

Adzuki Bean (*Vigna angularis*) Extract Inhibits 3T3-L1 Preadipocyte Differentiation

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Abstract The present study aimed to investigate the mechanism underlying the effect of adzuki bean extract on the differentiation of 3T3-L1 adipocytes. Treatment of these adipocytes with the extract significantly suppressed the expressions of the Ppar γ , C/ebp α , and Fabp4 genes that are involved in adipogenesis (adipocyte differentiation). Oil Red O staining revealed that fat accumulation within these adipocytes is reduced significantly when the cells are treated with the extract. To explore the impact of adzuki bean extract on the differentiation of 3T3-L1 adipocytes, we investigated the effect of adzuki bean extract on the expression of cycle-related factors. We observed reduced expression of cyclin D1 mRNA and phosphorylated Rb (pRb), a downstream factor of cyclin D1 at 24 h after the application of a differentiation-inducing stimulus. Meanwhile, at 72 h after the stimulation, the expression of pRb increased with an increase in the amount of adzuki bean extract. The proliferation of cells declined significantly on treatment with the extract at 16 h after the stimulation but accelerated significantly at 72 h after the stimulation. These results suggest that adzuki bean extract contains an active ingredient that suppresses the differentiation of adipocytes. As a possible mechanism, the extract may alter the mitotic clonal expansion (MCE) which is essential for adipogenesis.

Keywords: adzuki bean, obesity, 3T3-L1, differentiation, mitotic clonal expansion (MCE)

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1. Introduction

Obesity is a global health issue, and obese people are vulnerable to many lifestyle diseases (metabolic disorders), including type 2 diabetes, hypertension, and coronary artery disease [1,2]. Obesity is caused by not only the hypertrophy of adipose tissue but also the hyperplasia thereof [3]. Accordingly, suppressing adipogenesis is believed to be conducive in preventing obesity and subsequently other concomitant diseases. The process of adipogenesis, in which preadipocytes multiply and differentiate into mature adipocytes, can be simulated by the differentiation system of 3T3-L1 cells. This differentiation into adipocytes is regulated by transcription factors such as peroxisome proliferator-activated receptor γ (PPAR γ), fatty acid binding protein 4 (FABP4), and CCAAT/enhancer binding protein α (C/EBP α) [4,5]. Therefore, efforts are underway to effectively regulate these transcription factors and identify agents that can potentially suppress the process of differentiation of preadipocytes to adipocytes.

Many plant-derived foodstuffs have been reported to possess the properties to counter obesity [1]. The natural substances that can suppress adipogenesis are extremely

useful in combating obesity. Adzuki bean is produced and consumed primarily in East Asia, where it is regarded as a staple crop [6]. According to previous reports, adzuki bean extract possesses anticancer, antioxidant, and antidiabetic properties [7,8,9]. In addition, it helps in decreasing blood pressure and alleviating inflammation [10,11]. The extract exhibits various physiological activities and contains factors that prevent a number of diseases. Nevertheless, little is known as to how it affects the differentiation of adipocytes. In the present study, we examined the impact of adzuki bean extract on the process of adipogenesis using 3T3-L1 cells.

2. Materials and Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was a product of Microbiological Associates (Walkersville, MD). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and 4'6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (Tokyo, Japan). All other compounds were from Nacalai Tesque (Kyoto, Japan).

2.2. Sample Preparation

Adzuki beans (*V. angularis*, 500 g) harvested in Tokachi, Hokkaido, Japan, were immersed in distilled deionized water (2 L) at room temperature overnight, and then the mixture was filtrated through cotton. A 40% ethanol solution (4 L) was added to the red beans after the water extraction and allowed to stand at room temperature for 36 hours. After filtration of the ethanol extract with cotton, it was concentrated in *vacuo* at 45 ° C to yield the concentrate (22 g).

2.3. Cell culture and experiment

Low passage number 3T3-L1 cells were obtained from ATCC (Manassas, VA). 3T3-L1 cells were grown in DMEM containing 10% fetal bovine serum. For the induction of adipocyte differentiation, cells were treated with differentiation medium containing 10% fetal bovine serum, 0.5 mM IBMX, 1 μ M dexamethasone, and 1.7 μ M insulin. To examine the effect of Adzuki extract on adipocyte differentiation, the preadipocyte received 1, 10, 50, 100 and 300 μ g/ml Adzuki extract (in differentiation medium) every 2 days started at 2 days post confluence until the end of the experiment days. All cultures were maintained at 37 °C under 5% CO₂.

