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Inhibitory effect of enzyme-treated *Chrysanthmum Indicum* L. extract on lipid accumulation in *in vitro* and *in vivo* models

세포 및 동물 모델에서 효소처리 감국 추출물의 지방 축적 억제효과

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이 論文을 韓藥學碩士 學位論文으로 提出함

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ABSTRACT

Inhibitory effect of enzyme-treated *Chrysanthemum Indicum* L. extract on lipid accumulation in *in vitro* and *in vivo* models

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Obesity is a serious health epidemic caused by an imbalance in energy intake and output that is characterized by an increase in the number and size of adipocytes. In addition, obesity is associated with various metabolic disease such as hyperlipidemia, type 2 diabetes and cancer. In the USA, the most dire projections based on earlier secular trends point to over 85% of adults being overweight or obese by 2030. In addition, obesity prevalence in developing contries continues to trend upwards toward USA levels. *Chrysanthemum indicum* L. (CI) is perennial plant widely distributed in Korea, China and Japan. Until now, its flower has been reported to have biological functions including antibacterial, antiviral, antioxidant, immunomodulatory, and anti-obesity effects. However, previous studies have limitation because of the high concentration of CI extract. Therefore, it has to increase efficiency to use enzyme that method. In this study, anti obesity effect of enzyme-treated CI extract (CIVT) examined on *in*

vitro and *in vivo* models of obesity. As *in vitro* models, 3T3-L1 preadipocytes were treated with media to initiated differentiation (MDI) in presence or absence of CIVT with different concentrations. As *in vivo* model, C57BL/6 mice were fed high fat diet (HFD) and administered CIVT for 7 weeks. As a results, CIVT reduced lipid accumulation on 3T3-L1 preadipocytes on Oil Red O staining and triglyceride assay. Also, CIVT reduced protein expression of peroxisome proliferator activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein (C/EBP) and fatty acid synthase (FAS) in 3T3-L1. In addition, CIVT reduced body weight gain on HFD induced obesity mice. Furthermore, CIVT decreased the levels of serum total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-c), leptin, but increased high density lipoprotein cholesterol (HDL-c) and adiponectin. Therefore, CIVT could be used for prevent and improve obesity and metabolic syndrome.

Key words : Obesity; *Chrysanthemum indicum* L.; PPAR-γ; C/EBP-α; C/EBP-β; High fat diet; Lipid profiles

국문초록

세포 및 동물 모델에서 효소처리 감국 추출물의 지방 축적 억제효과

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지 도 교 수 이 영 미

비만은 과도한 에너지 섭취와 에너지 소비의 저하로 인한 열량대사의 불균형으로 인해 지방세포의 수와 크기가 증가하여 체내에 지방이 축적되고 이로 인한 고지혈증, 제 2 형 당뇨병 등 다양한 질병의 원인이 되는 질병이다. 비만은 세계적으로 증가하는 추세이며 미국에서는 2030 년에는 성인의 85% 이상이 과체중 혹은 비만이 될 것으로 예상되고 있다. 또한 개발도상국에서의 비만 유병률 또한 미국 수준으로 지속적으로 상승하는 추세이다. 감국(Chrysanthemum indicum L.)은 국화과(Compositae)에 속하는 다년생 초본으로 예로부터 차로 복용해왔고, 한방에서는 해열, 염증 및 호흡기계 질환에 사용해 왔다. 감국의 꽃은 항균, 항바이러스, 항산화, 면역 조절제, 항비만 효과 등의 생물학적 기능이 있다고 보고되어 있다. 선행연구에서 감국은 용량이 높기 때문에 비만 치료제로 사용하기에 한계가 있다. 따라서 비만 치료제로 사용하기 위해 효소를 사용해 효율을 높여야 하기 때문에 본 연구에서는 효소 처리된 감국 (CIVT) 추출물을 이용한 3T3-L1 세포와

고지방식이로 유도된 비만 마우스 모델에서 항비만 효과를 연구하였다. CIVT 의 세포독성을 확인하기 위해 지방세포 분화 기간 동안 CIVT 를 처리하여 사용한 모든 농도에서 세포독성이 없는 것을 확인하였다. 지방세포의 분화 억제를 확인하기 위해 분화 기간 동안 CIVT 를 농도별로 처리하였으며, 그 결과 지방세포 내 지방의 축적이 감소되는 것을 Oil Red O stain 및 triglyceride 측정법을 통하여 확인하였다. 또한, 지방세포 분화과정의 주요한 인자인 C/EBPα, PPARγ 및 FAS 를 확인한 결과, 대조군에 대비하여 단백질의 발현이 감소하였다. 또한, 고지방식이로 유도된 비만 마우스 모델에서의 CIVT 추출물은 용량 의존적으로 대조군에 비해 체중과 혈액 내 leptin 의 농도를 감소시키고, adiponectin 의 농도를 증가시켰다. 이상의 결과로부터, CIVT 는 비만 질환의 예방 또는 치료제의 소재로 활용될 수 있을 것으로 생각된다.

Ⅰ. INTRODUCTION

Obesity is a serious health problem caused by an imbalance in energy intake and output that is characterized by immoderate lipid accumulation and disfunction in adipose tissue[1]. Additionally, obesity is associated with many metabolic disease such as hyperlipidemia[2], type 2 diabetes[3], cancer[4,5], and it causes the pathology in many organs[6-8]. If secular trends continue, by 2030 an estimated 38% of the world's adult population will be overweight and another 20% will be obese[9]. In the USA, the most dire projections based on earlier secular trends point to over 85% of adults being overweight or obese by 2030[10]. While growth trends in overall obesity in most developed countries seem to have leveled off[11], morbid obesity in many of these countries continues to climb, including among children. In addition, obesity prevalence in developing countries continues to trend upwards toward US levels[12].

Plant walls were composed of glucoside like cellulose, hemicelluloses and lignan[13]. Because of cellulose being the most abundant, cellulases are important enzymes to increase the contents of bioactive components[14]. In recent study, viscozyme and tannase were used for carbohydrase and hydrolyzed tannin[13-16]. Carbohydrase were used for disintegration of cell wall, and facilitating of protein[15]. Tannase was characterized by their activity to complex polyphenolics, and hydrolyze the ester bond and depside bond of substrates such as tannic acid. In tea-processing, the enzymes improve the extract yield and cold-water extract ability[14].

Chrysanthemum indium (CI) flower is perennial plant widely distributed in Korea, China and Japan. Also, it is found in many tropical region such as Southeast Asia, Australia, Africa and USA[17]. CI is traditional herb that has been reported to have biological functions including various immune related disorders, several infectious disease and hypertension symptoms in Korea and China[18,19]. In recent study, CI has antibacterial, antiviral, antioxidant, immunomodulatory effects[20-24]. Recently, anti-obesity effect of CI ethanol extract and ethyl acetate fraction in high fat diet (HFD) induced obese mouse model was reported[25]. However, previous studies have limitation, because of the high concentration of CI extract. Therefore, it has to increase efficiency by using enzyme treated method. In this study, anti-obesity effect of enzyme-treated CI (CIVT) extract examined on *in vitro* and *in vivo* models of obesity. As *in vitro* model, 3T3-L1 preadipocytes were treated with 4, 20, and 100 μg/mL of CIVT extract. As *in vivo* model, C57BL/6 mice were treated with 4, 20, and 100 mg/kg of CIVT extract for seven weeks.

