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Research Article

Citrus sunki Peel Extract, Containing Nobiletin and Tangeretin, Enhances Proliferation and Differentiation in 3T3-L1 Adipocytes

Sehyuk Oh, Sanghun Park, Gyutae Park, Nayoung Choi, Chaeri Kim, Solhee Lee, and Jungseok Choi

Department of Animal Science, Chungbuk National University, Cheongju 28644, Republic of Korea

Correspondence should be addressed to Jungseok Choi; jchoi@chungbuk.ac.kr

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In this experiment, effects of *Citrus sunki* peel extract (CPE) on proliferation and differentiation of 3T3-L1 cells were analyzed. *Citrus sunki* peel was extracted with ethanol to obtain CPE. Results of measuring DPPH and hydrogen peroxide radical scavenging activity revealed that CPE had an antioxidant ability. The 3T3-L1 cells were cultured in the basal medium (C) or with 0.05% dimethyl-sulfoxide (CDMSO) or with 50, 100, 200, 300, and 400 μg/mL CPE (CPE50, CPE100, CPE200, CPE300, and CPE400, respectively). As a result of cell counting and MTS assay, CPE significantly enhanced proliferation capacity of 3T3-L1 cells. To analyze the effect of CPE on the differentiation capacity of 3T3-L1 cells, relative gene and protein expression levels of Cebpb, Cebpa, and FASN related to adipogenesis were measured by RT-qPCR and Western blot. CPE increased adipogenesis-related gene and protein expression in 3T3-L1 cells, with CPE300 being especially effective. In Oil Red O staining, adipogenesis was significantly greater in CPE200, CPE300, and CPE400 than in C. This was confirmed image wise through Nile Red staining. Through HPLC analysis, it was confirmed that nobiletin and tangeretin were most abundant in CPE. To analyze effects of nobiletin and tangeretin on 3T3-L1 cells, the cells were cultured with various concentrations of nobiletin, tangeretin, and both. As a result of cell counting and MTS assay, nobiletin and tangeretin significantly decreased the proliferation capacity of 3T3-L1 cells. In RT-qPCR, western blot, Oil Red O staining, and Nile Red staining to analyze differentiation capacity, nobiletin and tangeretin significantly enhanced the expression of genes and proteins related to adipogenic differentiation compared to the control group. In conclusion, CPE, which had high antioxidant capacity, enhanced proliferation and differentiation in 3T3-L1 adipocytes. This improvement in differentiation is due to the influence of nobiletin and tangeretin contained in CPE.

1. Introduction

Cultivated meat is edible meat produced via stem cell proliferation and differentiation to resolve challenges associated with traditional meat production [1]. Meat culture technologies are more environmentally sustainable than traditional meat production. They can contribute to animal welfare [2, 3]. Cultivated meat is emerging as a solution to the population's lack of food, especially protein sources. For this reason, many companies and researchers are working to obtain permission for research and production of cultivated meat in many countries and commercialization of cultivated meat is gradually taking place [4–6]. In cultivated meat production, efficient and rapid proliferation and differentiation of myosatellite cells or preadipocytes are essential. Research on the efficient cultivation of cells to be used in cultivated meat production is being actively conducted worldwide.

3T3-L1 cells are popularly used in preadipocyte proliferation and differentiation research as a preliminary study to improve the proliferation and differentiation ability of primary cells or immortal cells used to produce fat for cultivated meat. 3T3-L1 cells are an immortal cell line, can be easily cultured and differentiated in the laboratory, and is less expensive than other cell lines. Therefore, it is used in various adipogenic differentiation experiments [7, 8].

Citrus fruits contain beneficial phytochemicals [9]. Citrus fruits contain about 60 types of physiologically active substances such as flavonoids, carotenoids, coumarins, and phenylpropanoids, including polymethoxylated flavones such as hesperidin, naringin, tangeretin, and nobiletin [10]. Many types of bioactive substances that can be extracted from citrus fruits are being treated and studied in various cells. Research has shown that treating MCF-7 breast cancer cell line with nobiletin at a concentration of 100 μM reduces cell survival [11]. Natural citrus fruit extracts are rich in flavonoids, which have anti-inflammatory, antioxidant, and anticancer effects [12–14]. Tangeretin and nobiletin are unique components of citrus fruits. They possess excellent antioxidant, anti-inflammatory, and antiobesity effects [15, 16]. Instead of being found in the fruit pulp, these bioactive substances are actually present in the peel [17]. They are known to be more effective physiologically [18–20]. Nobiletin can improve cell viability by reducing ROS production in intracellular mitochondria and suppressing cell death signals. Additionally, nobiletin showed antioxidant effects by regulating mitochondrial cation channels [21]. Tangeretin, like nobiletin, is a natural product that has been pharmacologically studied for its neuroprotective effects [22]. Tangeretin can also inhibit cellular ROS production [23]. Nobiletin and tangeretin are especially abundant in *Citrus sunki*. *Citrus sunki* hybrid cultivated plant created through natural hybridization of *Citrus medica* and *Citrus reticulata* [24]. *Citrus Sunki* has been used in oriental medication since archaic times [10].

