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Anti-Adipogenic Effects of Ethanol Extracts Prepared from Selected Medicinal Herbs in 3T3-L1 Cells

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ABSTRACT: Obesity is a major risk factor for various metabolic diseases such as cardiovascular disease, hypertension, and type 2 diabetes mellitus. In this study, we prepared ethanol extracts from *Agastache rugosa* (ARE), *Chrysanthemum zawadskii* (CZE), *Mentha arvensis* (MAE), *Perilla frutescens* (PFE), *Leonurus sibiricus* (LSE), *Gardenia jasminoides* (GJE), and *Lycopus coreanus* (LCE). The anti-oxidant and anti-adipogenic effects were evaluated. The IC_{50} values for ascorbic acid and LCE against 2,2-diphenyl-1-picrylhydrazyl radicals were 246.2 μg/mL and 166.2 μg/mL, respectively, followed by ARE (186.6 μg/mL), CZE (198.6 μg/mL), MAE (337.1 μg/mL), PFE (415.3 μg/mL), LSE (548.2 μg/mL), and GJE (626.3 μg/mL). In non-toxic concentration ranges, CZE had a strong inhibitory effect against 3T3-L1 adipogenes (84.5%) than those of the other extracts. Furthermore, the anti-adipogenic effect of CZE is largely limited in the early stage of adipogenesis, and we revealed that the inhibitory role of CZE in adipogenesis is required for the activation of Wnt signaling. Our results provide scientific evidence that the anti-adipogenic effect of CZE can be applied as an ingredient for the development of functional foods and nutri-cosmetics for obesity prevention.

Keywords: *Chrysanthemum zawadskii*, adipognesis, obesity, Wnt signaling, medicinal herb

INTRODUCTION

Obesity is defined as a complex disorder characterized by excessive accumulation of body fat through an imbalance between energy intake and consumption (1). Obesity commonly leads various metabolic diseases such as hypertension, type 2 diabetes, and dyslipidemia. It has become one of the most critical health problems in adults as well as in children in modern society (2,3). Recent research has focused on preventing excessive weight and obesity through inhibition of adipogenesis and lipogenesis (4,5).

Adipocytes are the essential components of fat tissues, and they play an important role as a depot to store triglycerides. Adipogenesis is a complex process of differentiation from preadipocytes into adipocytes. It is closely connected to the development and stimulation of adipocytes depending on the systematic regulation of nutrients, internal hormones, and cytokines (6-8). In addition, previous studies demonstrated that adipogenic transcriptional factors such as CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor (PPAR) γ, sterol regulatory element-binding protein (SREBP)-1c, and lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA-carboxylase (ACC) are considered as key regulators of adipogenesis (9).

Previous studies showed that adipogenic transcriptional factors could be regulated by various cellular signaling pathways by either stimulating or inhibiting cell differentiation. It is well-known that PPARγ agonists such as rosiglitazone and troglitazone, insulin signaling, and cyclic adenosine monophosphate (cAMP) stimulate adipogenesis (10). In contrast, wingless-type mouse mammary tumor virus integration site family (Wnt) signaling, preadipocytes factor-1 (Pref-1), forkhead box protein (Foxo), tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, epidermal growth factor, prostaglandin F2α, and GATA binding proteins are known to play negative

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roles in adipogenesis (11). Thus, the inhibition of adipogenic transcriptional factors via activation of signaling pathways with negative role in adipogenesis can be one of the important strategies for obesity prevention.

Among negative factors, Wnt signaling has important roles in the development of tissue homeostasis, cell proliferation, and differentiation. Wnt signaling leads to increased levels of β-catenin and a concomitant decrease of transcriptional factors, PPARγ and C/EBPβ that activate the expression levels of adipogenic genes. Previous studies reported that the 3T3-L1 adipogenesis was inhibited by 6-gingerol treatment through activation of the Wnt, and 6-gingerol-inhibited 3T3-L1 differentiation was reversed after β-catenin knockdown (12). In addition, 3T3-L1 adipogenesis was suppressed by kirenol, a natural diterpenoid, treatment accompanied by down-regulating PPARγ, C/EBPα, SREBP-1c, FAS, and ACC (13). Taken together, previous reports indicate that Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcriptional factors.

Recent studies reported the potential of plant-based materials as an anti-obese functional ingredient in the development of functional foods and pharmaceuticals since they have physiological benefits while they possess relatively few side effects (14). In this study, we prepared 7 kinds of extracts from traditionally used medicinal herbs including wrinkled giant hyssop (*Agastache rugosa*), siberian chrysanthemum (*Chrysanthemum zawadskii*), peppermint (*Mentha arvensis*), perilla (*Perilla frutescens*), motherwort (*Leonurus sibiricus*), gardenia fruit (*Gardenia jasminoides*), and lycopus herb (*Lycopus coreanus*), and their anti-adipogenic effects were evaluated in 3T3-L1 cells.

