Biomedicine & Pharmacotherapy 106 (2018) 355–362

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Heshouwu (*Polygonum multiflorum* Thunb.) ethanol extract suppresses pre-adipocytes differentiation in 3T3-L1 cells and adiposity in obese mice

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ARTICLE INFO

*Keywords:*
Adiposity
Adipogenesis
Anti-obesity
Heshouwu
*Polygonum multiflorum* Thunb.

ABSTRACT

This study investigated whether Heshouwu (*Polygonum multiflorum* Thunb.) root ethanol extract (PME) has anti-obesity activity using 3T3-L1 cells and high-fat diet (HFD)-induced obese mice. Treatment with PME (5 and 10 μg/mL) dose-dependently suppressed 3T3-L1 pre-adipocyte differentiation to adipocytes and cellular triglyceride contents. In addition, PME inhibited mRNA and protein expression of adipogenic transcription factors such as CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ), which led to down-regulation of fatty acid synthase gene expression. After feeding mice PME (0.05%) with HFD for 12 weeks, their visceral fat mass, size and body weight were significantly reduced compared with the HFD group. Furthermore, PME supplementation significantly up-regulated the *PPARα*, *CPT1*, *CPT2*, *UCP1* and *HSL* mRNA levels compared with the HFD group, whereas it down-regulated expression of the *PPARγ* and *DGAT2* genes. Finally, HFD increased serum leptin, insulin, glucose and insulin and glucose levels; however, PME reversed these changes. These results demonstrated that PME might relieve obesity that occurs *via* inhibition of adipogenesis and lipogenesis as well as through lipolysis and fatty acid oxidation in 3T3-L1 cells and HFD-induced obese mice.

## 1. Introduction

Obesity is a worldwide epidemic with multiple associated health problems, including type 2 diabetes mellitus, dyslipidemia and steatosis [1]. Obesity is characterized by increased adipose tissue mass that results from both increased adipocyte number (hyperplasia) and increased adipocyte size (hypertrophy) [2,3]. The growth and expansion of adipose tissue have been found to be directly associated with the dysregulation of various transcription factors, such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα), which play important roles in maintaining the size and number of fat cells during adipogenesis [4,5]. Increased accumulation of adipose tissue is associated with deleterious disturbance including excess fatty acid secretion, increased production of inflammatory cytokines, and abnormal adipocyte hormone signaling, resulting in insulin resistance [1]. Therefore, targeting both adipogenesis and triglyceride (TG) synthesis genes may have promising effects in controlling adipose tissue growth and numerous metabolic disorders in obesity [4,6].

Many drugs have been used to treat obesity; however, most of those that have been approved and marketed have been withdrawn because of serious side effects [7]. A number of natural products, including crude extracts and isolated pure natural compounds can reduce body weight and prevent diet-induced obesity, which may be an alternative strategy for developing safe anti-obesity agents [8].

*Polygonum multiflorum* Thunb. has been used as a traditional medicinal herb in East Asia including China and Korea to treat many diseases such as constipation, early graying of the hair and hyperlipidemia [9,10]. The herb is known as Heshouwu in China and Fo-ti in North America [10,11]. Pharmacological studies and clinical practice have demonstrated that the roots of Heshouwu possess various biological activities, including anti-tumor, anti-HIV, antibacterial, hemostatic, spasmolytic, analgesic, immunological, and antioxidative properties, and that it lowers blood cholesterol and improves learning and memory [12–15]. Phenolic constituents such as anthraquinones, stilbene glycosides and tannins are thought to be the major active components [12]. However, few studies have compared the effects *in vitro* and *in vivo* of Heshouwu root ethanol extract (PME) against obesity.

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https://doi.org/10.1016/j.biopha.2018.06.140
Received 8 February 2018; Received in revised form 5 June 2018; Accepted 25 June 2018

Therefore, we investigated whether PME can modulate differentiation of 3T3-L1 adipocytes *in vitro*, then examined the effects of PME (0.05%) on fat mass and body weight in HFD-induced obese mice, which dose was equivalent to 1% whole Heshouwu/kg of diet. According to Chinese Pharmacopoeia, 3–6 g of raw Heshouwu is recommended for human clinical application, which corresponds to 0.625–1.25 g/kg for mice [16]. Li et al. [17] showed that 30 times (10 g/kg for rat in the experiment) the clinical dosage exerted no liver toxicity, which suggested the safety of raw Heshouwu with normal dosage. In this study, mice that received PME at a relatively low dose (61 mg/kg) showed lowered serum levels of the hepatotoxic marker, AST, compared to the HFD group.

## 2. Materials and methods

### 2.1. Preparation of PME

Roots of Heshouwu were purchased from Dong-Bu Herbal Market (Suncheon, Korea). For *in vitro* tests, Heshouwu (500 g) was extracted repeatedly with 70% ethanol (3 × 5 L) by exhaustive maceration under sonication for 2 h. The supernatant was then filtered using Whatman number 2 filter paper before being transferred into preweighed containers. Next, the fluid was concentrated with a rotary evaporator and freeze-dried to yield the crude extract (126 g). For the *in vivo* test, we extracted roots of Heshouwu using fermentation ethanol (95%, Ethanol Supplies World Co., Ltd., Jeonju, Korea), while the rest of the protocol was the same as for the *in vitro* test (yield 5.4%). Total polyphenol and flavonoid contents were determined as milligrams of tannic acid equivalent (TE) and rutin equivalent (RE). The total polyphenolic compound and flavonoid contents of PME were 229.59 ± 7.46 mg TE/g and 5.62 ± 0.17 mg RE/g, respectively.