Adipogenic transcription factors such as of the CCAAT/enhancer binding protein alpha (C/EBPα), nuclear receptor peroxisome proliferation-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein beta (C/EBPβ) play a key role in the transcriptional cascade during adipogenesis[26]. C/EBPβ is induced early phase of differentiation, however C/EBPα and PPARγ are expressed in late phase[27, 28]. During adipogenesis, inhibition of C/EBPα and PPARγ can inhibit lipid accumulation[29]. Also, the expression of these transcription factors stimulate expression of fatty acid synthesis (FAS), while synthesis of triglycerides[30, 31]. Two major adipokines, leptin and adiponectin are secreted by white adipose tissue

and essential for the regulation of metabolism. Leptin is satiety hormone to help regulation of energy balance[32,33]. In spite of high energy stored like obese, leptin sensitivity is reduced. Therefore, increase of leptin level signify overfeeding and obesity[32]. Adiponectin is a protein which acts as a crucial factor for improving insulin resistance[34,35]. In obese, serum adiponectin level is diagnostic marker of metabolic disease such as non-alcoholic steatohepatitis (NASH)[36,37].

Ⅱ. MATERIALS AND METHODS

2.1. Materials

All reagents were purchased from Sigma-Aldrich Co. (st. Louis, MO, USA). The 3T3-L1 pre-adipocytes cell stock was purchased from ATCC Co. (CL-173). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), bovine calf serum (BCS) and penicillin were purchased from LONZA (Basel, Switzerland). PVDF membrane, chemilumnescence (ECL) detection kit were purchased from Amersham Pharmacia biotech Co. (Piscataway, NJ, USA). The primary antibodies, mouse anti- PPAR-γ (sc-J3012), C/EBP-α (sc-65318), β-actin (sc-47778), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for FAS (C20G5) was purchased from Cell Signaling Technology (Beverly, MA, USA) and used at 1:1000 dilutions. The secondary antibody, goat-anti mouse IgG F(ab)2' HRP conjugate antibody, was purchased from Enzo Life Sciences (Enzo Life Sciences, Farmingdale, NY, USA). Secondary antibody was used at 1:5000 dilution.

2.2. Preparation of Ethanol Extract of Enzyme-treated *Chrysanthemum indicum* L. (CIVT)

Chrysanthemum indicum L. (CI) was provided by Ui-Seong, Korea. 1 liter of purified distilled water was added to 50 g of dried CI powder and boiled for 2 h at 90℃ in water bath. After cooling to 45℃, it was adjusted to pH 4.5 using citric acid solution (1N). Then, 0.75 g of viscozyme (Viscozyme-L. Novozyme, Denmark) and 100 mg of tannase (Tannase-KTFH, Kikkoman, Japan) were added

and the enzyme treatment was carried out with shaking at 100 rpm for 24 h at 45℃. After the enzyme treatment was completed, the enzyme was inactivated for 30 min at 90℃ and the primary extract was prepared by filtration using a standard sieve (No. 850, 75 μm). 1 liters of ethanol were added to the remaining residual substance and the mixture was extracted for 6 h at 70℃. Then, the substance was mixed with the primary extract, concentrated under reduced pressure, sterilized for 15 min at 121℃ and lyophilized to prepare a CIVT. The dried powders of CIVT was stored at 4℃.

Figure 1. Photograph of *Chrysanthemum indicum* L.

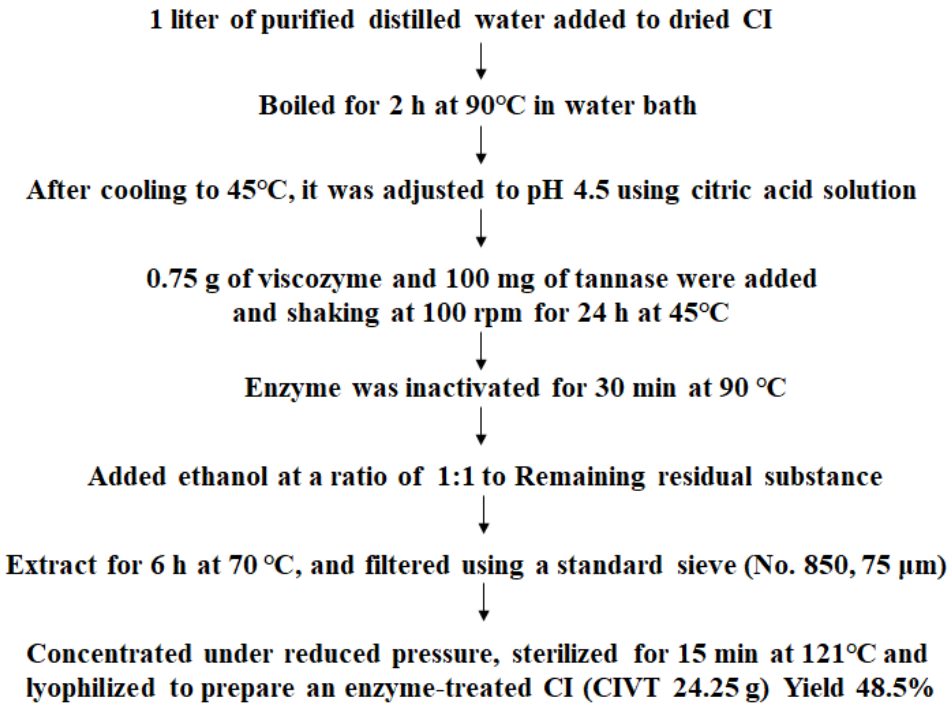


Figure 2. Preparation of Enzyme treated CI

2.3. Cell Viability Assay

The effects of CIVT on cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The end of experimental period (day 8), MTT (500 μg/mL) was added and the cells were incubated for 4 h at 37℃. After removal medium, the formazan were dissolved by DMSO. The absorbance of each well was measured at 540 nm using a enzyme-linked immunosorbent assay (ELISA) microplate reader (SpectraMax 190, Molecular Devices, California, USA).

2.4. Cell Culture and Differentiation of 3T3-L1 Preadipocytes

3T3-L1 preadipocytes were grown in DMEM supplemented with 2 mM glutamine, 1% penicillin/streptomycin (10,000 units of penicillin/mL and 10 mg sterptomycin/mL) and 10% BCS at 37℃ under a humidified 5% CO_2 atmosphere. For differentiation of 3T3-L1 preadipocytes, cells were seeded into 24-well plate or 6 cm dish and cultured as described above. Two days after confluence (define as day 0), cells were incubated in differentiation medium containing 1 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 5 μM dexamethasone in DMEM supplemented with 10% FBS and 1% penicillin for 2 days. The medium replaced by insulin in DMEM supplemented with 10% FBS and 1% penicillin for 2 days. After 4 days, the medium replaced by DMEM and cell lipid accumulation was measured by Oil Red O staining and TG assay.

