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## Roots extracts of *Adenophora triphylla* var. *japonica* improve obesity in 3T3-L1 adipocytes and high-fat diet-induced obese mice

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## ABSTRACT

**Objective:** To investigate the anti-obesity activity and the action mechanism of the roots of *Adenophora triphylla* var. *japonica* extract (ATE) in high-fat diet (HFD)-induced obese mice and 3T3-L1 adipocytes.

**Methods:** The roots of *Adenophora triphylla* were extracted with 70% ethanol. To demonstrate the compounds, linoleic acid was analyzed by using gas chromatography; and the anti-obesity effects and possible mechanisms of ATE were examined in 3T3-L1 adipocytes and HFD-induced obese mice.

**Results:** Treatment with ATE inhibited the lipid accumulation without cytotoxicity in 3T3-L1 adipocytes. Furthermore, 200 and 400 mg/kg ATE treatment significantly decreased the body weight gain, white adipose tissues (WATs) weight and plasma triglyceride level, while 100 and 200 mg/kg ATE treatment increased the plasma high-density lipoprotein cholesterol level in the HFD-induced obese mice, as compared with the HFD group. Treatment with 200 and 400 mg/kg ATE also lowered the size of adipocytes in adipose tissue and reduced the lipid accumulation in liver. ATE treatment showed significantly lower expression level of adipogenesis-related proteins, such as peroxisome proliferator-activated receptor  $\gamma$ , fatty acid binding protein (aP2), fatty acid synthase in 3T3-L1 adipocytes; and furthermore, decreased peroxisome proliferator-activated receptor  $\gamma$ , aP2, fatty acid synthase, sterol regulatory element binding protein-1c, and lipoprotein lipase mRNA expression levels in WAT of the HFD-induced obese mice.

**Conclusions:** These results suggested that the ATE has an anti-obesity effect, which may be elicited by regulating the expression of adipogenesis and lipogenesis-related genes and proteins in adipocytes and WAT of the HFD-induced obese mice.

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## 1. Introduction

*Adenophora triphylla* (*A. triphylla*) var. *japonica* (Korean name: Jan-dae, Japanese name: lady bell, English name: Three-leaf ladybell) belongs to the *Adenophora* species (Campanulaceae), which has been used as an oriental medicinal plant in Korea, China and Japan for anti-inflammation, anti-tussive [1] and hepatoprotective effects. Alkaloids, triterpenoids and essential oil components such as pyrrolidine, piperidine, triphyllol, nonacosane, heptacosane, lupenone and saponine have been identified from *A. triphylla*. These chemicals have anti-inflammatory and anti-oxidant properties, and are used to prevent obesity in Korea.

Obesity has become a significant clinical problem in recent decades since it could contribute to life-threatening diseases such as type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease [2]. It is characterized by increased fat mass, which is associated with increased cell number (hyperplasia) and sizes (hypertrophy) [3,4]. Adipocyte hypertrophy is caused by excessive accumulation of lipid such as triglyceride formed from energy intake [5]. Adipocytes hyperplasia results from a complex interaction between proliferation and differentiation in preadipocytes that is involved in the adipogenic process where undifferentiated preadipocytes are converted to differentiated adipocytes [6,7]. Therefore, adipogenesis and TG accumulation play an important role in fat mass increase. Accordingly, controlling adipogenesis and TG accumulation in adipocytes could help prevent obesity and its associated diseases.

There are many different pharmacological approaches to prevent and treat obesity, but they reportedly have a number of limitations such as undesirable side effects and adverse effects [8,9]. Recently, there is increasing public attention on using natural products to prevent and treat obesity with various plants such as fruits, vegetables, and herbs that have beneficial health effects and minimal side effects [10].

*A. triphylla* root extracts have anti-obesity and hypolipidemia effects [11,12], however, *A. triphylla* var. *japonica* have not yet been studied in adipocytes and high-fat diet (HFD)-induced obese mice related adipogenesis and lipogenesis mechanisms.

In the present study, we examined the effect of *A. triphylla* var. *japonica* extracts (ATE) on obesity and the underlying lipid mechanisms, lipid deposition and lipogenesis, in adipocytes and HFD-induced obese mice.

## 2. Materials and methods

### 2.1. Chemicals and reagents

5'-diallyl-2, 2'-biphenyldiol, 3-isobutylmethylxanthine, insulin, and dexamethasone were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Oil-Red O, isopropanol, and TRI reagent were purchased from Sigma-Aldrich (MO, USA). Polyclonal antibodies against peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), p2, and  $\alpha$ -tubulin were purchased from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-linked anti-rabbit IgG and HRP-linked anti-mouse IgG were purchased from Bio-Rad (CA, USA). SYBR Green reaction buffer was purchased from Takara (Shiga, Japan).

### 2.2. Preparation and component analysis of ATE

*A. triphylla* var. *japonica* was provided from DONG IL Pharmtec (Seoul, Korea). The dried *A. triphylla* var. *japonica* was extracted with 70% aqueous ethyl alcohol and subjected to vacuum evaporation. Gas chromatography was carried out with an Agilent 7890A GC-FID instrument for the analysis of linoleic acid as a reference compound. GC conditions were equipped on HP-INNOWAX (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) column. The GC oven temperature was maintained at 80 °C for 3 min and increased to 260 °C with helium as a carrier gas at a flow rate of 1.0 mL/min, and. Both injector and detector were set at 260 °C and 290 °C. The total analysis time was 25 min.

