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Asimina triloba (pawpaw) fruit extract suppresses adipocyte differentiation and lipogenesis-related protein expression in 3T3-L1 cells

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Abstract

Obesity is a health condition accompanied by life-threatening comorbidities; hence, there is an increasing need for anti-obesity agents. The anti-cancer effects of the leaves of *Asimina triloba* (pawpaw) has been reported. However, limited research has been conducted on the potential anti-obesity effects of *A. triloba* fruit. Therefore, this study aimed to explore the effects of *A. triloba* fruit extract on murine preadipocytes (3T3-L1). We specifically examined lipid droplet formation in these cells using Oil Red O solution and intracellular pro-adipogenic protein levels were examined using western blot analysis. The results revealed that treatment with *A. triloba* 70% ethanolic fruit extract effectively suppressed lipid droplet formation. Moreover, the expression of crucial proteins involved in adipogenesis, namely sterol regulatory element-binding protein 1, peroxisome proliferator-activated receptor γ, and fatty acid synthase, were significantly inhibited. These findings suggest that *A. triloba* fruit has the potential to prevent obesity by inhibiting fat synthesis and may serve as a natural source for anti-obesity functional agents.

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Keywords *Asimina triloba*, Pawpaw, 3T3-L1 cell, Obesity, Lipogenesis

Introduction

Obesity has recently emerged as a pressing global health issue fueled by contemporary lifestyles. This phenomenon is witnessing a surge in prevalence that surpasses historical levels, and more than half of the world's population will be affected by obesity by 2050 [1, 2]. The repercussions of this escalating obesity epidemic extend beyond merely weight gain, as it is invariably accompanied by a multitude of associated comorbidities, including type 2 diabetes, cardiovascular disease, stroke, arthritis, and various forms of cancer [3]. Therefore, the need for anti-obesity agents is increasing [4].

The differentiation of preadipocytes and the accumulation of lipids in mature adipocytes are intricately regulated processes that involve complex interactions among signal transduction pathways, adipogenic transcription factors, and adipocyte-specific genes. Adipose tissues prominently express transcription factors such as peroxisome proliferator-activated receptors (PPARs), CCTTA/enhancer-binding proteins (C/EBPs), and sterol regulatory element-binding protein 1 (SREBP1), which play crucial roles in driving adipocyte differentiation. Notably, PPARγ and C/EBPα are key regulators of adipogenesis and are primarily expressed during the advanced stages of cellular differentiation. These molecules regulate the activation of adipocyte-specific target genes, including leptin, adiponectin, and

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fatty acid synthase (FAS), which collectively govern various aspects of triglyceride synthesis and subsequent accumulation in differentiated adipocytes. Moreover, PPARγ influences insulin resistance and glucose metabolism, with its expression being facilitated by C/EBPα. Prior investigations have consistently reported the essentiality of C/EBPα and PPARγ in adipogenesis, as their reduction or knockdown impedes this process and hampers lipid accumulation. Thus, modulating the expression of these adipogenic proteins represents a promising strategy for treating obesity [5–10].