2.4. RNA extraction and qRT-PCR

Total RNA was isolated from mammary glands or 3T3-L1 cells using TRIzol (Invitrogen) according to the manufacture's protocol. For qRT-PCR, cDNA were synthesized from total RNA using a qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan).

qRT-PCR was performed in a Thermal Cycler Dice[®] Real Time System (TaKaRa Bio Inc., Shiga, Japan) by using SYBR Premix Ex TaqTM II (TaKaRa Bio Inc.). Relative expression was quantified using the standard curve method and data were normalized to GAPDH gene expression.

Primer sequences are as follows:

Ppar γ Forward: 5'-gatgactgctatgagcactt-3',

Reverse: 5'-agagtgccacagagctgattcc-3';

Fabp4 Forward: 5'-caccgcagacgacaggaag-3',

Reverse: 5'-gcacctgcaccagggc-3';

C/ebpa Forward: 5'-tgaccgtgaactggacacg-3',

Reverse: 5'-cagcctagatccagcgac-3';

Ccnd1 Forward: 5'-cggatgagaacaagcagacc-3',

Reverse: 5'-gcggtagcaggagaggaagt-3';

Gapdh Forward:

5'-ttgtgatgggtgtaaccacgag-3',

Reverse: 5'-catgagccctccacaatgcca-3'.

The sensitivity of the reaction and amplification of contaminating products, such as the extension of self-annealed primers, were evaluated by amplifying serial dilutions of cDNA. All data analysis was performed as recommended by the manufacturer.

2.5. Oil-red O Sating

Adzuki extract or a vehicle was added to the differentiation medium at day 0 and cells were cultured for 8 day. On day 8, 3T3-L1 cells were fixed with 10%

formalin in PBS for 10 min, rinsed with 60 % isopropanol, and stained by Oil-red O in 60 % isopropanol for 30 min. After the staining, cells were rinsed several times with PBS and subjected to microscopic analysis. Additionally, oil red O stain was eluted from the adipocyte using isopropanol (100 %) and measured at 490 nm.

2.6. MTT Cell Viability Assay

The 3T3-L1 preadipocytes were seeded at a density of 5 \times 10³ cells per well in 96-well plates and incubated in culture medium. The cells were then treated with Adzuki extract (10 or 100 μ g/ml). After 72 hr, cell viability was established using MTT Cell Viability Assay Kit (Biotium) according to manufacture's protocol. In brief; 10 μ l MTT solution was added to 100 μ l culture medium. After 4 hrs of incubation at 37°C, 200 μ l dimethylsulfoxide (DMSO) was added to each well. Absorbance was measured on a multimode microplate reader (iMark microplate reader, Bio-Rad) at 570 nm and a reference wavelength of 630 nm.

2.7. Western Blot Analysis

Cells were harvested in ice-cold phosphate buffered saline (PBS) and lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% Nonidet P-40, 10 mM β -glycerophosphate, 2.5 mM NaF, 0.1 mM Na₃VO₄) supplemented with protease inhibitors. Protein concentrations were measured using Bradford reagents (Pierce; Rockford, IL, USA). An equal amount of protein samples were separated by SDS-PAGE (7.5 %) and trans blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, USA). The immunoblotting was performed with rabbit anti-phospho Rb (Abcam, Camebridge, UK) and α -tubulin (MBL Co., Japan). ECLTM anti-rabbit IgG (GE Healthcare) was used as the secondary antibody. Labeled proteins were visualized using ECL Prime Western Blotting Detection Reagent kit (GE Healthcare) and detected with an ImageQuant LAS 500 (GE Healthcare).

2.8. EdU Proliferation Assay

At 16 h and 72 h after differentiation stimuli, cell proliferation was detected using incorporation of 5-ethynyl-2'-deoxyuridine (EdU) with the Click-iT EdU Cell Proliferation Assay Kit (Invitrogen). Briefly, cells were incubated with 10 μ M EdU for 1 h before fixation, permeabilization, and EdU staining, which were carried out according to the kit's protocol. The cells were incubated in a DAPI solution for 5 minutes. Fluorescence photographs were taken on EVOS[®] FL Auto (Life Technologies; Carlsbad, CA, USA).

2.9. Statistical Analysis

In all experiments, values are expressed as means \pm standard error of the mean, with at least 3 repeats in each experimental group. Student's *t*-test was applied to determine the statistical differences. The test was considered significant at *P* < 0.05.

