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Regulation Effects of *Crataegus pinnatifida* Leaf on Glucose and Lipids Metabolism

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ABSTRACT: The leaf of *Crataegus pinnatifida* (Rosaceae) is commonly consumed either raw or cooked to improve digestion and promote blood circulation in China. To investigate the regulation effects of it on glucose and lipid metabolism, the flavonoids fraction was prepared and analyzed by HPLC and LC−MS. *In vivo*, at doses of 250 and 500 mg/kg, the flavonoids fraction showed inhibitory effects on TG and glucose absorption, accelerating effects on gastrointestinal transit but no effect on gastric emptying. *In vitro*, treatment of 3T3-L1 preadipocytes with 30 μg/mL flavonoids fraction significantly suppressed the accumulation of TG and free fatty acid. It also suppressed the gene expressions of C/EBPα, PPARγ, SREBP 1c, aP2 and adiponectin but did not affect that of leptin. *C. pinnatifida* leaf may be useful for type 2 diabetics and hyperlipidemics as a foodstuff.

KEYWORDS: *Crataegus pinnatifida*, glucose and lipid absorption regulation, adiponectin, PPARγ, 3T3-L1 cell, flavonoids

INTRODUCTION

Diabetes mellitus, often simply referred to as diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin or because cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger), which also cause various complications.[1]

There has been increasing evidence that the postprandial state is an important contributing factor to the development of diabetes. In recent years, much emphasis has been placed on elevation of postprandial glucose. The avoidance of sharp increases in blood glucose concentration following a meal is a well-established principle in antidiabetic therapy. Some drugs have been developed to improve postprandial hyperglycemia by inhibiting intestinal α-glucosidase activity, such as acabose, voglibose and miglitol.[2]

Plants have been a rich source of postprandial hyperglycemia regulation. For example, methanol extract of *Rosa damascena* Mill. flowers showed a strong inhibitory effect on α-glucosidase. Oral administration of this plant extract significantly decreased blood glucose after maltose loading in normal and diabetic rats in a dose-dependent manner.[3] Red pepper extract showed inhibitory effects on α-glucosidase and amylase, which can be used for dietary management of type 2 diabetes.[4] Mulberry leaf aqueous extract reduced peak responses of blood glucose.[5]

Crataegus pinnatifida Bge (Rosaceae), also called hawthorn tree, is a perennial woody plant distributed widely in China (e.g., Yunnan, Guangxi, Hebei, Henan provinces). The leaf is commonly consumed either raw or cooked. The leaf of *C. pinnatifida* has been used as a foodstuff to improve digestion, remove retention of food, promote blood circulation, and resolve blood stasis in China for a long period.[6] Several polyphenols such as chlorogenic acid, vitexin-4″-*O*-glucoside, vitexin-2″-*O*-rhamnoside, vitexin, rutin, hyperoside, isoquercitrin, and quercetin have previously been isolated from leaf of *C. pinnatifida*,[7−10] and their pharmacologic activities such as cardiac function improvement,[11] antihypertensive action,[12] and anti-inflammatory, gastroprotective, free radical scavenging, and antimicrobial activities[13] were also reported.

In previous research work, we found that *C. pinnatifida* leaf flavonoids fraction showed inhibitory effects of α-glucosidase and scavenging effect of free radical.[14] In the present study we aimed to evaluate the regulation effect of *C. pinnatifida* leaf extract on glucose and lipid metabolism. In addition, we found that *C. pinnatifida* leaf flavonoids fraction could significantly inhibit TG accumulation in mature 3T3-L1 adipocytes. The potential mechanism was discussed.

MATERIALS AND METHODS

Plant Materials and Reagents. *C. pinnatifida* leaf was collected from Qingshan Mountain, Ji County, Tianjin, China, and identified by Dr. Liu Hong. The voucher specimen was deposited at the Academy of Traditional Chinese Medicine of Tianjin University of TCM.

Reference standards, rutin, viterxin, rhamnosyl vitexin, hyperoside, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. The purities of compounds were over 98% by HPLC analysis based on a peak area normalization method. The standard solution of each flavonoid was prepared by dissolving in 50% (v/v) acetonitrile to the proper concentration and storage at 4 °C until analysis.

HPLC-grade acetonitrile (Merck KGaA, Darmstadt, Germany) and formic acid (Tedia, Fairfield, OH, USA) were utilized for the UHPLC

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Table 1. Gene-Specific Primers Used for Quantitative Real-Time RT-PCR

gene name	forward	reverse
transcription factors		
c/EBPα	5′-GGTTTCGGGTCGCTGGAT-3′	5′-CGGCCTGACTCCCTCATCT-3′
PPARγ	5′-TCATCTACACGATGCTGGCCT-3′	5′-TCACATGCAGTAGCTGCAGCT-3′
SREBP 1c	5′-GCGCCATGGACGAGCTG-3′	5′-TTGGCACCTGGGCTGCT-3′
aP2	5′-TGTTGTCCACGTTGCACCTGGG-3′	5′-CGCAAAGTGCGGGCCTGAAGAG-3′
adipocytokines		
adiponectin	5′-GGTCTGCCTGTCCCCATGAGTACC-3′	5′-CATCTTCGGCATGACTGGGCA-3′
leptin	5′-GGTACACGCTTCGCTGCGGC-3′	5′-GCTGATGGCTTGCTTCAGATCCATC-3′
GADPH	5′-AACTTTGGCATTGTGGAAGG-3′	5′-GGATGCAGGGATGATGTTCT-3′

analysis. Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals used were of analytical grade.