Asimina triloba (Pawpaw) belongs to the Annonaceae family, a large family that encompasses numerous tropical and subtropical trees and shrubs. Pawpaws are known for their unique flavor, which is reminiscent of a blend of mangoes, bananas, and pineapple [11]. Furthermore, it is abundant in essential nutrients such as vitamins and minerals. In addition to North America, pawpaws are cultivated in several other countries, including Romania, Italy, Ukraine, Slovakia, Austria, Georgia, Russia, Japan, and Korea [12]. Since 2010, studies have focused on investigating the various health benefits and properties of different parts of pawpaw plants, including the roots, twigs, leaves, and seeds [13, 14]. Studies have explored their anticancer, antimalarial, anthelmintic, insecticidal, antiviral, and antimicrobial activities [15]. In Korea, a specific study focused on characterizing their properties [16]. This study involved conducting antioxidant activity tests and determining the total phenolic and flavonoid contents using 80% methanol and distilled water extracts of each plant part (roots, twigs, leaves, and fruit). Additionally, the phenolic compounds in each part were identified using high-performance liquid chromatography [17]. However, there is limited research on its inhibitory effects on adipocyte differentiation and the underlying mechanisms remain unexplored. Hence, the aim of this study was to assess the antiadipogenic potential of *A. triloba* fruits. In this study, the anti-adipogenic effects of *A. triloba* ethanolic fruit extract on murine preadipocytes (3T3-L1) were investigated. Intracellular protein levels were examined to elucidate the mechanism underlying the observed anti-adipogenic activity.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution, phosphate-buffered saline (PBS), and trypsin–EDTA solution were purchased from Gibco Co. (Grand Island, NY, USA). Oil Red O solution, dimethyl sulfoxide (DMSO), isopropanol, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, and-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). Mini-PROTEAN® TGX™ stain-free gels, Trans-Blot® Turbo™ ready-to-assemble transfer polyvinylidene fluoride (PVDF) kits, and detergent compatible (DC) protein assay kits were purchased from Bio-Rad (Hercules, CA, USA). FAS, SREBP1, PPARγ, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA). The 3T3-L1 cells were sourced from the American Type Culture Collection (Rockville, MD, USA). T-PER™ tissue protein extraction reagent and enhanced chemiluminescence (ECL) reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Goat anti-rabbit IgG-heavy and -light chain horseradish peroxidase-conjugated secondary antibodies were purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA).

Asimina triloba fruit extract preparation

The *A. triloba* fruits used in this experiment were harvested in September 2022 from Geochang, Gyeongsangnam-do, South Korea. The *A. triloba* fruits were washed, remove the seeds, freeze-dried, and then added to solvent. The ratio of the dried product to solvent (0%, 30%, 50%, 70%, or 99% ethanol) was 1:10 and extraction was performed twice for 3 h each. The extracts were concentrated using a concentrator, lyophilized, and stored at − 80 °C for subsequent use.

3T3-L1 cell differentiation and treatment with *A. triloba* fruit extracts

The 3T3-L1 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin and they were maintained at a temperature of 37 °C in a 5% CO_2 incubator. To induce adipocyte differentiation, cells were seeded in 24-well plates at a density of 1×10^5 cells/well. After 24 h of culture, the cells were allowed to reach full confluence, and the medium was replaced with a differentiation medium composed of 10% FBS and an adipogenic cocktail (10 μM DEX, 1 μg/mL insulin, and 0.5 mM IBMX). The cells were subsequently cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 1 μg/mL insulin for 2 days. The medium was replaced with fresh DMEM containing 10% FBS and 1% penicillin–streptomycin supplemented with insulin. This replacement step was repeated three times within a 2-day cycle. During adipocyte differentiation, the samples were treated with *A. triloba* ethanolic fruit extracts. The concentrations used for treatment were 1, 10, 25, 50, 100, and 200 μg/mL. The treatment of each sample coincided with the medium exchange for differentiation, which occurred

every 2 days. Treatment of 3T3-L1 cells with *A. triloba* ethanolic fruit extracts is shown in Fig. 1.

Measurement of cell viability

To evaluate the effect of *A. triloba* ethanolic fruit extracts on the viability of 3T3-L1 cells, the cells were seeded in a 96-well plate at a density of 2×10^4 cells/well and cultured for 3 days until they reached confluence. Simultaneously, in a humidified atmosphere with 5% CO_2 at 37 °C, the *A. triloba* fruit extracts were administered at varying concentrations of 1, 10, 25, 50, 100, and 200 μg/mL during the initiation of differentiation. On the 8th day after inducing differentiation, 10 μL of CCK-8 solution was added to each well and incubated in a 37 °C, 5% CO_2 condition for 1 h. Subsequently, the CCK-8 solution was completely removed, and 100 μL of DMSO was gradually added to dissolve the resulting formazan crystals. The absorbance was measured at 570 nm using a microplate reader (SpectraMax; Molecular Devices, Sunnyvale, CA, USA).