2.5. Oil Red O Staining

After lipid accumulation, the plate was stained with Oil Red O. Cells were washed PBS and fixed with 10% formalin for 1 h at room temperature. Cells were washed with 60% isopropyl alcohol. After washing, lipid droplets were stained with Oil Red O solution (3.5 mg/mL stock solution in 60% isopropyl alcohol, filtered through 0.45 μm filter) for 1 h. Then, cells were washed with 60% isopropyl alcohol for 2 times and the stained lipid droplets were extracted with isopropyl alcohol. Fat droplets in the cells were visualized using microscope and measured using ELISA reader at 490 nm filter.

2.6. TG Contents

3T3-L1 cell were homogenized in lysis buffer (iNtRON Biotech) on ice. After homogenate, that centrifuged at 4℃, 12,000 rpm for 10 min and transfer supernatants to fresh tube. TG contents were determined using a TG assay kit (Asan Pharmaceutical Co., Seoul, South Korea).

2.7. Western Blot Analysis

3T3-L1 cell were homogenized in lysis buffer (iNtRON Biotech) on ice. After homogenate, that centrifuged at 4℃, 12,000 rpm for 10 min and transfer supernatants to fresh tube. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Aliquots of 25 ~ 50 μg protein were resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes for 2 h. The transferred membranes were blocked in 5% skim milk in 1X PBST buffer for 2 h. And then, incubated primary antibodies (1:1000) for overnight at 4℃. After washing with PBST, the membrane were incubated with secondary antibodies for 1 h at room temperature; HRP-conjugated HRP-conjugated goat anti-mouse IgG, polyclonal antibody that 1:5000 dilution in 5% skim milk in PBST. Reactive bands were visualized using enhance chemiluminescence (ECL) reagents and protein expression were exposed by analyzing the signals captured on the PVDF membranes using a Fluor Chem E image analysis (Cell biosciences, CA, USA)

2.8. Animals and Treatment

Six-weeks-old, male C57BL/6J mice (22 ± 2g) were purchased from SAMTAKO Bio Korea (Osan, South Korea). Mice were housed in a controlled environment with constant humidity (50 ± 10%) and constant temperature (22 ± 2℃), under a 12-h dark/ 12-h light cycle, with a standard laboratory diet and water supply. After a one week acclimation period, mice were randomly divided into six groups of six mice each. ND, mice fed with normal chow diet; HFD, mice fed with high fat diet (HFD; 45% lard oil in normal chow diet); CIVT-4, mice fed with HFD and treated with CIVT (4 mg/kg); CIVT-20, mice fed with HFD and treated with CIVT (20 mg/kg); CIVT-100, mice fed with HFD and treated with CIVT (100 mg/kg); ORL, mice fed with HFD and treated with orlistat (20 mg/kg); Orlistat is an effective drug for obesity that inhibits lipid accumulation. Since many studies have reported that orlistat inhibited obesity, orlistat was selected as a positive control during the *in vivo* experiment. Each sample was dissolved in 0.3% carboxymethylcellulose (CMC) and orally administered once a day for seven weeks. Body weight was measured once a week over the seven-week experimental period.

2.9. Serum Preparation

Mice were anesthetized by exposure to diethyl ether and euthanized by rapid cervical dislocation. Blood samples were taken from the inferior vena cava of the mice. The collected blood samples were held for 1h at room temperature. Then, serum was obtained by centrifugation at 3,000 rpm for 10 min at 4℃. Serum was stored at -80℃ until use.

2.10. Serum Biochemical Analysis

Serum biochemical levels of triglyceride (TG), total cholesterol (TC), and high-density lipoprotein-cholesterol (HDL-c), were analyzed using assay kits (Asan Pharmaceutical Co., Seoul, South Korea). All of measurements were perfomed according to manufacturer's instruction. Low-density lipoprotein cholesterol (LDL-c) level was calculated using Friedewald's formula; LDL-c (mg/dL) = TC-(TG/5) - HDL-c.

2.11. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of leptin and adiponectin were analyzed using leptin ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) and adiponectin ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocols.

2.12. High Performance Liquid Chromatography analysis

HPLC was equipped with a vacuum degassed, a quaternary pump, and an automatic sample injection system. Chromatographic separation was performed using a Agilent ZORBAX Eclipse Plus C18 (4.6×250 mm, 5 μm, Agilent Technologies. Waldbronn, Germany) equipped with photodiode array detector with wavelength set at 330 nm. Temperature of column oven was 30°C and injection volume was 10 μL. The elution was performed at flow rate of 1.0 mL/min, using distill water (DW) with 0.1% H_3PO_4 (A) and acetonitrile (B) as mobile phase. The solvent gradient changed according to the conditions Table 1.

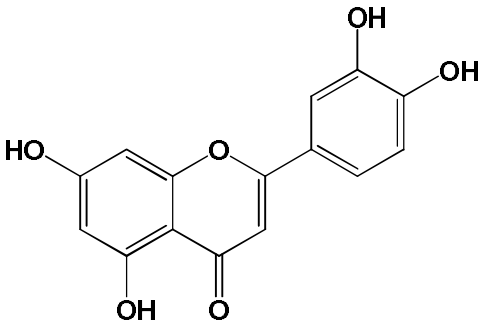


Figure 3. Chemical structure of Luteolin isolated from CIVT

Table 1. HPLC Conditions for analysing

Column	Agilent ZORBAX Eclipse Plus C18 (4.6 × 250 mm, 5 μm, Agilent Technologies)		
Detecting absortion	330 nm		
Column Temp.	30 ℃		
Flow rate	1.0 mL/min		
Injection volume	10 μl		
Elute A	0.1% H_3PO_4 in DW		
Elute B	Acetonitrile		
Gradient	Time	Elute A	Elute B
	0	85	15
	8.00	85	15
	25.0	65	35
	30.0	30	70

2.13. Statistical Analysis

Values are expressed as mean ± standard deviation (SD) for independent experiments, and statistical significance was determined using the Student's t-test. All statistical analysis was done with the SPSS statistical analysis software version 22 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $*p < 0.05$, $** p < 0.005$.

Ⅲ. RESULTS

3.1 The effect of CIVT on cell viability in 3T3-L1 cells.

The cytotoxicity of CIVT extracts on 3T3-L1 preadipocyte was measured with MTT assay. 3T3-L1 preadipocytes were seeded into 24 well plate at a density of $2x10^5$cell/well and incubation for 24 h. After incubation, 3T3-L1 cells were treated with concentration of 4, 20, and 100 μg/mL CIVT for 8 days. As shown in Figure 4, treatment with 4-100 μg/mL of CIVT extracts had no significant cell toxicity on 3T3-L1 preadipocytes. Thus, CIVT extracts were treated with 4, 20, and 100 μg/mL concentrations for all experiments.

