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Original Article

Extract of *Ranunculus sceleratus* Reduced Adipogenesis by Inhibiting AMPK Pathway in 3T3-L1 Preadipocytes

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3T3-L1 전구지방세포에서 개구리자리(*Ranunculus sceleratus*) 추출물의 AMPK 신호전달을 통한 지방생성 억제 효과

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Objectives: Adipogenesis is the process by which pre-adipocytes are differentiated into adipocytes. It also plays an important role in adipocyte formation and lipid accumulation. *Ranunculus sceleratus* (*R. sceleratus*) extracts are used for the treatment of various diseases such as hepatitis, jaundice, and tuberous lymphadenitis in oriental medicine. However, its effect on adipogenesis has not yet been studied. In this study, we investigated the effects of *R. sceleratus* on adipogenesis in 3T3-L1 cells.
Methods: Cells were treated with 50, 100, and 200 μg/ml of *R. sceleratus* and cell viability was evaluated. To differentiate the 3T3-L1 preadipocytes, a 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI) solution were used. The accumulation of lipid droplets was determined by Oil Red O staining. The expression levels of adipogenesis-related proteins were also determined.
Results: MDI solution differentiated the preadipocytes into adipocytes and accumulation of lipids was observed in the differentiated 3T3-L1 cells. Interestingly, the amount of lipid droplets was reduced after *R. sceleratus* treatment. In addition, the expression levels of key adipogenic transcription factors, such as CCAAT/enhancer-binding proteins-α (C/EBP-α) and peroxisome proliferator-activated receptors-γ (PPAR-γ) were also reduced after *R. sceleratus* treatment. Furthermore, *R. sceleratus* increased AMP-activated kinase (AMPK) phosphorylation and decreased sterol regulatory element-binding protein-1 expression.
Conclusions: Our results showed that *R. sceleratus* reduced preadipocyte differentiation by inhibiting C/EBP-α and PPAR-γ levels via the AMPK pathway. Therefore, we suggest that *R. sceleratus* may be potentially used as an anti-adipogenic agent.

Key Words: 3T3-L1 cells, Adipogenesis, CCAAT/enhancer-binding proteins-α, PPAR gramma, AMP-activated protein kinases, *Ranunculus sceleratus*

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Introduction

Obesity is a state of excessive accumulation of adipocyte during adipogenesis[1,2]. The obese population is rapidly increasing worldwide due to the irregular diet and unhealthy lifestyle of individuals[3,4]. In addition, obesity is a major

health problem and a risk factor for increasing the incidence of various human chronic diseases, such as hyperlipidemia, type 2 diabetes, heart disease, and cancer[5-8]. Several drugs have been developed to prevent and treat these obesity diseases. However, these drugs exert serious side effects, such as depression, gastrointestinal tract problems, and cardiovascular diseases[9]. Therefore, it is necessary to develop antiobesity products using natural extracts that possess good properties.

Excessive adipogenesis is a major cause of obesity. Adipogenesis is the process by which adipocytes develop and accumulate in the adipose tissue at various sites in the human body[10]. Adipocytes are formed by the proliferation and differentiation of preadipocytes into morphologically or biochemically mature adipocytes, which require the activation of important transcription factors. stimulation by hormones, including insulin, and regulation of adipocyte gene expression[11]. Therefore, the activity of transcription factors associated with the gene regulation of preadipocytes is also important for the regulation of obesity.

Ranunculus sceleratus Linn. (R. sceleratus, 石龍芮) is an annual or perennial herbaceous plant often found in riversides, ditches, and slow streams[12]. This species, the *Ranunculaceae* family, originated in the northern hemisphere and is distributed throughout the world[2,13]. *R. sceleratus* component include ranunculin, protoanemoninand anemonin[13]. Although the plant contains a toxic substance, protoanemonin, in the juice of its stems and leaves, it can be removed by heating or drying[2]. Anemonin is a known antipyretic, which along with protoanemonin, plays a major role in the sedating effect of this species[14]. This genus is unique, involving both its toxicological and pharmacological properties. In traditional medicine, *R. sceleratus* was used to treatment of various diseases, such as diabetes, arthritis, neuralgia, malaria, hepatitis, and jaundice[12,15,16]. All parts of the plant are poisonous when fresh, but the toxins are destroyed when the plant is heated or dried[2]. The heated or dried plant can be used to treat cancer of the esophagus and the breast[13]. Moreover, *R. sceleratus* exerts an anti-inflammatory effect. *R. sceleratus* have been reported to be effective in local anti-inflammatory in acute inflammatory models by inhibiting cyclooxygenase-1 and 12-lipoxygenas activity[2]. *R. sceleratus* have had many pharmacological effects, but the effect of *R. sceleratus* on adipogenesis has not yet been studied. Therefore, we examined whether *R. sceleratus* affect adipogenesis.