Oil red O staining and lipid accumulation determination

To assess the effect of *A. triloba* ethanolic fruit extract on the differentiation of 3T3-L1 cells and fat accumulation, we performed Oil Red O staining. Cells were seeded in a 6-well plate at a density of 5×10^5 cells/well. Concurrent with the initiation of differentiation, the cells were treated with different concentrations (1, 10, 25, 50, 100, and 200 μg/mL) of the extract. On the 8th day after inducing differentiation, the culture medium was removed, and the cells were rinsed with PBS. Subsequently, cells were fixed with 10% formalin for 15 min. After fixation, cells were washed with PBS and stained with Oil Red O solution for 15 min. Following three rinses with distilled water, the stained cells were dissolved in 100% isopropanol and the absorbance was measured at 490 nm.

Western blot analyses

The cells were washed with PBS and collected. After centrifugation, the cells were lysed using T-PER™ tissue protein extraction reagent. The protein concentration in each fraction was measured using the DC protein assay. Equal amounts of protein (10 μg) were electrophoresed on Mini-PROTEAN® TGX™ stain-free any kD gels and transferred onto PVDF membranes. Membranes were blocked with 5% skim milk and incubated with primary antibodies (1:1000 dilution). Subsequently, the membranes were incubated with goat anti-rabbit IgG-heavy and -light chain horseradish peroxidase-conjugated secondary antibodies. Antibody detection was performed using ECL reagent and visualized using a ChemiDoc imaging system (Bio-Rad). Band intensities were analyzed using Bio-Rad Image Lad Software.

Statistical analysis

The data was presented as mean ± standard deviation (SD), and statistical analysis was performed using GraphPad Prism software, version 7 (GraphPad Software, Boston, MA, USA). For comparisons involving three or more groups, data analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

Results

Extract yield and cytotoxicity

Asimina triloba fruits were extracted using solvent at various ethanol concentrations. The extraction yields were as follows: 28.20 ± 0.03% in water extraction, 46.50 ± 0.51% in 30% ethanol extraction, 49.19 ± 0.15% in 50% ethanol extraction, 75.00 ± 0.01% in 70% ethanol extraction, and 31.17 ± 0.18% in 99% ethanol extraction (Fig. 2a). To assess the cytotoxicity of *A. triloba* fruits, the CCK-8 method was employed to examine their effect on

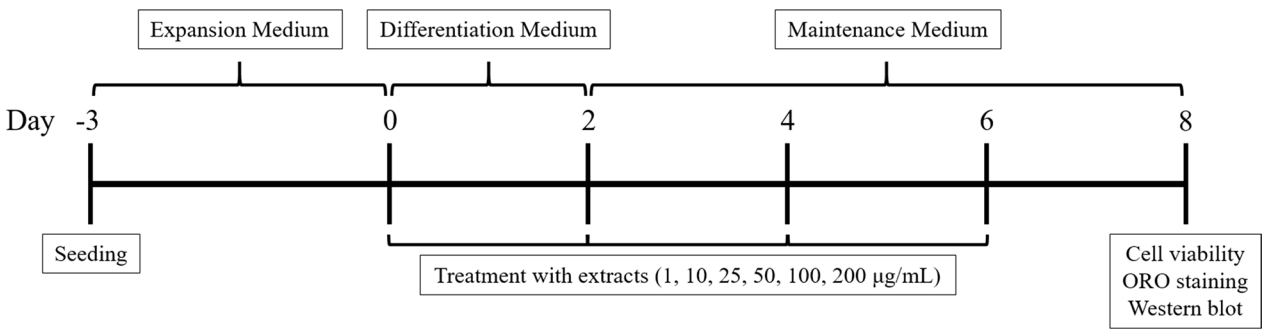

Fig. 1 Experimental scheme for murine preadipocyte (3T3-L1) differentiation, *Asimina triloba* fruit extract treatments of this study. ORO: Oil Red O