MATERIALS AND METHODS

Materials

Medicinal herbs including *Agastache rugosa*, *Chrysanthemum zawadskii*, *Mentha arvensis*, *Perilla frutescens*, *Leonurus sibiricus*, *Gardenia jasminoides*, and *Lycopus coreanus* were purchased from Gwangwoo Pharmaceutical (Changwon, Korea). Oil red O (ORO), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), insulin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 4-(1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-*N*-8-quinolinyl-benzamide (IWR-1) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, phosphate-buffered saline (PBS), and bovine calf serum (BCS) were obtained from Welgene Inc. (Daegu, Korea). Fetal bovine serum were purchased from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). TRIzol® was obtained from Life Technologies (Carlsbad, CA, USA), and the Maxime PCR PreMix (i-Taq) was purchased from Intron (Seongnam, Korea). CycleScript RT PreMix was obtained from Bioneer (Daejeon, Korea). All other chemicals were obtained from Sigma-Aldrich Co..

Preparation of extracts

Seven kinds of dried medicinal herbs including *Agastache rugosa*, *Chrysanthemum zawadskii*, *Mentha arvensis*, *Perilla frutescens*, *Leonurus sibiricus*, *Gardenia jasminoides*, and *Lycopus coreanus* were dried and ground into fine powders. Five grams of grounded medicinal herbs were extracted with 10 folds of 70% (v/v) ethanol using a shaking incubator (HST-201MS-2R, Hanbaek Scientific Technology, Bucheon, Korea) at 25°C for 24 h. Extracted medicinal herbs were evaporated using a rotary evaporator (Eyela N-100, Tokyo Rikakikai Co., Tokyo, Japan) under reduced pressure. The extracts of *Agastache rugose* (ARE), *Chrysanthemum zawadskii* (CZE), *Mentha arvensis* (MAE), *Perilla frutescens* (PFE), *Leonurus sibiricus* (LSE), *Gardenia jasminoides* (GJE), and *Lycopus coreanus* (LCE) were stored at −70°C until further analysis.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was measured according to the method of Chen et al. (15) with slight modifications. The DPPH was dissolved in ethanol, and the ethanol extracts from the medicinal herbs (10, 50, 100, 200, 400, and 800 μg/mL) were incubated with 200 μM DPPH solution for 30 min at 37°C. The absorbance was measured at 490 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The DPPH radical scavenging activity percentage was calculated using the following equation and ascorbic acid was used as a positive control: DPPH radical scavenging activity %=(absorbance of sample/absorbance of control) ×100. The IC_{50} values were deducted based on the scavenging activities of the extracts.

3T3-L1 cell culture and cytotoxicity assay

3T3-L1 cells, purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea), were maintained in DMEM supplemented with 10% BCS and 100 unit/mL penicillin-streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere. The medium was replaced every 2~3 days. To examine the cytotoxicities of medicinal herbs in 3T3-L1 cells, 3T3-L1 preadipocytes were treated with the indicated concentrations of extracts prepared from the medicinal herbs for 24 h and the medium was removed. The MTT-media (DMEM including 0.2 mg/mL of MTT) was added to each well. The plate was then stored in a CO_2 incubator for an additional 1 h. The reaction medium was completely removed, and the insoluble

formazan was dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a microplate reader (Molecular Devices), and the cell viability was calculated as a percentage of the untreated cells.

3T3-L1 adipocytes differentiation

Two-day after reaching confluency, designated as day 0, the cells were cultured with DMEM (differentiation medium; DM) supplemented with a hormonal cocktail of 500 μM IBMX, 5.2 μM DEX, and 167 nM insulin. After differentiation, the medium was replaced with DMEM (post-differentiation medium; post-DM) containing 167 nM insulin for another 2 days. Thereafter, the cells were cultured in normal DMEM, and the medium was changed every 2 days. The 3T3-L1 preadipocytes were treated with the indicated concentrations of extracts during day −2 to day 6 (Fig. 1).

ORO staining

The ORO staining was performed on day 6. Differentiated mature 3T3-L1 adipocytes were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde at room temperature for 30 min. The fixed adipocytes were then washed 3 times with tap water. The mature adipocytes were stained with 3 mg/mL ORO solution dissolved in isopropanol at room temperature for 15 min. After staining, the ORO-stainined 3T3-L1 cells were additionally washed 3 times with tap water and dried. The stained lipid droplets were dissolved in 300 μL DMSO and transferred to a 96-well microplate. The absorbance of the dissolved ORO was measured at 510 nm with a microplate reader (VersaMax, Molecular Devices).

Isolation of total RNA and RT-PCR analysis

The mRNA expression levels were estimated by RT-PCR analysis. The 3T3-L1 cells were treated with the indicated concentrations of CZE during day −2 to day 6. The total RNA was extracted from the 3T3-L1 cells with TRIzol® reagent. Briefly, the total RNA was dissolved in 40 μL of RNase-free water. Two micrograms of cDNA was then subjected to reverse transcription using the CycleScript RT PreMix for cDNA synthesis through 12 cycle reactions at 48°C. The mRNA expression levels were analyzed by electrophoresis using agarose gel, and β-actin was used as a control. For RT-PCR analysis, the primers for C/EBPβ, C/EBPα, PPARγ, FAS, and β-actin are shown in Table 1.

