experiments were carried out with various cycles to determine the nonsaturating conditions of the PCR amplification for all the genes studied. The primers are shown in Table 2.

The products were run on 2% agarose gels and stained with ethidium bromide. The relative density of the band was evaluated using AlphaDigiDoc 1201 software (Alpha Innotech, Co.). All the measured PCR products were normalized to the amount of cDNA of GAPDH in each sample.

Oral glucose tolerance test (OGTT). The normal mice (n = 5 or 6) were fasted for 15–18 h but were allowed access to 0.2 g/kg, 0.5 g/kg extract of *E. japonica*, or an equivalent amount of normal saline was given orally 30 min before an oral glucose load (1 g/kg body weight). Blood samples were collected at the time of the glucose administration (0) and every 30 minutes until 3 hours after glucose administration to determine the levels of glucose.

Preparation of the liver and adipose tissue for western immunoblotting analysis of phospho-AMPK (Thr172) proteins. Protein extractions and immunoblots for the determination of AMPK phosphorylation were carried out on frozen liver and adipose tissue from mice according to a previous report (Shen *et al*., 2005). Briefly, liver and adipose samples (0.1 g, 0.1 g, respectively) were powdered under liquid nitrogen and homogenized for 20 s in 500 μL buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 2% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 100 mM NaF, 2 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 10 μL/mL pepstatin (Raser *et al*., 1995; Veiseth *et al*., 2001). 40 μg of each homogenate was mixed with an equal amount of 2 × standard SDS sample loading buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.25% bromophenol blue, and boiled for 10 min before electrophoresis.

Proteins were separated by 12% SDS-PAGE according to the method of Laemmli (1970) and transferred by electroblotting onto PolyScreen PVDF transfer membrane (NEN) using a semi-dry transfer cell (Bio-Rad) according to the manufacturer's manual. The membrane was then treated sequentially with blocking solution (phosphate-buffered saline (PBS) containing 5% non-fat skim milk), with appropriate dilution of anti-phospho-AMPKα (Thr 172) antibody (Abcam Inc, USA), and with anti-(G6PD)G6PD (glucose 6 phosphate dehydrogenase antibody; Abcam Inc, USA) conjugated to peroxidase (Zymed). Finally, the membrane

was soaked in a chromogen/substrate solution (TMB single solution; Zymed) for color development.

Statistical analysis. The differences between groups were analysed by comparison, using one-way analysis of variance (ANOVA), and in the case of significance, a Dunnett's test was also applied.

RESULTS

Body weight, food intake, body weight gain and tissue weight

All group mice started with similar mean body weights (18.2 ± 0.2 g). At week 9 and 10, treatment with P1 and P3 significantly decreased the body weight ($p < 0.05$; $p < 0.05$, respectively) (Fig. 2A). Treatment with P3 for 1 week significantly reduced food intake ($p < 0.05$) (Fig. 2B). Treatment with P1, P2 and P3 from week 2 to 4 significantly decreased food intake (Fig. 2B). It was found that loquat had a significant suppressive effect on the 4-week cumulative food intake in HF-fed mice (Table 3). All the loquat-treated groups showed a significant reduction in body weight gain over 4 weeks compared with the HF group (Table 3). At week 10, the weights of absolute adipose tissue (epididymal, retroperitoneal WAT, and visceral fat) were markedly greater in the HF group than in the CON group (epididymal WAT 58.5%, retroperitoneal WAT 105.7% and visceral fat 67.4%). Loquat treatment significantly decreased the weights of absolute epididymal, mesenteric WAT, and visceral fat compared with the HF group. Treatment with P2 and P3 significantly decreased the liver weights compared with the HF group ($p < 0.05$) (Table 3).

Plasma glucose levels

At week 10, the glucose levels were significantly greater in the HF group than in the CON group ($p < 0.001$). After treatment, all the loquat- and ROS-treated groups showed a significant reduction in plasma glucose compared with the HF group (Table 3).

Plasma and liver lipid

As time passed, a hypercholesterolemic phenomenon was evident for the HF diet. At week 10, the levels of FFA, TG and TC were 52.0%, 35.7%, and 21.0% greater in the HF group than in the CON group ($p < 0.001$, $p < 0.05$, $p < 0.05$, respectively) (Table 3). Loquat and ROS suppressed the high-fat diet-induced increases in the concentrations of FFA, TG and TC (Table 3).

The liver total lipids and triacylglycerol concentrations were 71% and 150% greater, respectively, in the HF group than in the CON group (Table 3). Treatment with loquat and ROS significantly suppressed the HF diet-induced increase in the liver total lipids and triacylglycerol concentrations (Table 3).

Epididymal WAT histology

Feeding the HF diet induced hypertrophy of the adipocytes (Fig. 3B) compared with the CON group (Fig. 3A) in epididymal WAT. Afterwards, treatment with loquat (P1, P2 and P3) decreased the hypertrophy compared with the HF group (Fig. 3C, 3D and 3E). The results obtained from the other mice were similar to those shown in Fig. 3.