### 2.2. Cell culture and SRB assay

3T3-L1 pre-adipocyte cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 1% antibiotic-antimycotic (Gibco) and 10% newborn calf serum (Gibco) at 37 °C in 5% $CO_2$ atmosphere until confluence. A sulforhodamine B (SRB) assay was performed as previously described [18]. Briefly, cells ($3 \times 10^3$ cells/well) were seeded in 96-well plates and treated with various concentrations (10, 30, 50 and 100 μg/mL) of PME for 48 h. The medium was then replaced with 50 μL of 12% trichloroacetic acid (TCA), after which samples were incubated at 4 °C for 1 h. Samples were then washed with distilled water, followed by staining of cells with 50 μL SRB for 30 min. Dyed cell was subsequently washed three times with 1% acetic acid, then solubilized with 100 μL of 10 mM Tris buffer. Next, solution was transferred to a new plate and the absorbance at 540 nm was measured using a microplate reader (Molecular Devices, LLC., Sunnyvale, CA, USA).

### 2.3. Cell differentiation and Oil Red O staining

To induce differentiation, 2-day post-confluent pre-adipocytes (designated day 0) were cultured in differentiation medium with DMEM containing 10% fetal bovine serum (FBS, Gibco), 10 μg/mL insulin (Sigma-Aldrich, Saint Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 0.25 μM dexamethasone (Sigma-Aldrich) for 2 days. The cells were cultured for an additional 2 days in maturation medium (DMEM containing 10% FBS and 10 μg/mL insulin), then maintained in DMEM medium containing 10% FBS, which was replaced every two days for an additional four days (designated days 4–8) until the cells were harvested. PME was treated with medium at concentrations of 5 and 10 μg/mL on days 0–8. After induction of differentiation, cells were washed with phosphate-buffered saline (PBS), fixed with 10% formaldehyde for 1 h, then washed with distilled water. The cells were then stained with Oil Red O solution for 15 min, after which they were washed three times with distilled water and viewed under a Leica microscope Type 090–135.002 (Leica Microsystems GmbH, Wetzlar, Germany) at 200× magnification. Stained Oil Red O was also eluted with isopropanol and quantified by measuring the absorbance at 540 nm.

### 2.4. TG contents

Differentiated 3T3-L1 cells were washed in PBS and lysed with trypsin-EDTA. Thereafter, cells were centrifuged at 1,200 rpm for 5 min, then washed two times with PBS. Finally, cells were lysed in lysis buffer for 3 h and centrifuged at 10,000 rpm for 5 min, after which the TG concentration was determined using a commercial kit (Asan Pharmaceutical Co., Ltd., Seoul, Korea).

### 2.5. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from 3T3-L1 cells and epididymal adipose tissue using Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). Total RNA (1 μg) was reverse-transcribed into cDNA using a ReverTra Ace qPCR RT master mix (Toyobo Co., Ltd., Osaka, Japan). The mRNA expression was quantified by real-time quantitative PCR using a SYBR green PCR kit (Qiagen, Hilden, Germany) and CFX96TM real-time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cycle thresholds were determined based on the SYBR green emission intensity during the exponential phase. The sequences of forward and reverse primers are shown in Table 1. The relative expression of target transcripts were calculated from each sample after normalization by *GAPDH* or *RPLP0*. Fold changes were calculated using the $2^{-\triangle\triangle CT}$ method [19].

**Table 1**
Primer sequences for real-time RT-PCR.

| Gene | Full name | Forward/Reverse(5'-3') |
|---|---|---|
| *C/EBPα* | CCAAT/enhancer binding protein (C/EBP), alpha | GCGCAAGAGCCGAGATAAA/GGTGAGGACACAGACTCAAATC |
| *CPT1* | Carnitine palmitoyltransferase 1b | TGGCTACGGGGTCTCTTACA/AAGTTCGGCGATGTCCAACA |
| *CPT2* | Carnitine palmitoyltransferase 2 | GCCTGCTGTTGCGTGACTG/TGGTGGGTACGATGCTGTGC |
| *DGAT2* | Diacylglycerol O-acyltransferase 2 | CTGGCTGATAGCTGTGCTCTACTTC/TGCGATCTCCTGCCACCTTTC |
| *FAS* | Fatty acid synthase | TTGGAGCTAAGGCATGGTGG/GCAGTTGTCCTCTGGATGCT |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | AAGGTCATCCCAGAGCTGAA/CTGCTTCACCACCTTCTTGA |
| *GLUT4* | Glucose transporter type 4 | GGCTTTGTGGCCTTCTTTGAG/GACCCATAGCATCCGCAACAT |
| *IRS1* | Insulin receptor substrate 1 | ACGAACACTTTGCCATTGCC/CCTTTGCCCGATTATGCAGC |
| *HSL* | Lipase, hormone sensitive | GTGAATGAGATGGCGAGGGTC/TGAGGAGTCGCGTTAGAGTC |
| *PPARα* | Peroxisome proliferator activated receptor alpha | GCTGGAGGGTTCGTGGAGTC/CGGTGAGATACGCCCAAATGC |
| *PPARγ* | Peroxisome proliferator activated receptor gamma | TCGCTGATGCACTGCCTATG/GAGAGGTCCACAGAGCTGAT |
| *RPLP0* | Ribosomal protein, large, P0 | GCAGGTGTTTGACAACGGCAG/GATGATGGAGTGTGGCACCGA |
| *SREBP1* | Sterol regulatory element binding transcription factor 1 | AACCTCATCCGCCACCTG/TGGTAGACAACAGCCGCATC |
| *UCP1* | Uncoupling protein1 (mitochondrial, proton carrier) | CCTGCCTCTCTCGGAAACAA/TCTGGGCTTGCATTCTGACC |