About a third of citrus fruit production is used for processing, and more than 80% of such processing is used to produce orange juice. However, a large amount of peel generated for citrus juice production is generally considered as agroindustrial waste [25]. Citrus peel can cause economic and environmental problems due to fermentation and microbial decay [9, 26]. An increased utilization of citrus peel can resolve these problems.

There are many experimental results in which various cells were treated with citrus fruit extracts. However, there are many different types of citrus fruit, and each type shows different results. Moreover, there are a limited number of studies that have explored the use of natural product extracts for the purpose of boosting the growth and differentiation of adipocytes. The purpose of this study was to determine how *Citrus sunki* peel extract affects 3T3-L1 preadipocyte. Furthermore, the study analyzed the effects of nobiletin and tangeretin, two major flavonoids present in Citrus sunki peel extract, on 3T3-L1 cells.

2. Materials and Methods

2.1. Cell Line. The 3T3-L1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Extraction of Citrus sunki Peel. Commercially available *Citrus sunki* peels (Jeju Kong xin cai, Seogwipo, Korea) were purchased and used. The number of samples of *Citrus sunki* peels used in the experiment was three. First, *Citrus sunki* peel was crushed into small pieces. A 50 g of *Citrus sunki* peel was transferred to 1 L of 60% ethanol and extracted for 6 h in a 70°C water bath. After filtering through a 0.4 μm filter, the CPE was obtained at 40°C using an Eyela rotary vacuum evaporator (NE-1, Eyela, Seongnam, Korea) for 1 day. The extract was powdered using a freeze dryer (FDU-1100, Eyela, Seongnam, Korea) for 3 days to remove moisture.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis. A sample was prepared by adding 1.2 mg CPE to 6 mL ethanol. It was separated using a 150 mm × 4.6 mm, 5 μm Zorbax Eclipse XDB-C18 column (Agilent, Santa Clara, USA) at a column temperature of 30°C. The mobile phase included methanol (A) and water (pH adjusted to 3.0 with glacial acetic acid) (B). Gradient elution program included 0–24 min, 25%–40% (A); 24–35 min, 40%–62% (A); 35–44 min, 62% (A); 44–50 min, 62%–85% (A); and 50–60 min, 85%–100% (A). The detection wavelength was 330 nm. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. To determine contents of nobiletin and tangeretin in CPE, samples were prepared at concentrations of 1, 2, 5, 10, 25, 50, and 100 ppm. A standard curve representing the content versus the area of the chromatogram was obtained.

2.4. DPPH Radical Scavenging Activity. The antioxidant activity of each sample was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Samples were prepared by dissolving 50, 100, 200, 300, and 400 μg/mL of CPE in methyl alcohol. Blank that is 100% methyl alcohol and reference that is adding 1 part DPPH solution (890132, Biozoa Biological Supply, Seoul, KR) to 1 part methyl alcohol were also prepared. Once the light was obstructed, the samples were kept in a dimly lit room at normal temperature for 20 minutes. The solution's absorption was gauged at 517 nm using a microplate spectrophotometer (Microdigital Co., Ltd., Seongnam, Korea). The scavenging activity of each sample against DPPH radical was calculated with the following formula:

$$\text{DPPH radical scavenging activity } (\%) = \left(1 - \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of reference} - \text{Absorbance of blank}}\right) \times 100. \quad (1)$$

2.5. MTS Assay for H_2O_2 (Hydrogen Peroxide) Scavenging Activity. The 3T3-L1 cells were seeded into a 96-well plate at a density of 10,000 cells/cm^2. Cells were grown in Dulbecco's modified eagles medium (DMEM; 11995-065, Gibco, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-amphotericin (PSA) for 24 h. DMEM added 1% PSA without FBS was used as the control (C) general medium. The control medium added with 0.05% DMSO was CDMSO. Experimental groups included treatment with 50, 100, 200, 300, and 400 μg/mL of CPE (CPE50, CPE100, CPE200, CPE300, and CPE400, respectively). 3T3-L1 cells were treated with each culture medium for 2 h. After removing the medium, cells were treated with DMEM with 700 μM·H_2O_2 without FBS. After treating cells for 24 h, cell activity was measured through 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