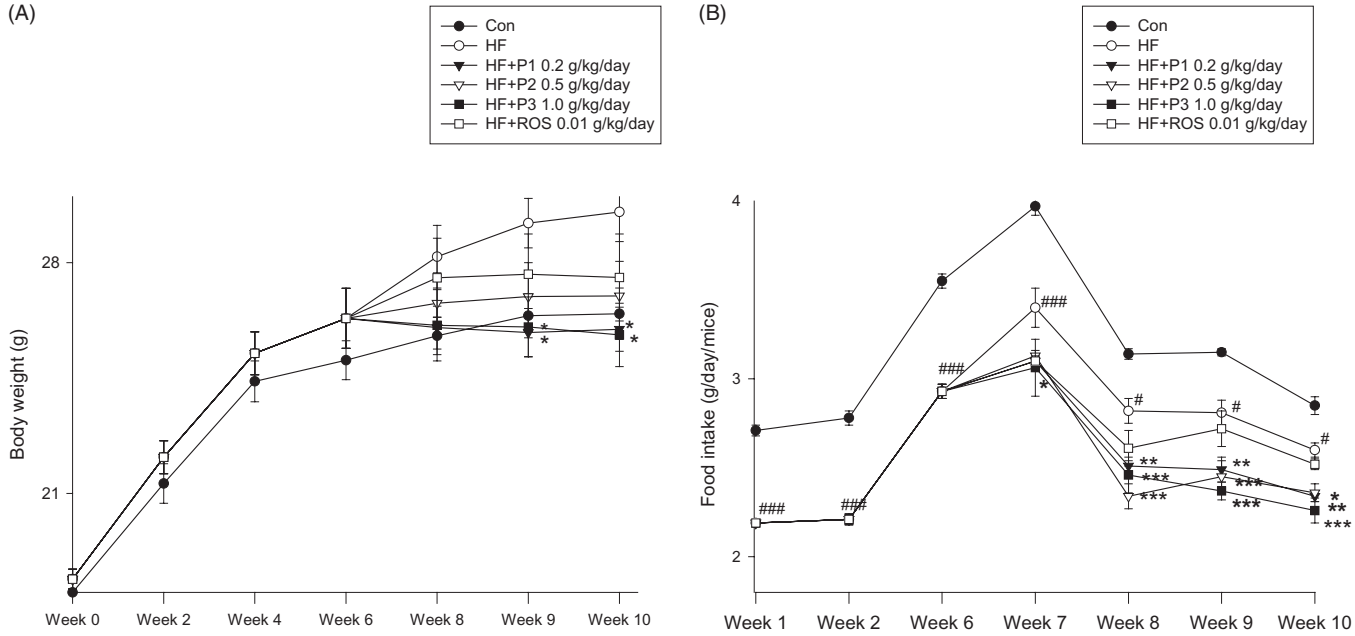


Figure 2. (A) Body weight change. (B) Food intake. Mice were fed a 45% high-fat diet (HF) or low-fat diet (CON) for 10 weeks. At 6 weeks post-HF, the HF mice were treated with vehicle (water; p.o.), or extracts of *Eriobotrya japonica*, or rosiglitazone (p.o.) accompanied with HF diet for 4 weeks. All values are mean ± SE ($n = 9$). There were no significant differences in body weight between the control group and the HF group observed after 6 weeks of HF treatment. # $p < 0.05$, ### $p < 0.001$ compared with the control (CON) group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the high-fat + vehicle (distilled water) (HF) group by ANOVA coupled with Dunnett's test. P1, P2, P3, extracts of *Eriobotrya japonica*.

Table 3. Absolute tissue weight, 4-week cumulative food intake (g/mouse), body weight gain, liver lipids and blood profiles

Parameter	CON	HF	HF + P1 0.2 g/kg/day	HF + P2 0.5 g/kg/day	HF + P3 1.0 g/kg/day	HF + ROS 0.01 g/kg/day
Absolute tissue weight (g)						
EWAT	0.694 ± 0.070	1.100 ± 0.089[c]	0.684 ± 0.047[f]	0.816 ± 0.074[d]	0.634 ± 0.071[f]	1.070 ± 0.118
MWAT	0.397 ± 0.035	0.478 ± 0.030	0.351 ± 0.019[d]	0.359 ± 0.027[d]	0.364 ± 0.034[d]	0.476 ± 0.089
RWAT	0.158 ± 0.023	0.325 ± 0.045[b]	0.224 ± 0.029	0.249 ± 0.031	0.158 ± 0.020[e]	0.283 ± 0.017
Visceral fat	0.852 ± 0.089	1.426 ± 0.119[c]	0.908 ± 0.073[f]	1.065 ± 0.103[d]	0.792 ± 0.079[f]	1.353 ± 0.135
BAT	0.129 ± 0.012	0.135 ± 0.010	0.113 ± 0.006	0.107 ± 0.009	0.108 ± 0.017	0.141 ± 0.023
Liver (g)	1.339 ± 0.061	1.287 ± 0.031	1.138 ± 0.037	1.100 ± 0.046[d]	1.067 ± 0.046[d]	1.136 ± 0.046
Food intake (g)	79.44 ± 0.65	73.59 ± 1.74[a]	66.03 ± 1.08[e]	65.08 ± 1.38[f]	64.28 ± 2.26[f]	69.07 ± 1.71
Body weight (g)	26.45 ± 0.78	28.84 ± 0.90	25.98 ± 0.67[d]	26.99 ± 1.05	25.81 ± 0.96[d]	27.55 ± 1.32
Weight gain (g)	0.86 ± 0.20	0.84 ± 0.10	–0.71 ± 0.19[f]	0.12 ± 0.18[d]	–0.73 ± 0.27[f]	–0.02 ± 0.54
Liver lipids						
Total lipid (mg/g)	54.6 ± 2.6	93.2 ± 5.6[c]	68.4 ± 3.9[e]	65.08 ± 4.3[e]	62.4 ± 4.8[e]	60.3 ± 5.1[e]
Triacylglycerol (μmol/g)	29.7 ± 3.5	74.3 ± 8.2[c]	56.3 ± 4.9[e]	46.5 ± 5.2[f]	43.6 ± 6.6[f]	40.5 ± 3.6[f]
Blood profiles						
Glucose level (mg/dL)	88.6 ± 3.5	110.6 ± 2.5[c]	94.0 ± 2.0[d]	91.9 ± 4.2[e]	91.7 ± 4.3[e]	85.7 ± 2.0[f]
FFA (meq/L)	0.644 ± 0.071	0.979 ± 0.046[c]	0.477 ± 0.059[f]	0.550 ± 0.045[f]	0.384 ± 0.067[f]	0.458 ± 0.033[f]
TG (mg/dL)	103.2 ± 11.1	140.1 ± 10.9[a]	64.3 ± 7.3[f]	63.3 ± 7.4[f]	57.8 ± 5.6[f]	52.5 ± 12.0[f]
TC (mg/dL)	160.7 ± 8.3	204.4 ± 11.2[a]	154.6 ± 8.6[e]	152.6 ± 10.8[e]	161.7 ± 10.3[d]	140.5 ± 12.5[d]
Leptin (μg/mL)	1.49 ± 0.29	6.35 ± 0.83[c]	2.70 ± 0.36[e]	4.99 ± 1.04	4.37 ± 0.78[d]	4.36 ± 0.87[d]
Insulin (μg/L)	0.579 ± 0.029	1.117 ± 0.403[c]	0.706 ± 0.090[f]	0.821 ± 0.056[e]	0.836 ± 0.052[d]	0.660 ± 0.034[f]
Adiponectin (ng/mL)	10.26 ± 0.23	9.04 ± 0.52	10.87 ± 0.46[d]	11.19 ± 0.35[e]	10.06 ± 0.62	12.20 ± 0.82[d]