In this study, we investigated the effect of the *R. sceleratus* extracts on lipid accumulation in 3T3-L1 preadipocytes.

Materials and Methods

1. Materials

R. sceleratus extract was obtained from the Nakdoggang National Institute (Freshwater Bioresources Culture Collection, Sangju, Korea). *R. sceleratus* was collected from Sangju, Gyeongsangbuk-do in 2017. The whole plant was hot air-dried at 60°C for 72 hours and ground. Samples are extracted twice using 70% ethanol (4023-4110; Daejung, Siheung, Korea) to 20 times of sample weight at room temperature. After extraction, it was filtered using ADVANTEC No. 2 filter paper and then concentrated under reduced pressure (N-1100S, Rotary Evaporator Vertical; EYELA, Tokyo, Japan). 20 mg of the prepared extract was dissolved in 1 ml of dimethyl sulfoxide (Sigma Aldrich Co., Ltd., St. Louis, MO, USA) to prepare a concentration of 20 mg/ml.

2. Cell culture

The 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Korea) supplemented with 10% bovine calf serum (BCS; Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin (P/S; Gibco) in a humidified incubator at 37°C in 5%.

3. Differentiation of 3T3-L1 preadipocytes

The 3T3-L1 pre-adipocytes were distributed in a 6-well plate at a density of 5× cells/well in DMEM supplemented with 10% BCS and 1% P/S at 37°C in 5%. After incubating until confluency, the 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), and insulin (MDI) solution contain-

ing 1 μM DEX (Sigma Aldrich Co., Ltd.), 0.5 mM IBMX (Sigma Aldrich Co., Ltd.), and 10 μg/ml insulin (Sigma Aldrich Co., Ltd.) was used to induce the differentiation of cells in DMEM with 10% fetal bovine serum (FBS) for two days. After two days of differentiation induction, the medium was replaced with DMEM supplemented with 10% FBS (Gibco) and 10 μg/ml insulin every two days. This was followed by cell differentiation and incubation for 10 d. Every time the medium was changed, the cells were treated at 50, 100, and 200 μg/ml with *R. sceleratus*.

4. Cell viability assay

The 3T3-L1 pre-adipocytes were seeded at a density of 1 × cells/well in a 96-well plate and incubated at 37°C in 5% for 24 h. Next, 3T3-L1 cells were treated with different concentrations (0, 50, 100, and 200 μg/ml) of *R. sceleratus* for 24, 48, and 72 h. EZ-cytox cell viability assay solution (Dugen, Seoul, Korea) was added to each well, and the cells were incubated for 1 h. Cell viability was measured at a wavelength of 450 nm using a microplate reader (Bio-TEK, Senergy HT; BioTek, Santa Clara, CA, USA).

5. Oil Red O staining

After 10 days of differentiation, the cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin (Sigma Aldrich Co., Ltd.) for 1 h at room temperature. The cells were washed with 60% isopropyl alcohol (Fujifilm, Osaka, Japan) and stained for 10 minutes with 0.5% Oil-Red O solution (Sigma Aldrich Co., Ltd.). The Oil Red O solution was then removed and the cells were washed twice with distilled water. After Oil Red O staining, the cells were observed under an inverted microscope. Stained lipid droplets were dissolved in isopropanol and quantified at 500 nm using a microplate reader.

6. Western blotting analysis

The differentiated 3T3-L1 cells were washed once with cold PBS and dissolved with radioimmunoprecipitation assay lysis buffer (cell signaling) and centrifuged at 12,000 rpm for 15 minutes at 4°C to obtain the supernatant. The quantified protein was transferred to a polyvinylidene fluoride membrane after electrophoresis on 6-12% sodium dodecyl sulfate-polyacrylamide gel. The membrane was blocked with 5% nonfat milk in PBS-T buffer with 20% tween-20 for 1-2 h and incubated overnight with the following primary antibodies: anti-peroxisome proliferator-activated receptor-γ (PPAR-γ; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CCAAT/enhancer-binding protein-α (C/EBP-α; Santa Cruz Biotechnology), anti-sterol regulatory element-binding protein-1 (SREBP-1; Santa Cruz Biotechnology), anti-glyceraldehyde-3-phosphate dehydrogenase (Ab Frontier; GW Vitek, Seoul, Korea), anti-AMP-activated protein kinase (AMPK; Cell Signaling, Danvers, MA, USA), and anti-phosphorylated AMPK (p-AMPK; Cell Signaling) antibodies. After washing with PBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or mouse anti-goat secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 2 h at room temperature. Proteins were visualized using a detection kit (Amersham BioSciences UK Ltd., Little Chalfont, UK) and quantified using a CS analyzer (ATTO, Tokyo, Japan).

