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# *Epimedium koreanum* Nakai and its main constituent icariin suppress lipid accumulation during adipocyte differentiation of 3T3-L1 preadipocytes

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**[ABSTRACT]** Obesity is associated with a number of metabolic abnormalities such as type 2 diabetes and has become a major health problem worldwide. In the present study, we investigated the effects of *Epimedium koreanum* Nakai (Herba Epimedii, HE) and its main constituent icariin on the adipocyte differentiation in 3T3-L1 preadipocytes. HE extract and icariin significantly reduced lipid accumulation and suppressed the expressions of PPAR$\gamma$, C/EBP$\alpha$, and SREBP-1c in 3T3-L1 adipocytes. They also inhibited fatty acid synthase (FAS), acyl-Co A synthase (ACS1), and perilipin. Moreover, HE extract and icariin markedly increased the phosphorylation of AMPK. These results indicated that HE extract and icariin can inhibit the adipocyte differentiation through downregulation of the adipogenic transcription factors, suggesting that HE containing icariin may be used as a potential therapeutic agent in the treatment and prevention of obesity.

**[KEY WORDS]** *Epimedium koreanum* Nakai; Herba Epimedii; Icariin; 3T3-L1 cells; Obesity

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## Introduction

The prevalence of obesity has been increased over recent years in most developed and industrialized countries [1]. Obesity is characterized by an increase in the number or size of adipocytes and results from an imbalance between energy intake and consumption [2]. Increased adipocytes are closely associated with the occurrence of diabetes mellitus (DM), severe cardiovascular disease, dyslipidemia, atherosclerosis, hypertension, stroke, and certain types of cancer [3-4]. Therefore, identifying molecular basis for controlling adipocytes has an implication in therapeutic modalities of obesity and obesity-related metabolic disorders [5-6].

Adipogenesis is a differentiation process of adipocytes that converts preadipocytes to mature adipocytes and is characterized by the formation of lipid droplets and changes in gene expression and cellular morphology [6]. During adipogenesis, many key transcriptional factors such as peroxisome proliferator-activated receptor gamma (PPAR$\gamma$), CCAAT element binding protein alpha (C/EBP$\alpha$) and sterol regulatory element-binding protein-1c (SREBP-1c) are involved in adipocytes [7]. SREBP-1c promotes preadipocyte differentiation by increasing expression of the PPAR$\gamma$ and C/EBP$\alpha$ [2]. These transcription factors control the expression of adipocyte specific genes such as fatty acid synthase (FAS), acyl-Co A synthase (ACS1), and perilipin, leading to fat droplet formation [8]. Adenosine monophosphate-activated protein kinase (AMPK), a central sensor of cellular energy, also controls adipocyte differentiation through regulating the expression of

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SREBPs and its target genes such as FAS [5, 9]. Thus, the regulation of these factors may be a key mechanism for inhibition of adipogenesis.

The upper parts of *Epimedium koreanum* Nakai (Berberidaceae, Herba Epimedii, HE) including leaves are commonly used in the treatment of cardiovascular diseases and many inflammatory disorders in traditional Korean Medicine [10]. It has been demonstrated to have a wide range of pharmacological and biological activities, such as anti- tumor, anti-cancer, anti-proliferative activities and improved sexual function [11-12]. HE has been known to have various chemical constituents such as icariin, anhydroicaritin-3-*O*-*α*-L- rhamnopyranoside, quercetin, epimedin A, epimedin B, ikarisoside, *β*-sitosterol, daucosterol, campesterol, epimediphine [13], and two flavonol glycosides icarisids B and C [14]. Icariin, a major active flavonoid isolated from plants in the *Epimedium* family, has been reported to have inhibitory effects on LPS-induced acute inflammatory responses [15], streptozotocin- induced diabetic nephropathy [16], and *β*-amyloid production in Alzheimer's diseases [17]. Even though numerous biological activities of HE and icariin have been reported, there is limited evidence for its anti-adipogenic effects. In the present study, we evaluated the anti-adipogenic effects of HE extract and icariin, focusing on the differentiation of adipocytes from preadipocytes.

## Materials and Methods

### *Preparation of HE extract and icariin*

HE extract was prepared by water extraction. Briefly, 200 g of HE was soaked in water for 3 h at room temperature. The extract was filtered through a two-layer mesh and concentrated in a boiling water bath to obtain the residue (yields of 22.5%). The HE extract was stored at −20 °C until use. Icariin was purchased from Sigma-Aldrich Chemical (St Louis, MO, USA).