### 2.3. Animal studies

Male C57BL/6J mice were purchased from Samtaco (Gyeonggi-do, Korea) at 4 wk of age. The mice were housed individually in stainless steel cages and maintained under a temperature of (23  $\pm$  3) °C in a humidity-controlled room with a 12 h-light/dark cycle. Mice were given free access to water and food (Certified irradiated global 18% protein diet, 2918C, Harlan Laboratories, Inc., Indianapolis, Indiana, USA). After acclimatization for 1 wk, they were fed with either the normal-fat diet (NFD,  $n$  = 8, Rodent diet with 10% kcal fat, D12450B) or HFD ( $n$  = 32, Rodent diet with 60% kcal fat, D12492) purchased from Research Diet Inc., (New Brunswick, NJ, USA) for 4 wk to induce obesity. After obesity induction, the mice were divided into 4 subgroups ( $n$  = 8/group) that were matched with body weight. The following 4 groups were studied for 6 wk: HFD; HFD with oral administration of ATE at 100 mg/kg of body weight (HFD + ATE 100); HFD with ATE at 200 mg/kg of body weight (HFD + ATE 200); and HFD with ATE at 400 mg/kg of body weight (HFD + ATE 400). The body weight of the mice was measured once weekly for 6 wk. Food intake was measured every 5 d on a per-cage basis throughout the study. Food intake (g/mouse/day) was determined by subtracting the remaining food weight from the initial food weight of the previous feeding day, and dividing by the number of mice housed in the cage. At the end of the experiment, the mice were sacrificed, and the liver, kidney, and white adipose tissue (WAT) were excised immediately. The parts were then rinsed, weighed, frozen in liquid N<sub>2</sub>, and stored at -80 °C until analysis. The experimental design was approved by The Animal Experiment Committee of GyeongGi Bio Center, and the mice were maintained in accordance with their guidelines.

### 2.4. Plasma biochemical analysis

After 6 wk of feeding, 4 h-fasted mice were anesthetized using ether and blood was collected. Plasma was obtained from the blood by centrifugation at 800 g for 10 min at 4 °C for biochemical analyses of plasma parameters. The separated plasma was stored at -80 °C until analysis. Plasma total cholesterol (T-CHO), TG, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, and glucose levels were determined enzymatically using commercial assay kits (Asan Chemical, Seoul, Korea). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercial assay kits (Sigma-Aldrich, MO, USA).

## 2.5. Histological analysis

Histological examination of liver was conducted, and epididymal adipose tissue were dissected and fixed in 10% neutral formalin solution and embedded in paraffin. The tissues were studied under hematoxylin and eosin staining. The sections were viewed with a light microscope (Olympus, Tokyo, Japan) and photographed at  $\times 200$  magnification.

## 2.6. Cell culture

Mouse 3T3-L1 preadipocytes obtained from the Dr. Rhee, Sang Dal (Korea Research Institute of Chemical Technology, Daejeon, Korea), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics [100 U/mL penicillin and 100 g/mL streptomycin (Gibco, Carlsbad, CA, USA)] at 37 °C under a humidified 5% CO<sub>2</sub> atmosphere. For differentiation of 3T3-L1 preadipocytes to mature adipocytes, preconfluent cells (defined as day 0) were cultured in differentiation medium containing 0.5 mM 3-isobutylmethylxanthine, 5  $\mu$ g/mL insulin, and 1  $\mu$ M dexamethasone in DMEM supplemented with 10% FBS (MDI differentiation medium). After 3 d, the cell culture medium was changed to DMEM containing 5  $\mu$ g/mL insulin and 10% FBS. The medium was replaced again with DMEM containing 10% FBS after another 2 d. To determine the effect of ATE on adipogenesis, differentiated preadipocytes were treated with presence or absence of ATE for 5 d.

## 2.7. Oil-red O staining

After differentiation, the cells were fixed with 4% formaldehyde solution in phosphate-buffered saline for 1 h, and were subsequently washed thrice with water. The cells were stained with oil-red O (0.5% in 60% isopropanol) for 1 h and washed thrice with distilled water. Stained cells were photographed under an optical microscope (Olympus, Tokyo, Japan). Stained lipid droplets were extracted with isopropanol and the absorbance was measured at 520 nm with a microplate reader (Tecan, Mannedorf, Switzerland).

## 2.8. Cell cytotoxicity assay

For cell cytotoxicity, the cell were treated for 24 h with various concentrations (0, 100, 300, and 500  $\mu$ g/mL) of ATE extracts in DMEM containing 10% FBS. Filtered MTT reagent (5 mg/mL) was added to the cells to reach a final concentration of 0.5 mg MTT/mL, and the cells were further incubated for 4 h at 37 °C. The violet formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance was measured at 570 nm by using a microplate reader. The cell viability (%) was

calculated by comparing the absorbance of the samples and the control (untreated cell).

## 2.9. Protein extraction and Western blot analysis

Cold phosphate-buffered saline washed 3T3-L1 cells and epididymal adipose tissue were homogenized in RIPA buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 1% of protease inhibitor cocktail (Sigma–Aldrich, MO, USA). The homogenates were centrifuged at 12000 rpm for 15 min at 4 °C, and supernatant was collected. The protein concentrations were calculated by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% of nonfat dried skim milk and then incubated with appropriate antibodies. The membranes were washed with wash buffer and incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 1 h. The immunoreactive bands were enhanced by chemiluminescence reagents and detected by chemi-luminometer (CLINX Science Instruments Co., Ltd., Shanghai, China).

## 2.10. Isolation of total RNA and quantitative real-time PCR (qRT-PCR)

Total RNA of epididymal adipose tissue was isolated by TRI reagent (Sigma–Aldrich, MO, USA) according to the manufacturer's protocols. Total RNA was reverse transcribed to cDNA by using random primers and a reverse transcription system (Promega, Piscataway, NJ) according to manufacturer's recommendations. The mRNA expression levels of adipogenesis-related genes were analyzed by real-time PCR iQ<sup>TM</sup>5 system (Bio-Rad Laboratories, Hercules, CA, USA) using a SYBR Green Master PCR Kit (Takara, Shiga, Japan) according to the manufacturer's protocols. The real-time PCR cycling parameters were as follows: 2 min at 95 °C; 45 cycles of 20 s at 95 °C, 20 s at 60 °C, 40 s at 72 °C, and 30 s at 72 °C; and a final extension for 5 min at 72 °C, followed by a melting curve analysis. All samples were normalized to the expression level of  $\beta$ -actin, and the results were expressed as the fold changes relative to the HFD group. The primers used in the experiments were listed in Table 1.