7. Statistical analysis

All experimental data were presented as the mean±standard error of the mean of triplicate experiments. Data analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and Sigma Plot software (Systat Software Inc., Chicago, IL, USA). The significance of differences was determined using a one-way analysis of variance by post-hoc Tukey's test when relevant. P-value <0.05 was considered to be statistically significant.

Result

1. Effects of *R. sceleratus* on the cell viability of 3T3-L1 preadipocytes

The effect of *R. sceleratus* on the cell viability of 3T3-L1 preadipocytes was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay with 50, 100, and 200 μg/ml of *R. sceleratus* extract. As shown in Fig. 1, *R. sceleratus* extract showed no significant effects on via-

bility after 24, 48, and 72 hours.

2. Effect of *R. sceleratus* extracts on the differentiation of 3T3-L1 cells

Fig. 2A shows the differentiation of the lipid droplet experimental protocol for 3T3-L1 preadipocytes. *R. sceleratus*-mediated regulation of lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes for 10 days was examined via Oil Red O staining. As shown in Fig. 2B, the concentration of *R. sceleratus* was increased, with a decrease in the rate of differentiation of 3T3-L1 preadipocytes; control (+) showed an increase in the size of the

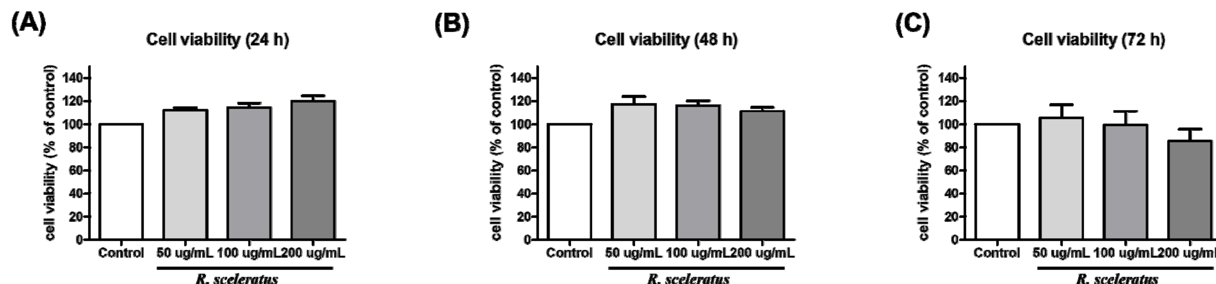

Fig. 1. Effect of *R. sceleratus* extract on the cell viability of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with various concentrations (50~200 μg/ml) of *R. sceleratus* (A) Cell viability of 3T3-L1 preadipocytes after 24 h. (B) Cell viability of 3T3-L1 preadipocytes after 48 h. (C) Cell viability of 3T3-L1 preadipocytes after 72 h.