Sample Preparation. Hawthorn leaf was extracted with 70% EtOH for 2 h under reflux twice. Combined 70% EtOH extract was concentrated in a rotary evaporator *in vacuo* at 40 °C to give a crude extract. The crude extract was subjected to a D-101 resin column chromatography ($H_2O \rightarrow$ 70% EtOH $\rightarrow$ 95% EtOH) to give 3 fractions. The 70% EtOH fraction was used as the hawthorn leaf flavonoids fraction (4.6%).

HPLC Condition. Reversed phase high-performance liquid chromatography (HPLC) was used for analysis via an Agilent 1100 system with DAD detector. The hyperoside content was determined by HPLC analysis under the following conditions: column, Hypersil SB C18 (4.6 mm × 250 mm, 5 μm); UV detector at 363 nm; column temperature, 25 °C; mobile phase, CH_3CN−MeOH−tetrahydrofuran−0.5% HAc (1:1:19.4:78.6, v/v/v/v); flow rate 1.0 mL/min.

Determination of Total Flavonoids Content. Total flavonoids content was determined by the aluminum chloride colorimetric method.[15] An 65% EtOH solution (2 mL) of extracts or standard solution of hyperoside was added to a 25 mL volumetric flask containing 1 mL of 5% $NaNO_2$. After 6 min, 1 mL of 10% $AlCl_3$ was added. At the 6th min, 10 mL of 1 M NaOH solution was added and the total volume was made up to 25 mL with 65% EtOH. The solution was mixed well, and the absorbance was measured against prepared reagent blank at 510 nm. Sample was analyzed in triplicate.

LC−MS Analysis of Extract from *C. pinnatifida*. An aliquot of extract from *C. pinnatifida* leaves was dissolved in 50% (v/v) acetonitrile solution. After centrifugation at 14000 rpm for 10 min, the supernatant was transferred to an autosampler vial for UHPLC-DAD-Q-TOF-MS analysis. The injection volume was 1 μL.

UHPLC Conditions. UHPLC analyses were performed on an Agilent 1290 UHPLC instrument (Agilent, Waldbronn, Germany) coupled to a binary pump, a diode-array detector, an autosampler, and a column thermostat. The sample was separated on an Agilent RRHD SB-C18 column (1.8 μm, 100 × 2.1 mm; Agilent Technologies, made in USA). The mobile phase consisted of CH_3CN (solvent A) and H_2O (containing 0.1% formic acid; solvent B). A gradient program was used according to the following profile: 0−20 min, 10%-23% A; 20−30 min, 23%-70% A; 30−35 min, decreased to 10% A; 35−40 min, 10% A. The flow rate was 0.3 mL/min and the column temperature was 25 °C.

Q-TOF-MS Parameters. Aglient 6520 Q-TOF mass spectrometer (Agilent Corp., Santa Clara, CA, USA) was connected to the Agilent 1290 UHPLC instrument via an ESI interface. The acquisition parameters were as follows: drying gas (N_2) flow rate, 8.0 L/min; temperature, 350 °C; nebulizing, 30 psig; capillary, 4000 V; fragmentor, 200 V; skimmer, 65 V; OCT RF V, 750 V. Each sample was analyzed in both positive and negative ion mode to provide complementary information for molecular formulas and structural identification. Mass range recorded: m/z 100−1700.

3T3-L1 Cell Differentiation. 3T3-L1 cells (Cell Resource Center, IBMS, CAMS/PUMC) were maintained in low-glucose Dulbecco's modified Eagle's medium (Thermo Scientific, USA) supplemented with 10% calf serum (Thermo Scientific, USA). Confluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes according to the literature.[16] Briefly, 1 day after confluence, the cells were treated with high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Thermo Scientific, USA), 1 μM dexamethasone (Sigma, USA), 0.5 mM 3-isobutyl-methylxanthine (Sigma, USA) and 10 μg/mL insulin (Sigma, USA) for 3 days. At the same time, cells were treated with or without 30 μM sample DMSO solution (final DMSO concentration was 0.5%). Subsequently, cells were then switched to 10% FBS/DMEM media containing 5 μg/mL insulin for 3 more days, and then switched to 10% FBS/DMEM media without insulin. Differentiated cells were used when at least 95% of the cells showed an adipocyte phenotype by accumulation of lipid droplets.

Measurement of Lipolytic Activity. Confluent cultures of 3T3-L1 cells in 48-well plates (Costar, USA) were induced as previously described. The amounts of intracellular triglycerides and nonesterified fatty acid (NEFA) were determined with the triglycerides kit (BioSino Biotechnology and Science Inc., China) and NEFA-C kit (NEFA C-test, Wako, Japan) after cell lysis, respectively. TG and NEFA values were corrected by their protein content.

RNA Extraction and cDNA Synthesis. Confluent cultures of 3T3-L1 cells in 6-well plates (Costar, USA) were induced as previously described. Total RNA was isolated from 3T3-L1 adipocytes with TRIzol reagent (Invitrogen, USA). One microgram of RNA was reverse transcribed by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) to obtain cDNA according to the protocols provided by the manufacturer. Briefly, the total reaction volume was 20 μL with the reaction incubated as follows in a PE-480 HYBAID (Perkin-Elmer, USA): 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, hold at 4 °C.