Inhibitor assay

The Wnt specific inhibitor, IWR-1 was used for examination of the effect of signaling inhibitor on CZE-suppressed 3T3-L1 adipogenesis. 3T3-L1 cells were exposed to 50 μg/mL of CZE in the presence or absence of 10 μM IWR-1 from day −2 to day 2. Intracellular lipid accumulation was visualized using ORO staining.

Statistical analysis

All data are presented as the mean±standard deviation (SD). Statistical analysis was performed using the SPSS software (SPSS Inc., Chicago, IL, USA). The significance of differences between the groups was assessed with one-way analysis of variance (ANOVA) followed by Student's *t*-test. A P-value<0.05 was used for significance.

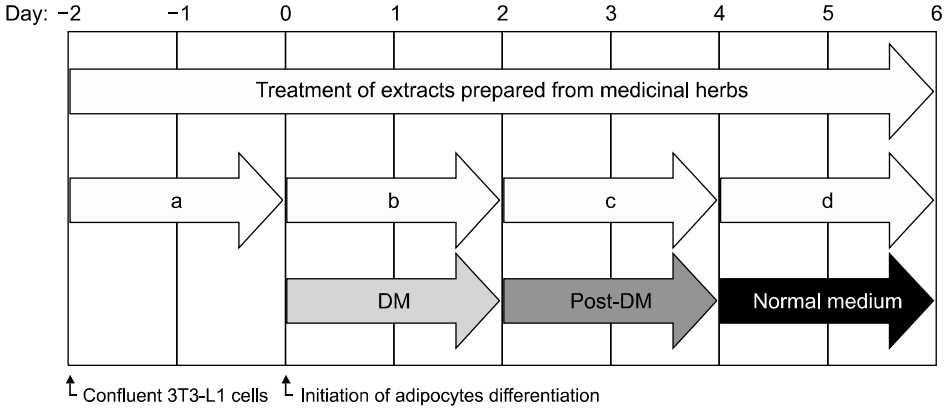

Fig. 1. Scheme of 3T3-L1 differentiation and the treatment of extracts from medicinal herbs. The 3T3-L1 cells were treated with ethanol extracts of medicinal herbs during day −2 to day 6. DM, differentiation medium consist of fetal bovine serum (FBS)-Dulbecco's modified Eagle's medium (DMEM) including 3-isobutyl-1-methylxanthine (500 μM), dexamethasone (5.2 μM), and insulin (167 nM); Post-DM, post-differentiation medium consist of FBS-DMEM including insulin (167 nM).

Table 1. Primer sequences for PCR analysis

Gene	Forward (5′→3′)	Reverse (5′→3′)
C/EBPβ	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
C/EBPα	CCAGAGGATGGTTTCGGGTC	TCCCCAACACCTAAGTCCCT
PPARγ	CCATTCTGGCCCACCAACTT	CCTTCTCGGCCTGTCGATCC
FAS	GTGCACACAGTGCTCAAAGG	GGTATAGACGACGGGCACAG
β-Actin	AGGGAAATCGTGCGTGACAT	AGCTCAGTAACAGTCCGCCT

Table 2. The extraction yields and anti-oxidant activites of ethanol extracts prepared from medicinal herbs

Medicinal herbs	Extract	Extraction yield (%, w/w-dry weight)	DPPH radical scavenging activity (IC_{50}, μg/mL)
Agastache rugosa	ARE	10.0	186.6
Chrysanthemum zawadskii	CZE	5.9	198.6
Mentha arvensis	MAE	12.0	337.1
Perilla frutescens	PFE	8.3	415.3
Leonurus sibiricus	LSE	9.3	548.2
Gardenia jasminoides	GJE	33.0	626.3
Lycopus coreanus	LCE	13.2	166.2
Ascorbic acid	—	—	246.2

RESULTS

Extraction yields and anti-oxidant activities of medicinal herb extracts

The extraction yields of extracts prepared from medicinal herbs are presented in Table 2. The extraction yield of GJE was 33.0%, followed by LCE (13.2%), MAE (12.0 %), ARE (10.0%), LSE (9.3%), PFE (8.3%), and CZE (5.9%). The anti-oxidant activities of the medicinal herb extracts were examined by the DPPH assay. The IC_{50} values of ascorbic acid, a well-known anti-oxidant and LCE against DPPH radicals were 246.2 μg/mL and 166.2 μg/mL, respectively, followed by ARE (186.6 μg/mL), CZE (198.6 μg/mL), MAE (337.1 μg/mL), PFE (415.3 μg/mL), LSE (548.2 μg/mL), and GJE (626.3 μg/mL).

Cytotoxicities of medicinal herb extracts in 3T3-L1 cells

The effects of extracts prepared from 7 kinds of medicinal herbs on the viability of 3T3-L1 preadipocytes, were evaluated by the MTT assay. The 3T3-L1 preadipocytes were treated with 10, 50, 100, and 200 μg/mL of extracts for 24 h. Exposure to the extracts had no toxic effect on the viability of 3T3-L1 cells up to a concentration of 100 μg/mL as shown in Table 3. Among the concentrations tested, 200 μg/mL CZE treatment significantly reduced the viability of 3T3-L1 cells. Thus, up to 100 μg/mL of extracts were chosen for further experiments. We also observed that up to 10 μg/mL LCE and 100 μg/mL GJE significantly exerted the 3T3-L1 cell growth and proliferation when compared to non-treated 3T3-L1 cells.