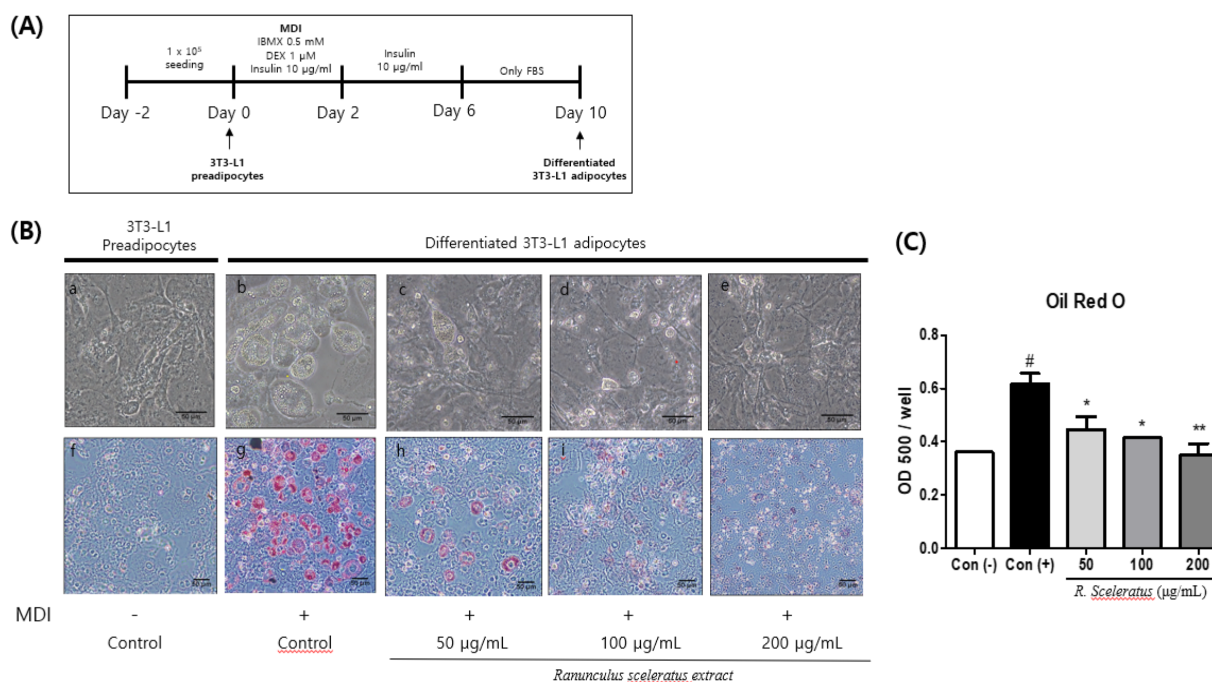

Fig. 2. Effect of *R. sceleratus* extract on the differentiation of 3T3-L1 adipocytes via Oil Red O staining. 3T3-L1 preadipocytes were treated with various concentrations (50~200 μg/ml) of *R. sceleratus*. (A) Cell culture and differentiation protocol. (B) Intercellular lipid droplets were stained with Oil Red O. After 10 days differentiation, representative phasecontrast photomicrographs at 200x and 400x magnification depictied of 3T3-L1 adipocyte. Control (−) include a and b, control (+) include b and g, 50 μg/ml, include c and h, 100 μg/ml include d and i, 200 μg/ml include e and j. (C) Quantification of lipid accumulation by eluting with isopropanol. The Data were presented as mean±standard error of the mean from three independent experiments. Con (−): control without MDI media, Con (+): control with MDI media, MDI: 0.5 mM IBMX, 1 μM DEX, 10 μg/ml insulin, *R. sceleratus*: *Ranunculus sceleratus*, IBMX: 3-isobutyl-1-methylxanthine, DEX: dexamethasone, FBS: fetal bovine serum. #P<0.05 compared with the control (−); *P<0.05, **P<0.01 compared with the control (+).

lipid droplets compared to the control (-). However, *R. sceleratus* reduced the accumulation of lipid droplets compared to the control (+) at 50, 100, and 200 μg/ml concentrations (Figs. 2B a-e). To confirm lipid droplets, we performed Oil Red O staining. We observed an increase in red-colored lipid droplets in the control (+) (Figs. 2B f-j). As shown in Fig. 2C, the concentration of *R. sceleratus* was increased, along with a significant decrease in the rate of differentiation of 3T3-L1 preadipocytes: the differentiation rate at 50, 100, and 200 μg/ml declined by 27.83, 32.77, and 43.45% respectively. These results confirm that the *R. sceleratus* extract inhibits the accumulation of lipid droplets in cells.

3. Effect of *R. sceleratus* on the regulation of PPAR γ and C/EBP-α transcription factors

The effects of *R. sceleratus* on adipogenesis-related transcription factors (C/EBP-α and PPAR-γ) were determined via western blotting analysis. As shown in Fig. 3, control (+) increased compared to control (-). *R. sceleratus* reduced the expression levels of PPAR-γ in 3T3-L1 cells compared to those in the control (+). Similarly, protein levels of C/EBP-α were decreased with 200 μg/ml of *R. sceleratus* (except 50 and 100 μg/ml concentrations) in 3T3-L1 cells compared to those in the control (+).