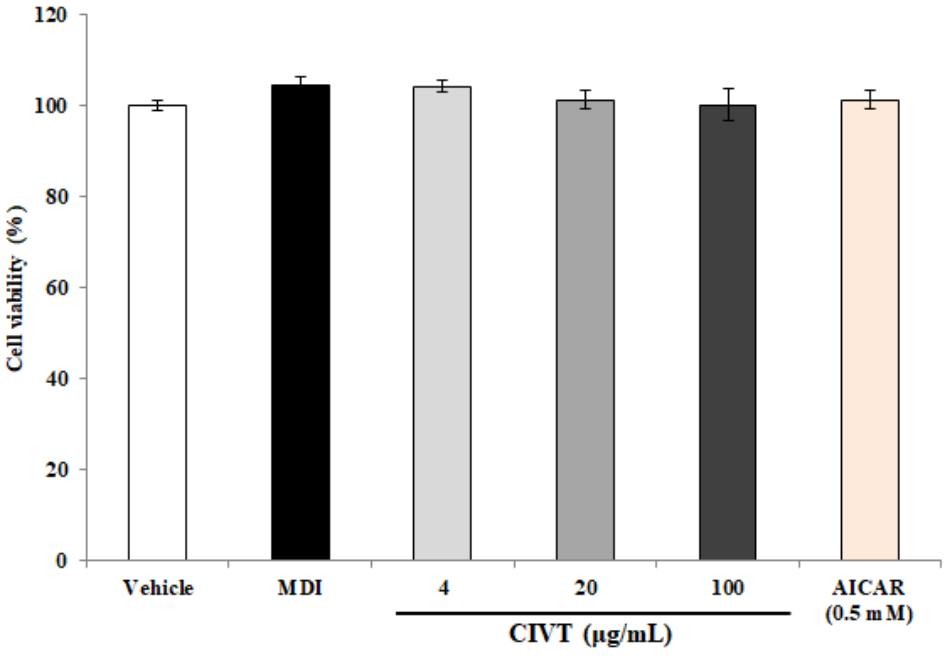


Figure 4. Effect of CIVT on cell viability in 3T3-L1 cells.

3T3-L1 cells were treated with various concentrations of CIVT extract. As a result, CIVT showed no cytotoxicity in 3T3-L1 preadipocytes. Data are represented as mean ± SD from three separate experiments.

3.2. The effect of CIVT on lipid accumulation in 3T3-L1 cells.

The lipid accumulation of CIVT was measured using Oil Red O staining in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated for 2 days after seeding, and treated with 4, 20, and 100 μg/mL of CIVT for 2 to 8 days. After 8 days, lipid accumulation measured using Oil Red O staining. As shown in Figure 5 and 6, CIVT reduced intracellular lipid accumulation in 3T3-L1. Especially, CIVT reduced lipid accumulation approximatively 30% than control at 100 μg/mL, while AICAR (positive control) reduced 33% than control.

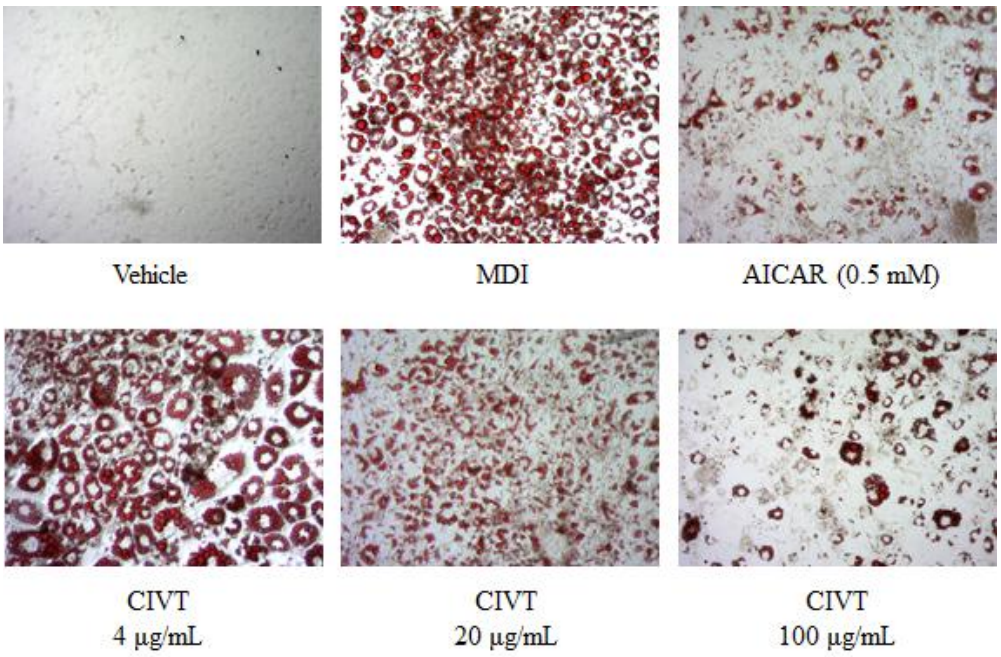


Figure 5. Effect of CIVT on morphological change in 3T3-L1 cells.

CIVT suppressed lipid accumulation and adipocyte differentiation in various concentrations in 3T3-L1 cells as measured using Oil Red O staining (magnification 200X). AICAR was used as positive control.

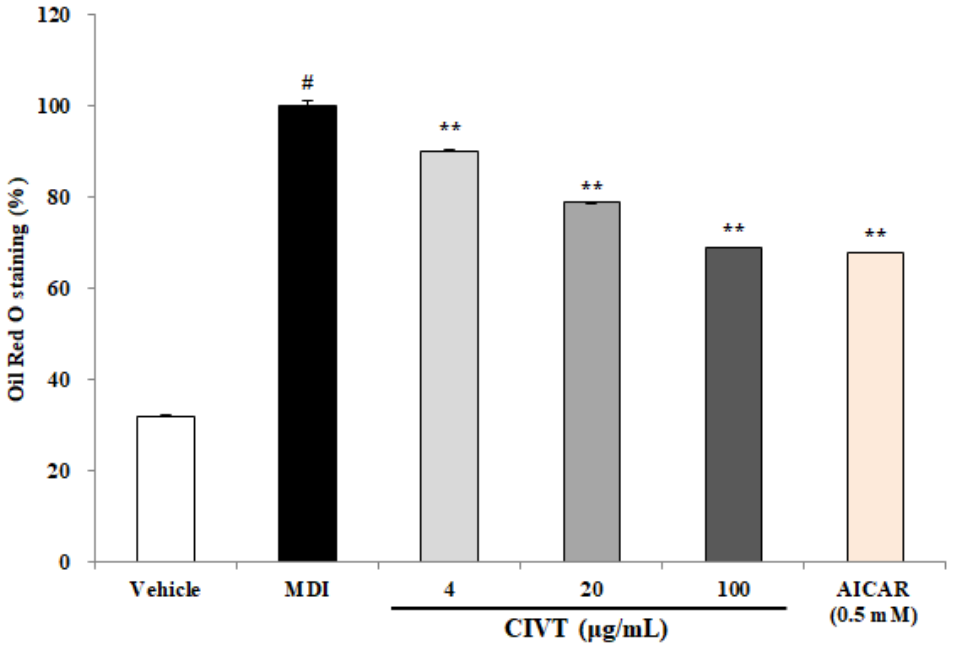


Figure 6. Effect of CIVT on lipid accumulation in 3T3-L1 cells.

3T3-L1 cells were treated with various concentrations of CIVT. After 8 days, lipid accumulation was measured using Oil Red O staining. AICAR was used as positive control. Data are represented as mean ± SD from three separate experiments. #$p < 0.05$ vs Vehicle, *$p < 0.05$, **$p < 0.01$ vs MDI.