## 2.11. Statistical analysis

Statistical evaluation of the data was expressed as the mean  $\pm$  standard error (SEM). The statistical significance of differences between the mean values for the treatment groups was analyzed by Student's *t*-tests and one-way analysis of

**Table 1**

Primer sequences used for quantitative real-time PCR.

Gene name	Accession no.	Forward primer	Reverse primer
<i><math>\beta</math>-actin</i>	NM_007393	5'-TGTCCACCTTCCAGCAGATGT-3'	5'-AGCTCAGTAACAGTCCGCCTAGA-3'
<i>PPAR<math>\gamma</math></i>	NM_011146	5'-TCACAAGAGGTGACCCAATG-3'	5'-CCATCCTTCAACAAGCATGAA-3'
<i>aP2</i>	NM_011547	5'-CCAATGAGCAAGTGGCAAGA-3'	5'-GATGCCAGGCTCCAGGATAG-3'
<i>FAS</i>	NM_007988	5'-TGGTGGGTTTGGTGAATTGTC-3'	5'-GCTTGTCTGCTCTAACTGGA-3'
<i>SREBP-1c</i>	NM_011480	5'-GGCACTAAGTGCCCTCAACCT-3'	5'-GCCACATAGATCTCTGCCAGTGT-3'

variance (ANOVA) using the software Origin 7 (Microcal Software, USA). Values of  $P < 0.05$  were considered as statistical significance.

### 3. Results

#### 3.1. Linoleic acid content of *A. triphylla*

The linoleic acid content in *A. triphylla* was elucidated by comparing the chromatographic profile of standard on gas chromatography analysis. We confirmed that linoleic acid content was approximately 2.0% (Figure 1), and the optimized *A. triphylla* was used in all subsequent experiments.

#### 3.2. Effects of ATE on adipogenesis of 3T3-L1 preadipocytes

To determine the effect of ATE on lipid accumulation during differentiation, 3T3-L1 preadipocytes were induced to differentiate into adipocytes in the presence of ATE for 5 d. Accumulated lipid droplets were measured by Oil-Red O staining. ATE inhibited the lipid accumulation in 3T3-L1 adipocytes (Figure 2A); especially, 500  $\mu\text{g}/\text{mL}$  ATE treatment significantly reduced the lipid content by 49.6%, as compared to the control ( $P < 0.05$ ) (Figure 2B). The cytotoxic effect of ATE on 3T3-L1 adipocytes was evaluated by the MTT assay. ATE had no cytotoxic activity at various concentrations *i.e.*, 100–500  $\mu\text{g}/\text{mL}$  for 24 h (Figure 2C). To investigate how ATE inhibits lipid accumulation, we measured the expression levels of adipogenesis-related proteins using Western blotting. As shown

in Figure 2D, PPAR $\gamma$  protein expression level decreased in 300 and 500  $\mu\text{g}/\text{mL}$  of ATE treatment, as compared to the control. Target proteins *i.e.*, aP2 and fatty acid synthase (FAS) protein expression level also decreased as compared to the control.

#### 3.3. Effects of ATE on body weight and food intake

ATE inhibited lipid accumulation in 3T3-L1 cells. Based on these results, we investigated the effect of ATE on anti-obesity in HFD-induced obese mice. The body weight gain and food intakes were shown in Figure 3. The body weight gain of the HFD group was significantly increased, as compared to the NFD group after 6 wk of feeding; whereas administration of ATE at 100, 200, and 400 mg/kg showed a tendency to decrease body weight gain than the HFD group (Figure 3A). Food intake did not display significant group wise differences (Figure 3B).

#### 3.4. Effects of ATE on organs weight

The relative weight of liver, epididymal, perirenal, and mesenteric WAT were compared to those in the HFD group (Figure 4). Supplementation with ATE did not show significant differences in kidney weight in mice fed with HFD (Figure 4A). However, the HFD plus ATE presented a decreased liver weight when compared to the mice fed HFD (Figure 4B). Epididymal, perirenal, mesenteric, and total WAT weights in the HFD group were higher than those of the NFD group (Figure 4C). ATE significantly decreased epididymal, perirenal, mesenteric, and total WAT weights in HFD plus ATE 200 and 400 mg/kg fed mice, respectively.