4. Effect of *R. sceleratus* on the regulation of the AMPK/SREBP-1 signaling pathway

The differentiated control (+) increased compared to the undifferentiated control (-); however, AMPK levels were increased in the *R. sceleratus*-treated group compared to the control (+). As shown in Fig. 4, we confirmed the effect of *R. sceleratus* via the AMPK/SREBP-1 signaling pathway. *R.*

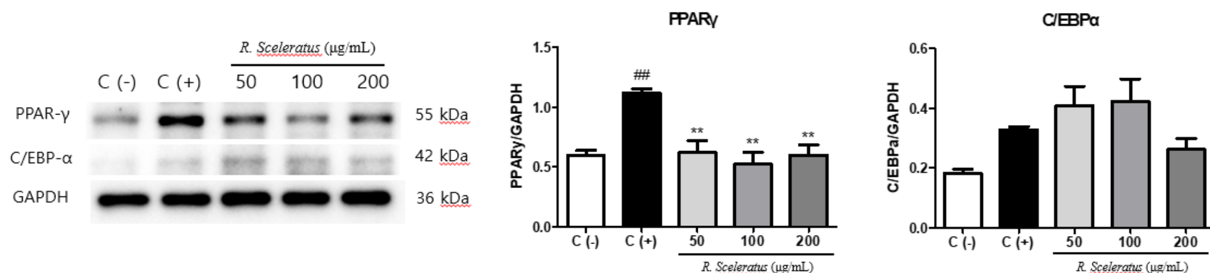

Fig. 3. Effect of *R. sceleratus* on the expression levels of the C/EBP-α and PPAR-γ in 3T3-L1 cells. 3T3-L1 preadipocytes were treated with various concentrations (50~200 μg/ml) of *R. sceleratus*. GAPDH was used as a loading control. The Data were presented as mean±standard error of the mean from three independent experiments. PPAR-γ: peroxisome proliferator-activated receptor-γ, C/EBP-α: CCAAT/enhancer-binding proteins-α, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, C (-): control (-), C (+): control (+). ##P<0.01 compared with the control (-); **P<0.01 compared with the control (+).

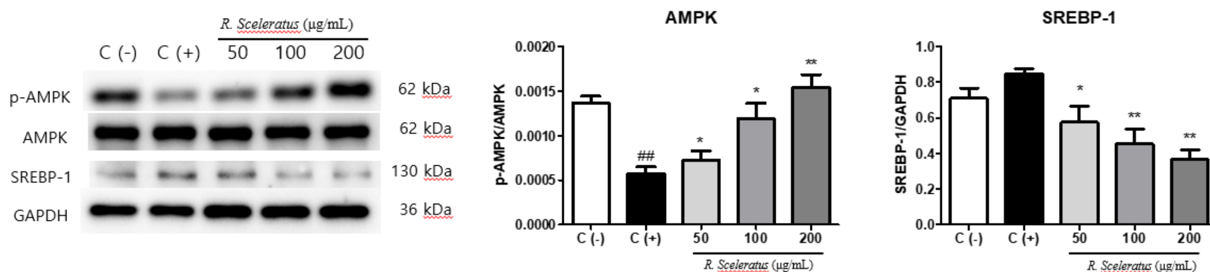

Fig. 4. Effect of *R. sceleratus* on the expression of AMPK/SREBP-1 signaling pathway in 3T3-L1 cells. 3T3-L1 preadipocytes were treated with various concentrations (50~200 μg/ml) of *R. sceleratus*. GAPDH was used as a loading control. The Data were presented as mean±standard error of the mean from three independent experiments. AMPK: AMP-activated protein kinase, p-AMPK: phosphorylated AMPK, SREBP-1: sterol regulatory element-binding protein-1, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, C (-): control (-), C (+): control (+). ##P<0.01 compared with the control (-); *P<0.05, **P<0.01 compared with the control (+).

sceleratus increased AMPK levels and decreased SREBP-1 levels compared to those in the control (+). We confirmed that *R. sceleratus* induced the phosphorylation of AMPK.

Discussion

Our study aimed to determine the ameliorative effect of the *R. sceleratus* extract on adipogenesis. Initially, we determined the cytotoxicity of *R. sceleratus* in 3T3-L1 preadipocytes. We also examined the effect of *R. sceleratus* via Oil Red O staining of 3T3-L1 preadipocytes. The number of fat droplets decreased in the group treated with *R. sceleratus* compared to that in the control (+) *R. sceleratus* effectively inhibited lipid droplet accumulation.