Real-Time PCR. Real-time PCR was performed with an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) using Power SYBR Green PCR master mix (Applied Biosystems, USA) according to the protocols provided by the manufacturer. Briefly, PCR was performed in a final volume of 20 μL including 10 ng of sample cDNA, 5 μM specific forward and reverse primers, and 10 μL of Power SYBR green PCR Master Mix. PCR reactions consisted of an initial denaturating cycle at 95 °C for 10 min, followed by 40 amplification cycles: 15 s at 95 °C and 1 min at 60 °C. The primers used were as in Table 1. The relative amounts of PPARγ were calculated by using the $2^{-\Delta\Delta CT}$ method.[17] Analysis was carried out in triplicate.

Animals. Male Wistar rats (130−170 g) and ddY mice were purchased from Shanchuanhong Laboratory Animal Co., Ltd., Tianjin, China. The animals were housed at a temperature of 23 ± 2 °C and were fed a standard laboratory chow [provided by The Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College, China]. The animals were fasted for

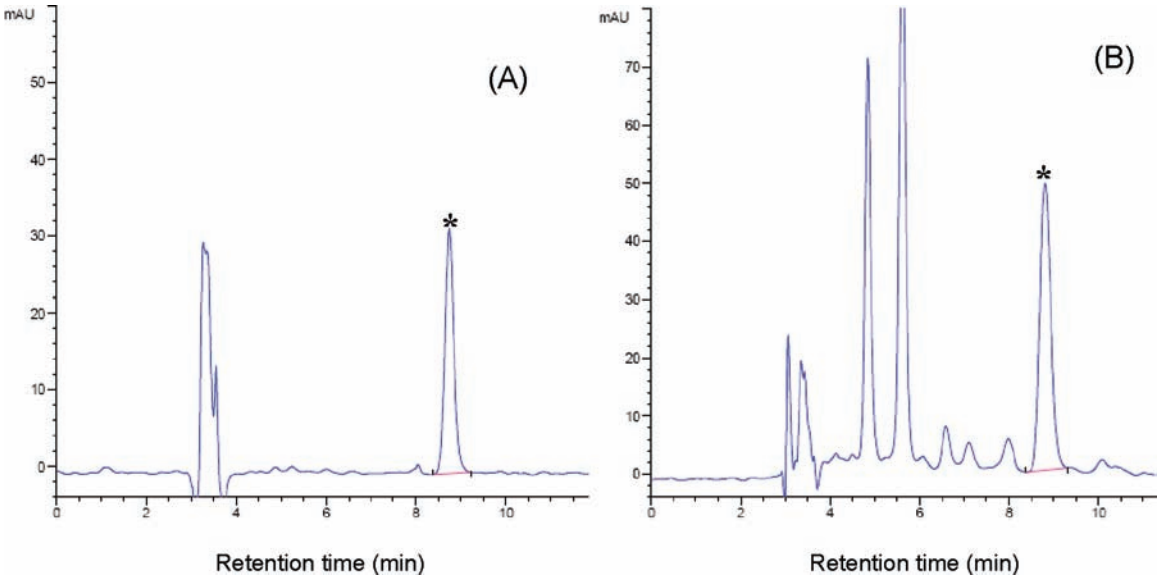

Figure 1. HPLC chart of *C. pinnatifida* leaf and the flavonoids fraction. (A) *C. pinnatifida* leaf; (B) *C. pinnatifida* leaf flavonoids fraction; (*) hyperoside; column, Hypersil SB C18 (4.6 mm ×250 mm, 5 μm); UV detector at 363 nm; column temperature, 25 °C; mobile phase, CH_3CN−MeOH−tetrahydrofuran−0.5%HAc (1:1:19.4:78.6, v/v); flow rate 1.0 mL/min.

24−26 h prior to the experiment, but were allowed free access to tap water. The experimental protocol was approved by the Experimental Animal Research Committee at Tianjin University of TCM.

Effects on Serum Glucose Levels in Sucrose-Loaded Rats. The Male Wistar rats were divided into 6 groups (normal, control, sample 125 mg/kg, 250 mg/kg, 500 mg/kg and positive control) of 6−8 animals each. The test samples were suspended in 5% acacia solution (5 mL/kg), and then given orally to the rats. Normal and control groups were administered 5% acacia water solution only. Thirty minutes thereafter, a water solution (5 mL/kg) of sucrose (1.0 g/kg) was administered orally, and normal group was administered 5 mL/kg distilled water. Blood (*ca.* 0.4 mL) was collected from infraorbital venous plexus under ether anesthesia at 0.5, 1.0, and 2.0 h after test sample administration. The serum glucose levels were determined by the glucose-oxidase method (Glu, BioSino Biotechnology and Science Inc.). 5% acacia water solution of tolbutamide, a potassium channel blocker, sulfonylurea oral hypoglycemic drug (purchased from Tianjin Yifang S&T Co. Ltd.) was used as positive control (25 mg/kg, po).