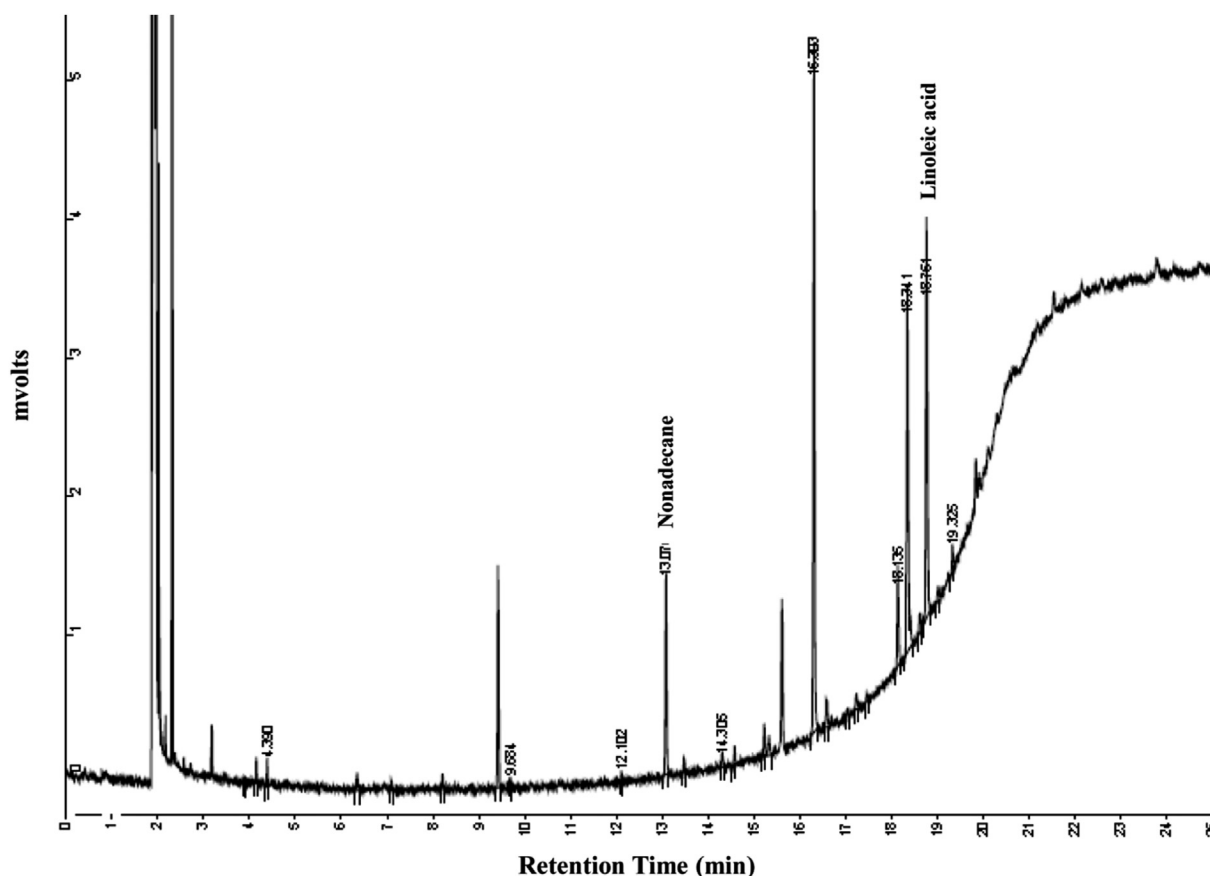
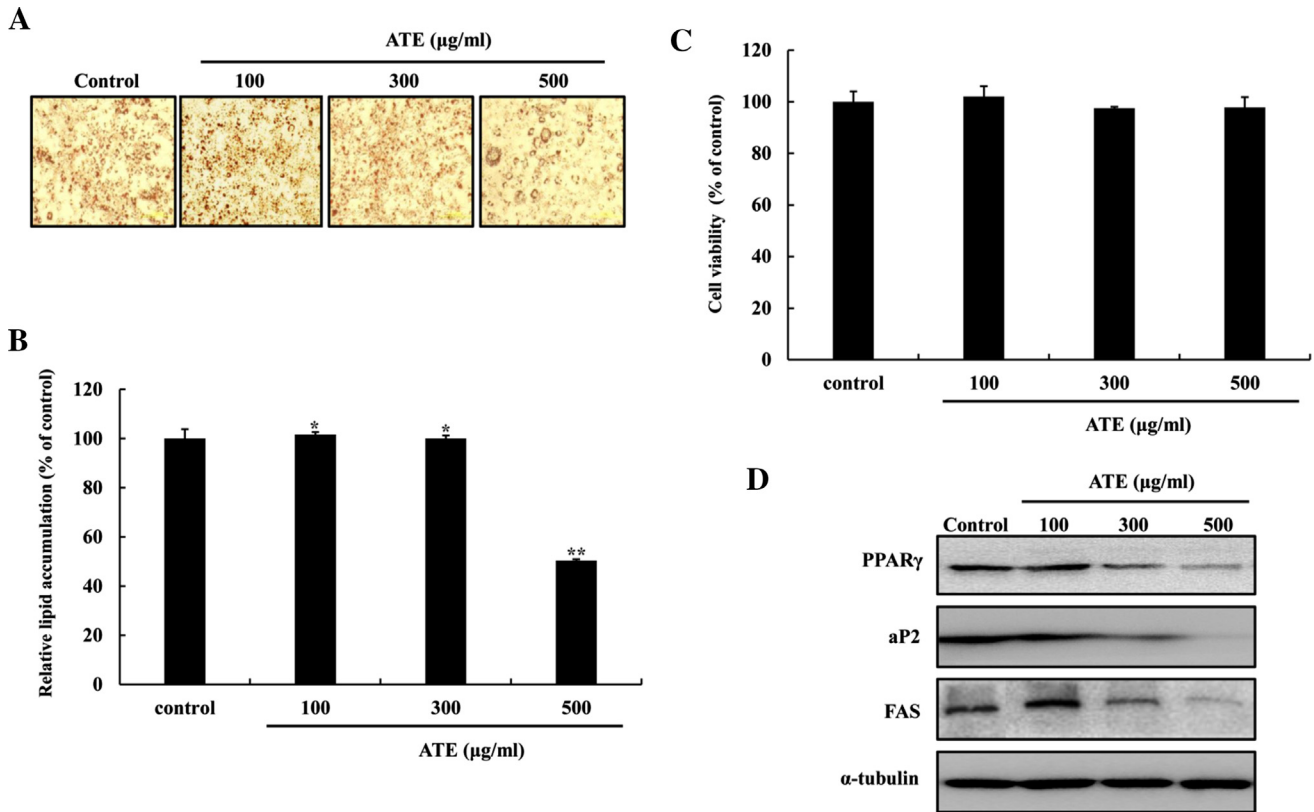


Figure 1. Gas chromatogram of linoleic acid from ATE.