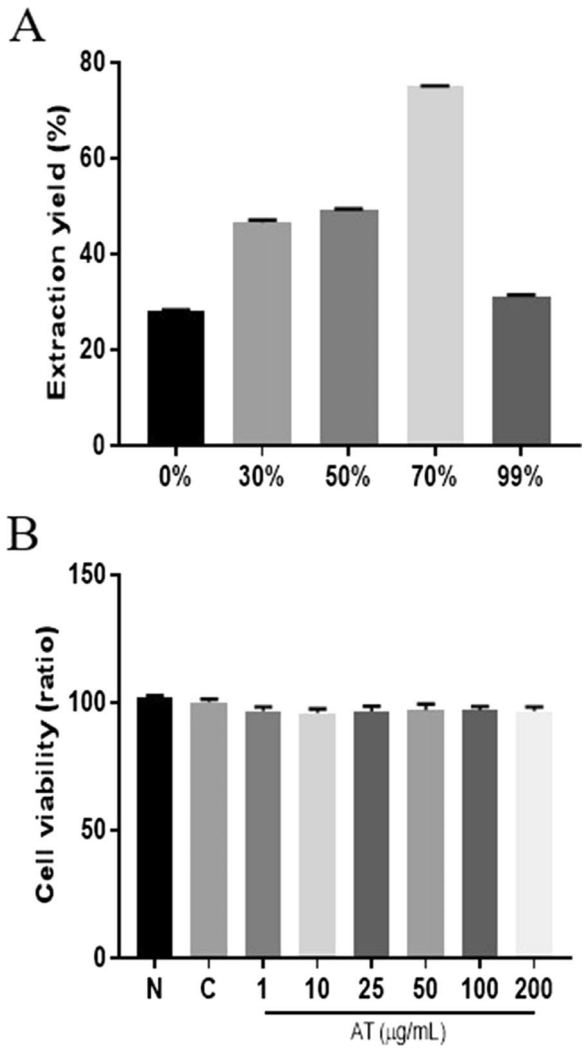

Fig. 2 Extraction yield of *Asimina triloba* fruits and effects on cell viability. **A** Yield of AT ethanol extraction. **B** effects on cell viability of 70% ethanolic AT extract in 3T3-L1 cells. N: untreated group. C: differentiated adipocytes. 3T3-L1 cells were differentiated using the *Asimina triloba* fruit extracts. The data represents the mean ± S.E. of three experiments

cell survival rate of both pre-adipocytes and adipocytes at various concentrations at the 8-day point. The results are presented in Fig. 2b. Compared with the control group, *A. triloba* fruit 70% ethanolic extracts (AT) ranging from 1 to 200 μg/mL exhibited no toxicity towards preadipocytes (Fig. 2b). The AT had the highest yield and was not toxic to 3T3-L1 cells; therefore, subsequent experiments were conducted using AT.

Effect of AT on preadipocyte differentiation

We examined the effect of AT on lipid accumulation in 3T3-L1 cells. Figure 3a illustrates an increase in the quantity and size of lipid droplets in the control group compared with those in the normal group. Lipid droplet accumulation was not observed in untreated 3T3-L1 preadipocytes (N), whereas the control group (C) showed high lipid accumulation. Moreover, AT significantly inhibited lipid accumulation in a dose-dependent manner. In terms of relative lipid content, C (321.43 ± 1.59%) increased by approximately 3.21-fold compared with N (100.00 ± 3.17%). When treated with 1 μg/mL AT (156.24 ± 3.08%), the lipid content decreased by 2.06-fold compared with that in C, and at 200 μg/mL AT (129.44 ± 10.64%), it was similar to N. This physiological transformation is supported by the quantitative analysis shown in Fig. 3b.

Effect of AT on protein expression levels

In the western blot assay, three high concentrations were selected and protein extraction was performed. Figure 4 shows the results of the protein expression levels of fat synthesis-related factors such as FAS, fat cell differentiation factors such as SREBP1, PPARγ and C/EBPα, energy metabolism regulators such as adiponectin, and phospho-AMP-activated protein kinase (p-AMPK) in lipid metabolism. As a result of inducing 3T3-L1 differentiation into adipocytes, FAS, SREBP1, PPARγ, C/EBPα, and adiponectin were all increased in the control group compared with those in the normal group. When treated with AT, the protein expression levels decreased in a concentration-dependent manner. In addition, p-AMPK protein expression, a representative factor of energy metabolism regulation, increased after AT treatment. Overall, treatment with AT inhibited the differentiation of 3T3-L1 preadipocytes and lipid synthesis by reducing the levels of proteins related to adipocyte differentiation and adipogenesis, and by regulating energy metabolism to inhibit lipid accumulation.