Effects on Serum Triglyceride Levels in Olive Oil-Loaded Mice. The male ddY mice were divided into 6 groups (normal, control, sample 125 mg/kg, 250 mg/kg, 500 mg/kg and positive control) of 8 animals each. After animals were fasted for 20 h, the test sample suspended in 5% acacia solution was given orally, and normal and control groups were administered 5% acacia water solution only. Thirty minutes later, olive oil was given (5 mL/kg, po), and normal group was administered 5 mL/kg distilled water. Blood samples were collected from the infraorbital venous plexus at 2, 4, and 6 h after feeding of olive oil. Serum TG levels were determined using a commercial kit (TG, BioSino Biotechnology and Science Inc.). 5% acacia water solution of orlistat, a pancreatic lipase inhibitor (purchased from Tianjin Yifang S&T Co. Ltd.), was used as positive control (12.5 mg/kg, po).

Effect on Gastric Emptying in Rats. The male Wistar rats were divided into 4 groups (normal, control, sample 250 mg/kg and 500 mg/kg) of 8 animals each. A solution of 10% glucose containing 0.05% phenol red as a marker was given orally (0.75 mL/rat) to rats. Thirty minutes later, the animals were sacrificed by cervical dislocation under ether anesthesia. The abdominal cavity was opened, and the gastresophageal junction and pylorus were clamped, then the stomach was removed, weighed and placed in 0.1 M NaOH (50 mL) and homogenized. The suspension was allowed to settle for 1 h at room temperature, and 5 mL of the supernatant was added to 20% trichloroacetic acid (0.5 mL) and then centrifuged at 3000 rpm for 20 min. The supernatant (2 mL) was mixed with 0.5 M NaOH (2 mL), and the amount of phenol red was determined from the absorbance at 560 nm. Phenol red recovered from animal scarified immediately after the administration of 10% glucose containing 0.05% phenol red was used as a standard (0% emptying). The gastric emptying (%) during the 30 min period was calculated by the following equation: gastric emptying (%) = (1 − amount of test sample/amount of standard) × 100. The test sample was given orally by a metal orogastric tube 30 min prior to the administration of 10% glucose containing 0.05% phenol red.

Effect on Gastrointestinal Transit. The male ddY mice were divided into 3 groups (control, sample 250 mg/kg and 500 mg/kg) of 11−12 animals each. Mice received orally a black marker (5% charcoal suspension in 5% gum arabic, 1.0 g/kg), and 30 min later the mice were killed and the gastrointestinal tract was removed. The distance traveled by the marker was used to calculate the percentage of the total length of the small intestine from pylorus to cecum.

Statistical Analysis. Values are expressed as mean ± SD. All the grouped data were statistically performed with SPSS 11.0. Significant differences between means were evaluated by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

In the Chinese Pharmacopoeia, the quality of *C. pinnatifida* leaf was evaluated according to the contents of hyperoside.[18] Hyperoside contents of *C. pinnatifida* leaf and its flavonoids fraction were determined by HPLC method. The retention time of hyperoside was 8.382 min with a linearity in a range of 8−320 $\mu g \cdot mL^{-1}$. The purity of hyperoside peak was checked by HPLC/DAD (Agilent 1100 G1315A DAD detector); no overlapped peak was detected at 220, 254, and 280 nm (Figure 1). The regression equation was y (area) = 17997 × (concentration, $\mu g \cdot mL^{-1}$) + 35.17 with the correlation coefficient $r = 0.9995$. Intraday precision was less than 4.0%. The average recovery fell in the range of 96.0−104.1% (RSD < 2.0%, $n = 5$).

Table 2. ESI-TOF-MS Data of Compounds Identified from *C. pinnatifida* Leaf Flavonoids Fraction

				m/z				
peak	RT (min)	formula	selected ion	exptl	calcd	error (ppm)	UV (λ_{max}/nm)	tentative identification
1	3.50	$C_{32}H_{36}O_{18}$	$[M-H]^-$	707.1792	707.1829	5.25	280, 320	
			$[M+Na]^+$	731.1797	731.1794	−0.35		
2	4.79	$C_{30}H_{26}O_{12}$	$[M-H]^-$	577.1338	577.1351	2.38	238, 328	isovitexin-2″-*O*-rhamnoside
			$[M+H]^+$	579.1504	579.1497	−1.1		
3	5.53	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0714	289.0718	1.25	275, 330	cyanidol
			$[M+H]^+$	291.0869	291.0863	−2.13		
4	7.80	$C_{33}H_{40}O_{20}$	$[M-H]^-$	755.2039	755.2040	0.12	258, 355	rutin-4‴-*O*-rhamnoside
			$[M+H]^+$	757.2210	757.2186	−3.19		
5	8.40	$C_{26}H_{44}O_{16}$	$[M-H]^-$	611.2552	611.2557	0.8	240, 365	eriodietyol-5,3′-diglucoside
			$[M+Na]^+$	635.2531	635.2522	−1.43		
6	9.36	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.1449	609.1461	1.92	258, 357	
			$[M+H]^+$	611.1613	611.1607	−1.02		
7	9.76	$C_{27}H_{30}O_{15}$	$[M-H]^-$	593.1502	593.1512	1.72	270, 340	vitexin-4″-*O*-glucoside
			$[M+H]^+$	595.1673	595.1657	−2.56		
8	10.40	$C_{27}H_{30}O_{14}$	$[M-H]^-$	577.1554	577.1563	1.61	270, 344	vitexin-2″-*O*-rhamnoside
			$[M+H]^+$	579.1721	579.1708	−2.21		
9	10.89	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.1443	609.1461	2.96	262, 344	rutin
			$[M+H]^+$	611.1613	611.1607	−0.99		
10	11.13	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.0866	463.0882	3.42	258, 355	hyperoside
			$[M+H]^+$	465.1036	465.1028	−1.73		
11	11.65	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.0865	463.0882	3.71	258, 355	quercetin-4′-*O*-glucoside
			$[M+H]^+$	465.1034	465.1028	−1.46		
12	13.62	$C_{27}H_{30}O_{15}$	$[M-H]^-$	593.1484	593.1512	4.77	266, 348	vitexin-2″-*O*- glucoside
			$[M+H]^+$	595.1662	595.1657	−0.83		
13	14.07	$C_{30}H_{32}O_{17}$	$[M-H]^-$	663.1535	663.1567	4.72	266, 350	
			$[M+H]^+$	665.1716	665.1712	−0.61		
14	19.15	$C_{20}H_{18}O_9$	$[M-H]^-$	401.0873	401.0878	1.23	266, 348	
			$[M+Na]^+$	425.0850	425.0843	−1.83		
15	22.26	$C_{15}H_{10}O_7$	$[M-H]^-$	301.0349	301.0354	1.5	258, 373	quercetin
			$[M+H]^+$	303.0505	303.0499	−1.77		