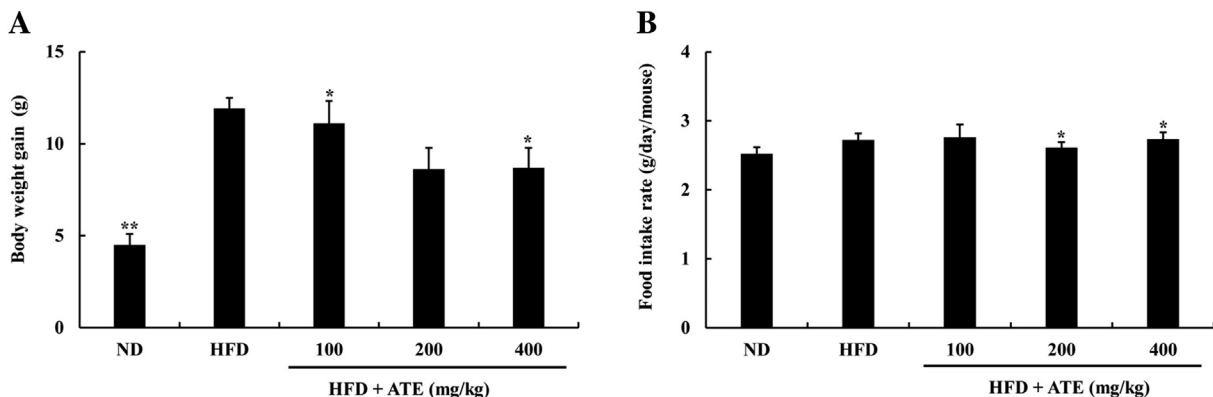
**Figure 2.** Effects of ATE on lipid accumulation.

(A) Lipid droplets were photographed after Oil-Red O staining. (B) The stained lipid content was quantified by measuring absorbance. Each percentage value in treated cells was calculated with respect to control cells. (C) The cytotoxicity of 3T3-L1 cells was determined using MTT assay at 24 h and expressed as a percentage of the optical density. (D) ATE-treated cells were subjected to Western blot analysis to determine the effects on adipogenesis-related protein expression. \* $P < 0.05$  and \*\* $P < 0.01$ , when compared to the control group.

### 3.5. Effects of ATE on the plasma biochemical levels

The plasma biochemical parameters were compared to the HFD fed group. As shown in Table 2, glucose, T-CHO, and TG levels in the HFD group were significantly increased, as compared to the ND fed mice group. In HFD plus ATE groups, decreased HFD-induced plasma glucose, T-CHO; and TG levels showed a propensity to decrease in a dose-dependent manner, although not significantly, as compare to the HFD group. However, TG level in the HFD plus ATE 200 and 400 groups were significantly decreased, as compared to the HFD group. HDL-cholesterol levels in HFD fed with ATE groups were

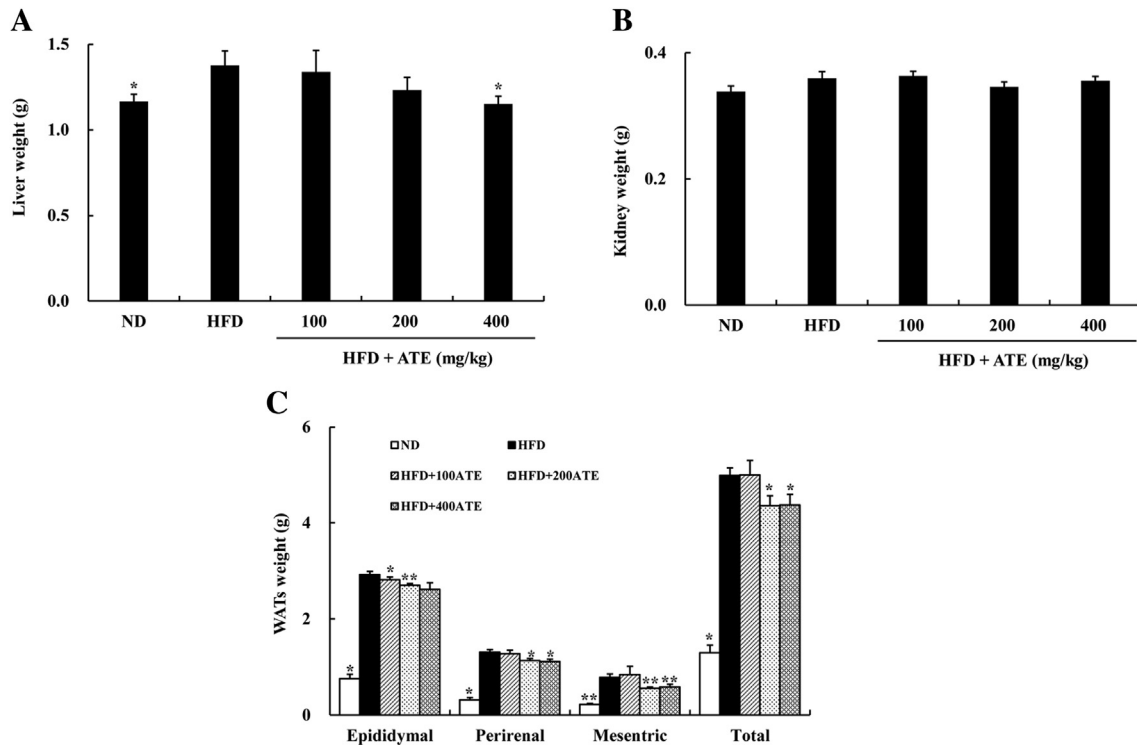
significantly increased, when compared to the HFD fed group. There were no significant differences in the LDL cholesterol level, among the HFD and ATE-treated groups, but the ATE fed groups presented a tendency towards decreased levels in. ALT level in the HFD fed group was more significantly increased than that of the ND fed group. The HFD + ATE 200 and 400 groups exhibited tendency of decreased ALT, although there were no statistically significant differences, as compared to HFD fed group. AST level in the NFD and HFD + ATE 100 group did not differ, as compared to the HFD group, but showed a significant reduction in HFD fed with 200 and 400 mg/kg of ATE groups.



**Figure 3.** Effects of ATE on body weight gain and food intake rate in HFD-induced obese mice for 6 wk.

(A) Body weight gain. (B) Food intake rate. \* $P < 0.05$  and \*\* $P < 0.01$ , when compared to the HFD group ( $n = 8$  per group).