3.3. The effect of CIVT on TG accumulation in 3T3-L1 cells.

3T3-L1 cells were treated with various concentrations of CIVT. 3T3-L1 preadipocytes were incubated for 2 days after seeding, treated with 4, 20, and 100 μg/mL of CIVT for 2 to 8 days. After 8 days, 3T3-L1 cells were homogenized in lysis buffer. TG contents in cell lysate was measured using TG assay kit. As shown in Figure 7, CIVT reduced intracellular triglyceride accumulation in 3T3-L1. Especially, CIVT reduced TG accumulation approximatively 60% than control at 100 μg/mL, while AICAR (positive control) reduced 65% than control.

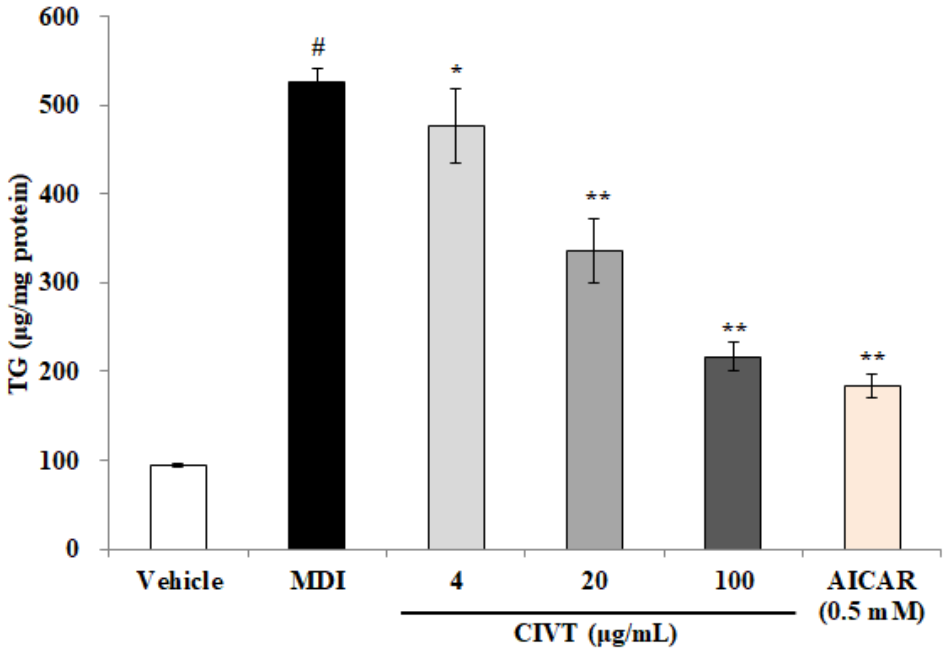


Figure 7. Effect of CIVT on TG accumulation in 3T3-L1 cells.

3T3-L1 cells were treated with various concentrations of CIVT. After 8 days, triglyceride accumulation measured using TG assay kit. AICAR was used as positive control. Data are represented as mean ± SD from three separate experiments. #$p < 0.05$ vs Vehicle, *$p < 0.05$, **$p < 0.01$ vs MDI.

3.4. The effect of CIVT on the expression of early adipogenic transcription factors in 3T3-L1 cells.

In early phase of adipocyte differentiation, MDI induces confluent preadipocytes to one or two of decision, which is a precondition for differentiation into adipocytes. C/EBPβ plays early catalytic action in the differentiation pathway followed by expression levels of C/EBPα in 3T3-L1. To demonstrate the effect of CIVT on early process differentiation, 3T3-L1 cells were incubated for 2 days after seeding, treated 4, 20, and 100 μg/mL of CIVT in MDI cocktail medium to the cells. As shown in Figure 8, the expression levels of C/EBPβ in 3T3-L1 were decreased compared to control. Especially, CIVT reduced the expression levels of C/EBPβ in 3T3-L1 approximatively 85% than control at 100 μg/mL, while AICAR (positive control) reduced 90% than control.

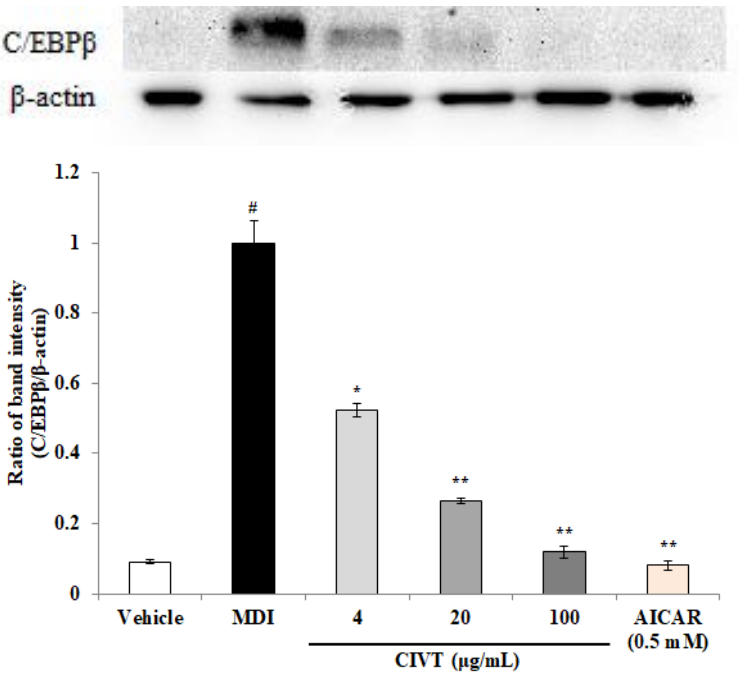


Figure 8. Inhibitory effect of CIVT on the expression of C/EBPβ in early phase differentiation on 3T3-L1 cells.

Protein expression levels of C/EBPβ in 3T3-L1 were determined using western blot analysis and expressed as percent of control (MDI). AICAR was used as positive control, and β-actin was used as a loading control. Data are represented as mean ± SD from three separate experiments. #$p < 0.05$ vs Vehicle, *$p < 0.05$, **$p < 0.01$ vs MDI.

3.5. The effect of CIVT on the expression of adipogenic transcription factors in 3T3-L1 cells.

3T3-L1 preadipocytes were incubated for 2 days after seeding, and treated 4, 20, and 100 μg/mL of CIVT for 2 to 4 days with MDI cocktail medium. As a result, the expression levels of C/EBPα in 3T3-L1 were decreased compared to control. Especially, CIVT reduced expreesion levels of C/EBPα in 3T3-L1 approximatively 55% than control at 100 μg/mL, while AICAR (positive control) reduced 70% than control (Figure 9). Also, the protein expression levels of PPAR γ in 3T3-L1 were decreased compared to control. Especially, CIVT reduced expression levels of PPARγ in 3T3-L1 approximatively 75% than control at 100 μ g/mL, while AICAR (positive control) reduced 90% than control (Figure 10).