Discussion

Fat accumulation in the body occurs through enlargement and an increase in the number of adipocytes in the tissues [18]. Adipocyte hyperplasia, resulting from the proliferation and activation of adipocyte differentiation, contributes to an increase in adipocyte number and the accumulation of lipids through enhanced lipogenesis in differentiated cells [19]. The regulation of adipogenesis involves hyperplasia of adipocytes and proliferation and differentiation of preadipocytes. The balance between fat synthesis and lipolysis determines the hypertrophy of adipocytes [20].

To investigate the anti-obesity effect of *A. triloba* fruit, we examined the transcriptional and protein expression of key factors involved in adipogenesis, including PPARγ, C/EBPα, SREBP1, AMPK, and FAS in 3T3-L1 cells

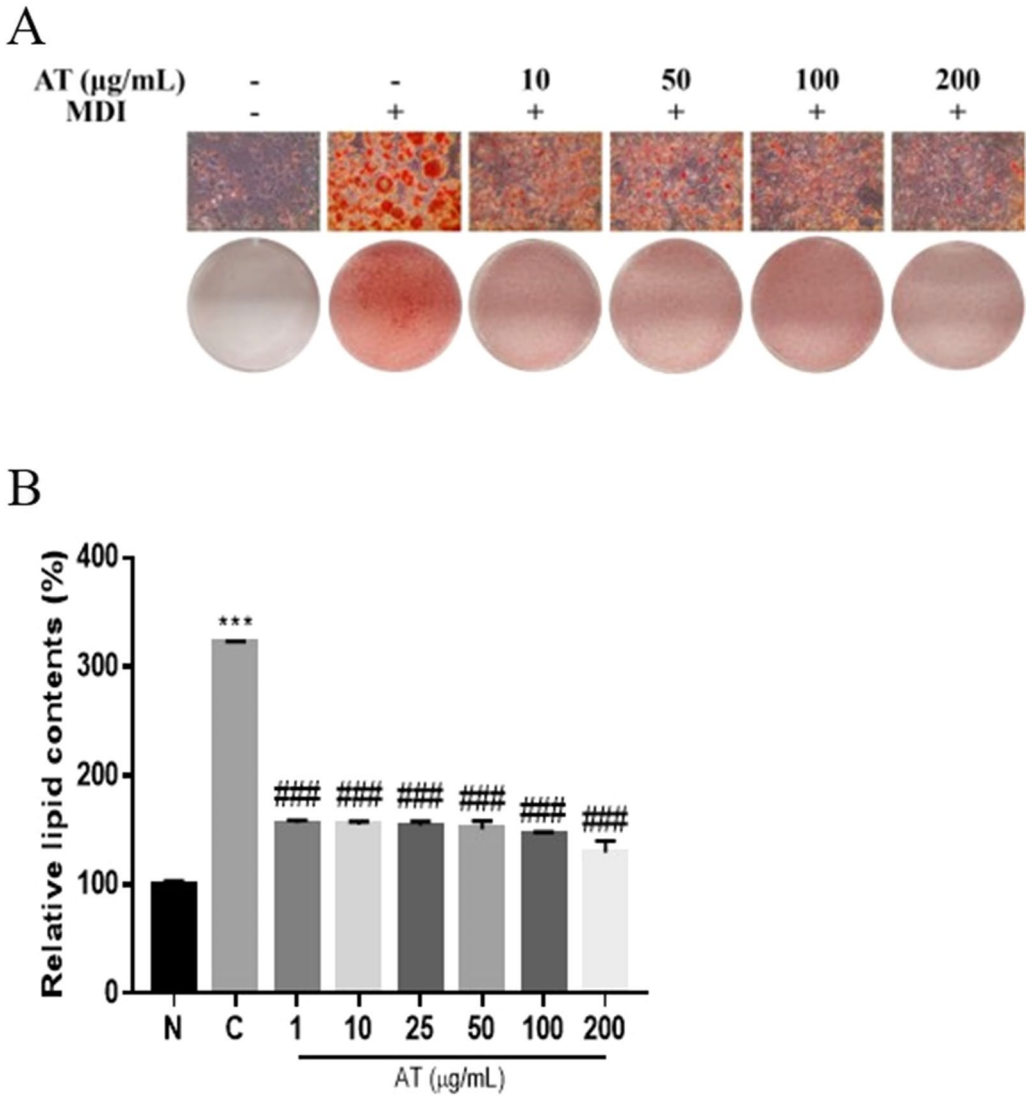

Fig. 3 Effect of AT on lipid accumulation in differentiated murine preadipocytes (3T3-L1). **A** Cells were fixed and stained with Oil Red O to visualize lipid droplets by light microscopy; Magnification, × 200. **B** Relative lipid content at different concentrations. N: untreated group. C: differentiated adipocytes. The data represents the mean ± S.E. of three experiments. ***$p < 0.001$ compared with normal group. ###$p < 0.001$ compared with control group

(Fig. 4). PPARγ and C/EBPα are critical transcription factors that regulate early adipocyte differentiation. Their expression influences the expression of adipogenic genes, leading to increased fatty acid synthesis and triglyceride production in adipose tissue [21]. Consequently, the suppression of PPARγ and C/EBPα expression reduces adipogenesis. We observed a significant decrease in the expression of PPARγ and C/EBPα in AT-treated cells compared with that in the control, indicating that AT

(See figure on next page.)

Fig. 4 Effect of AT on signaling protein expression in 3T3-L1 cells. **A** Representative immunoblots. **B** Relative protein expression levels of fatty acid synthase (FAS)/glyceraldehyde 3-phosphate dehydrogenase (GAPDH), **C** sterol regulatory element-binding protein 1 (SREBP1)/GAPDH, **D** peroxisome proliferator-activated receptor (PPAR)γ/GAPDH, **E** CCTTA/enhancer-binding protein (C/EBP)α/GAPDH, **F** Adiponectin/GAPDH, and **G** phospho-AMP-activated protein kinase (p-AMPK)/AMP-activated protein kinase (AMPK). N: untreated group. C: Differentiated adipocytes. The data represents the mean ± S.E. of three experiments. ***$p < 0.001$ compared with normal group. ###$p < 0.001$ compared with control group