Table 3. Effects of ethanol extracts prepared from medicinal herbs on the 3T3-L1 viability (unit: %)

Extract[1)]	Concentration (μg/mL)			
	10	50	100	200
ARE	97.0±2.5	98.1±3.6	113.5±2.2*	110.9±6.1
CZE	96.5±2.9	97.8±5.1	101.1±2.8	90.7±4.0*
MAE	99.9±3.9	103.7±5.7	112.6±3.2*	113.3±7.2
PFE	97.3±6.6	103.8±1.6	114.6±2.1***	107.5±7.8
LSE	98.9±1.3	96.2±2.0	96.0±2.6	97.0±2.2
GJE	103.2±2.2	107.4±4.7	114.4±0.4***	111.6±2.6**
LCE	109.5±1.4**	114.0±3.9**	125.3±3.0***	114.9±3.7**

Significant differences based on Student's *t*-test (*P<0.05, **P<0.01, and ***P<0.001 versus untreated 3T3-L1 cells, the data of untreated 3T3-L1 viabilities are not shown).
[1)]ARE, *Agastache rugose* ethanol extract; CZE, *Chrysanthemum zawadskii* ethanol extract; MAE, *Mentha arvensis* ethanol extract; PFE, *Perilla frutescens* ethanol extract; LSE, *Leonurus sibiricus* ethanol extract; GJE, *Gardenia jasminoides* ethanol extract; LCE, *Lycopus coreanus* ethanol extract.

Anti-adipogenic effects of medicinal herb extracts

To examine the anti-adipogenic effects of extracts prepared from medicinal herbs on 3T3-L1 adipogenesis, 3T3-L1 cells were treated with 10 and 50 μg/mL of ARE, CZE, MAE, PFE, LSE, GJE, and LCE during day −2 to day 6 (Fig. 1). The inhibitory effects of medicinal herb extracts on 3T3-L1 adipogenesis were evaluated by ORO staining on day 6. A dramatically decrease in lipid accumulation by extract treatments in 3T3-L1 adipocytes was observed (Fig. 2A). The 50 μg/mL of CZE treatment strongly inhibited the levels of intracellular lipid accumulations by 84.5%, followed by 75.0% (LSE), 66.4% (PFE), 48.9% (GJE), 48.2% (MAE), 43.9% (LCE), and 20.4% (ARE) (Fig. 2B). Among those tested, CZE showed the strongest anti-adipogenic effect in 3T3-L1 differentiation than those of the other extracts.

The CZE-inhibited lipid accumulation is strongly limited in the early stage of 3T3-L1 adipogenesis

From the anti-adipogenic effects of extracts prepared from 7 kinds of medicinal herbs, our study focused on elucidating the role and function of CZE in 3T3-L1 adipogenesis. As illustrated in Fig. 1, we first examined the inhibitory effects of CZE on adipogenic stages of 3T3-L1 cells; 3T3-L1 cells were treated with 50 μg/mL CZE divided by 4 stages of adipogenesis including pre-adipogenic stage (a: day −2∼0), early stage (b: day 0∼2), intermediate stage (c: day 2∼4), and terminal stage (d: day 4∼6). The levels of intracellular lipid accumulation were visualized by ORO staining on day 6. Lipid accumulations of 3T3-L1 adipocytes treated with 50 μg/mL CZE during a, b, and c stages were significantly inhibited by 62.0%, 85.9%, and 55.2%, respectively (Fig. 3A). However, we did not observe a significant difference between