3. Results

3.1. Adzuki Extract Restrains the Expression Levels of Differentiated-associated Genes in 3T3-L1 Cells

We performed quantitative real-time PCR to examine the effect of adzuki bean extract on the expression of genes related to adipogenesis. After adding the extract to the differentiation medium at varying concentrations (0–300 µg/mL), gene expressions in cells were analyzed at 3 days after treatment. At concentrations of 100 µg/mL or above, a significant decrease in the *Pparγ* expression was observed (Figure 1). The expression of *Fabp4*, on the other hand, was found to be significantly reduced at all concentrations. Further, a significant decline in the expression of *C/ebpa* was observed at concentrations of 50 µg/mL or above.

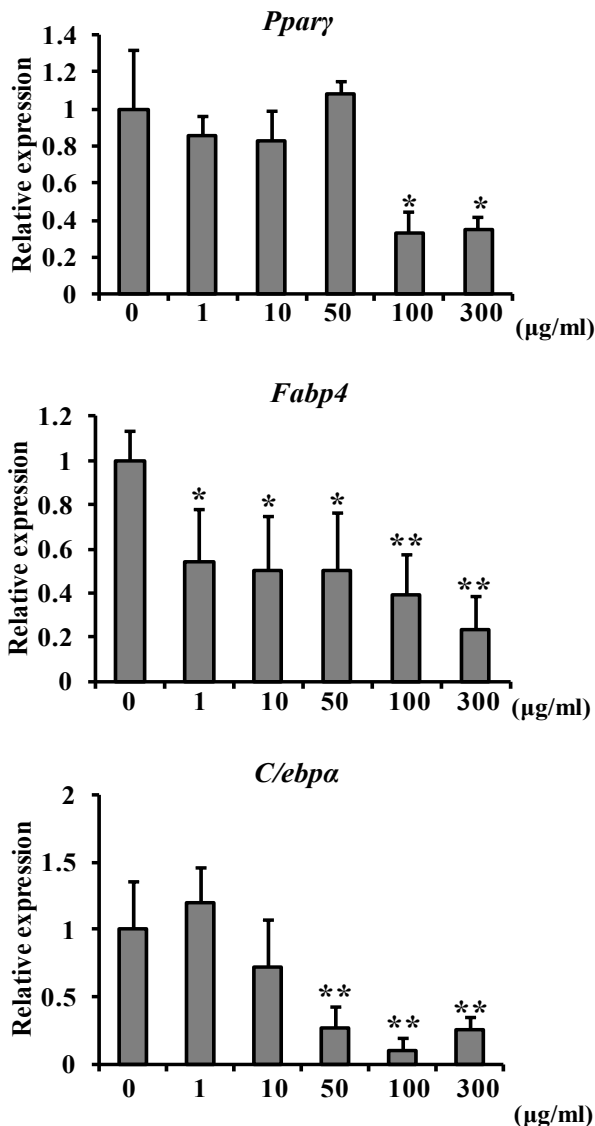


Figure 1. Adzuki extract restrains the expression levels of differentiated-associated genes in 3T3-L1 cells. Adzuki extract or a vehicle was added to the differentiation medium as day 0 and cells were cultured for 3 day. The mRNA expression of PPAR γ , FABP4 and C/EBP α was determined by real-time PCR and normalized by GAPDH. The results are expressed as means \pm SEM for three independent determinations. * P <0.05, ** P <0.01 compared with control

3.2. Adzuki Extract Impairs the Lipid Accumulation in Differentiating 3T3-L1 Cells

Oil Red O staining was performed to evaluate the impact of adzuki bean extract on the differentiation of adipocytes. We added the extract to the differentiation medium and examined cells at 8 days after treatment. Compared with control cells, extract-treated cells contained fewer fat droplets that were dyed red inside Petri dishes (Figure 2A). A colorimetric assay revealed a significant decrease in the accumulation of fat droplets in the extract-treated cells (Figure 2B). We also performed an MTT assay to assess the impact of the extract on the viability of 3T3-L1 cells, but no cytotoxicity that would adversely affect cell survival was detected (Figure 2C).