2.6. 3T3-L1 Cell Culture. The flask was covered in collagen for proliferation. The collagen mixture (5 mg/mL) was thinned with distilled water to a 0.5% concentration, and then 1M acetic acid was added to the collagen coating to make it 2% soluble. The collagen solution was poured into a flask and then placed in an incubator at 37°C for a minimum of 16 hours. Prior to conducting the experiment, the flask was purged of the coating solution through suction and then rinsed twice with 1X phosphate buffered saline (PBS; Cytiva HyClone™, Logan, Utah, USA). Subsequently, it was dried for use in the experiment. The growth medium (GM) used for cell growth was DMEM with 10% FBS and 1% PSA for cell proliferation. For cell proliferation during experiments, 3T3-L1 cells were seeded at a density of 2,000 cells/cm^2 and incubated at 37°C with 5% CO_2 for 4 days.

After 3T3-L1 cells were more than 80% confluent, cells were differentiated in the first differentiation medium (DM1) containing 10% FBS, 1% PSA, 10 μg/mL insulin (I0516, Sigma, UK), 1 μM dexamethasone (D-085, Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; I5879, Sigma) in DMEM for 3 days. The differentiation medium was then replaced with the second differentiation medium (DM2) containing 2% FBS, 1% PSA, and 10 μg/mL insulin in DMEM every 3 days. Differentiation was continued for a total of 9 days.

2.7. Cell Counting. Cells were detached with trypsin. The trypsin was neutralized using a trypsin neutralization solution (TNS) of 2% FBS in PBS. After staining with trypan blue solution (T8154, Sigma), the cell number was counted using a cell counter (Countess® cell FL automated cell counter, Invitrogen, USA).

2.8. MTS Assay. The 3T3-L1 cells were cultured in 96-well plates. The cell proliferation assay was performed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) utilizing MTS, which was reduced to formazan by dehydrogenases in viable cells. Cells in 96-well plates were treated with MTS (final concentration, 0.33 mg/mL MTS) and incubated at 37°C for 2 h. The absorbance was measured at 490 nm.

2.9. Oil Red O Staining and Nile Red Staining. To perform Oil Red O staining, the differentiated 3T3-L1 cells had their medium removed and were washed with PBS two times. Paraformaldehyde (2%) was used to fix cells for 1 h. Paraformaldehyde was removed, and the 3T3-L1 cells were washed twice with distilled water. Isopropanol (70%) was added and incubated for 5 min. It was then replaced with Oil Red O working solution. The Oil Red O working solution was obtained by adding 3 parts of Oil Red O solution (01391, Sigma) to 2 parts of distilled water, followed by filtration with a 0.45 μm syringe filter (6784-2504, Whatman, Maidstone, UK). Cells were incubated in 37°C for 20 min with Oil Red O working solution. The Oil Red O solution was removed, and cells were washed five times with distilled water and observed under a microscope. To quantitatively measure Oil Red O staining, 3T3-L1 cells were washed with 70% isopropanol three times with gentle shaking for 5 min each time. Then, Oil Red O staining was extracted with 100% isopropanol for 5 min with gentle shaking. As a background control, 100% isopropanol was used to subtract background signals. All treatments had absorbance readings at 492 nm.

For Nile Red staining, the medium was removed from differentiated 3T3-L1 cells and washed twice with PBS. Paraformaldehyde (2%) was used to fix cells for 1 h. Paraformaldehyde was removed, and the 3T3-L1 cells were washed twice with distilled water. Nile Red working solution was prepared by diluting nile red dye (GC15539, GLPBIO, Montclair, CA) in PBS to a concentration of 1 μg/mL. Cells were treated with nile red working solution for 30 min at room temperature.

2.10. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). The 3T3-L1 cells were differentiated in an incubator at 37°C with 5% CO_2 for 9 days. After removing the culture medium, differentiated cells were washed with 1X PBS. Cell samples were collected into a flask with cell scrapers. TRIzol reagent of RNA extraction kit (17221, Intron Biotechnology, Seongnam, KR) was used for RNA extraction. Then, cDNA was prepared using a Reverse Transcription Master Premix (ELPIS-BIOTECH, Korea) with a template RNA Primer Mixture and 1.0 μg of RNA as a template according to the manufacturer's protocol, followed by incubation at 60°C for 60 min and 94°C for 5 min. Expression levels of related genes were analyzed by RT-qPCR. *Actb* gene was used as an internal control for the analysis of target gene expression. The RT-qPCR test was carried out using a 20 μL mixture. This mixture contained 1 μL of cDNA as a template, 10 μL of EzAmp™ FAST qPCR 2X Master Mix (ELPIS - BIOTECH, Korea), and 1 μL of each primer. The amplification process consisted of heating at 95°C for 10 minutes, followed by 40 cycles of heating at 95°C for 10 seconds and 60°C for 20 seconds. Primer sequences used in the RT-qPCR are listed in Table 1.

