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Research Article

Effect of *Aporosa villosa* Stem Ethanolic Extract on Adipogenesis in 3T3-L1 Adipocytes

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ABSTRACT

Background: An excessive fat accumulation is related to development of obesity. Obesity is associated with the induction of insulin resistance and diabetes mellitus conditions. *Aporosa villosa* is a plant that found in the Northern and Northeastern region of Thailand. **Objective**: The present study used 3T3-L1 adipocytes for investigating the effect of *Aporosa villosa* stem ethanolic extract (AS) on adipogenesis. **Materials and Methods**: 3T3-L1 adipocytes were used for measuring the cytotoxicity of AS at a concentration range of 3-100 µg/mL. After adipocyte cells treated with AS (3-100 µg/mL) for 8 days, the lipid accumulation was detected by Oil Red O staining and adipogenic gene expression were determined by quantitative real-time PCR. **Results**: AS extracts (3-100 µg/mL) did not show cytotoxicity on cell proliferation. After 8 days of treating 3T3-L1 adipocytes with AS at doses of 3, 10, 30 and 100 µg/mL, the lipid droplets were reduced as compared to non-treated cells. Furthermore, the adipogenic genes were measured. The regulators of adipogenesis, CCAAT/enhancer-binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element binding protein 1c (SREBP1c) were found decreasing in AS extracts. The downstream target genes of these regulators cluster of differentiation (CD) 36, fatty acid synthase (FAS) and lipoprotein lipase (LPL) were also reduced by AS treatments. **Conclusion:** These findings indicate that AS extract has an inhibitory activity on adipogenesis in 3T3-L1 adipocytes via suppressing C/EBPα, PPARγ and SREBP1c.
Key words: *Aporosa villosa*; Adipogenesis; Obesity.

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INTRODUCTION

Obesity is responsible for the development of many diseases including hypertension, type 2 diabetes mellitus and atherosclerosis.[1] Adipogenesis is the state of excess fat accumulation in adipocytes during the preadipocyte differentiation process[2], and there is a study showing that increased adipocyte number related to obesity.[3] Thus, the regulation of adipocyte number may be helpful for people who are obese. Several transcriptional factor genes such as CCAAT/enhancer-binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element binding protein 1c (SREBP1c) are associated with the adipogenesis process.[4] C/EBPα and PPARγ participate in a modulation of downstream lipogenic genes such as acetyl-CoA carboxylase (ACC), adipocyte fatty acid-binding protein 2 (aP2), cluster of differentiation (CD) 36, fatty acid synthase (FAS) and lipoprotein lipase (LPL).[5]

Aporosa villosa is a plant that found in the Northern and Northeastern region of Thailand. It is used as a traditional medicine in some areas of Thailand, such as Ubon Ratchathani, for treating jaundice. However, this plant has limited pharmacological data. As obesity is an important condition that associated with the progression of several diseases, any novel substance or medicine, especially from natural sources, that can tackle this problem would become beneficial. Therefore, the present study interested to investigate whether *Aporosa villosa* extract has an anti-obesity action. The 3T3-L1 adipocyte cells were used as a model for determination of *Aporosa villosa* stem ethanolic extract on adipogenesis process. This study may provide supportive data for future study in animal and human models.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, sodium pyruvate and L-glutamine were obtained from Corning (Glendale, AZ, USA). Bovine calf serum (BCS), fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). 3T3-L1 adipocyte (ATCC CL-173) was purchased from American Type Culture Collection (Manassas, VA, USA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). High Capacity cDNA Reverse Transcription Kit was purchased from Applied Biosystems (Foster City, CA, USA). LightCycler® 480 SYBR Green I Master was purchased from Roche Molecular Systems (Pleasanton, CA, USA). Other chemicals were obtained from Sigma-Aldrich (St. Louise, MO, USA).

Plant materials

Aporosa villosa stem (AS) was collected from Ubon Ratchanthani. The identification (voucher specimen: AV-UBUPH00624) was approved by Faculty of Pharmaceutical Sciences, Ubon Ratchathani University.