3.3. Effect of Adzuki Extract on Expression of Cell Cycle-related Molecules in 3T3-L1 Adipocytes

To undergo normal functional differentiation, 3T3-L1 cells essentially require a cell proliferation process called mitotic clonal expansion (MCE) in the early stages of differentiation. When 3T3-L1 cells that have become confluent and stopped cell growth are subjected to induction of differentiation, they undergo two mitoses in succession over approximately 2 days after the induction. Such instances of mitosis, which apparently precede the expression of differentiation-related genes, are referred to as MCE [12]. The first and second mitoses are completed in 24–36 and 48–60 h after being subjected to the induction of differentiation, respectively [13]. For cells to initiate adipogenesis, it is a prerequisite that MCE is induced immediately after subjecting to a differentiation stimulus and completed by 60 h after the induction and termination of cell proliferation [14]. In the present study, we examined the impact of adzuki bean extract treatment on cyclin D1 mRNA, a factor regulating the cell cycle. Compared with control cells, the 3T3-L1 cells treated with adzuki bean extract simultaneously with the induction of differentiation displayed significantly lower levels of cyclin D1 mRNA expression after 24 h (Figure 3A). Furthermore, a western blot analysis of the phosphorylated Retinoblastoma (pRb) protein expression revealed that the expression of inactive Rb decreased, albeit slightly, in the extract-treated cells at 24 h after the induction. Meanwhile, at 72 h after the stimulation, the expression of pRb increased in response to adzuki bean extract in a dose-dependent manner (Figure 3B).

3.4. Effect of Adzuki Extract on Proliferation of 3T3-L1 Adipocytes during Early Differentiation

Finally, we performed EdU staining to investigate how treatment with adzuki bean extract would affect the proportion of proliferated 3T3-L1 cells at 16 and 72 h after the induction of differentiation. Although numerous control cells were observed to have proliferated at 16 h after the induction, only a small proportion of the extract-treated cells had multiplied. At 72 h after the

induction, on the other hand, the control cells had entered the G₀ phase, thus halting cell proliferation, whereas

many of the extract-treated cells were still multiplying (Figure 4).