**Figure 4.** Effects of ATE on organ weights in HFD-induced obese mice during 6 wk. (A) Liver weight. (B) Kidney weight. (C) WATs weight. WAT: white adipose tissue. \* $P < 0.05$  and \*\* $P < 0.01$ , when compared to the HFD group ( $n = 8$  per group).

**Table 2**

Effects of ATE on plasma biochemical parameters in HFD-induced obese mice.

Indexes	ND	HFD	HFD + ATE 100	HFD + ATE 200	HFD + ATE 400
Glucose (mg/dL)	164.21 ± 15.00*	264.71 ± 16.72	248.39 ± 11.37	248.81 ± 8.06	238.70 ± 4.75
T-CHO (mg/dL)	135.63 ± 5.51*	184.14 ± 9.03	177.80 ± 5.05	172.80 ± 9.00	169.33 ± 7.59
TG (mg/dL)	66.78 ± 3.57*	78.67 ± 3.55	72.75 ± 2.71	68.75 ± 0.86*	64.56 ± 3.02**
LDL (mg/dL)	37.14 ± 5.35	46.43 ± 3.30	42.00 ± 3.12	43.17 ± 1.96	41.14 ± 2.46
HDL (mg/dL)	83.59 ± 5.33**	130.21 ± 6.09	132.52 ± 4.07	130.25 ± 5.29	129.21 ± 5.10
ALT (U/L)	37.79 ± 4.10	50.29 ± 4.82	44.70 ± 7.00	39.06 ± 2.72	36.20 ± 2.62
AST (U/L)	61.61 ± 3.33	71.58 ± 3.88	70.20 ± 5.64	59.84 ± 1.02*	57.37 ± 2.15**

The data are presented as means ± SEM. \* $P < 0.05$  and \*\* $P < 0.01$  and, when compared to the HFD group ( $n = 8$  per group).

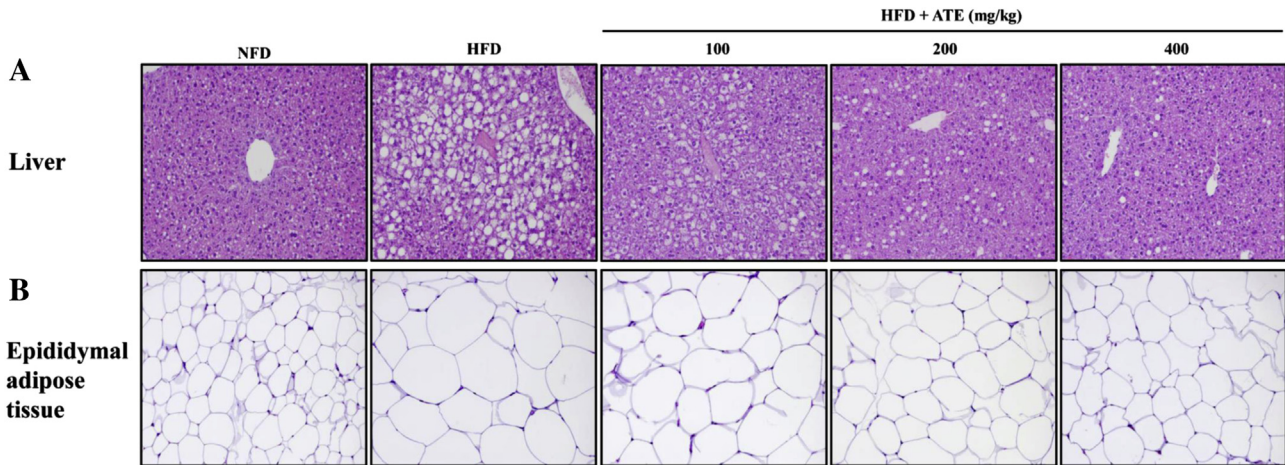
**3.6. Effects of ATE on liver and epididymal adipose tissue morphology**

Histological observations of liver and epididymal adipose tissue were conducted through 5 groups as follows: NFD; HFD; HFD + ATE 100, 200, and 400 groups (Figure 5). Sections of liver and epididymal adipose tissue were stained with hematoxylin and eosin to examine the lipid accumulations and cellular morphology. In WAT, adipocytes size of the HFD group showed an increase, as compared to the NFD group; however, adipocytes size of the HFD + ATE 200 and 400 groups were reduced, as compared to the HFD group. In the liver, lipid droplet sizes of the HFD group were larger than that of the NFD group; however, ATE treatment decreased the lipid droplet size, in a dose-dependent manner.