The plant was dried and extracted with 95 % ethanol by maceration (48 h × 3 times) at room temperature.

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The extracts were evaporated under reduced pressure to obtain the dry extracts. The yields of the dry powder were 8.22%.

Phytochemical screening

The crude ethanolic extracts of AS were tested for the presence of phenolic contents by using high-performance liquid chromatography with diode array detection and mass spectrometry detector (HPLC-DAD/MSD) method.[6] Caffeic acid, catechin, courmaric acid, ferulic acid, gallic acid, protocatechuic acid, quercetin, rutin, sinapic acid and vanillic acid were used as phenolic standards.

3T3-L1 adipocyte culture

The experiments of 3T3-L1 adipocytes were approved by the Thammasat University Institutional Biosafety Committee (TU-IBC 030/2562). The preadipocytes were plated in 24-well plates (2×10^5cells/well), cultured in DMEM with 10% BCS, penicillin and streptomycin at 37°C in 5% CO_2. After 2 days of cell confluence, the induction of cell differentiation was started by incubating in DMEM/10% FBS/penicillin/streptomycin with dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and insulin for 48 h. Then, cells were maintained and re-fed every 2 days with DMEM/10% FBS/penicillin/streptomycin/insulin. To determine the effect of AS on adipocyte differentiation, the cells were treated with AS at a concentration range of 3-100 µg/mL compared with the cells treated with dimethyl sulfoxide (DMSO) as a control group. Treatments of AS were started from the first day of the induction of cell differentiation (day 0) to the end of the experiment on day 8. Three independent experiments were performed, each in triplicate.

Oil Red O staining

At the end of the experimental period, cells were fixed with 10% formalin for 1 h and washed 3 times with phosphate-buffered saline (PBS). Then, cells were stained with 0.6% Oil Red O solution for 1 h and washed again with PBS 3 times. The stained cells were photographed by Primovert (Carl Zeiss, NY, USA) at ×200 magnification. After that, the stained Oil Red O was eluted with isopropanol and quantified by measuring absorbance at 540 nm.

Cell viability assay

The 3T3-L1 cells (1×10^4 cells/well) were plated into a 96-well plate and treated with serial dilutions of AS (0-100 µg/mL). After AS incubation for 48 h, the cell viability was measured with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. The absorbance of the sample was determined at 570 nm.[7] Three independent experiments were performed, each in triplicate.

Measurement of quantitative real-time PCR

Total RNA of 3T3-L1 cells was extracted using TRIzol reagent and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit. The SYBR Green-based quantitative real-time PCR was performed using a LightCycler® 480 Instrument II (Roche Molecular Systems, Pleasanton, CA, USA). The primer sequences were mouse C/EBPα (Fwd: 5′-TGCCTATGAGCACTTCACAA-3′, Rev: 5′-AACTCCAGCACCTTCTGTTG-3′), mouse PPARγ (Fwd: 5′-TGGGAACCTGGAAGC TTGTCTC-3′, Rev: 5′TGTGGTA-AAGGG CTTGATGT-3′), mouse SREBP1c (Fwd: 5′-GGGCTCT-GCTGGACCAC-3′, Rev: 5′-TGGCCTTGTCAATGGAACTG-3′), mouse CD36 (Fwd: 5′-TCTGAAGAGACCTTACATTGTACC-TA-3′, Rev: 5′-CAATCCCAAGTAAGGCCATC-3′), mouse FAS (Fwd: 5′-GCACCTATGGCGAGGACTT-3′, Rev: 5′-ATGGATGAT-GTTGATGATGGA-3′), mouse LPL (Fwd: 5′-GGCCAGATTCAT-CAACTGGAT-3′, Rev: 5′-GCTCCAAGGCTGTACCCTAAG-3′) and mouse GAPDH (Fwd: 5′-CTGGAGAAACCTGCCAAGTA-3′, Rev: 5′-AGTGGGAGTTGC TGTTGAAG-3′). Relative expressions were calculated with normalization to GAPDH with the formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (Systat version 4.0, CA, USA) were used for investigating the significance difference among experimental groups. A statistical significance was set at $P < 0.05$.

