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# Eupatilin inhibits adipogenesis through suppression of PPARγ activity in 3T3-L1 cells

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ABSTRACT

Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) is a flavonoid compound from *Artemisia* species that possesses beneficial biological activities such as anti-cancer, anti-oxidation, and anti-inflammatory activities. However, an anti-adipogenic effect has not yet been reported. In this study, we found that eupatilin significantly inhibited the adipogenesis of 3T3-L1 adipocytes. Eupatilin decreased intracellular lipid accumulation and suppressed the expression level of key adipogenic regulators in 3T3-L1 adipocytes, including peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα), in a concentration-dependent manner. These results show that eupatilin significantly inhibits 3T3-L1 cell differentiation and suggest that it has potential as a novel anti-obesity therapy.

## 1. Introduction

Obesity is a major health problem worldwide, and can induce various diseases such as type 2 diabetes, cardiovascular disease, and cancer [1]. The prevalence of obesity, and the number of patients with metabolic diseases caused by obesity, are increasing in most countries [2,3]. Although the development of anti-obesity drugs continues, and some were approved and marketed, most such drugs have been withdrawn because of serious side effects [4]. Therefore, there is a need for alternative drugs for treating obesity without side effects.

Adipogenesis is the process by which cells differentiate into adipocytes. This process involves the conversion of preadipocytes into mature adipocytes with intracellular lipid accumulation [5]. It is regulated by key adipogenic transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα). PPARγ and C/EBPα also accelerate adipogenesis by stimulating the expression of adipokines including adipocyte fatty acid-binding protein (aP2), lipoprotein lipase, and adiponectin [6,7]. Because adipocytes play an important role in regulating adipokine secretion which promotes adipogenesis, understanding the molecular mechanisms that regulate adipogenesis is important for exploring anti-obesity therapy [8].

*Artemisia* species grow in the northern hemisphere and have been used for centuries as a source of traditional Chinese medicine because they contain a high content of flavonoids [9,10]. Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) (Fig. 1), a flavonoid compound from *Artemisia* species, was reported to possess several beneficial biological activities including neuroprotection, anti-cancer, antinociception, anti-oxidation, and anti-inflammation [11–13]. However, an anti-adipogenic effect of eupatilin has not yet been investigated. Therefore, the present study investigated the anti-adipogenic effect underlying the action of eupatilin in 3T3-L1 adipocytes.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Eupatilin was purchased from LKT Laboratories (St Paul, MN, USA). Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), and newborn calf serum were obtained from Gibco Life Technologies (Grand Island, NY, USA). Insulin, indomethacin, dexamethasone, 3-isobutyl-1-methylxanthine, and Oil Red O solution were obtained from Sigma-Aldrich (St Louis, MO, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). Primers were obtained from Macrogen (Seoul, Korea). Antibodies against C/EBPα and β-actin were purchased from

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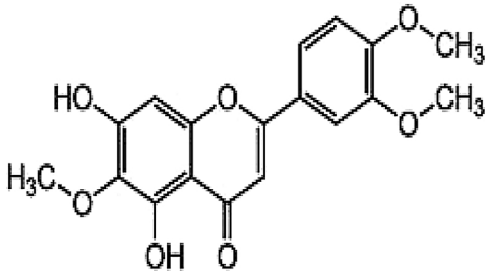

**Fig. 1.** The chemical structure of eupatilin.

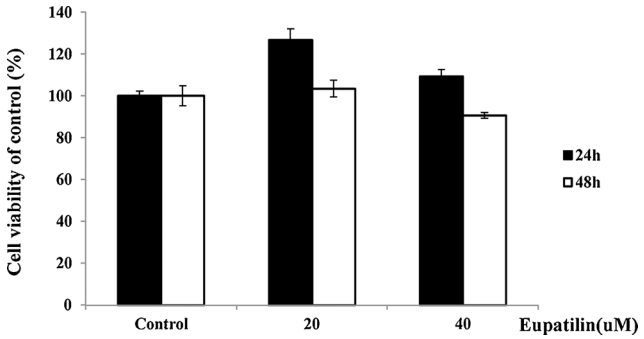


**Fig. 2.** Cytotoxicity of eupatilin on 3T3-L1 cells. Cell viability was investigated using the MTT assay. 3T3-L1 preadipocytes were treated with 20 or 40 μM eupatilin for 24 or 48 h, respectively. The formazan formed was measured using spectrophotometry. All treatments were repeated in triplicate. Bars represent means ± SD from three independent experiments.

Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Antibodies against PPARγ and adiponectin were obtained from Abcam (Cambridge, UK).

### 2.2. 3T3-L1 cell differentiation

Mouse 3T3-L1 preadipocytes were purchased from the Korean Cell Line Bank (Seoul, Korea). They were maintained in DMEM supplemented with 10% newborn calf serum and antibiotics (1% penicillin/streptomycin), and cultured at 37 °C in a humidified 5% $CO_2$ incubator. After confluence was maintained for two days, the cells were exposed to differentiation medium consisting of DMEM with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/mL insulin, 1 μM dexamethasone, and 100 μM indomethacin. After two days, the culture medium was replaced with DMEM supplemented with 10% FBS and insulin, and changed every two days from days two to eight.

### 2.3. Cell viability assay

3T3-L1 cells ($2 \times 10^3$ cells/well) were seeded in 96-well plates. After 24 h, cells were treated with eupatilin at a concentration of either 20 or 40 μM. After 24 or 48 h of incubation, respectively, the medium was removed and MTT solution (0.5 mg/mL) was added to each well. After incubation for 3 h, isopropanol was added to each well and the absorbance was measured at 495 nm.

### 2.4. Oil Red O staining and triglyceride (TG) colorimetric assay

3T3-L1 cells were treated with eupatilin (20 or 40 μM) for eight days or were left untreated. On day eight, the medium was removed and the cells were washed with 20% isopropanol, then fixed with 5% formalin for 1 h. Intracellular lipid droplets were stained with Oil Red O solution for 20 min and then dissolved in 100% isopropanol. The absorbance of each sample was measured at 495 nm. Total amounts of TG were determined using a Triglyceride Quantification Colorimetric/Fluorometric Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol.

### 2.5. Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted by the TRIzol® method according to the protocol recommended by the manufacturer (TaKaRa Bio, Otsu, Shiga, Japan). Complimentary DNA (cDNA) was synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa Bio). Quantitative RT-PCR was conducted with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green (TOYOBO, Osaka, Japan). All reactions were performed in triplicate. The primer sequences are shown in Table 1.

### 2.6. Western blot analysis

3T3-L1 adipocytes were lysed using radioimmunoprecipitation assay lysis buffer (Biosesang, Korea). Equal amounts of protein were separated on 12% sodium dodecyl sulfate polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked in 5% skim milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 1 h, and incubated overnight with primary antibodies against PPARγ, C/EBPα, and adiponectin at 4 °C. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Proteins were detected using an enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK).

### 2.7. Statistical analysis

Statistical analysis of all data was performed using SPSS version 20.0 (IBM, Chicago, IL, USA). Significant differences between groups were determined by using a one-way analysis of variance (LSD). P values of $< 0.05$ were considered significant.

## 3. Result

### 3.1. Effect of eupatilin on 3T3-L1 cell viability

To investigate the cytotoxic effect of eupatilin on 3T3-L1 preadipocytes, we used the MTT assay. As shown in Fig. 2, cytotoxicity was not observed in 3T3-L1 preadipocytes treated with 20 or 40 μM eupatilin for 24 h or 48 h, respectively. Therefore, we performed subsequent experiments at concentrations of 20 and 40 μM.

**Table 1**
Gene-specific primers used for reverse transcription polymerase chain reaction.

| Gene name | Accession no. | Forward primer | Reverse primer |
|---|---|---|---|
| PPAR γ | AB644275.1 | 5′-GGAAGACCACTCGCATTCCTT-3′ | 5′-GTAATCAGCAACCATTGGGTCA-3′ |
| C/EBPα | NM_001287523.1 | 5′- CAAGAACAGCAACGAGTACCG-3′ | 5′-GTCACTGGTCAACTCCAGCAC3′ |
| aP2 | NM_024406.2 | 5′-GATGCCTTTGTGGGAACCT-3′ | 5′-CTGTCGTCTGCGGTGATTT-3′ |
| Adiponectin | NM_009605.4 | 5′- GATGGCACTCCTGGAGAGAA-3′ | 5′-TCTCCAGGCTCTCCTTTCCT-3′ |
| β-actin | NM_007393.4 | 5′- CGTGCGTGACATCAAAGAGAA-3′ | 5′-GCTCGTTGCCAATAGTGATGA-3′ |

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