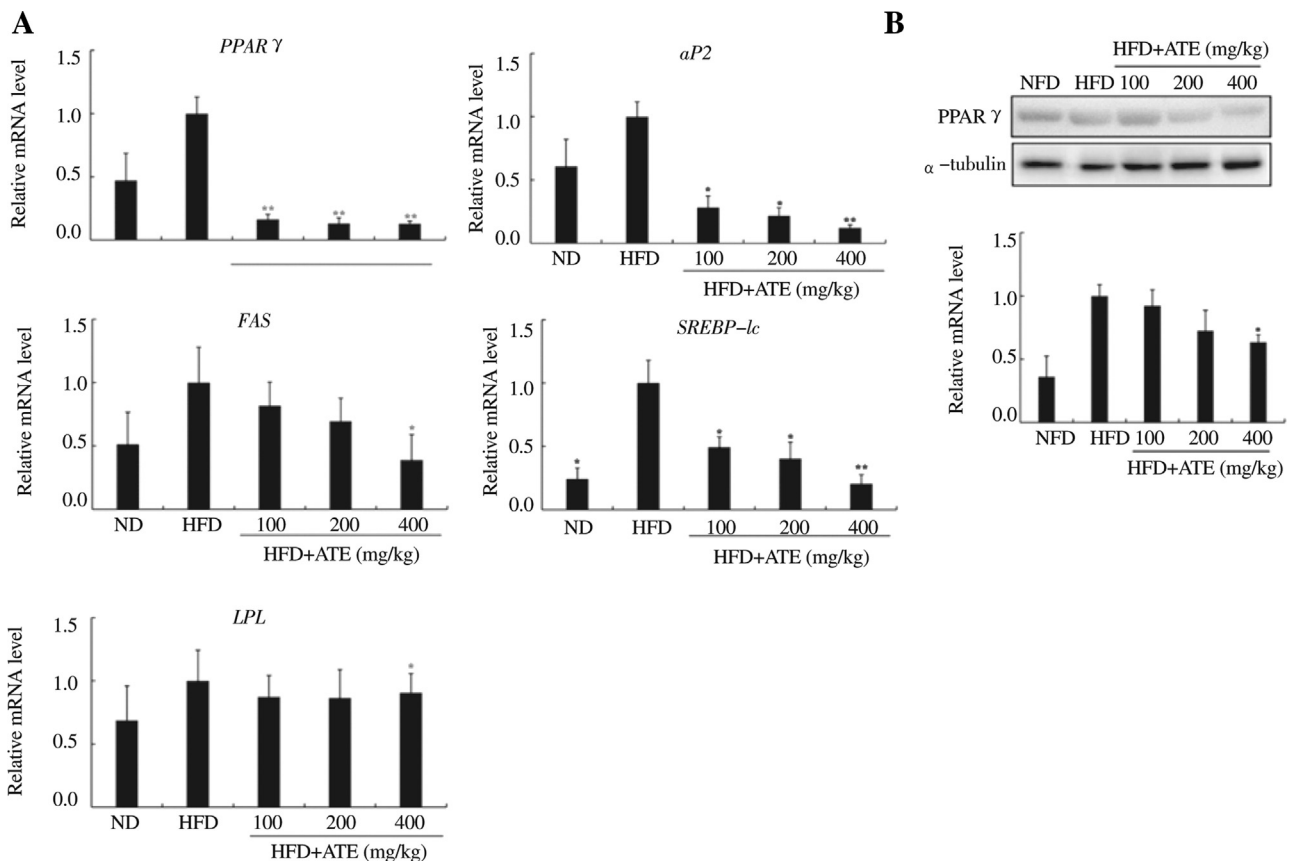
**3.7. Effects of ATE on expression levels of adipogenesis-related genes and proteins**

Of the adipogenesis-related protein expression levels in ATE-treated 3T3-L1 adipocytes, we found that PPARγ, aP2, and FAS

protein expression levels were decreased in a dose-dependent manner. We determined whether ATE affects the protein expression of adipogenesis-related genes and protein in epididymal adipose tissues of HFD-induced obese mice, using the quantitative real-time PCR and Western blot. As shown in Figure 6A, PPARγ, aP2, FAS, sterol regulatory element binding protein-1c (SREBP-1c), and LPL mRNA expression levels in the HFD fed group were increased than those of the NFD fed group, but the difference was not statistically significant. However, the mRNA expression level was significantly decreased in the HFD plus ATE groups, as compared to the HFD group. SREBP-1c and FAS mRNA expression level in the HFD group was significantly increased, as compared to the NFD group, whereas SREBP-1c mRNA expression level significantly decreased in 3 ATE-treated groups. FAS mRNA expression level showed a tendency of decrease, and was significantly decreased in the HFD plus 400 mg/kg ATE group, as compared to the HFD group. Additionally, ATE treatments increased LPL mRNA level, in a dose-dependent manner. Consistently, PPARγ protein expression level in the HFD plus ATE groups was decreased in a dose-dependent manner, and it especially showed statically



**Figure 5.** Histological observations of liver section and epididymal adipose tissue. (A) Epididymal adipose tissue and (B) liver were stained with hematoxylin and eosin, and were viewed under a microscope ( $\times 200$ ). Representative sections are from 3 mice of each treated group.



**Figure 6.** Effects of ATE on expression level of adipogenesis-related genes and protein in epididymal adipose tissues from 8 mice. (A) The mRNA expressions level of adipogenesis-related genes was evaluated by quantitative real-time PCR. (B) PPAR $\gamma$  protein expression level was detected by Western blot analysis. \* $P < 0.05$  and \*\* $P < 0.01$ , when compared to the HFD group ( $n = 8$  per group).

significant difference in the HFD + ATE 200 and 400 groups (Figure 6B).

#### 4. Discussion

Long-chain polyunsaturated fatty acid, linoleic acid, has less known effects when derived from plants, especially from roots of *A. triphylla* [14]. Polyunsaturated fatty acid is a potent compound with biological responses that are required for health. In the present study, we demonstrated the 18-carbon polyunsaturated fatty acid, linoleic acid (LA, 18:2n-6, the

precursor of arachidonic acid) as a reference compound by gas chromatography analysis with 2% from ATE. Linoleic acid is an essential fatty acid that is synthesized in the human body as an unsaturated fatty acid for membrane phospholipids. Recent publications have shown that linoleic acid has anti-bacterial [15], anti-oxidative [16], anti-inflammatory [17] activities and attenuates the development of obesity-related pathogenesis, such as insulin resistance [18,19].

Recently, several studies showed that *A. triphylla* has compounds including saponine,  $\beta$ -sitosterol, cycloartenyl acetate, and taraxenone [11,13]. It has been reported that ATE and isolated

compounds show anti-inflammatory, hepatoprotective, and anti-tumor effects. Based on these results, the present study confirmed an anti-obesity effect including lipid synthesis of ATE and elucidated the mechanisms underlying such effects in 3T3-L1 adipocytes and HFD-induced obese mice.