All values are mean ± SE ($n = 9$). [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared with the control (CON) group; [d] $p < 0.05$, [e] $p < 0.01$, [f] $p < 0.001$ compared with the high-fat + vehicle (distilled water) (HF) group. P1, P2, P3, extracts of *Eriobotrya japonica*. BAT, brown adipose tissue; EWAT, epididymal white adipose tissue; RWAT, retroperitoneal white adipose tissue; MWAT, mesenteric white adipose tissue; EWAT+ RWAT, visceral fat; FFA, plasma free fatty acid; TC, total cholesterol; TG, triglyceride.

Table 4. Semiquantitative RT-PCR analysis for mRNA expression in liver, white adipose tissue and brown adipose tissue

Parameter	CON	HF	HF + P1 0.2 g/kg/day	HF + P2 0.5 g/kg/day	HF + P3 1.0 g/kg/day	HF + ROS 0.01 g/kg/day
Liver						
PPARα	2.67 ± 0.26	1.65 ± 0.15	3.46 ± 0.21[a]	8.28 ± 0.55[c]	11.19 ± 0.92[c]	–
ACC	6.84 ± 1.51	4.89 ± 1.24	3.91 ± 0.22	1.24 ± 0.27[a]	0.83 ± 0.24[a]	0.82 ± 0.28[a]
SREBP-1c	1.56 ± 0.28	1.66 ± 0.29	0.32 ± 0.12[c]	0.61 ± 0.14[b]	1.31 ± 0.22	0.89 ± 0.26
AMPK	1.86 ± 0.48	1.17 ± 0.18	4.51 ± 1.71[a]	1.89 ± 0.92	1.10 ± 0.16	1.03 ± 0.41
White adipose tissue						
PPARγ	2.60 ± 0.14	1.36 ± 0.16[d]	3.61 ± 0.27[c]	6.89 ± 0.55[c]	7.94 ± 0.30[c]	6.14 ± 0.77[c]
LPL	7.67 ± 1.92	4.53 ± 1.15	4.56 ± 1.56	10.16 ± 1.42	13.81 ± 4.39[a]	13.23 ± 4.27[a]
aP2	1.12 ± 0.18	1.49 ± 0.31	0.17 ± 0.04[c]	0.39 ± 0.12[c]	0.47 ± 0.13[b]	0.42 ± 0.16[b]

All values are mean ± SE ($n = 9$). [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared with the high-fat + vehicle (distilled water) (HF) group. [d] $p < 0.05$ compared with the control (CON) group. Total RNA (1 μg) isolated from tissue was reverse transcripted by MMLV-RT, 10 μL of RT products were used as templates for PCR. Signals were quantitated by image analysis; each value was normalized by GAPDH. P1, P2, P3, extracts of *Eriobotrya japonica*.

Leptin concentration, the expressions of PPARγ and leptin in EWAT

At week 10, the concentrations of leptin were greater in the HF group than in the CON group ($p < 0.001$) (Table 3). Treatment with P1, P3 and ROS significantly decreased the concentration of leptin ($p < 0.01, p < 0.05, p < 0.05$, respectively) (Table 3). At week 10, the mRNA levels of leptin were greater in the HF group than in the CON group ($p < 0.001$) (Fig. 4A), whereas the mRNA levels of PPARγ were lower in the HF group than in the CON group ($p < 0.05$) (Table 4). Treatment with P1, P2, P3 and ROS significantly decreased the mRNA level of leptin ($p < 0.05, p < 0.001, p < 0.01, p < 0.01$, respectively) (Fig. 4A). After treatment, P1, P2, P3, and ROS significantly increased the mRNA level of PPARγ ($p < 0.001$, $p < 0.001, p < 0.001, p < 0.001$, respectively) (Table 4).

Expressions of FAS, aP2 and LPL in EWAT

At week 10, no significant difference in the expression of FAS (fatty acid synthase), aP2 (adipocyte fatty acid binding protein) and LPL (lipoprotein lipase) was observed in the HF group compared with the CON group. After treatment, the FAS and aP2 mRNA levels were lower in all the loquat-treated groups than in the HF group, whereas the LPL expressions were higher in the HF + P3 and HF + ROS groups than in the HF group (Fig. 4B and Table 4).