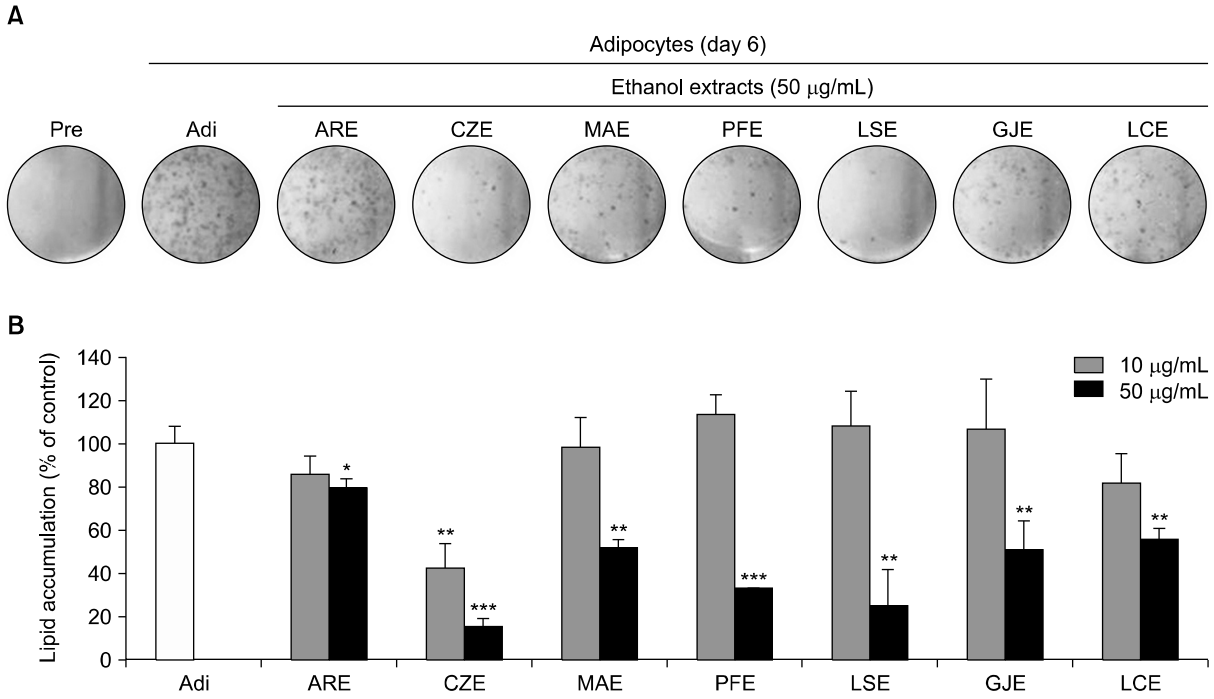

Fig. 2. Effects of ethanol extracts from medicinal herbs treatment on the adipogenesis of 3T3-L1 cells. The 3T3-L1 cells were treated with 10 and 50 μg/mL concentration in the presence or absence of differentiation media during day −2 to day 6. The size and number of lipid droplets were observed (A). The levels of lipid accumulation were quantified by Oil red O staining using microplate reader at 510 nm (B). Significant differences by one-way ANOVA followed by Student's *t*-tests (**P*<0.05, ***P*<0.01, and ****P*<0.001 versus Adi). Pre, 3T3-L1 preadipocytes; Adi, mature 3T3-L1 adipocytes, ARE, *Agastache rugose* ethanol extract; CZE, *Chrysanthemum zawadskii* ethanol extract; MAE, *Mentha arvensis* ethanol extract; PFE, *Perilla frutescens* ethanol extract; LSE, *Leonurus sibiricus* ethanol extract; GJE, *Gardenia jasminoides* ethanol extract; LCE, *Lycopus coreanus* ethanol extract.

CZE-treated adipocytes and mature adipocyte control on day 6. Among the CZE treatment in a, b, and c stages, the CZE treatment in stage b showed a stronger inhibitory effect than the CZE treatment in a and c stages. Similarly, decreaed size and number of lipid droplets were observed in 3T3-L1 adipocytes treated with CZE during a, b, and c stages (Fig. 3B).

The CZE treatment suppresses adipogenic transcriptional factors via activation of Wnt signaling

The 3T3-L1 adipogenesis is accompanied by enhanced expression of transcriptional factors and adipocytes-specific genes. mRNA expression levels of transcriptional factors (C/EBPβ, C/EBPα, and PPARγ) and lipogenic enzyme (FAS) were analyzed by RT-PCR in 3T3-L1 adipocytes in the presence or absence of CZE. mRNA expression levels of C/EBPβ, C/EBPα, PPARγ, and FAS were strongly suppressed by CZE treatment (10～100 μg/mL) in PCR analysis (Fig. 4). We further examined whether CZE-inhibited adipogenesis is mediated by Wnt signaling. Selective inhibitor for Wnt signaling, IWR-1, was treated together with 50 μg/mL CZE in 3T3-L1 cells from day −2 to day 2. As expected, 50 μg/mL CZE treatment dramatically suppressed 3T3-L1 adipogenesis, whereas 3T3-L1 cells co-treated with CZE and IWR-1 showed that the CZE-inhibited lipid accumulation was reversed (Fig. 5).

DISCUSSION

Numerous studies reported that anti-oxidant effects of various medicinal herbs were correlated with their phytochemical composition, such as phenolic acids, flavonoids, stilbenes, and lignans (16). Reduction of oxidative stress by medicinal herbs has been attributed to prevention and treatment of many human diseases such as cancer, cardiovascular diseases, diabetes, obesity, inflammatory diseases, osteoporosis and neurodegenerative diseases (17,18). According to Lee et al. (19), increased lipid accumulation in 3T3-L1 adipocytes was in concordance with elevated ROS levels. In addition, methanol extracts prepared from herbs in Korea effectively inhibited both lipid accumulation and ROS levels (19). Previous reports led us to hypothesize that medicinal herbs possessed anti-oxidant activities that could effectively inhibit the adipogenic program of 3T3-L1 cells. To test this, we first prepared ethanol extracts from 7 kinds of medicinal herbs, *Agastache rugosa*, *Chrysanthemum zawadskii*, *Mentha arvensis*, *Perilla frutescens*, *Leonurus sibiricus*, *Gardenia jasminoides*, and *Lycopus coreanus*, and tested their scavenging activities against DPPH radicals. In this study, we obtained over 10% (w/w) extraction yields from 6 kinds of medicinal herbs, whereas CZE showed relatively low extraction yield (5.9%), compared to others tested. Kim et al. (20) previously obtained a 6.4% (w/w) extraction