RESULTS

Contents of phenolic compounds

Contents of phenolics were found in AS extract as shown in Table 1. The three main phenolics found in AS were rutin (199.20 µg/g), vanillic acid (106.91 µg/g) and catechin (98.60 µg/g).

Lipid accumulation

At day 8 of AS incubation, the microscopic observations of the Oil Red O staining showed that the AS at 3-100 µg/mL could inhibit lipid accumulation as compared to the untreated adipocyte group (Figure 1A). The absorbance value revealed that the lipid accumulation in 3T3-L1 adipocytes was significantly reduced by AS extracts (3-100 µg/mL) compared with the untreated adipocyte group (Figure 1B). Moreover, the inhibition of lipid accumulation of AS extracts did not cause cytotoxicity (Figure 1C).

Adipogenic gene expression

In comparison with the untreated adipocyte group, AS extracts (10-100 µg/mL) significantly decreased the gene expressions of PPARγ and C/EBPα (Figure 2A and 2B). SREBP1c was also inhibited by AS (3-100 µg/mL) (Figure 2C). Moreover, the AS extracts (3-100 µg/mL) significantly reduced the level of CD36, FAS and LPL genes, compared with untreated adipocyte group (Figure 2D, 2E and 2F).

DISCUSSION/CONCLUSION

Obesity is a metabolic syndrome that is characterized by the excessive fat accumulation in adipose tissue.[8] The present study investigated the effect of *Aporosa villosa* stem ethanolic extract in 3T3-L1 adipocytes. AS extracts (3-100 µg/mL) were able to suppress lipid accumulation and adipogenic gene expressions in 3T3-L1 adipocytes model. Thus, inhibition of excessive lipid storage in adipocytes may have a positive effect in preventing obesity.

There are several stages in processing adipogenesis from preadipocytes to differentiated mature adipocytes.[9] In this study, AS (3-100 µg/mL) could inhibit lipid storage without showing cytotoxicity in adipocyte cells. There is a report indicating that the transcription factors C/EBPα, PPARγ and SREBP1c are key regulators for the differentiation of preadipocytes to mature adipocytes.[5] PPARγ is involved in the late stage of adipocytes differentiation, and its absence can block lipid droplet formation.[10-11] The present result showed that AS at the concentration of 10, 30 and 100 µg/mL could inhibit PPARγ gene in 3T3-L1 cells. It has been reported that C/EBPs and SREBPs are associated with regulating early stage of adipocyte differentiation process.[12]Thus, the

Table 1: Phenolic contents of AS.

Phenolic compound contents	µg/g
Gallic acid	3.14
Protocatechuic acid	Not detectable
Vanillic acid	106.91
Caffeic acid	Not detectable
Courmaric acid	13.67
Ferulic acid	6.97
Sinapic acid	20.82
Catechin	98.60
Rutin	199.20
Quercetin	10.81