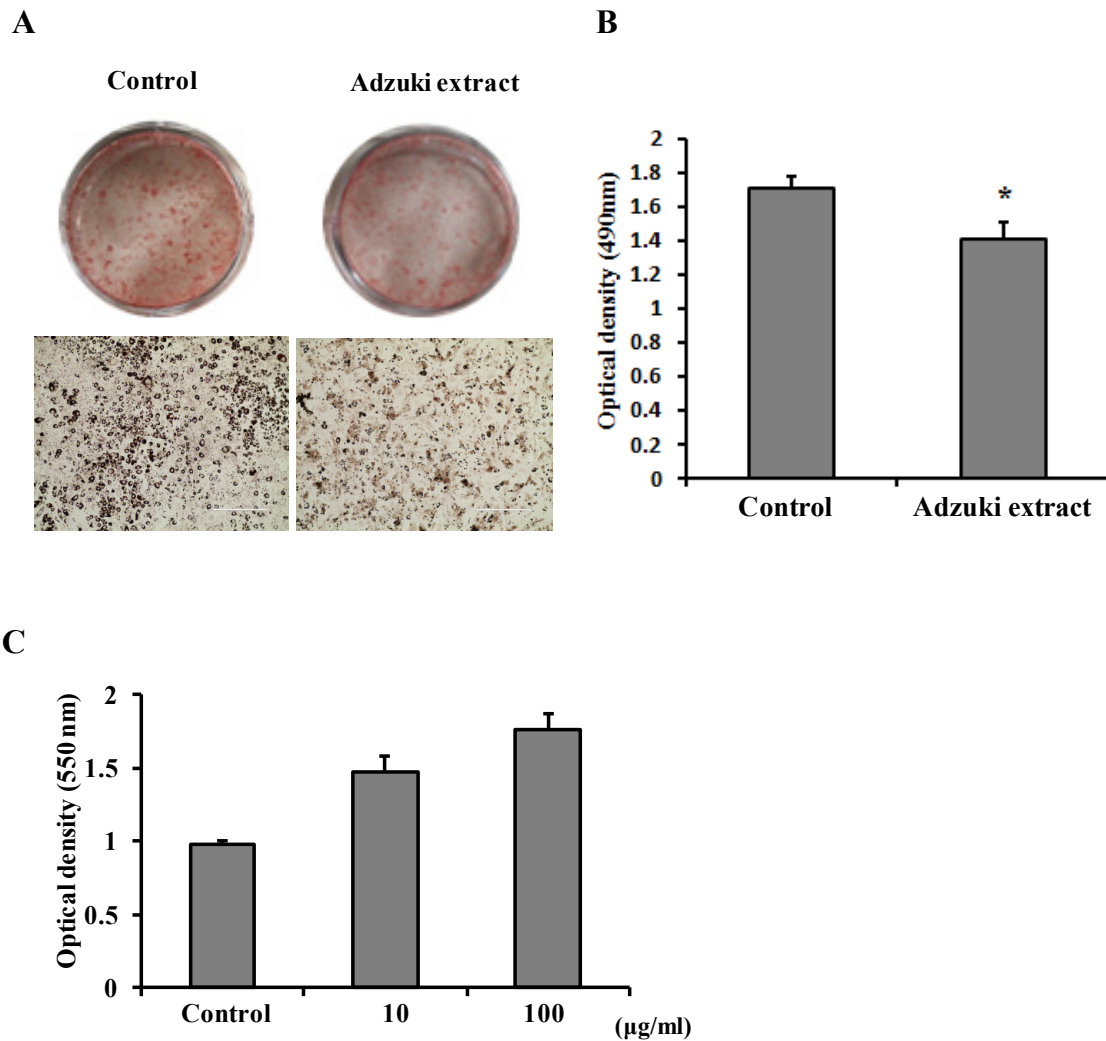


Figure 2. Effect of Adzuki extract on lipid accumulation in 3T3-L1 adipocytes. (A) Adzuki extract or a vehicle was added to the differentiation medium at day 0 and cells were cultured for 8 day. Cells were stained with Oil Red O and observed under the microscope. (B) Oil Red O-stained intracellular lipids were then extracted using isopropanol and quantified by measuring the optical density at 490 nm. The results are expressed as means \pm SEM for three independent determinations. * P <0.05 compared with control. (C) Undifferentiated 3T3-L1 cells were treated with Azuki extract or a vehide. Cell viability was measured by the MTT assay after 72 hr. The results are expressed as means \pm SEM for three independent determinations.

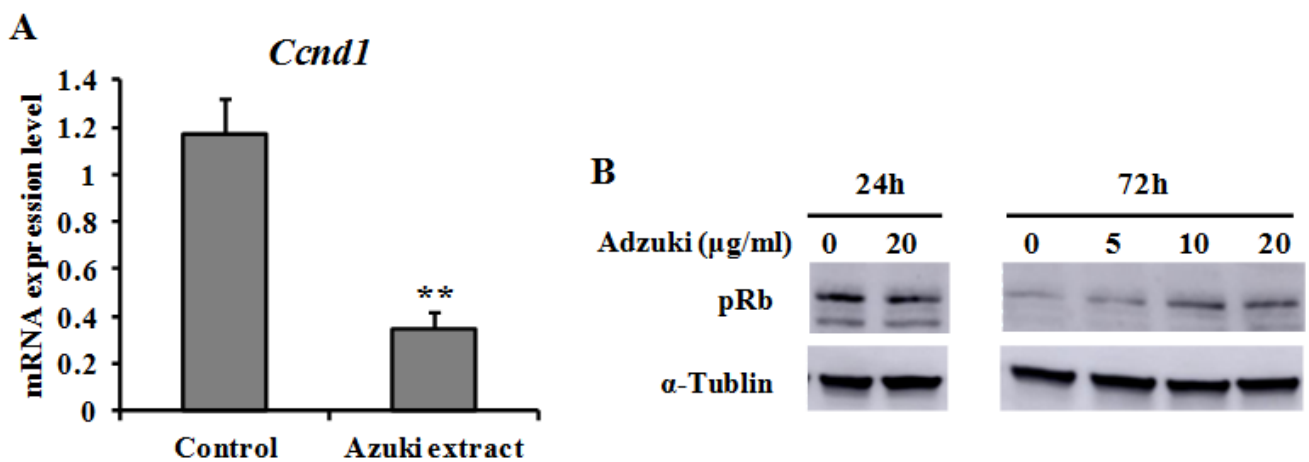


Figure 3. Effect of Adzuki extract on expression of cell cycle-related molecules in 3T3-L1 adipocytes. (A) Adzuki extract or a vehicle was added to the differentiation medium at day 0 and cells were cultured for 24 h. The mRNA expression of *Ccnd1* was determined by real-time PCR and normalized by GAPDH. The results are expressed as means \pm SEM for three independent determinations. ** P <0.01 compared with control. (B) Immunoblotting of 3T3-L1 cell lysates with phosphor Rb polyclonal antibody. Adzuki extract or a vehicle was added to the differentiation medium at day 0 and cells were cultured for 24 h or 72h. The results are representative of three identical experiments