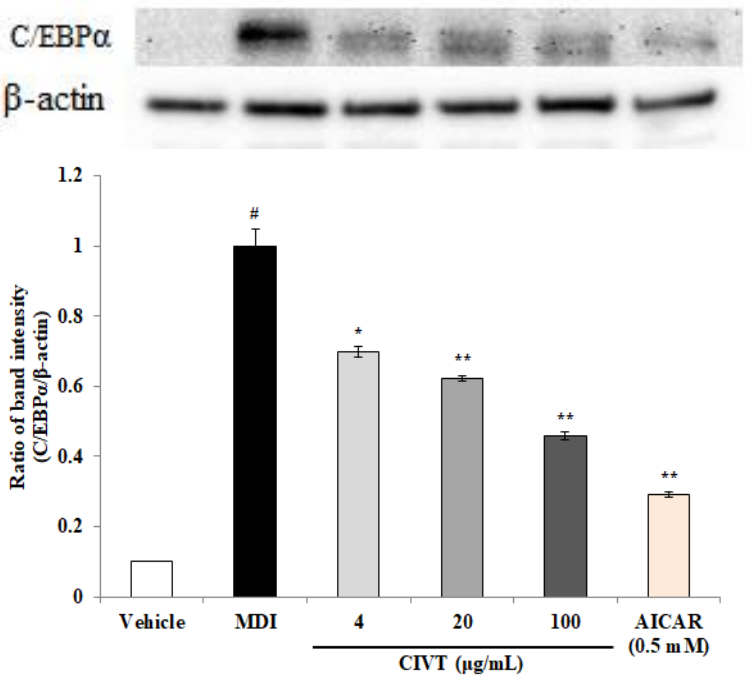


Figure 9. Inhibitory effect of CIVT on the expression of C/EBPα in differentiation on 3T3-L1 cells.

Protein expression levels of C/EBPα in 3T3-L1 were determined using western blot analysis and expressed as percent of control (MDI). AICAR was used as positive control, and β-actin was used as a loading control. Data are represented as mean ± SD from three separate experiments. #$p < 0.05$ vs Vehicle, *$p < 0.05$, **$p < 0.01$ vs MDI.

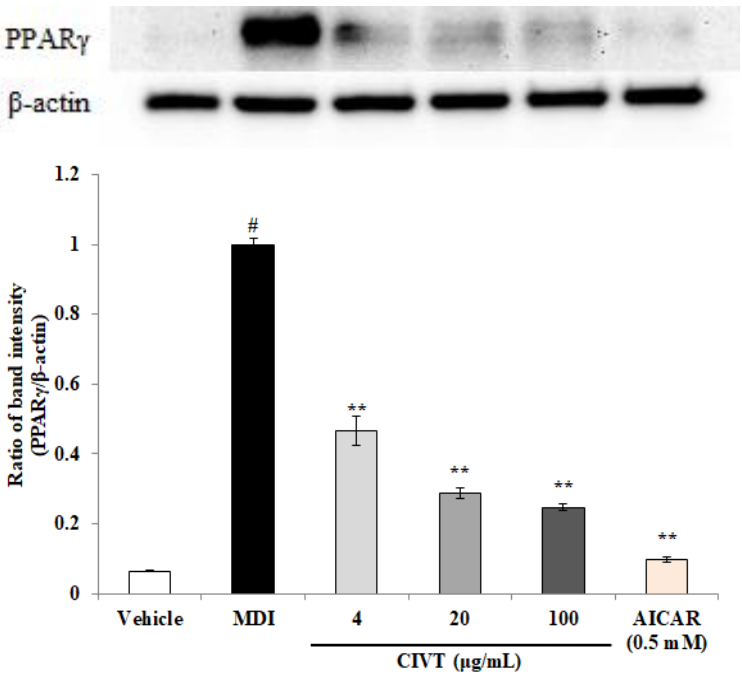


Figure 10. Inhibitory effect of CIVT on the expression of PPARγ in differentiation on 3T3-L1 cells.

Protein expression levels of PPARγ in 3T3-L1 were determined using western blot analysis and expressed as percent of control (MDI). AICAR was used as positive control, and β-actin was used as a loading control. Data are represented as mean ± SD from three separate experiments. #$p < 0.05$ vs Vehicle, *$p < 0.05$, **$p < 0.01$ vs MDI.

3.6. The effect of CIVT on the expression of fatty acid synthase in 3T3-L1 cells.

3T3-L1 preadipocytes were incubated for 2 days after seeding, and treated 4, 20, and 100 μg/mL of CIVT for 2 to 8 days with MDI cocktail medium. As shown in Figure 11, the expression levels of FAS in 3T3-L1 were decreased compared to control. Especially, CIVT reduced expression of FAS approximatively 60% than control at 100 μg/mL, while AICAR (positive control) reduced 40% than control.

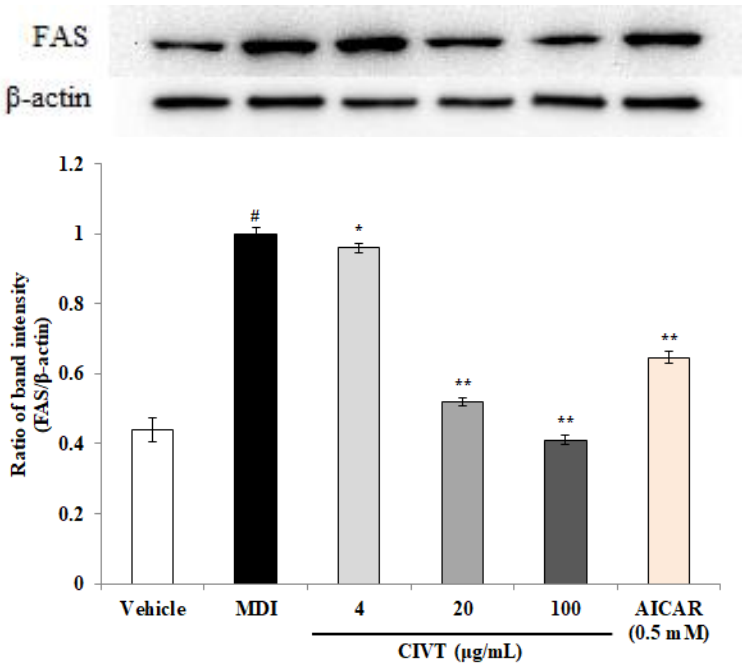


Figure 11. Inhibitory effect of CIVT extracts on the expression of FAS in differentiation on 3T3-L1 cells.

Protein expression levels of FAS in 3T3-L1 were determined using western blot analysis and expressed as percent of control (MDI). AICAR was used as positive control, and β-actin was used as a loading control. Data are represented as mean ± SD from three separate experiments. #$p < 0.05$ vs Vehicle, *$p < 0.05$, **$p < 0.01$ vs MDI.

3.7. The effect of CIVT on body weight change in HFD-induced obese mice.

To examine the anti-obesity effect of CIVT, body weight were measured weekly. On the first day of the experiment (week 0), the average body weights of all groups were similar. The final average body weights of all groups had increased from the start day. In particular, body weight gain in the HFD group was far higher than that in ND group. As shown in Figure 12, the mean body weight gain value was increased in HFD group (14.20 ± 0.71) compared to ND group (6.95 ± 0.46). CIVT reduced body weight gain in CIVT-20 (6.58 ± 0.47), CIVT-100 (6.35 ± 0.57) groups better than positive control, ORT (7.15 ± 0.22).