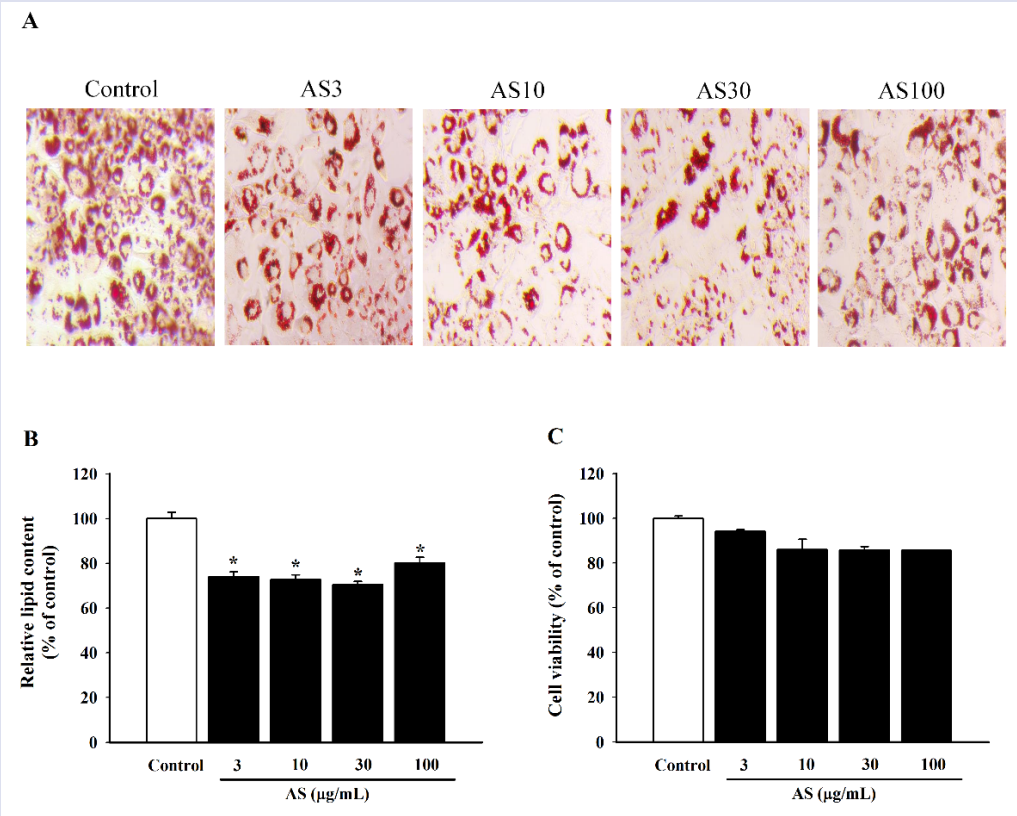

Figure 1: Effect of AS on lipid accumulation in 3T3-L1 adipocyte cells. At day 8 of treatment with 3-100 µg/mL AS, the adipocyte cells were stained with Oil Red O (A). Quantification of the lipid accumulation was based on the OD values at 540 nm of destained Oil Red O from the adipocytes (B) and cell viability (C). Values are expressed as mean ± SEM (n=3). * *P*<0.05 vs. the control group (untreated adipocyte cells). AS: *Aporosa villosa* stem ethanolic extract.