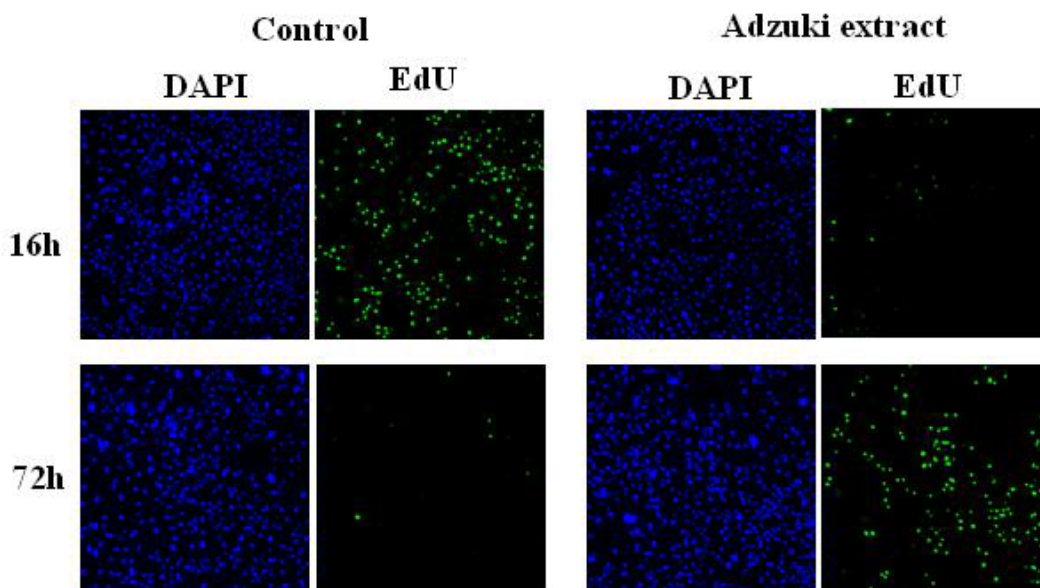


Figure 4. Effect of Adzuki extract on proliferation of 3T3-L1 adipocytes during early differentiation. Adzuki extract or a vehicle was added to the differentiation medium at day 0 and cells were cultured for 16 h or 72 h. Then, the cells were incubated in EdU and DAPI solution. Images showing EdU incorporation (green) and DAPI (blue) in 3T3-L1 cells. Representative images from at least three independent experiments are shown

4. Discussion

The present study demonstrated that adzuki bean extract possesses properties to suppress adipogenesis. It has been reported that adzuki bean extract contains some familiar physiologically active substances, such as catechin and saponin [15,16,17,18]. Catechin and epigallocatechin gallate (EGCG) are also reported to inhibit the differentiation of 3T3-L1 adipocytes [19,20]. The next step is to identify the active ingredients within the extract.

Treatment with adzuki bean extract suppressed the expressions of adipogenesis-related genes: *Ppar γ* , *C/abpa*, and *Fabp4*. The terminal differentiation of preadipocytes to adipocytes commences with the preadipocyte-mediated activation of the transcription factors *C/EBP α* and *PPAR- γ 2* [21,22]. The expression of the *FABP4* gene is regulated by *PPAR- γ 2* [22]. *FABP4*, a protein that affects insulin sensitivity and lipid metabolism, is reported to be involved in the induction of TG synthetase and the cellular uptake of fatty acids [23,24,25]. These reports suggest that adzuki bean extract exerts influence on the initial stages of preadipocyte differentiation.

Cyclin D1 is a protein essential for advancing the cell cycle from the G1 phase onward. It becomes functional by forming a dimer with *CDK4/6* [26]. The cyclin D1-*CDK* complex then targets *Rb* and positively regulates the cell cycle by inactivating *Rb*, which suppresses the cell cycle [27]. When *Rb* is inactivated through phosphorylation, the cell cycle progresses beyond the G1 phase, thereby enabling progression of DNA replication. In the present study, treatment with adzuki bean extract resulted in reduced expression of cyclin D1 mRNA as well as phosphorylated *Rb*, a downstream factor of cyclin D1, at 24 h after the application of a differentiation-inducing stimulus. Moreover, the proportion of proliferated cells was significantly lesser in the extract-treated cells than that in the control cells. Following differentiation-inducing stimulation, preadipocytes essentially need to undergo a

mitotic process called MCE before initiating adipogenesis [12]. During MCE, 3T3-L1 cells are blocked from entering the S phase, thereby inhibiting the differentiation [12]. Given that cells would normally undergo MCE 24 h after the induction of differentiation, the treatment with adzuki bean extract might have suppressed MCE in the present case. Meanwhile, the degree of phosphorylation in *pRb* was accelerated at 72 h after induced differentiation with the increase in the concentration of adzuki bean extract. Moreover, the proportion of proliferated cells was significantly higher in the extract-treated cells than that in the control cells. Normally, MCE would have been completed by 72 h after the induction of differentiation, with the cells having already exited the cell cycle. According to previous reports, MCE that occurs in the early stages of adipogenesis is intricately connected to the production of *PPAR* ligands, and hence, inhibiting MCE prevents cells from differentiating [28]. Accordingly, the treatment with adzuki bean extract might have inhibited MCE and prevented differentiation, leaving the cells in the cell cycle.