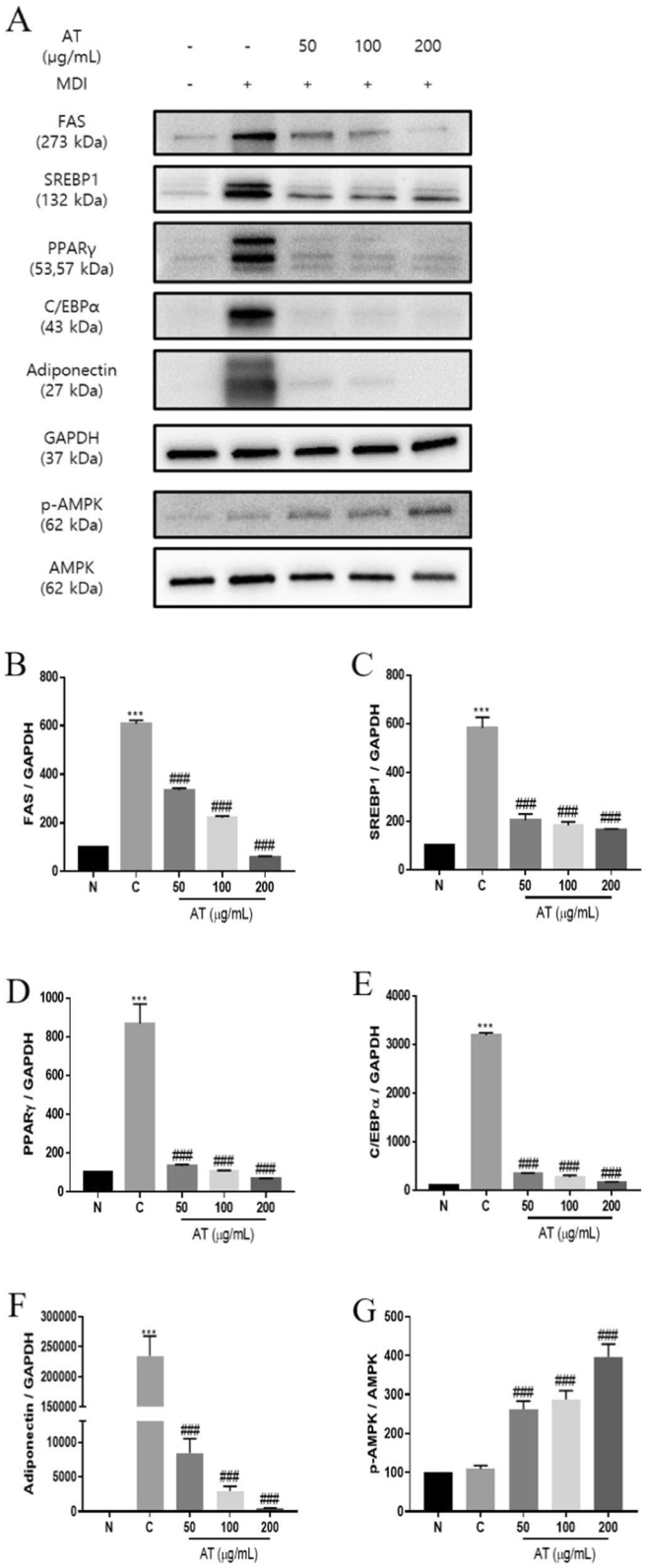

Fig. 4 (See legend on previous page.)