Hyperoside contents of *C. pinnatifida* leaf and its flavonoids fraction were 0.16% and 2.35%, respectively.

Total flavonoids contents of *C. pinnatifida* leaf flavonoids fraction were determined by aluminum chloride colorimetric method. The standard sample of hyperoside showed a linearity in the range of 0.2−1.0 mg. The regression equation was Y (mg) = 1.2949 × (A) + 0.0091, with the correlation coefficient r = 0.9994. Intraday precision was less than 1.8%. The average recovery was 102.6% (n = 6). Total flavonoids contents of *C. pinnatifida* leaf flavonoids fraction were 74.9 ± 0.4% (w/w).

To characterize some major compounds in *C. pinnatifida* leaf flavonoids fraction, a flavonoid profiling was obtained by using UHPLC-DAD-Q-TOF-MS. Based on retention time, UV and MS spectra of the reference compounds (Table 2) and the data in the literature,[19] 15 compounds were detected, and among them, 11 were tentatively identified (Figure 2).

We examined the effects of the *C. pinnatifida* leaf flavonoids fraction on plasma glucose levels in sucrose-loaded rats. As shown in Table 3, the flavonoids fraction significantly inhibited the plasma glucose increase at doses of 500 mg/kg after 0.5 h administration of sucrose. Both 250 mg/kg and 500 mg/kg suppressed the plasma glucose elevation although the inhibition was not statistically significant.

The effects of the *C. pinnatifida* leaf flavonoids fraction on plasma TG levels increase in olive oil-loaded mice were examined. As shown in Table 4, it significantly inhibited plasma TG elevation at doses of 250 and 500 mg/kg.

In our previous studies,[14] the inhibitory effects of *C. pinnatifida* leaf flavonoids fraction on enzyme activities of rat intestinal α-glucosidase (sucrase) and porcine pancreatic lipase were examined, and the IC_{50} were 243.8 μg/mL and 324.0 μg/mL, respectively, which suggested that the sucrase and lipase inhibitory activities might be involved in the inhibitory effects of the extract of *C. pinnatifida leaf* on blood glucose and triglyceride elevation in sucrose- and olive oil-loaded animals.

Improvement of gastrointestinal transit is believed to be beneficial for the treatment of postprandial hypertriglyceridemia in diabetic patients[20] possibly due to a decrease in the rate of gluconeogenesis (Table 5). In gastric emptying experiment, extract of *C. pinnatifida* leaf flavonoids fraction showed no effect on gastric emptying (Table 6). At the dosage of 500 mg/kg, it was found to exhibit accelerating effects on gastrointestinal transit in mice.

Postprandial lipidemia is prolonged and exaggerated in patients with type 2 diabetes mellitus, with an accumulation of atherogenic triglyceride-rich lipoprotein remnants. *C. pinnatifida* leaf has a long history of use in traditional Chinese medicine, as a treatment