PPAR-γ and C/EBP-α are transcription factors that play important roles in adipogenesis during the differentiation of preadipocytes to adipocytes[17,18]. C/EBP-α plays an important role in the late differentiation process of adipogenesis in adipocytes[19]. PPAR-γ is responsible for regulating the differentiation of adipocytes and its expression is associated with adipogenesis and fat storage[20]. PPAR-γ and C/EBP-α are master regulators of adipogenesis[21-23]. Our results showed that *R. sceleratus* significantly decreased the expression levels of PPAR-γ and C/EBP-α compared to those in the MDI-induced adipocytes. This indicates that *R. sceleratus* inhibits adipogenesis in 3T3-L1 cells by downregulating PPAR-γ and C/EBP-α expression levels.

Additionally, we assessed the AMPK signaling pathway, which regulates the transcription factors. Oxidation of fatty acids, lipid hydrolysis of triglycerides, and adipogenesis by adipocytes regulate the AMPK pathway[24-26]. AMPK is involved in adipocyte differentiation and adipogenesis regulation, and the activation of AMPK suppresses adipogenesis[27,28]. Activation of the regulatory pathway requires AMPK phosphorylation which inhibits lipid synthesis and upregulates lipid hydrolysis and fatty acid oxidation[26,29,30]. Several studies have shown that AMPK inhibits adipogenesis by inactivating SREBP-1, a transcription factor that regulates lipid homeostasis and metabolism[31-34]. SREBP-1 induces gene expression associated with the regulation of PPAR-γ transcriptional activity and accumulation of lipids[35]. SREBP-1 is quickly induced in the early stages of preadipocyte differentiation and plays a role in promoting preadipocyte differentiation along with PPAR-γ. SREBP-1 promotes lipid metabolism by increasing the expression levels of several genes involved in lipid metabolism[36,37]. These results suggest that AMPK regulates various transcription factors, such as PPAR-γ, C/EBP-α, and SREBP-1, that are responsible for adipocyte differentiation and inhibition of adipogenesis[34]. Our results showed that *R. sceleratus* significantly increased AMPK phosphorylation and decreased the expression levels of SREBP-1 compared to those in the MDI-induced adipocytes, and that *R. sceleratus* inhibited adipogenesis in 3T3-L1 cells by upregulating AMPK expression (Fig. 5).

Obesity is an underlying condition for inflammatory and metabolic diseases and is often accompanied by a low-grade chronic inflammation[38]. Anemone, one of the main components in *R. sceleratus,* plays an important role in sedation[2]. Also, It is has been reported to be effective in anti-inflammatory[2]. Therefore, we think that anemone is effective in anti-obesity because it is effective in anti-inflammatory. However, we haven't confirmed which ingredient is the active ingredient in *R. sceleratus*. In a future study, we should be confirmed which ingredients have an anti-obesity effect in *R. sceleratus* extract. In addition, further studies should be conducted

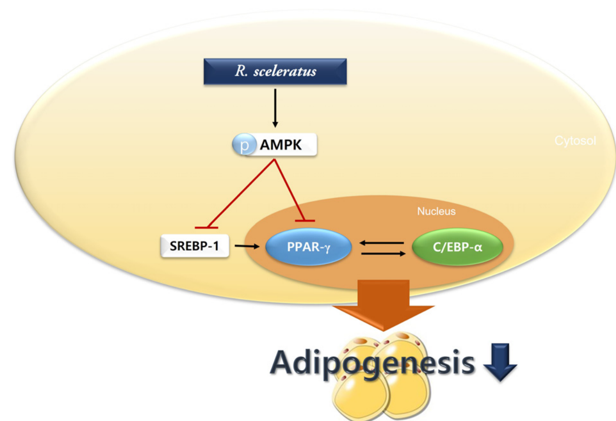

Fig. 5. AMPK signaling pathway of adipogenesis inhibition of *R. sceleratus* in 3T3-L1. PPAR-γ: peroxisome proliferator-activated receptor-γ, C/EBP-α: CCAAT/enhancer-binding proteins-α, AMPK: AMP-activated protein kinase, SREBP-1: sterol regulatory element-binding protein-1.

to confirm anti-obesity activity through animal experiments.

In summary, we demonstrated that *R. sceleratus* inhibits adipogenesis in 3T3-L1 cells by activating the AMPK pathway and suppressing the expression levels of adipogenic transcription factors. Based on these data, this study suggests that the *R. sceleratus* could be used as an alternative therapeutic agent to prevent and ameliorate obesity.

Conclusions

R. sceleratus exerts anti-adipogenic effects on differentiating 3T3-L1 preadipocytes. In addition, it downregulates the expression levels of PPAR-γ and C/EBP-α by inhibiting the AMPK/SREBP-1 signaling pathway. These results suggest that *R. sceleratus* can potentially be used as an anti-adipogenic agent.

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