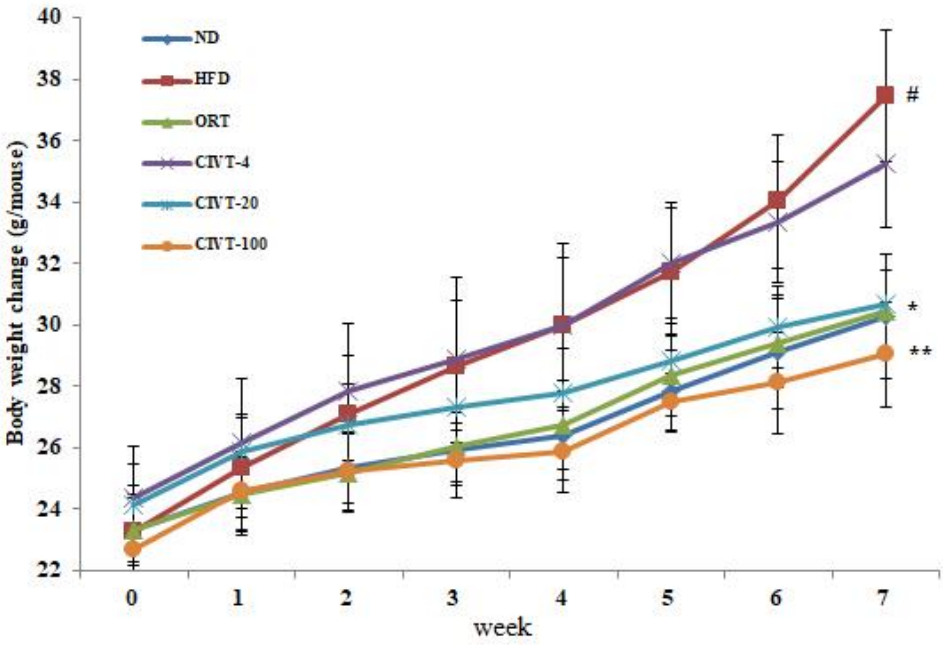


Figure 12. Effect of CIVT on body weight change in HFD-induced obese mice.

Body weight were measured weekly for 7 weeks. ND ; mice fed with normal diet, HFD ; mice fed with HFD, CIVT-4 ; mice fed with HFD and treated with CIVT (4 mg/kg), CIVT-20 ; mice fed with HFD and treated with CIVT (20 mg/kg), CIVT-100 ; mice fed with HFD and treated with CIVT (100 mg/kg), ORT ; mice fed with HFD and orlistat (20 mg/kg). Orlistat was used as positive control. Data are represented as mean ± SD. #$p < 0.05$ vs ND, *$p < 0.05$, **$p < 0.01$ vs HFD.

3.8. Effect of CIVT on the serum lipid profiles in HFD-induced obese mice.

In obese persons, often results in lipid profiles such as increased serum TG, TC, LDL-c and decreased HDL-c levels. Therefore, to investigate effect of CIVT on obese mice, serum lipid profiles were measured. TC, TG, and LDL-c levels were increased in HFD group compare with ND group. And HDL-c levels were decreased in HFD groups compare with ND group. As shown in Table 2, CIVT oral administration inhibited TC, TG, LDL-c levels and increased HDL-c levels, compare with HFD group more than ORT.

Table 2. Effect of CIVT on the serum lipid profiles in HFD-induced obese mice.

Groups	ND	HFD	HFD+ORT	HFD+CIVT (mg/kg)		
				4	20	100
Triglyceride (TG, mg/dL)	110 ± 4.2	160.5 ± 28.3 #	85.9 ± 23.1 *	160.2 ± 23.2	104.8 ± 7.8 *	94.87 ± 6.7 *
Total cholesterol (TC, mg/dL)	107.8 ± 7.0	171.5 ± 3.9 ##	115.0 ± 18.8 *	149.1 ± 8.3 *	109.7 ± 7.3 **	106.3 ± 13.4 *
LDL-cholesterol (LDLc, mg/dL)	44.8 ± 5.5	63.9 ± 10.9 #	35.9 ± 7.0 *	54.3 ± 12.2	38.5 ± 6.4 *	33.48 ± 5.1 *
HDL-cholesterol (HDLc, mg/dL)	41.0 ± 2.9	81.3 ± 8.4 ##	61.8 ± 10.0 *	78.6 ± 20.6	54.71 ± 6.3 **	55.38 ± 3.2 **

ND ; mice fed with normal diet, HFD ; mice fed with HFD, CIVT-4 ; mice fed with HFD and treated with CIVT (4 mg/kg), CIVT-20 ; mice fed with HFD and treated with CIVT (20 mg/kg), CIVT-100 ; mice fed with HFD and treated with CIVT (100 mg/kg), ORT ; mice fed with HFD and orlistat (20 mg/kg). Orlistat was used as positive control. Data are represented as mean ± SD. #$p < 0.05$ vs ND, *$p < 0.05$, **$p < 0.01$ vs HFD.

3.9. Effect of CIVT on serum adipokines in HFD-induced obese mice.

In obese persons, often results in adipokines such as increased serum leptin and decreased adiponectin levels. Therefore, to investigate effect of CIVT on obese mice, serum adipokines were measured. Serum leptin level was increased in HFD group compared with ND group. However, serum adiponectin level was decreased in HFD group compared with ND group. As shown in Table 3, CIVT oral administration inhibited leptin level, whereas adiponectin levels were increased.

Table 3. Effect of CIVT on serum adipokines in HFD-induced obese mice.

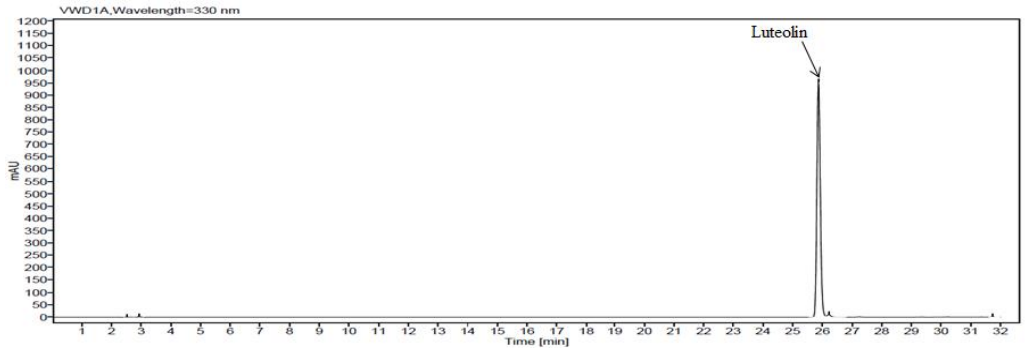
Groups	ND	HFD	HFD+ORT	HFD+CIVT (mg/kg)		
				4	20	100
Leptin (pg/mL)	1329 ± 166	24192 ± 3044 ##	12700 ± 3121 *	24184 ± 3938	11725 ± 3962 **	6518 ± 6237 *
Adiponectin (ng/mL)	32.07 ± 1.5	29.14 ± 3.5	38.71 ± 5.3	35.69 ± 3.6 *	35.49 ± 5.4	40.29 ± 6.6

ND ; mice fed with normal diet, HFD ; mice fed with HFD, CIVT-4 ; mice fed with HFD and treated with CIVT (4 mg/kg), CIVT-20 ; mice fed with HFD and treated with CIVT (20 mg/kg), CIVT-100 ; mice fed with HFD and treated with CIVT (100 mg/kg), ORT ; mice fed with HFD and orlistat (20 mg/kg). Orlistat was used as positive control. Data are represented as mean ± SD from six separate experiments. #$p < 0.05$ vs ND, *$p < 0.05$, **$p < 0.01$ vs HFD.