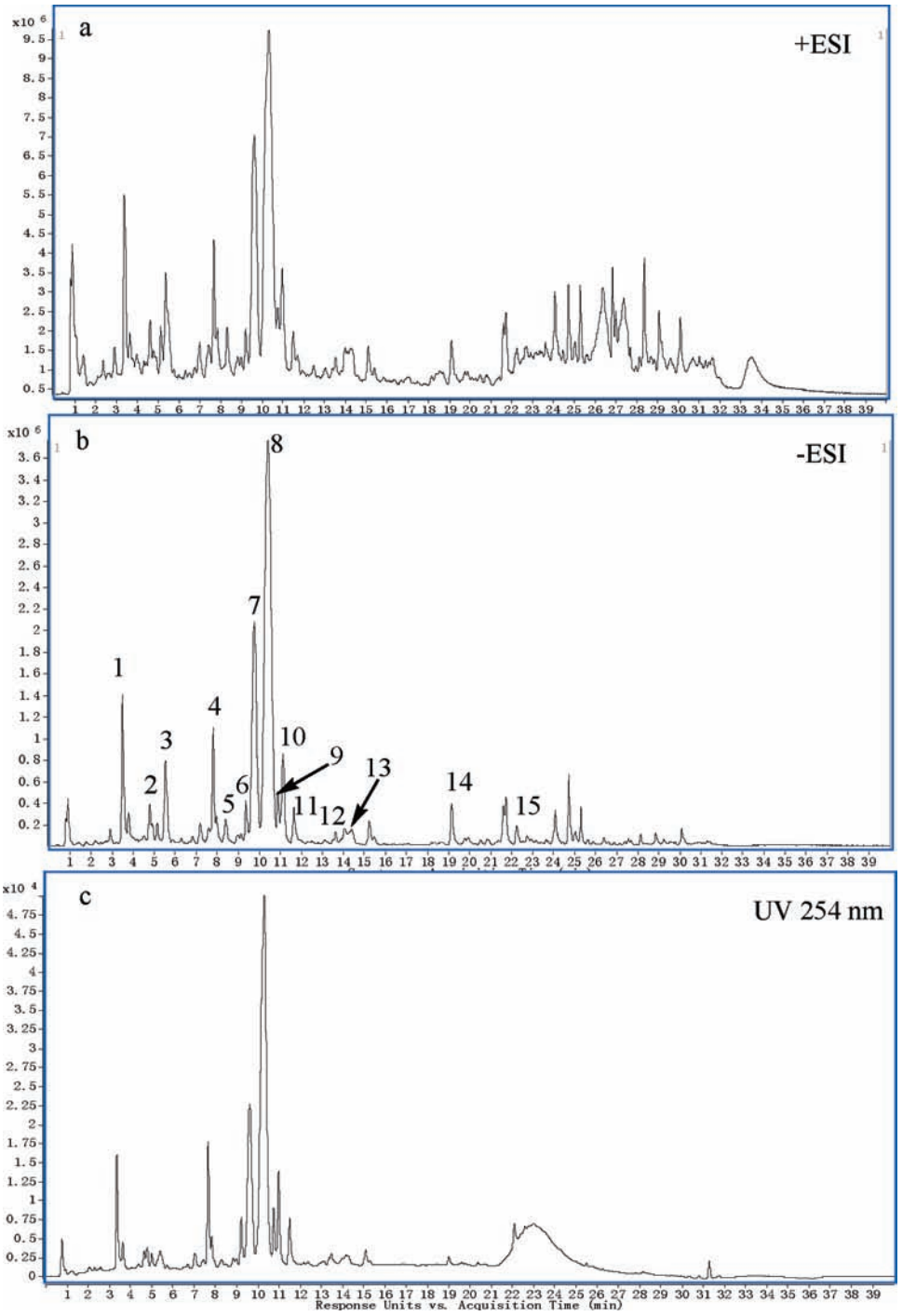

Figure 2. Chromatograms of *C. pinnatifida* leaf flavonoids fraction: (a) Total ion current (TIC) chromatogram in positive mode, (b) TIC in negative mode, (c) UV (254 nm) chromatogram.

for digestive disorders. In this study, we suggested that the flavonoids fraction may contain the effective constituents responsible for glucose-, lipid-absorption inhibition and gastrointestinal transit acceleration.

Adipocytes play an important role in regulating adipose mass, obesity and diabetes, in relation not only to lipid homeostasis and energy balance but also to secret transcription factors. Adipocytes are derived from mesodermal multifunctional stem cells. The mature process of adipocytes contains several steps including somatic stem cell, mesenchymal stem cell, preadipocyte and mature adipocyte.[21] Except the mature adipocyte, the other states are jointly called preadipocyte. Differentiation from preadipocytes to adipocytes results in morphological, and biochemical changes where the cells become rounded in shape, and begin to accumulate triacylglycerol and lipid vacuoles.[22]

Various hormones and growth factors that affect adipocyte differentiation in a positive or negative manner have been identified. In the process of differentiation, preadipocyte is regulated by two major transcription factors, peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT/enhancer binding protein

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Table 3. Inhibitory Effects of *C. pinnatifida* Leaf Flavonoids Fraction on Plasma Glucose Elevation in Sucrose-Loaded Rats

	dose (mg/kg, po)	*n*	plasma glucose levels[a] (mg/dL) 0.5 h	1 h	2 h
normal		6	84.3 ± 8.9**	93.0 ± 14.9**	95.1 ± 7.2
control	0	7	190.2 ± 12.7	167.3 ± 11.9	106.4 ± 10.0
sample	125	8	188.3 ± 5.4	155.1 ± 4.8	113.5 ± 7.2
	250	8	182.0 ± 11.0	158.2 ± 24.9	105.1 ± 11.3
	500	6	161.8 ± 25.0*	146.0 ± 30.1	110.8 ± 7.1
tolbutamide	25	6	138.1 ± 3.5**	106.3 ± 6.3**	102.3 ± 6.5

[a] Values represent the means ± SE. Significantly different from the control group, $^{**}p < 0.01$, $^{*}p < 0.05$.