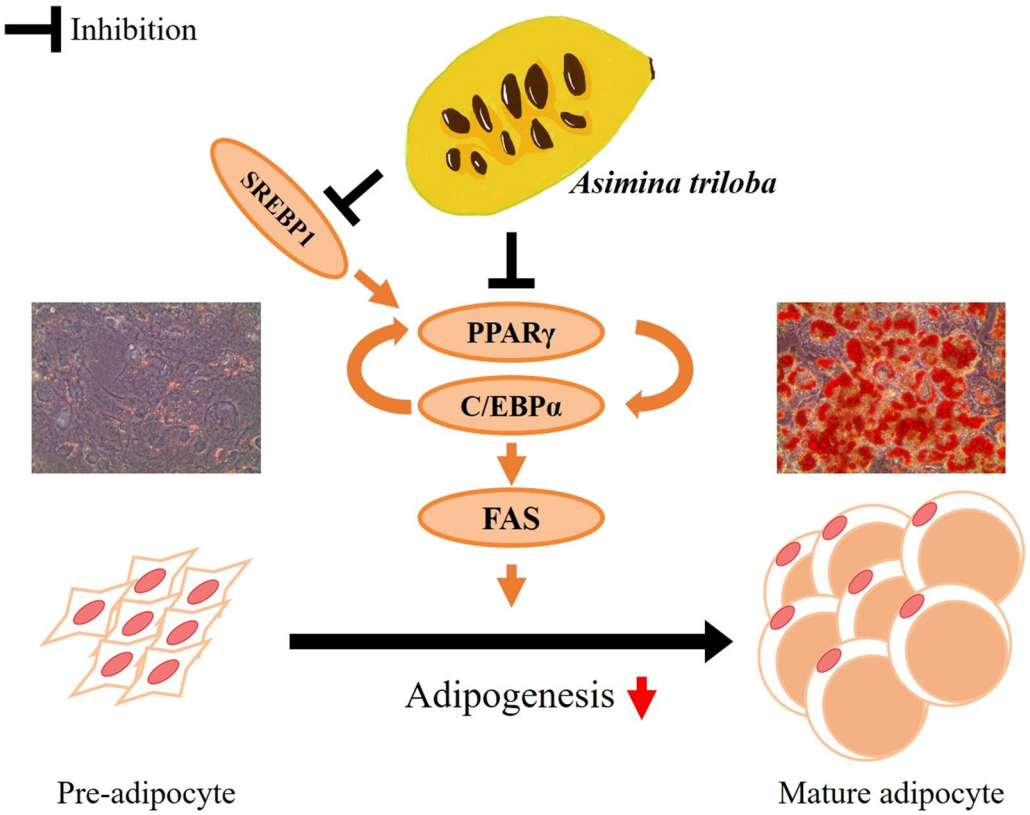

Fig. 5 Molecular mechanism for anti-adipogenic effect of AT in 3T3-L1 cells. SREBP1: sterol regulatory element-binding protein 1; PPAR: peroxisome proliferator-activated receptor; C/EBP: CCTTA/enhancer-binding protein; FAS: fatty acid synthase

inhibits adipogenesis and fat accumulation in adipocytes (Fig. 5).

Moreover, the activation of PPARγ and C/EBPα promotes the activity of SREBP1 and FAS, further promoting adipogenesis [22]. Thus, the notable reduction in SREBP1 and FAS during AT treatment can be attributed to the significant decrease in PPARγ and C/EBPα expression. Additionally, the transcription levels and phosphoprotein activity of AMPK was significantly increased in AT-treated cells compared with those in the control. AMPK, an enzyme crucial for cellular homeostasis [23], plays a vital role in enhancing glucose and fatty acid oxidation, inhibiting fat synthesis and gluconeogenesis in the liver, and regulating insulin secretion in the pancreas. When comparing the peel, seeds, and pulp of *A. triloba* fruit, the types and contents of phenolic compounds were the lowest in the pulp [24]. Nevertheless, the anti-obesity effect of the fruit was demonstrated in our results. According to the following research, *A. triloba* pulp contains phenolic acid (caffeic acid, chlorogeinc acid, coumaric acid, ferulic acid), flavonols (hyeroside, isoquercitrin, quercetin, quercitrin), benzoic acid (ellagic acid, gallic acid), and catechins (catechin, epicatechin) and vitamin C [13]. These polyphenols are known to have excellent antioxidant properties, and studies have shown that they can help with anti-obesity [25]. Additionally, according to a recent study, the seeds of the *A. triloba* fruit have the effect of inhibiting lipogenesis in 3T3-L1 cells [26]. In our study did not contain seeds, its potent anti-lipid production effect and no cell toxicity was confirmed, so it is believed that *A. triloba* fruit useful in anti-obesity. The study's limitations include that there may be other compounds that have not been studied in the pulp and peel of *A. triloba* fruit used in the experiment and there may be anti-obesity effects due to metabolites. Therefore, animal experiments and substance analysis will be conducted in future studies.

Based on these findings, we established that the optimal ethanol concentration for *A. triloba* fruit extraction was 70%, which demonstrated both the highest extraction yield and non-cytotoxic properties. AT exhibited dose-dependent inhibition of lipid accumulation in 3T3-L1 cells, as measured by Oil Red O staining. Moreover, treatment with AT effectively suppressed the expression of key adipogenesis markers, including PPARγ, SREBP1, and C/EBPα. These results suggest the potential of *Asimina triloba* fruit as a

valuable natural source for the development of anti-obesity functional agents.

Abbreviations

PPAR	Peroxisome proliferator-activated receptor
C/EBP	CCTTA/enhancer-binding protein
SREBP1	Sterol regulatory element-binding protein 1
3T3-L1	Murine preadipocytes
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
IBMX	3-Isobutyl-1-methylxanthine
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PVDF	Polyvinylidene fluoride
DC	Detergent compatible
ECL	Enhanced chemiluminescence
AT	*Asimina triloba* Fruit 70% ethanolic extract
p-AMPK	Phospho-AMP-activated protein kinase

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Author contributions
Laboratory experiments: CJL, JH; Data analysis: CJL, YSK, SYC, GY; Wrote the manuscript: CJL, SYC; Designed and supervised the study: SYC, YSK. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used in this study are available from the corresponding author on reasonable request.

Declarations

Competing interests
All authors declare that there are no competing interests.

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