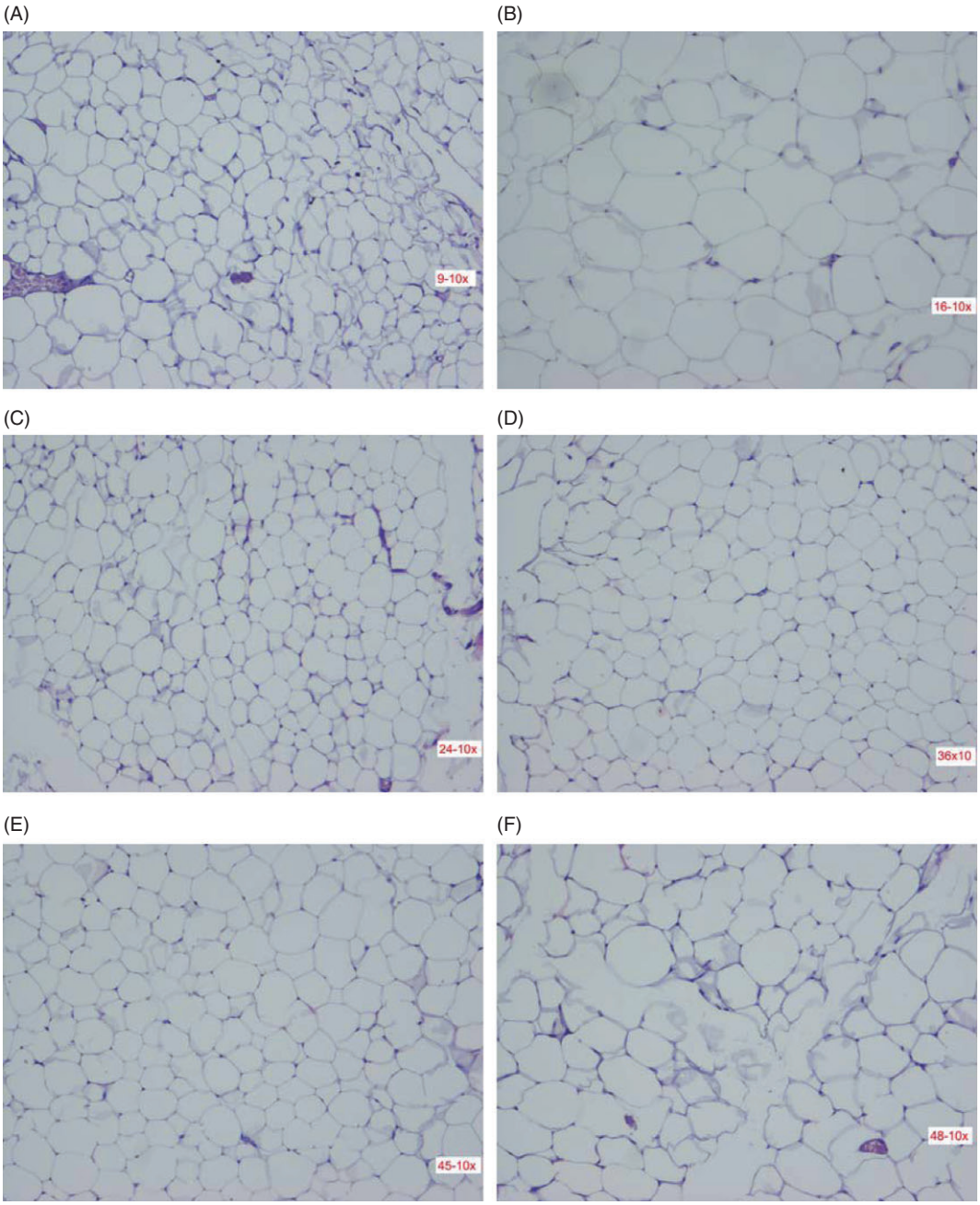


Figure 3. Histology of the epididymal white adipose tissue (WAT) of mice in the (A) Low-fat (LF), (B) High-fat (HF), (C) HF + P1, (D) HF + P2, (E) HF + P3, or (F) HF + ROS groups. Each presented is typical and representative of nine mice. This figure is available in colour online at http://wileyonlinelibrary.com/journal/ptr

Expressions of PPARα, ACC1, SREBP-1c, AMPK, FAS and CPT-1 in liver

At week 10, there was no significant difference in PPARα, ACC1 (acetyl-coenzyme A carboxylase-1), SREBP-1c, AMPK, FAS and CPT-1 (carnitine palmitoyl transferase 1) expression of mRNA in the HF group compared with the CON group (Table 4, Fig. 4C and 4D). After treatment, the mRNA level of PPARα was greater in all loquat-treated groups than in the HF group (Table 4), whereas the FAS mRNA level was lower in all the loquat-treated groups than in the HF group (Table 4 and Fig. 4D). Treatment with P2, P3 and ROS decreased the ACC mRNA level compared with the HF group, and treatment with P1 and P2 decreased the SREBP-1c mRNA level compared with the HF group. Treatment with P1 increased the AMPK mRNA level compared with the HF group ($p < 0.05$) (Table 4).