Table 4. Inhibitory Effects of *C. pinnatifida* Leaf Flavonoids Fraction on Plasma TG Elevation in Olive Oil-Loaded Mice

	dose (mg/kg, po)	*n*	plasma TG levels[a] (mmol/L) 2 h	4 h	6 h
normal		8	1.83 ± 0.16**	1.84 ± 0.33**	1.35 ± 0.12**
control	0	8	10.42 ± 2.48	9.59 ± 3.95	7.08 ± 3.11
sample	125	8	5.38 ± 3.09*	6.89 ± 5.53	4.82 ± 4.30
	250	8	5.06 ± 1.46**	2.92 ± 2.66**	2.07 ± 1.94**
	500	8	2.24 ± 0.65**	1.03 ± 0.16**	1.06 ± 0.24**
orlistat	6.25	8	3.45 ± 0.84**	2.33 ± 1.32**	2.01 ± 0.98**

[a] Values represent the means ± SE. Significantly different from the control group, $^{**}p < 0.01$, $^{*}p < 0.05$.

(C/EBP).[23] Expression of PPARγ and C/EBP activates the expression of genes involved in adipogenesis such as aP2 (activating protein 2), LPL (lipoprotein lipase), PEPCK (phosphoenolpyruvate carboxykinase) and SREBP 1c (sterol regulatory element-binding protein 1c)[24] to trigger cell differentiation progress, and finally result in synthesis of TG (triglyceride). Activating protein 2 (aP2) is a mammalian transcription factor and a carrier protein for fatty acids. In the mature adipocyte, expression of aP2 mRNA and protein levels markedly increased following the increase in PPARγ gene expression, while the increase in aP2 expression occurred earlier than that of the C/EBPα expression. It is likely that aP2 protein levels are regulated at the transcriptional level by intranuclear PPARγ in mammals.[25]

Accordingly, adipocytes are emerging as a potential therapeutic target for obesity, type 2 diabetes, and cardiovascular disease. Recently, PPARγ ligands have been established as a target for type 2 diabetes therapy.[26] The medications that target PPARγ reduce insulin resistance by inducing differentiation of preadipocytes into small adipocytes.

3T3-L1 preadipocytes were treated with the flavonoids fraction at doses of 30 μg/mL. Fourteen days after induction of differentiation, the cells were fixed and stained with Oil Red O. In normal groups, 3T3-L1 preadipocytes were cultured without induction medium or any samples, and no significant lipid droplets were observed. In control groups, cells were cultured with induction medium for 14 days, and many lipid droplets were

Table 5. Promotion Effects of *C. pinnatifida* Leaf Flavonoids Fraction on Small Intestinal Transit

	dose (mg/kg, po)	*n*	promote rate[a] (%)
control	0	12	52.2 ± 12.8
sample	250	12	61.8 ± 12.7
	500	11	63.7 ± 6.5*

[a] Values represent the means ± SE. Significantly different from the control group, $^{*}p < 0.05$.

Table 6. Promotion Effects of *C. pinnatifida* Leaf Flavonoids Fraction on Gastric Emptying

	dose (mg/kg, po)	*n*	promote rate[a] (%)
normal		8	100 ± 11.6
control	0	8	91.3 ± 3.1
sample	250	8	86.0 ± 7.8
	500	8	83.9 ± 12.3

[a] Values represent the means ± SE.

observed. Compared to untreated cells, the flavonoids fraction significantly suppressed the accumulation of TG (42.75 ± 6.00%) and free fatty acid (50.22 ± 2.89%) in mature 3T3-L1 cells respectively. We also tested the activity of major constituents vitexin-4″-*O*-glucoside (14.8%, HPLC analysis) and vitexin-2″-*O*-rhamnoside (45.1%) in *C. pinnatifida* leaf flavonoids fraction. Compared with the flavonoids fraction, both of them showed weaker inhibitory effect on TG accumulation in mature 3T3-L1 cells at the dose of 30 μg/mL (vitexin-4″-*O*-glucoside, 32.82 ± 4.31%; vitexin-2″-*O*-rhamnoside, 28.54 ± 5.62%). But, minor constituents rutin and hyperoside (each less than 5%) which were also isolated from *Abelmoschus manihot* flowers showed strong inhibitory effect (64.3 ± 6.0% and 79.1 ± 9.8%, respectively) using the same active evaluate system in our previous research.[27] The above data suggested that its biological activities are likely due to a synergistic action of multiple components, a phenomenon supported by traditional Chinese medicine theory and by modern combinatorial chemistry practice.

In the present study, the flavonoids fraction suppressed the gene expressions of C/EBPα, PPARγ, SREBP 1c, aP2 and adiponectin, but did not affect that of leptin (Figure 3).

Maturation of 3T3-L1 preadipocytes is accompanied by transcriptional remodeling and activation of numerous adipose function-related genes. In the induction process, the expression of adipogenesis inhibitor preadipocyte factor 1 (Pref-1) is first down regulated by stimulatory agents (such as insulin, dexamethasone and IBMX) while concomitantly C/EBPβ and δ expressions are increased.[28] Successively, key transcription factors PPARγ and C/EBPα are activated. Between days 3 and 4 after induction, C/EBPα undifferentiated protein (CUP) activity decreases precipitously and reaches a minimum. At this time, there is a concomitant increase in transcription of the C/EBPα gene.[29] We partly revealed that the inhibitory effect of hawthorn leaf flavonoids fraction on TG accumulation in 3T3-L1 was concerned with PPARγ, C/EBPα, SREBP 1c and aP2 gene expression down regulation. This result is in agreement with the "multi-component and multi-target action" therapy theory of traditional Chinese medicine.[14]