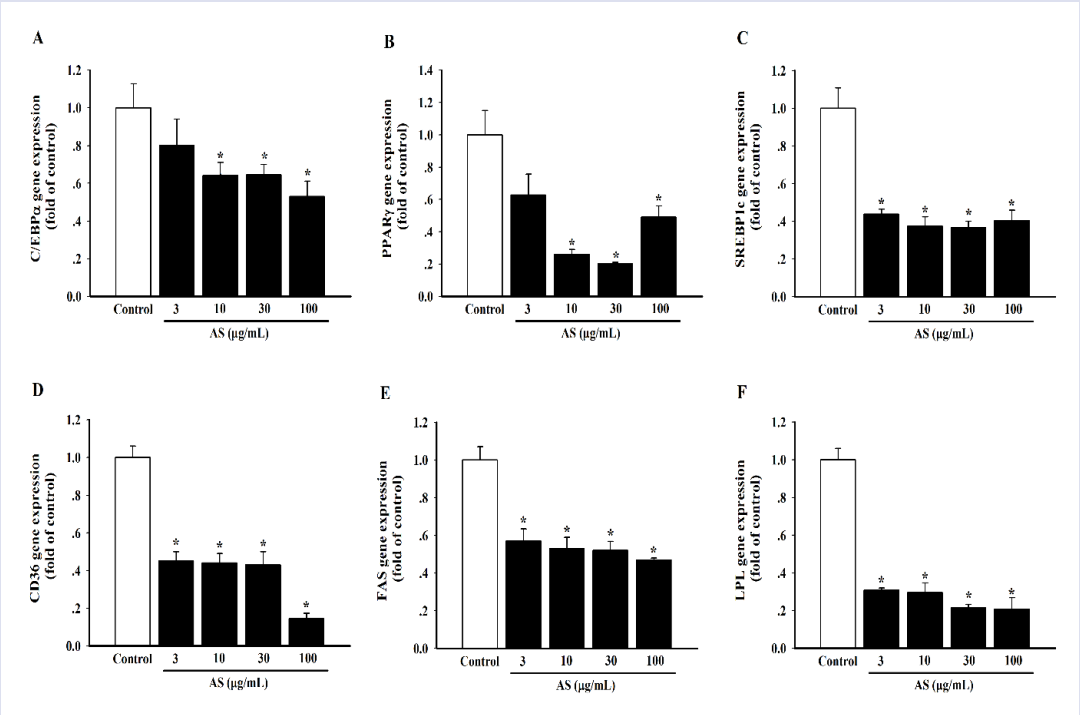

Figure 2: Effect of AS on the expression of the adipogenic gene expressions of C/EBPα (A), PPARγ (B), SREBP1c (C), CD36 (D), FAS (E) and LPL (F) in 3T3-L1 adipocyte cells. Values are expressed as mean ± SEM (n=3). * *P*<0.05 vs. the control group (untreated adipocyte cells). AS: *Aporosa villosa* stem ethanolic extract.

gene expressions of C/EBPα and SREBP1c were investigated in this study. The results showed that the AS treatment could reduce both genes in 3T3-L1 cells. These data demonstrated that AS treatment could inhibit the expression of transcription factor genes regulating adipogenesis.

The major transcription factors C/EBPα, PPARγ and SREBP1c involving in adipogenesis process regulate the adipocyte-specific markers such as LPL, FAS and CD36.[5] LPL is a major lipid synthesis enzyme which hydrolyzes lipoproteins from chylomicrons to free triglyceride in serum.[13] Overexpression of LPL is related to the initiation of lipid accumulation.[14] FAS is also a lipid synthesis enzyme. It can induce fatty acid synthesis and lipid accumulation.[15] CD36 plays a role in adipocyte cholesterol and adipogenesis metabolism.[16-17] Deficiency of CD36 could reduce the size and differentiation of adipocyte, and lipid accumulation in mice.[18] This study showed that AS extracts could decrease expression of these aforementioned genes. We speculate that AS inhibited the expressions of C/EBPα, PPARγ and SREBP1c genes, resulting in the suppression of the adipocyte-specific markers CD36, FAS and LPL genes in 3T3-L1 adipocytes.

Aporosa villosa is a plant that found in the Northern and Northeastern region of Thailand. Although it has been used in regional traditional medicinal practices, its systematic pharmacological data is limited. Generally, phenolic compounds widely found in plants possess antioxidant and anti-obesity activities.[19] In the present study, the AS extract was found to contain various phenolics, especially rutin, vanillic acid and catechin. These three main compounds have been previously reported to have an anti-obesity effect.[19-21] It is thus likely that these main phenolic compounds in the AS extract play a role in suppressing adipogenesis process in 3T3-L1 adipocytes by regulating C/EBPs/PPARγ/SREBP1c signaling pathways.

In conclusion, this study shows the effect of AS on adipocyte differentiation and lipid accumulation. AS extract can inhibit adipogenesis process by decreasing the lipid accumulation and suppressing the expression of C/EBPα, PPARγ and SREBP1c genes and their downstream target genes CD36, FAS and LPL. Overall, these results suggest that AS has an anti-adipogenic activity in the 3T3-L1 adipocyte model, which implies that it may have potential as an anti-obesity agent.

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CONFLICTS OF INTEREST

None.