The phospho-AMPK (Thr172) protein contents in white adipose and liver tissue

There was no significant difference either in white adipose or liver tissue in the HF group compared with the CON group. After treatment, the contents of phospho-AMPK protein increased in the P2, P3 and ROS-treated groups compared with the HF group in adipose tissue ($p < 0.001, p < 0.05, p < 0.05$, respectively) (Fig. 5A). After treatment, the contents of phospho-AMPK protein increased in all the loquat and ROS-treated groups compared with the HF group in liver

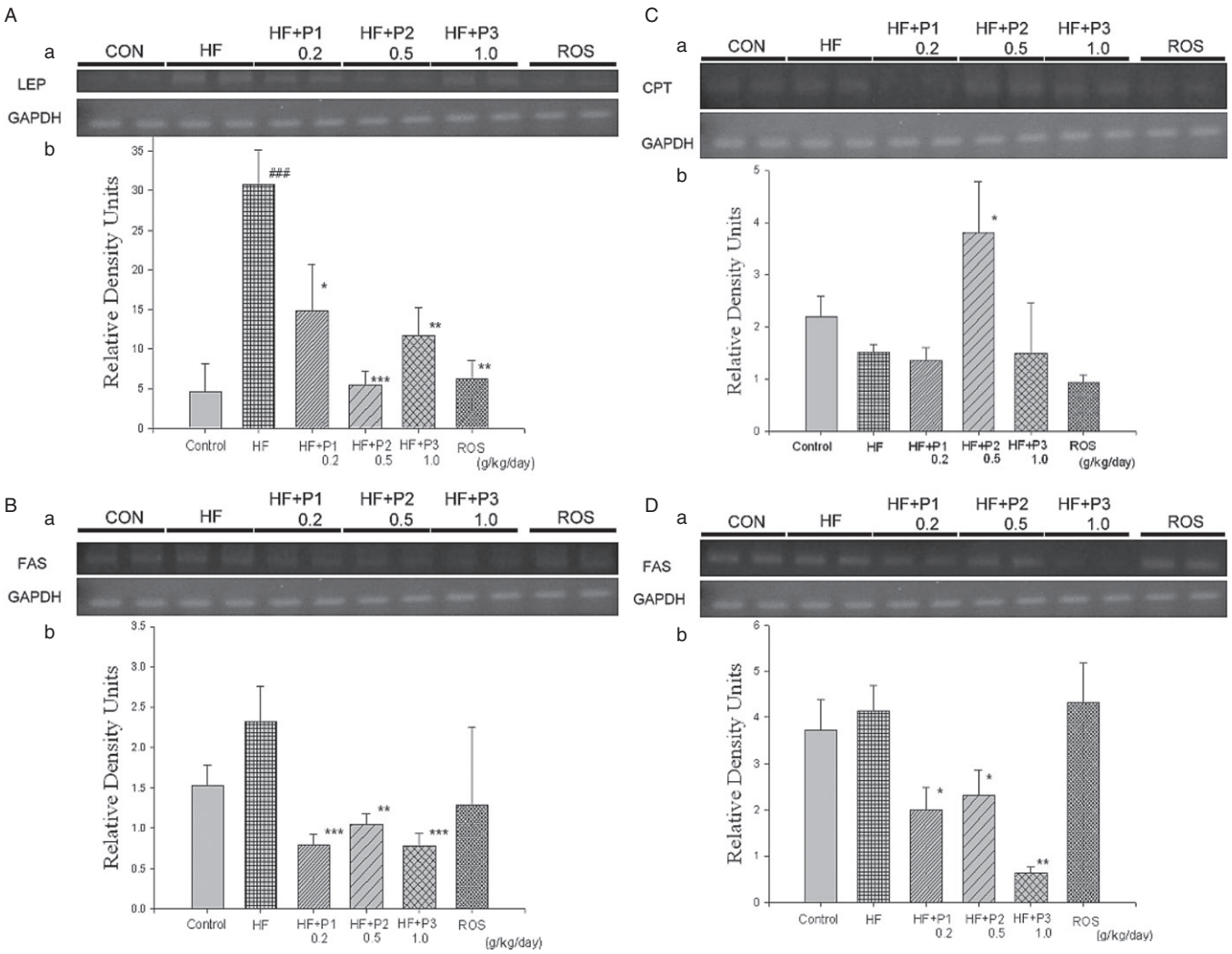


Figure 4. Semiquantitative RT-PCR analysis for (A) leptin, (B) FAS in adipose tissue and (C) CPT-1, (D) FAS mRNA expression in liver tissue of the mice by oral gavage extracts of *Eriobotrya japonica* for 4 weeks. All values are mean ± SE ($n = 9$). ### $p < 0.001$ compared with the control (CON) group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the high-fat + vehicle (distilled water) (HF) group. Total RNA (1 μg) isolated from tissue was reverse transcripted by MMLV-RT, 10 μL of RT products was used as template for PCR. Signals were quantitated by image analysis; each value was normalized by GAPDH. P1, P2, P3, extracts of *Eriobotrya japonica*.

tissue ($p < 0.05, p < 0.01, p < 0.001, p < 0.05$, respectively) (Fig. 5B).

Oral glucose tolerance test

The effect of extract of *E. japonica* on OGTT is shown in Fig. 6. In the mice treated with 0.2 g/kg extract of *E. japonica* the increased blood glucose levels were significantly suppressed after 30, 120 and 180 min. The extract of *E. japonica* (0.5 g/kg) significantly decreased blood glucose levels at 30, 60 and 90 min after glucose-loading when compared with the control.