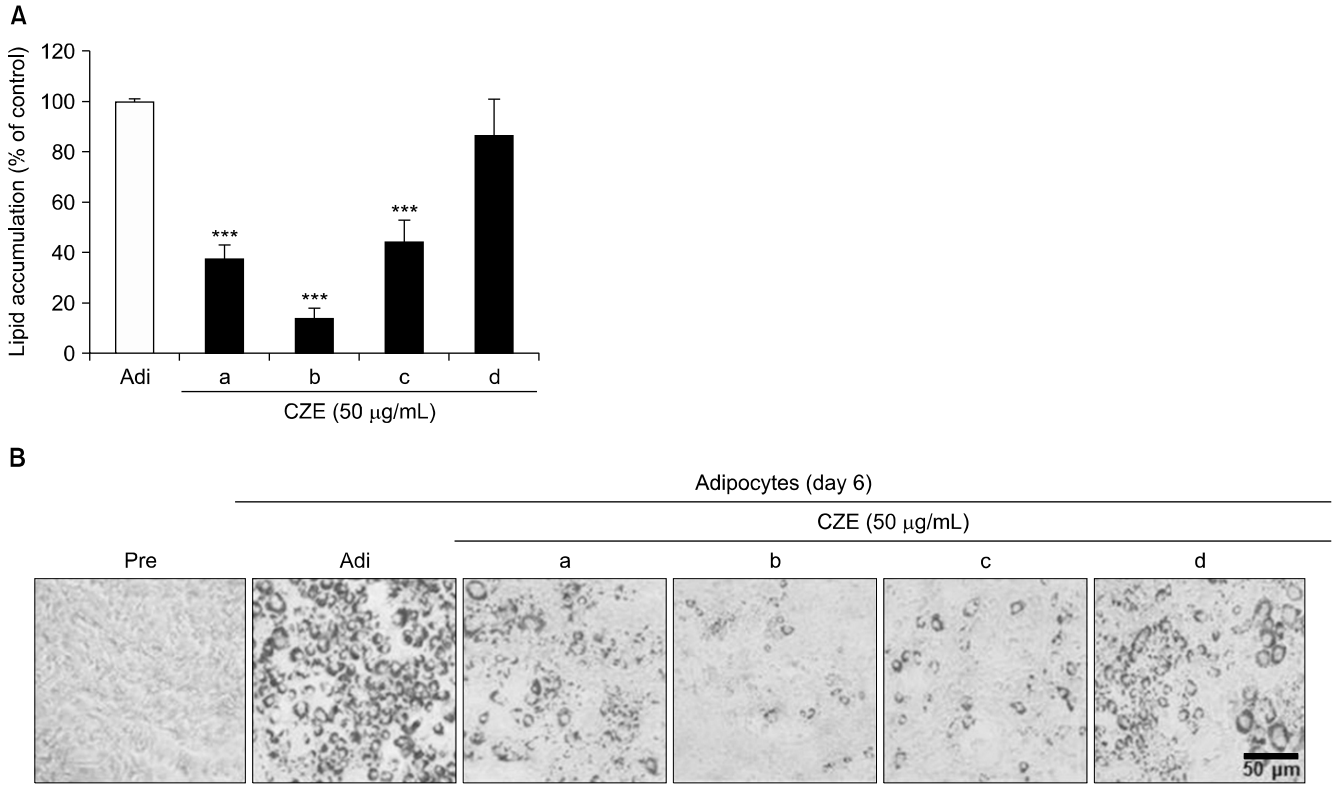

Fig. 3. Effects of *Chrysanthemum zawadskii* ethanol extract (CZE) treatment of various stages on the lipid accumulation of 3T3-L1 cells. The CZE treatment were divided into four stages, pre-adipogenic stage (a: day −2∼0), early stage (b: day 0∼2), intermediate stage (c: day 2∼4), and terminal stage (d: day 4∼6). (A) Levels of lipid accumulation were evaluated by ORO staining and quantified using microplate reader at 510 nm. (B) The number and size of lipid droplets were observed using inverted microscope. Significant differences by one-way ANOVA followed by Student's *t*-tests (***P<0.001 versus Adi). Pre, 3T3-L1 preadipocytes; Adi, mature 3T3-L1 adipocytes.

yield from *Chrysanthemum zawadskii*, suggesting that *Chrysanthemum zawadskii* possesses lower lipid-soluble components than those of other medicinal herbs. In the DPPH assay, the IC_{50} value for DPPH radical scavenging activities of medicinal herb extracts exhibited the following order: LCE> ARE> CZE> MAE> PFE> LSE> GJE. The anti-adipongeic effects were in the following order: CZE ≥ PFE> LSE≥ MAE≥ GJE≥ LCE> ARE. CZE was chosen for further experimentation based on the results of these studies, however, we did not observe a clear positive correlation between anti-oxidant activities and anti-adipogenic effects of the selected medicinal herb extracts in this study. The DPPH assay is widely used since it is simple and rapid.