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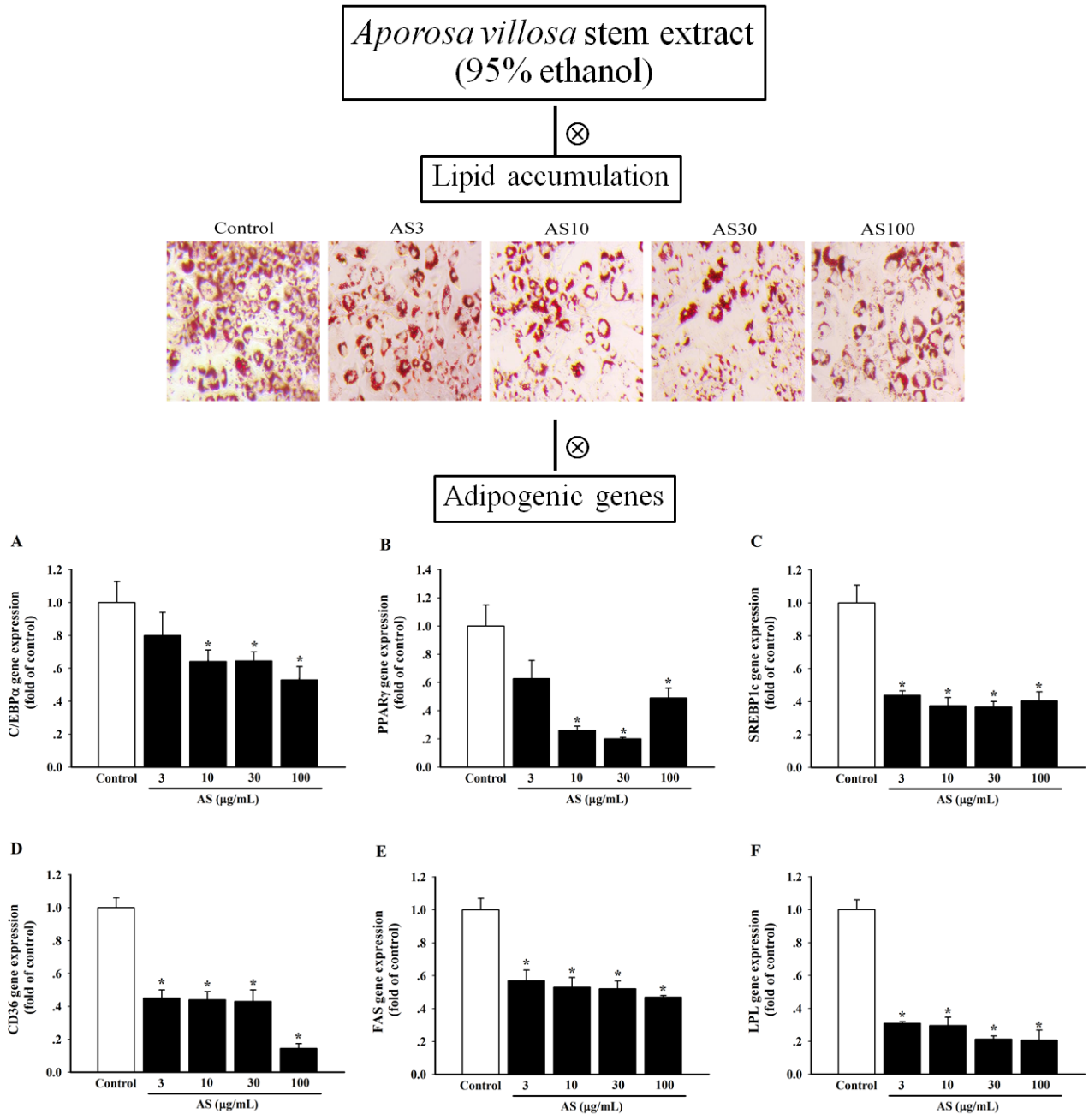
GRAPHICAL ABSTRACT



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