DISCUSSION

The present study proved that loquat is effective in improving insulin resistance in a mouse model of Type 2 diabetes. It was demonstrated that loquat could influence both PPARα/PPARγ and mediated gene expression, leading to a suppression of body weight gain and a reduction of visceral fat accumulation, and decreased the levels of TG and FFA. It is speculated that loquat could not only regulate the PPARα-mediated pathway, which induces liver fatty acid oxidation, thereby lowering the blood lipid effect, but also influence the PPARγ-mediated pathway, which regulates adipocytokine gene expression, resulting in improved insulin resistance and effectively controlling hyperglycemia. Since a HF-induced obesity and Type 2 diabetes animal model was used, we speculated that loquat might be another choice for protecting against visceral obesity and Type 2 diabetes.

The study results demonstrated that loquat extract effectively controlled hyperglycemia and hyperinsulinemia by significantly reducing the blood glucose and insulin levels in C57BL/6J mice on a HF diet. Moreover, loquat exerted its effects similar to insulin sensitizers, which act as PPARα and PPARγ agonists.

PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily, and PPAR ligands include fatty acids and eicosanoids

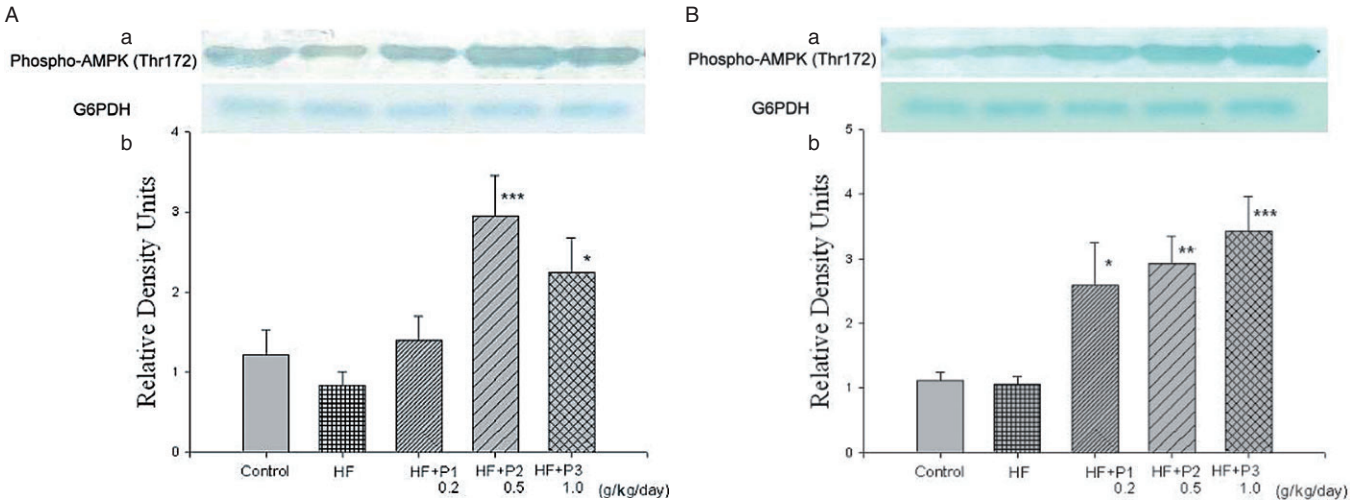


Figure 5. The phospho-AMPK (Thr172) protein contents in (A) white adipose and (B) liver tissue of the mice by oral gavage extracts of *Eriobotrya japonica* for 4 weeks. Protein was separated by 12% SDS-PAGE detected by western blot. All values are mean ± SE ($n = 9$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the high-fat + vehicle (distilled water) (HF) group by ANOVA coupled with Dunnett's test. P1, P2, P3, extracts of *Eriobotrya japonica*. This figure is available in colour online at http://wileyonlinelibrary.com/journal/ptr

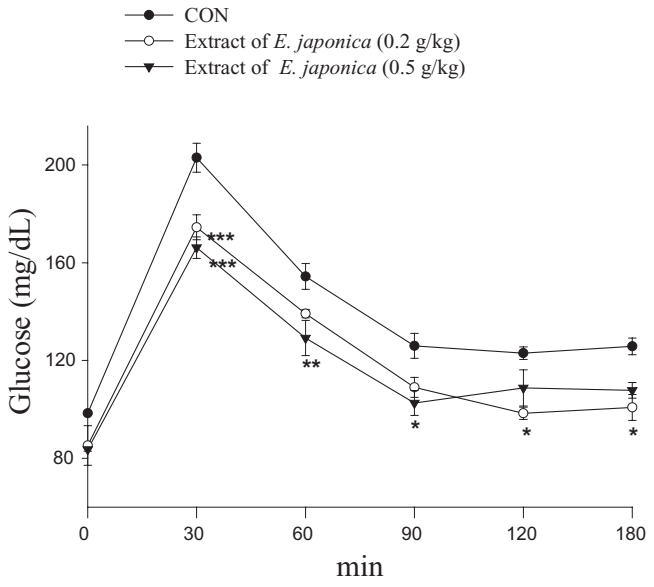


Figure 6. Effects of extract of *Eriobotrya japonica* on oral glucose tolerance in normal mice. Animals in all groups received oral glucose 30 min after the extract administration. Blood samples were collected and centrifuged at 3000 rpm for 10 minutes. Each point is the mean ± SE of 5–6 separate mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different compared with the control group in the same time by ANOVA coupled with Dunnett's test.

(Verges, 2004). PPARγ is mainly present in adipose tissue, and plays a key role in glucose homeostasis and the differentiation of fat cells (Chawla *et al*., 1994). PPARγ ligand activates PPARγ. Thiazolidinedione, a synthetic PPARγ ligand, significantly increased insulin sensitivity via PPARγ, actually displaying improved insulin sensitivity on a HF diet (Kubota *et al*., 1999). In Type 2 diabetes patients, TZD, a synthetic PPARγ ligand, significantly increased insulin sensitivity (Sood *et al*., 2000). Lee *et al*. (2003) have suggested that PPARγ ligand up-regulated the expression of genes involved in the glucose uptake of adipocytes and lipid storage of adipocytes. It was observed that loquat increased adipose tissue PPARγ mRNA expressions. Therefore, it was assumed that loquat used in our study behaved similarly to several PPARs ligands. Also, a significant decrease in blood TG and FFA by loquat extract is possibly mediated by increased expression of PPARs.