Obesity, characterized as an accumulation of fat due to an increase in the size and number of adipocytes, has become a serious public health problem and an important worldwide issue (21). Regular meals and exercise would be recommended for the prevention of metabolic disorders such as obesity. Previous studies have reported the anti-obesity activity of medicinal herb extracts, and it is important to inhibit adipogenesis for anti-obesity research (22). Recent studies reported that the anti-adipogenic activities of plant-based material such as amur grape root (*Vitis amurensis* Ruprecht), chocolate vine leave (*Akebia quinata* D.), and black ginseng (23,24). Furthermore, Shon et al. reported that 20 types fruits and vegetables exhibited the anti-obesity effects via inhibition of lipid accumulation in 3T3-L1 adipocytes (25).

The 3T3-L1 cell line is one of the cell culture models most widely used for evaluating the anti-obesity activity. It is stimulated to accumulate intracellular lipid droplets by metabolic regulating hormone such as IBMX, DEX, insulin, and various growth factors (26). Adipogenic hormones exert the induction of the expressions of transcription factors in 3T3-L1 adipocytes. Various intracellular genes are expressed in each of the differentiation stages through up- and down-regulation of the adipogenesis. The activations of various signaling pathway such as extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), Akt, glycogen synthase kinase (GSK) 3β, and phosphoinositide 3-kinase were associated with the development of adipogenesis. Previous reports showed that the activation of Brd2 nuclear serine /threonine kinase inhibited levels of lipid accumulation in 3T3-L1 adipocytes through down-regulation of signaling pathways such as ERK1/2 (27). Schisandrae fructus (*Schisandra chinensis*) caused the inhibition of 3T3-L1 adipogenesis by inhibiting levels of Akt and GSK3β phosphorylation and its inhibitory activities induced the