### *Cell culture and differentiation assay*

The 3T3-L1 cells (ATCC, Manassas, VA, USA), a murine preadipocyte cell line, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated bovine calf serum (BCS; WelGENE biopharmaceuticals, Daegu, Korea) in a 5% $CO_2$ humidified atmosphere at 37 °C. Two days after confluence (designated as Day 0), the cells were switched to differentiation medium containing 0.5 mmol·L$^{-1}$ of 3-isobutyl-1-methylxanthine (IBMX), 1 μmol·L$^{-1}$ of dexamethasone and 5 μg·mL$^{-1}$ of insulin in DMEM supplemented with 10% fetal bovine serum (FBS; WelGENE Biopharmaceuticals) for 2 days. Thereafter, the cell culture medium was replaced with DMEM containing 10% FBS and 5 μg·mL$^{-1}$ of insulin and incubation was continued for an additional 1 day. HE extract and icariin were dissolved in phosphate-buffered saline (PBS) and filtered through 0.2-μm-pore syringe filters. The cells were treated every 2 d with HE extract or icariin at appropriate concentrations in differentiation media for 6 days.

### *Cell viability assay*

Cell viability was measured using the 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Roche, Mannheim, Germany). The cells were cultured in DMEM containing 10% BCS and various concentrations of HE extract or icariin for 24 h. Thereafter, the medium was removed, the MMT solution was added, and the purple formazan crystals were dissolved in DMSO. The optical density (OD) was measured at 550 nm on a microplate reader (GENios, TEKAN Instruments, Inc., Männedorf, Switzerland).

### *Oil red O staining*

The lipid accumulation in adipocytes was measured by Oil red O staining. After differentiation, the cells were washed with 1 × PBS and fixed with 10% formalin for 1 h at room temperature. After the 10% formalin was removed, the plates were stained with Oil red O dye (Sigma-Aldrich Chemical) for 30 min. The plates were washed four times with distilled water and the morphological characteristics of lipid accumulation in cells were observed by Olympus microscopy (Tokyo, Japan) at 50 × magnification. To quantify the amount of lipid accumulations, stained oil droplets were eluted with isopropyl alcohol, and the absorbance was measured at 490 nm on a microplate reader (GENios).

### *Reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from the 3T3-L1 cells using TRIzol reagent (Invitrogen, Calsbad, CA, USA) and the cDNA was generated from 5 μg of total RNA. RT reaction was performed in a reaction mixture containing RNA, 1 × reverse transcriptase buffer (Promega, Madison, WI), 0.5 mmol·L$^{-1}$ of dNTP (deoxynucleotide triphosphate), 3 mmol·L$^{-1}$ of $MgCl_2$, 5 U of RNase inhibitor (Amersham, Piscataway, NJ), 0.5 μmol·L$^{-1}$ of oligo-dT primer, and 5 U of Superscript Reverse Transcriptase (Promega, Madison, WI, USA) in a total volume of 20 μL. Then the mRNA expression level was performed by PCR using the prepared cDNA as a template with the following cycle parameters: 94 °C, 2 min, 30−35 cycles; 94 °C, 30 s; 56−59 °C, 30 s; 72 °C, 1 min; and 92 °C, 10 min. The PCR products were then resolved on 1% agarose gels at 100 V and specific genes were verified by assessing their predicted sizes under UV light. The oligonucleotide primers used in the PCR were as follows. PPAR*γ* (accession No. NM 011146) Fw: 5′-GAA AGA CAA CGG ACA AAT CAC C-3′ and Rv: 5′-GGG GGT GAT ATG TTT GAA CTT G-3′; C/EBP*α* (accession No. NM 007678.3) Fw: 5′-TTA CAA CAG GCC AGG TTT CC-3′ and Rv: 5′-AAC TCC AGT CCC TCT GGG AT-3′; SREBP-1c (accession No. NM 011480.3) Fw: 5′-TCA TGC CCT CCA TAG ACA CA-3′ and Rv: 5′-AGC TCA AAG ACC TGG TGG TG-3′; perilipin (accession No. NM 175640.1) Fw: 5′-AAG GAT CCT GCA CCT CAC AC-3′ and Rv: 5′-CCT CTG CTG AAG GGT TAT CG-3′; ACS1 (accession No. NM 007981.3) Fw: 5′-CAA CCC AGA ACC ATG GAA GT-3′ and Rv: 5′- CCT CTG CTG AAG GGT TAT CG-3′; FAS (accession No. MMFASC) Fw: 5′-GCC CGG TAG CTC TGG GTG TA-3′ and Rv: 5′-TGC TCC CAG CTG CAG GC-3′; and GAPDH