Our results demonstrated that ATE treatment dose-dependently inhibited the differentiation and lipid accumulation in 3T3-L1 adipocytes. This result was further supported by *in vivo* experiment, demonstrating that ATE treatment for 6 wk significantly reduced the body weight gain in HFD-induced obese mice. These results are in accordance with previous reports that *A. triphylla* root ethanol extract has anti-obesity effects [11,12]. Additionally, ATE treatment did not affect the food intake in HFD-induced obese mice. These results suggested that ATE treatment has anti-obesity effects, and reduction in weight gain induced by ATE treatment is related to decreasing food efficiency and not decreasing food intake.

Obesity is characterized by adipose tissue remodeling associated with hyperplasia and hypertrophy [20,21]. Adipocytes hypertrophy is caused by an excessive accumulation of TG from the intake of excess energy, and it results from an energy imbalance. Since adipocytes hyperplasia results from a complex interaction between proliferation and differentiation in preadipocytes [22]. It is examined by 3T3-L1 adipocytes differentiation system. To differentiate from preadipocytes into adipocytes, proliferation of preadipocytes is a prerequisite [23]. Adipogenic signals MDI differentiation medium, initiating the differentiation after mitotic clonal expansion [24]. It has been suggested that inhibition of proliferation may be the potential mechanism for reducing the adipocytes number [25]. We confirmed that ATE treatment inhibited lipid accumulation during differentiation in 3T3-L1 adipocytes without cytotoxicity. Also, ATE with HFD fed mice exhibited the tendency to decrease the epididymal, perirenal, and total WAT weights in HFD-induced obese mice. Moreover, histological data showed a decrease in the adipocytes size of adipose tissue in ATE-treated mice. These results indicated that ATE treatment may affect both the cell number and sizes of adipocytes in adipose tissue, which is likely to be associated with reduced WATs weight. However, cell proliferation and cell cycle need to be further investigated.

Fatty liver results from increased free fatty acid uptake and endogenous hepatic fatty acid synthesis [26]. Histological data of liver revealed that ATE treatment reduced lipid droplet size and number, indicating that ATE treatment was responsible for the inhibition of lipid accumulation in liver. In our study, ATE treatment lowered plasma TG level, while 100 and 200 mg/kg ATE treatment increased plasma HDL-cholesterol level in HFD-induced obese mice, as shown in previous reports [11,12]. These results collectively suggest that ATE treatment reduces the accumulation in liver, resulting in the improved hyperlipidemia in HFD-induced obese mice.

Excessive fat intake can cause the oxidative stress, which induces dysfunction and fatty degeneration in the liver through abnormal activation of AST and ALT. Previous reports have demonstrated that various extracts of *A. triphylla* have an antioxidant effect that reduces plasma ALT and AST levels in HFD-induced obese mice [11,12]. In our study, 200 and 400 mg/kg ATE treatment showed a tendency to decrease plasma ALT and AST levels in HFD-induced obese mice, as compared to the HFD group. These results showed that ATE treatment may improve the liver function.

To examine the underlying mechanism, we measured the expression levels of adipogenesis-related genes and proteins in 3T3-L1 adipocytes and WAT in HFD-induced obese mice. Adipogenesis is closely associated with etiologies of obesity, in which the preadipocytes are differentiated into mature adipocytes. Adipogenesis is regulated by sequential expression of adipogenic and lipogenic genes [27]. PPAR $\gamma$  is a transcription factor that regulates adipocytes differentiation and adipogenesis. C/EBP $\alpha$  promotes adipocytes differentiation through cooperation with PPAR $\gamma$ , which increases the expression of adipocytes-specific genes such as aP2 [28,29]. SREBP-1c is also a transcription factor that plays key roles in lipid metabolism, by increasing the expressions of several lipogenic genes such as FAS in adipose tissue and liver. It activates PPAR $\gamma$  through regulation of its expression and production of an endogenous PPAR $\gamma$  ligand [30,31]. The lipoprotein lipase is a key regulator of blood TG levels via decomposition of lipoprotein triglyceride. It plays a critical roles in transporting the fats and hydrolysis fat-carried lipoproteins [32]. Combined action of these transcription factors causes expression of target genes and proteins that are responsible for TG synthesis and storage, which leads to accelerated adipogenesis. We found that the high concentration of ATE treatment reduced the PPAR $\gamma$ , C/EBP $\alpha$ , aP2, and FAS protein expression level in 3T3-L1 adipocytes. Furthermore, PPAR $\gamma$  and SREBP-1c mRNA expression level were significantly decreased in epididymal adipose tissue of 100, 200, and 400 mg/kg ATE-treated mice. Additionally, aP2 and FAS mRNA expression level was decreased in 400 mg/kg ATE-treated mice. PPAR $\gamma$  protein expression levels tended to be reduced specifically in adipose tissues of HFD fed with ATE groups, and significant down-regulation was observed in the 200 and 400 mg/kg ATE treatment groups. On the other hand, the reduction in body weight gain and total WATs weight were unfortunately not observed in the 100 mg/kg of ATE treatment group, despite the down-regulation of adipogenesis-related genes. There is a possibility that there are differences in PPAR $\gamma$  protein expression levels in ATE-treated groups. However, more study is needed on the expression levels of other genes related to adipogenesis in adipose and other tissues. Based on our results, ATE treatment affects the expression levels of adipogenesis-related genes and proteins. Furthermore, it is likely that the anti-obesity effects of ATE treatment are caused by suppression of gene expressions and proteins involved in adipogenesis.

In conclusion, our results showed that ATE treatment inhibited the lipid accumulation in 3T3-L1 adipocytes and reduced the body weight gain, WATs weight, adipocytes sizes, and plasma TG levels, without any toxic effects in HFD-induced obese mice. These effects were at least partially accompanied by the regulation of gene expressions and proteins associated with adipogenesis/lipogenesis, in 3T3-L1 adipocytes and HFD-induced obese mice. These findings indicated that ATE prevents the development of obesity and hyperlipidemia in 3T3-L1 adipocytes and HFD-induced obese mice, which suggest the possibility of its use for the prevention of obesity and obesity-related diseases.

### Conflict of interest statement

We declare that we have no conflict of interest.



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