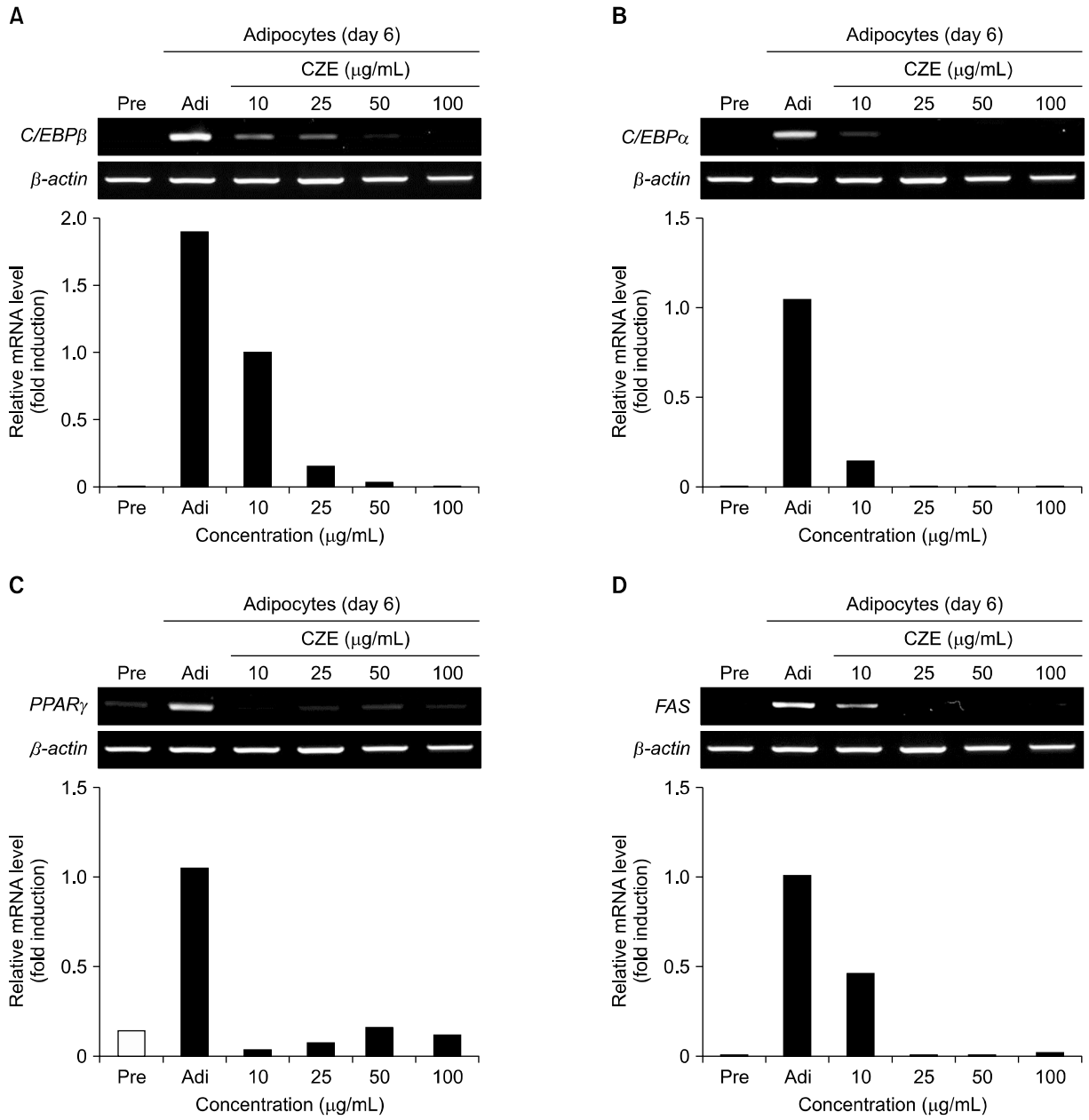

Fig. 4. The *Chrysanthemum zawadskii* ethanol extract (CZE) treatment downregulate mRNA levels of adipogenic transcriptional factors in 3T3-L1 cells. The mRNA expression levels of adipogenic transcriptional factors such as CCAAT/enhancer-binding protein (C/EBP)β (A), C/EBPα (B), peroxisome proliferator-activated receptor (PPAR) γ (C), and fatty acid synthase (FAS) (D) were estimated by RT-PCR analysis. β-Actin was used as a control. Pre, 3T3-L1 preadipocytes; Adi, mature 3T3-L1 adipocytes.

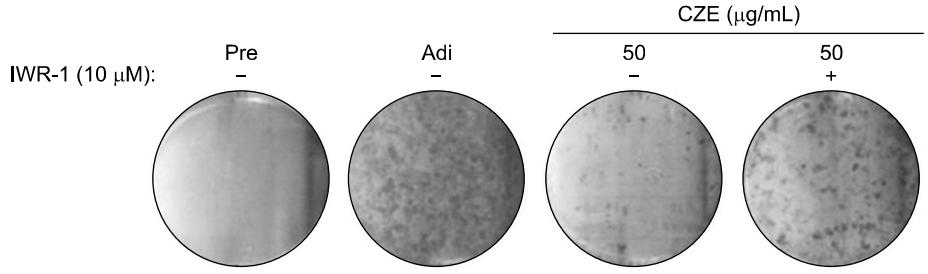

Fig. 5. The anti-adipogenic effect of *Chrysanthemum zawadskii* ethanol extract (CZE) is required the Wnt signaling. 3T3-L1 cells were exposed to 50 μg/mL of CZE in the presence or absence of 10 μM 4-(1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-benzamide (IWR-1) from day −2 to day 2. Intracellular lipid accumulation was visualized using Oil red O staining. Pre, 3T3-L1 preadipocytes; Adi, mature 3T3-L1 adipocytes.

down-regulation of C/EBPα and PPARγ (28). The activation of Wnt, Pref-1, forkhead-containing transcription factors (FoxA1 and FoxA2), and the GATA binding proteins in the pre-adipogenic and early stages resulted in the inhibition of adipogenesis. Previous studies demonstrated that curcumin-induced suppression of 3T3-L1 adipogenesis was associated with activation of Wnt/β-catenin with Wnt-binding protein such as Wnt10b, Fz2 (Wnt direct receptor), and low-density lipoprotein receptor-related protein 5 (Wnt coreceptor) and inhibition of mitogen-activated protein kinase (MAPK; ERK, JNK, and p38) phosphorylation (29). Taken together, these results indicate that the inhibitory effect of CZE in 3T3-L1 adipogenesis would be associated with anti-adipogenic activity through up- and down-regulation of adipogenic transcriptional factors and their down-stream targets.

Differentiation of mature adipocytes from preadipocytes are controlled by 2 key families of adipogenic transcription factors, C/EBP and PPARγ. They promote downstream adipose specific gene expression, such as adipocyte protein 2 (aP2), lipoprotein lipase, and FAS, to trigger the synthesis of fatty acids and triglycerides (30,31). According to Park et al. (32), methanol extract of CZE also demonstrated antiobesity activity through dose-dependent suppression of C/EBPβ, C/EBPα, and PPARγ and reduction of triglyceride contents in 3T3-L1 adipocytes. Blueberry peel extract exhibited DPPH radical scavenging activity and the expression of aP2 and FAS were reduced through dose-dependent suppression of C/EBPβ, C/EBPα, and PPARγ expression in 3T3-L1 adipocytes (33). Although, the bioactive components including estragole, 6-methoxy luteolin, menthiol, rosmarinic acid, leosibirin, crocin, and aglycone forms of rosmarinic acid in ARE, CZE, MAE, PFE, LSE, GJE, and LCE were reported, a comparative study of anti-adipogenic activity and the molecular mechanisms were not conducted. Taken together, we suggest that the CZE inhibited 3T3-L1 adipogenesis by downregulation of the mRNA expression levels of transcriptional factors and adipocyte-specific gene such as C/EBPβ, C/EBPα, PPARγ, and FAS. In addition, our inhibitor assay revealed the Wnt signaling is required for CZE inhibition of 3T3-L1 adipogenesis. In conclusion, CZE has potential applicability as an ingredient for development of functional foods and nutricosmetics for obesity prevention.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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