Adiponectin is a collagen-like protein that is solely secreted by adipocytes and acts as a hormone with anti-inflammatory and

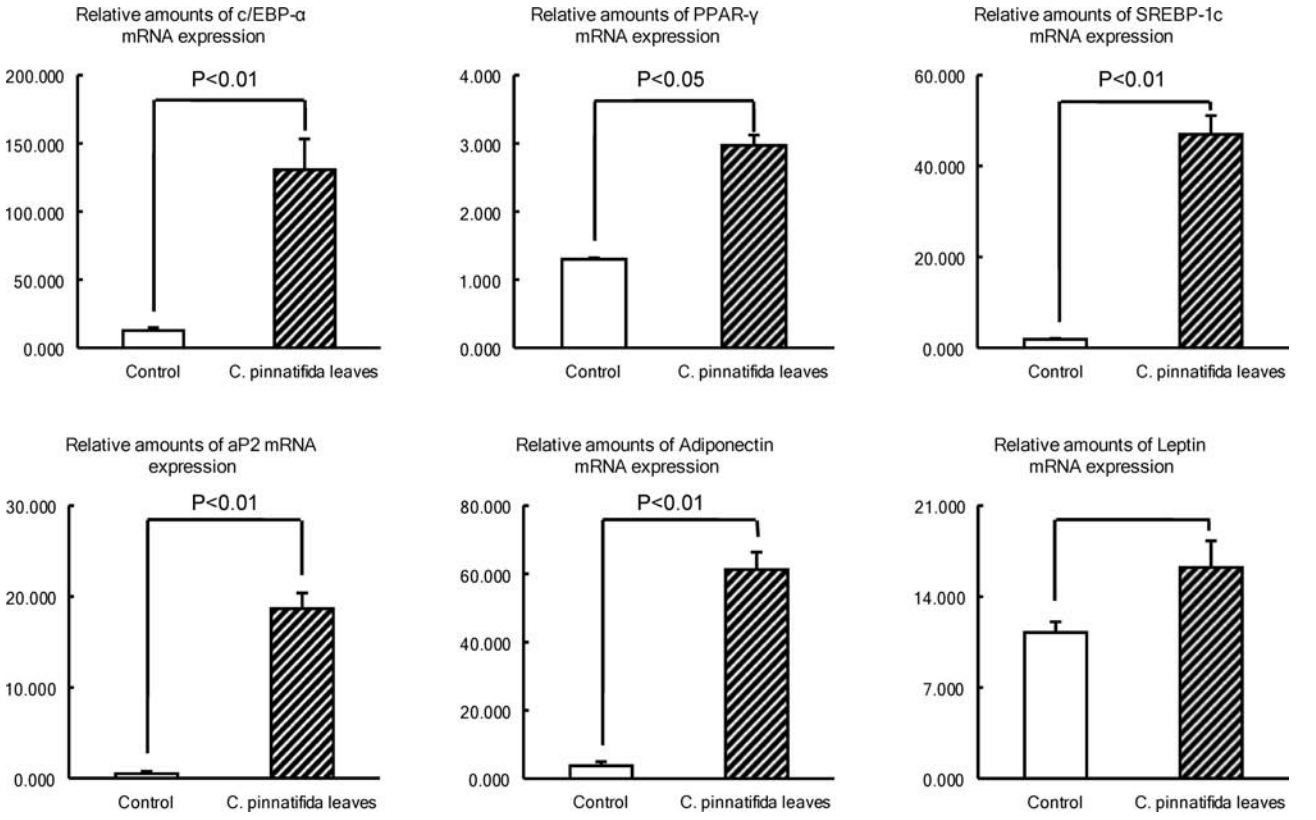

Figure 3. Inhibitory effects of mRNA expression in mature 3T3-L1 cells. 3T3-L1 cells were seeded in 6-well plates and induced as previously described. Briefly, fourteen days after induction of differentiation, total RNA was isolated as previously described (Materials and Methods). We used the $2^{-\Delta\Delta CT}$ method for analysis of real-time quantitative PCR data. Values represent the mean ± SD of three determinations.

insulin sensitizing properties. Adiponectin may decrease the risk of type 2 diabetes, including suppression of hepatic gluconeogenesis, stimulation of fatty acid oxidation in the liver, stimulation of fatty acid oxidation and glucose uptake in skeletal muscle, and stimulation of insulin secretion.[30] In the present study, we observed an increase in adiponectin levels in differentiated 3T3-L1 adipocytes treated with hawthorn leaf flavonoids fraction.

In conclusion, this study presents for the first time that hawthorn leaf flavonoids fraction might exert its antidiabetic effects through enhanced adiponectin secretion, c/EBPα, PPARγ, SREBP 1c, aP2 translocation. Hawthorn leaf flavonoids fraction also exhibited significant inhibitory effects on absorption of triglyceride and glucose in olive oil-loaded mice and sucrose-loaded rat, respectively. In addition, hawthorn leaf flavonoids fraction showed accelerating effects on gastrointestinal transit, but no effect on gastric emptying in mice. Hawthorn leaf may be useful to develop potent lead compounds for the treatment of diabetes through promoting the adiponectin secretion regulation of glucose and lipid absorption.

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