An improvement of insulin resistance is important in preventing the development of Type 2 diabetes. It is apparent that insulin sensitivity is regulated by adipocytokine, a wide group of bioactive proteins produced by adipocyte. The PPAR regulates a large amount of genes homeostasis involved in glucose and lipid metabolism. PPARγ plays an important role in insulin sensitivity. PPARγ represses the gene expression of leptin, which has been implicated in insulin resistance (Rangwala and Lazar, 2004). Thus, it was speculated that loquat might have the same mode of action as TZDs on the improvement of insulin sensitivity. Moreover, loquat prevented HF diet-induced increases in epididymal adipose depot mass and visceral fat weight. Since visceral obesity is thought to play a major role in the pathogenesis of metabolic syndrome (Kissebah, 1997), loquat is likely to be useful in the treatment of metabolic syndrome associated with visceral adiposity, such as hyperlipidemia, insulin resistance and Type 2 diabetes.

One of the findings of this study is that treatment of mice with loquat enhanced adiponectin levels. An increase in the level of adiponectin will be beneficial for insulin sensitizing and the control of obesity. Loquat could provide a unique therapeutic advantage in the regulation of the adipocyte function to improve insulin sensitivity.

Wu *et al.* (2003) demonstrated that treatment of rat adipocytes with the globular domain of adiponectin increased glucose uptake and AMPK activation. AMPK also plays an important role in the regulation of whole-body energy metabolism. Adiponectin activates AMPK in the liver, increasing glucose utilization and fatty acid oxidation, and inhibiting glucose production in the liver (Yamauchi *et al*., 2002). It is noteworthy and a novel finding of the present study that the treatment with loquat markedly increased the phosphorylation of

AMPK. Based on the reports of Wu *et al*. (2003) and Minokoshi *et al*. (2002), the AMPK phosphorylation by loquat may be linked to adiponectin and/or leptin secretion and gene expression. There are two possibilities that loquat could directly activate AMPK, or increase plasma adiponectin concentration by inducing AMPK activation. The target molecule for loquat should be identified.

The suppression of body fat accumulation was partly due to a reduction of energy intake and an increase of energy expenditure. Therefore, loquat extract may have potential in counteracting obesity. There is no consistent correlation between the doses of loquat and body weight, gene expressions and serum leptin level. After treatment with P2, there was a significant reduction in food intake, but no significant reduction in body weight (Fig. 2A). This is also contradictory to the dramatic change in CPT-1 expression levels in HF + P2 mice (Fig. 4C) because one would expect more energy expenditure would lead to an increased CPT-1 levels and phospho-AMPK protein contents in adipose tissue, thus leading to decreasing body weight. The resistance to the gain of body weight may be due to diverse mechanisms including food malabsorption or decreased total energy storage. Furthermore, loquat inhibited the high-fat diet-induced increases in epididymal weights. Theoretically speaking, loquat might be related to energy homeostasis if the relationship between WAT and energy storage is considered (Eckel, 1989). Nevertheless, loquat has no dose-dependent effect in body weight and CPT-1 expression levels. One possible explanation is due to the complex ingredients, good absorption, or good energy storage in the HF + P2 group. Moreover, the current result shows that the circulating levels of leptin do not always correlate well with gene expression in white adipose tissue. This is due to the fact that the production of the adipocytokines is affected by more complex factors *in vivo* (Rossi *et al*., 2005).

PPARα agonists are known to stimulate the mitochondrial oxidation and cellular uptake of FFA by modifying the expression of genes such as acyl-CoA synthetase gene and fatty acid transport protein gene (Schoonjans *et al*., 1995; Reddy and Hashimoto, 2001). Ersten *et al*. (1999) reported that, in a fasting state, the cellular uptake and oxidation of fatty acids liberated from fat tissues occurs with increased liver PPARα expression. Pharmacological stimulation with synthetic PPARα ligands such as fibrates also up-regulates the genes involved in fatty acid oxidation and the cellular uptake of FFA (Chou *et al*., 2002; Kim *et al*., 2003). PPARα ligands also increase the expression of the LPL gene (Staels *et al*., 1998), resulting in a hypotriglyceridemic effect. The results suggest that loquat improves plasma lipid profiles by stimulating fatty acid oxidation through PPARα-mediated pathways.

The results demonstrate that loquat substantially reduces adipose tissue mass in mice fed a HF diet. PPARα has been proposed to play a central role in a pathway that, under conditions of excess dietary energy, serves to minimize fat storage in the central organs at the expense of white adipose tissue (Unger and Orci, 2001). Due to the involvement of PPARα in energy homeostasis, we speculate that loquat may activate PPARα in liver, leading to reductions in adipose mass, body weight gain and hyperlipidemia in C57BL/6J mice fed a HF diet. Since lipids that accumulate in adipose tissue are largely derived from circulating triacylglycerols (Bourgeois *et al*., 1983) and the liver is a major target tissue for lipid and lipoprotein metabolism, loquat may be able to mobilize fat from adipose tissue by increasing fat catabolism in the liver. According to our results, the increased fatty acid oxidation and possibly decreased triacylglycerol synthesis in liver effectively decreased adipose tissue mass, resulting in the regulation of visceral obesity.