5. Conclusions

Accordingly, suppressing adipogenesis is believed to be conducive in preventing obesity and subsequently other concomitant diseases. The natural substances that can suppress adipogenesis are extremely useful in combating obesity.

Our results confirm the existence of active ingredients in adzuki bean extract that suppress adipogenesis. As an underlying mechanism, the active ingredients likely alter the MCE which is essential for the differentiation of adipocytes. The results of the present study not only demonstrate the presence of physiologically active substances within adzuki beans but also propose a novel molecular mechanism for suppressing adipogenesis.

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Conflicts of Interest

The authors declare no conflict of interest.

References

- [1] Rayalam, S., Della-Fera, M.A. and Baile, C.A., "Phytochemicals and regulation of the adipocyte life cycle," *Journal of nutritional biochemistry*, 19 (11). 717-26. May 2008.
- [2] Gariballa, S., Afandi, B., Abualtem, M., Yassin, J., Habib, H. and Ibrahim, W., "Oxidative damage and inflammation in obese diabetic Emirati subjects supplemented with antioxidants and B-vitamins: a randomized placebo-controlled trial," *Nutrition & Metabolism (Lond)*, 10(1). 21. Feb 2013.
- [3] de Ferranti, S. and Mozaffarian, D., "The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences," *Clinical Chemistry*, 54 (6). 945-55. Apr 2008.
- [4] Tang, Q.Q. and Lane, M.D., "Adipogenesis: from stem cell to adipocyte," *Annual Reviv of Biochemistry*, 81. 715-36. Apr 2012.
- [5] Rosen, E.D., Walkey, C.J., Puigserver, P and Spiegelman, B.M., "Transcriptional regulation of adipogenesis," *Genes & Development*, 14 (11). 1293-307. Jun 2000.
- [6] Itoh, T., Itoh, Y., Mizutani, M., Fujishiro, K., Furuichi, Y., Komiya, T. and Hibasami, H., "A hot water extract of Adzuki (*Vigna angularis*) induces apoptosis in cultured human stomach cancer cells," *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi*, 49 (5). 339-44. 2002.
- [7] Itoh, T., Umekawa, H. and Furuichi, Y., "Potential ability of hot water adzuki (*Vigna angularis*) extracts to inhibit the adhesion, invasion, and metastasis of murine B16 melanoma cells," *Bioscience, Biotechnology, and Biochemistry*, 69 (3). 448-54. Mar 2005.
- [8] Itoh, T., Kita, N., Kurokawa, Y., Kobayashi, M., Horio, F. and Furuichi, Y., "Suppressive effect of a hot water extract of adzuki beans (*Vigna angularis*) on hyperglycemia after sucrose loading in mice and diabetic rats," *Bioscience, Biotechnology, and Biochemistry*, 68 (12). 2421-6. Dec 2004.
- [9] Itoh, T., Kobayashi, M., Horio, F. and Furuichi, Y., "Hypoglycemic effect of hot-water extract of adzuki (*Vigna angularis*) in spontaneously diabetic KK-A(y) mice," *Nutrition*, 25 (2). 134-41. Feb 2009.
- [10] Sato, S., Mukai, Y., Yamate, J., Kato, J., Kurasaki, M., Hatai, A. and Sagai, M., "Effect of polyphenol-containing azuki bean (*Vigna angularis*) extract on blood pressure elevation and macrophage infiltration in the heart and kidney of spontaneously hypertensive rats," *Clinical and Experimental Pharmacology & Physiology*, 35 (1). 43-9. Jan 2008.
- [11] Mukai, Y. and Sato, S., "Polyphenol-containing azuki bean (*Vigna angularis*) seed coats attenuate vascular oxidative stress and inflammation in spontaneously hypertensive rats," *Journal of Nutritional Biochemistry*, 22 (1). 16-21. Jan 2011.
- [12] Tang, Q.Q., Otto, T.C. and Lane, M.D., "Mitotic clonal expansion: a synchronous process required for adipogenesis," Proceedings of the National Academy of Science of the United States of America, 100 (1). 44-9. Jan 2003.