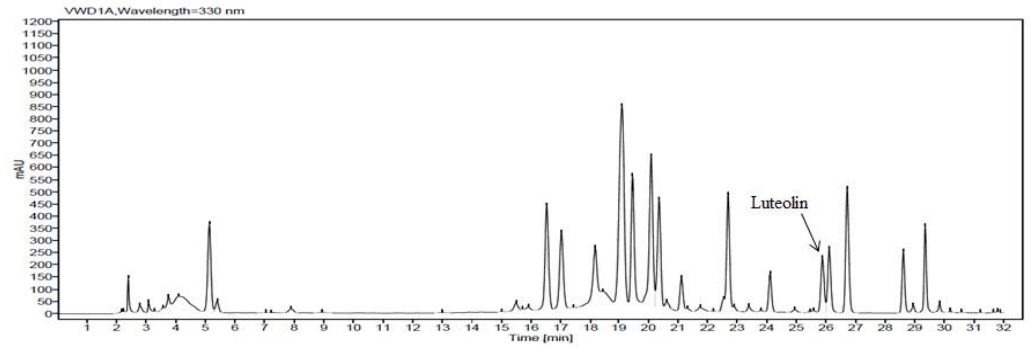
3.10. HPLC analysis of CI ethanol extract (CIEE) and CIVT.

The chemical structure of luteolin is shown Figure 3. The data from the quantitative analysis of the luteolin, CIEE and CIVT made using HPLC with Photodiode-Array detector (PDA), while the chromatograms with detector responses at 330 nm. As shown Figure 13. luteolin was identified at a retention time of 25.852 min and it is contained 1.31 ± 0.02 mg/g in CIEE. However, luteolin was identified at a retention time of 25.843 min and it is contained 3.09 ± 0.03 mg/g in CIVT. It was confirmed that CIVT contained compounds 2.3 fold higher than CIEE

(A)



(B)



(C)

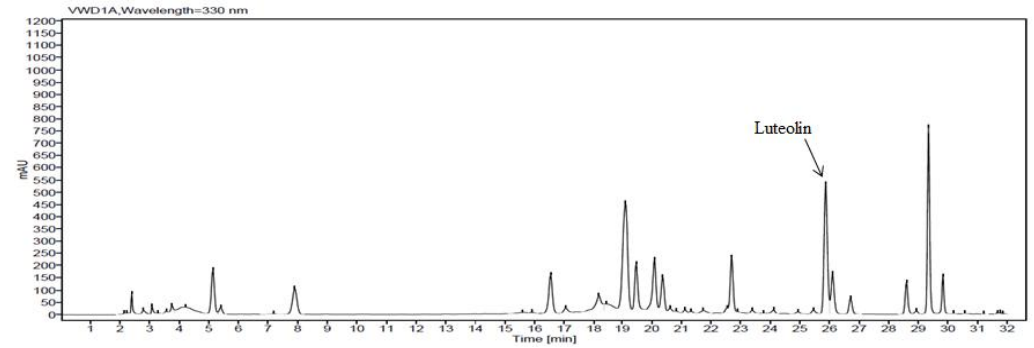


Figure 13. HPLC analysis of CIEE and CIVT.

HPLC chromatogram of CIEE and CIVT at 330 nm. HPLC chromatogram of Luteolin standard (A), CIEE (B) and CIVT (C)

Ⅵ. DISCUSSION

Recently, study about obesity are ongoing worldwide, and a variety results of research are emerging. To maintain health and improve metabolic disease lead to obesity, many drugs have been developed. However, treatment of obesity drugs, including orlistat (gastrointestinal lipid uptake inhibitor), sibutramine (appetite suppressor), fibrates (peroxisome proliferator-activated receptor-α agonists) have been reported to undesirable side effects such as elevated blood pressure, dry mouth, anorexia, constipation, insomnia, nausea and dizziness[44]. Thus, the research has been increased for stable and effective anti-obesity reagents. Therefore, it is necessary to develop the useful agent to improve, prevent and treat obesity form natural products.

As shown in Figure 5-7, CIVT extract significantly and dose-depentently inhibited lipid droplet accumulation in 3T3-L1 preadipocytes. As shown in Figure 8-11, protein expression levels of C/EBPs, PPARɣ and FAS were decreased compared with control. C/EBPs and PPARɣ are the most important factors on differentiation pathway of 3T3-L1 preadipocytes. Especially, expression levels of C/EBPβ is increased rapidly at early phase of differentiation caused by hormonal stimulation. Subsequently, expression levels of C/EBPα and PPARɣ are induced after expression of C/EBPβ is decreased sequentially. However, C/EBPβ plays early catalytic roles in preadipocyte differentiation[26]. PPARɣ and C/EBPα induce expression of genes involved in lipogenesis like FAS, causing the synthesis of triglyceride and fatty acid[31]. These results indicate that CIVT extract inhibit adipogenesis through decrease protein expression of C/EBPs and PPARɣ, regulate

early and middle phase of adipocyte differentiation.

In the *in vivo* level, as shown in Figure 12, CIVT extract administration in HFD-induced obese mice for 7 weeks, significantly and dose-dependently decreased body weight gain more than orlistat. In the liver, it occurs accumulation of TG, because of the use of liver TG due to use of very low density lipoproteins (VLDLs)[38]. In addition, TG level was increased when the same time accompanied by decrease in HDL-c level, which used as a marker of insulin resistance, type 2 diabetes[39]. As shown in Table 2, lipid profiles in blood serum, such as TC, TG, and LDL-c levels were significantly reduced in treatment of CIVT extract, similar levels of orlistat. On the other hand, CIVT increased HDL-c. These results indicate that CIVT extract suppresses hyperlipidemia in HFD-induced obese mice. Two major adipokines, leptin and adiponectin, are secreted by white adipose tissue and essential for the regulation of metabolism. Leptin plays a key role in controlling the size of adipose tissue. Another adipokine, adiponectin is known to contribute to insulin sensitivity and fatty acid oxidation[40-43]. As shown in Table 3, serum leptin level significantly increase in HFD-induced obese mice, but lowered in CIVT treated mice. And serum adiponectin level was raised by CIVT treatment. These results indicate that CIVT efficiently regulates metabolic homeostasis in HFD-induced obese mice.

The major compound of the CI extract are reported as luteolin[45]. Luteolin was known to improve obesity by inhibiting adipogenesis and lipid accumulation[46]. By treating the CI extract with enzymes, flavonoid glycosides to aglycones, increased bioavailability. These results support that enzyme treatment of CI extract leads to better anti obesity effect by increasing the contents of anti

obesity components.

In conclusion, CIVT inhibits lipid accumulation in 3T3-L1 cells by suppressing C/EBPs, PPARɣ and FAS expression. Also, CIVT decrease body weight gain and TC, TG, LDL-c and leptin in serum, and increase HDL-c and adiponectin in serum through increase major compounds, luteolin. Therefore, CIVT could be used to prevent and improve obesity and metabolic syndrome.

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