Moreover, aP2 deficiency was reported to protect mice with dietary or genetic obesity from the development of insulin resistance, hyperglycemia and hypertriglyceridemia (Hotamisligil *et al*., 1996; Uysal *et al*., 2000). Loquat results in decreased adipose tissue aP2 expression, thus has a favorable impact on multiple components of metabolic syndrome by protecting from diet-induced obesity, insulin resistance, Type 2 diabetes and fatty liver disease.

To clarify the mechanism for suppressing the development of obesity of loquat, the study focused on the expression of fatty acid and triacylglycerol metabolic enzymes. Fatty acid synthase (FAS) is the key enzyme in fatty acid synthesis (Wakil, 1989). Acetyl-CoA carboxylase (ACC) catalyses the formation of malonyl-CoA from acetyl-CoA. Acyl-CoA is partitioned between β-oxidation and triacylglycerol synthesis. When adequate energy is present, acyl-CoA is utilized mainly for triacylglycerol synthesis in the liver and adipocytes. Carnitine palmitoyl transferase I (CPT-1) is the rate-limiting enzyme for mitochondrial fatty acid oxidation, permitting their entry into the mitochondria for fatty acid oxidation (McGarry and Brown, 1997). The gene expression of these lipogenic enzymes is affected by the nutritional state. Insulin up-regulates the enzymes, and sterol regulatory elements binding protein-1c (SREBP-1c) may be a major transcriptional factor involved in the insulin regulation of the lipogenic enzyme expression. Loquat extract significantly decreased the FAS mRNA level in the liver and epididymal WAT compared with the HF group. This decrease may have contributed to the suppression of triacylglycerol accumulation in all the loquat-treated groups. ACC mRNA levels were found to be the rate-limiting enzyme for fatty acid synthesis (McGarry and Brown, 1997). The present study showed that the ACC mRNA level was markedly decreased in the liver after loquat treatment. The malonyl-CoA synthesized by ACC can be used for triacylglycerol synthesis in the liver. In addition, it is noteworthy that the CPT-1 mRNA level was significantly higher in the loquat-treated group than in the HF group, thus leading to the increased β-oxidation. These results suggest that such changes also suppressed triacylglyerol accumulation in the liver.

SREBP (SREBP-1a, -1c, and -2) are synthesized as precursors bound to the endoplasmic reticulum; they regulate the gene expression of enzymes involved in lipogenesis and cholesterol biosynthesis (Brown and Goldstein, 1999). They are released from the membrane by a sequential two-step proteolytic cleavage. This released mature form enters the nucleus and promotes transcription of target genes. SREBP-1c plays an important role in the response to activation of lipogenic enzyme expression, fatty acid synthesis and triglyceride accumulation (Horton *et al*., 1998; Shimomura *et al*., 2000). Ide *et al*. (2000) clearly showed that dietary sesamin decreased lipogenic enzyme gene expression

through the reduction of the SREBP-1c mRNA level and protein content of the precursor and its mature forms. Loquat could have modulated the release of the mature form and resulted in a reduction of the FAS mRNA level without the elevation of its mRNA level. The present study demonstrated that loquat significantly reduced the SREBP-1c mRNA level in liver tissue, suggesting that loquat would at least down-regulate the gene expression of these enzymes through a reduction in the SREBP-1 mRNA level, thus reversing these metabolic abnormalities.

Inactivation of acetylCoA carboxylase (ACC) decreases during malonyl-CoA synthesis; it shifts the balance toward fatty acid oxidation and theoretically decreases hepatic fat accumulation. The glucose-induced FAS mRNA level was down-regulated by AMPK (Foretz *et al*., 1998; Muoio *et al*., 1999), suggesting that loquat might accelerate direct or AMPK activation. Zhou *et al*. (2001) showed that metformin, which is a drug used for the therapy of Type 2 diabetes mellitus, down-regulated the SREBP-1c expression, thereby reducing the FAS mRNA level through AMPK activation. Therefore, there is a possibility that loquat inactivated these enzymes and/or down-regulated gene expression through AMPK activation.

Loquat reduces hepatic SREBP-1c levels and lowers lipid accumulation in the livers of HF mice. Loquat increased phospho-AMPK protein content. AMPK is a major cellular regulator of lipid and glucose metabolism. By increasing the phosphorylation of AMPK in liver and adipose tissue, loquat should decrease the hepatic ACC expression and thereby increase fatty acid oxidation and theoretically decrease hepatic fat accumulation. Increasing the phosphorylation of AMPK by loquat also suppresses the expression of SREBP-1c. Theoretically, the activation of AMPK provides an explanation for many of the pleiotropic beneficial effects of loquat.

Moreover, loquat reduced food intake also contributing to a reduced fat intake, which may have reduced the calorie intake. It is tempting to speculate, therefore, that metformin, TZDs, calorie restriction and loquat activate AMPK, and in turn modulate the action of SREBP-1c.

Loquat extract significantly suppressed the development of obesity and ameliorated hyperglycemia induced by HF diet feeding in mice. Loquat suppressed the mRNA levels of the enzymes involved in fatty acid and triacylglycerol synthesis accompanied by a reduction of the SREBP-1c mRNA level in liver tissue. These down-regulations with loquat treatment may contribute to the suppression of triacylglycerol accumulation.

As one of the possible mechanisms, AMPK activation would be associated with these changes, nevertheless, the AMP: ATP ratio is now being studied. Loquat has the potential of a unique therapeutic advantage to improve insulin sensitivity and the target molecule. Our findings provide a biochemical basis for the use of loquat, which could also have important implications for preventing obesity and diabetes.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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