- [13] Choi, H., Lee, H., Kim, T.H., Kim, H.J., Lee, Y.J., Lee, S.J., Yu, J.H., Kim, D., Kim, K.S., Park, S.W. and Kim, J.W., "G0/G1 switch gene 2 has a critical role in adipocyte differentiation," *Cell Death and Differentiation*, 21 (7). 1071-80. Jul 2014.
- [14] MacDougald, O.A. and Lane, M.D., "Transcriptional regulation of gene expression during adipocyte differentiation," *Annual Review of Biochemistry*, 64. 345-73. 1995.
- [15] Hirao, K., Yumoto, H., Nakanishi, T., Mukai, K., Takahashi, K., Takegawa, D. and Matsuo, T., "Tea catechins reduce inflammatory reactions via mitogen-activated protein kinase pathways in toll-like receptor 2 ligand-stimulated dental pulp cells," *Life Sciences*, 86 (17-18). 654-60. Apr 2010.
- [16] Takahashi, S., Hori, K., Hokari, M., Gotoh, T. and Sugiyama, T., "Inhibition of human renin activity by saponins," *Biomedical Research*, 31 (2). Apr 2010.
- [17] Tamura, S., Yoshihira, K., Fujiwara, K. and Murakami, N., "New inhibitors for expression of IgE receptor on human mast cell," *Bioorganic & Medicinal Chemistry Letters*, 20 (7). 2299-302. Apr 2010.
- [18] Zhang, W. and Popovich, D.G., "Group B oleanane triterpenoid extract containing soyasaponins I and III from soy flour induces apoptosis in Hep-G2 cells," *Journal of Agricultural and Food Chemistry*, 58 (9). 5315-9. May 2010.
- [19] Furuyashiki, T., Nagayasu, H., Aoki, Y., Bessho, H., Hashimoto, T., Kanazawa, K. and Ashida, H., "Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells," *Bioscience, Biotechnology, and Biochemistry*, 68 (11). 2353-9. Nov 2004.
- [20] Hung, P.F., Wu, B.T., Chen, H.C., Chen, Y.H., Chen, C.L., Wu, M.H., Liu, H.C., Lee, M.J. and Kao, Y.H., "Antimitogenic effect of green tea (-)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the ERK and Cdk2 pathways," *American Journal of Physiology. Cell Physiology*, 288 (5). C1094-108. May 2005.
- [21] Cao, Z., Umek, R.M. and McKnight, S.L., "Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells," *Genes & Development*, 5 (9). 1538-52. Sep 1991.
- [22] Tontonoz, P., Hu, E. and Spiegelman, B.M., "Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor," *Cell*, 79 (7). 1147-56. Dec 1994.
- [23] Boord, J.B., Fazio, S. and Linton, M.F., "Cytoplasmic fatty acid-binding proteins: emerging roles in metabolism and atherosclerosis," *Current Opinion in Lipidology*, 13 (2). 141-7. Apr 2002.
- [24] Spiegelman, B.M., Frank, M. and Green, H., "Molecular cloning of mRNA from 3T3 adipocytes. Regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development," *Journal of Biological Chemistry*, 258 (16). 10083-9. Aug 1983.
- [25] Waggoner, D.W. and Bernlohr, D.A., "In situ labeling of the adipocyte lipid binding protein with 3-[125I]iodo-4-azido-N-hexadecylsalicylamide. Evidence for a role of fatty acid binding proteins in lipid uptake," *Journal of Biological Chemistry*, 265 (10). 11417-20. Jul 1990.
- [26] Baldin, V., Lukas, J., Marcote, M.J., Pagano, M. and Draetta, G., "Cyclin D1 is a nuclear protein required for cell cycle progression in G1," *Genes & Development*, 7 (5). 812-21. May 1993.
- [27] Matsushima, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F. and Sherr, C.J., "Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins," *Cell*, 1992;71(2). 323-34. Oct 1992.
- [28] Hallenborg, P., Petersen, R.K., Feddersen, S., Sundekilde, U., Hansen, J.B., Blagoev, B., Madsen, L. and Kristiansen, K., "PPARgamma ligand production is tightly linked to clonal expansion during initiation of adipocyte differentiation," *Journal of Lipid Research*, 55 (12). 2491-500. Dec 2014.