Table 1: Primer sequences used in RT-qPCR.

Gene accession number	Primer	Direction	Sequence ($5' \longrightarrow 3'$)
NM_007393.5	*Actb*	F	AGCCATGTACGTAGCCATCC
		R	CTCTAGCTGTGGTGGTGAA
NM_001287739.1	*Cebpb*	F	CAAGCTGAGCGACGAGTACA
		R	AGCTGCTCCACCTTCTTCTG
NM_007678.4	*Cebpa*	F	TTACAACAGGCCAGGTTTCC
		R	CTCTGGGATGGATCGATTGT
NM_007988.3	*Fasn*	F	TGGGTTCTAGCCAGCAGAGT
		R	ACCACCAGAGACCGTTATGC

2.11. Western Blot. The 3T3-L1 cells were placed into T25 flasks that had already been coated. They were then cultured at a temperature of 39°C with a 5% concentration of CO^2. Once the cells were more than 80% full in T25 flasks, the GM was substituted with DM1 for a period of 3 days, followed by DM2 for 6 days. Samples of the differentiated 3T3-L1 cells from the T25 flasks were collected for Western blotting. These T25 flasks were kept in cold conditions and rinsed with cold 1× PBS. A 1X radioimmunoprecipitation assay (RIPA) lysis buffer (Rockland, Gilbertsville, PA, USA) was poured, and cells were detached from the flask using a cell scraper. Protein concentrations in the samples were determined using the Bradford assay. Following quantitative analysis, approximately 10 μg protein samples were loaded onto TGX Precast Gel (Bio-Rad, USA). The electrophoresis process was used to divide the samples, which were then moved onto an Immun-Blot PVDF membrane (Bio-Rad, USA). To prevent any interference, the membrane was covered with EveryBlot Blocking Buffer (Bio-Rad, Japan) and left at room temperature for 5 min. The proteins in 3T3-L1 cells were then exposed to primary antibodies that targeted actin β (Actb; 42 kDa, 1 : 2000, PA1-46296, Invitrogen), CAAT enhancer binding protein β (Cebpb; 38 kDa, 1 : 10000, NBP1-46179, Novus Biologicals, CO, USA), CCAAT enhancer binding protein α (Cebpa; 41 kDa, 1 : 2000, MBS9610673, MyBioSource, San Diego, USA), and fatty acid synthase (FASN; 273 kDa, 1 : 2500, MA5-14887, Invitrogen) for one day at 4°C. The primary antibody was diluted in blocking buffer. The membranes underwent four washes in TBS with 0.1% Tween® 20 (TBST, Bio-Rad, USA) at room temperature for 10 minutes. Afterward, they were exposed to Affinity Purified Goat Anti-Rabbit IgG (H + L) HRP-conjugated antibody (1 : 10000, SA002-500, GenDEPOT, TX, USA) at room temperature for 1 hour. After washing four times with TBST again (10 min each wash), Clarity western ECL substrate (Bio-Rad, USA) was used to detect proteins. Band images were taken using a Western blot imager (Amersham™ ImageQuant™ 800, Cytiva, Amersham, England), and densitometry was quantified using the ImageQuant TL analysis software (Cytiva).

2.12. Statistical Analysis. Each measurement was conducted a minimum of three times. The statistical software SPSS 28 was employed to determine the significance of the results. To identify significant variances among the measured values, the Duncan multiple range test was conducted with a significance level of $P < 0.05$.

3. Results and Discussion

3.1. Extraction and Ingredient Analysis of Citrus sunki Peel. CPE was determined using HPLC. Figure 1 presents the HPLC chromatogram of CPE and structures of nobiletin and tangeretin. The marked portion in Figure 1 represents nobiletin and tangeretin [27], and the area indicates 8543.73 mVs and 18300.68 mVs. Substituting the standard curve of nobiletin and tangeretin contents versus the area of the chromatogram, CPE contained 5006.35 ppm of nobiletin and 10796.55 ppm of tangeretin as a result of HPLC analysis of 11 types of flavonoids from 67 types of citrus fruits. Overall, there were more flavonoids in the peel than in the flesh [17]. Contents of nobiletin, tangeretin, and hesperidin in the extract of *Citrus sunki* peel accounted for more than 70% [28]. It has been reported that the nucleic acid and chloroform layers contain high levels of tangeretin and nobiletin and that hesperidin appears in ethyl acetate, butanol, and water layers. This separation is due to difference in polarity. Tangeretin and nobiletin have a methoxyl group (CH_3O-). They belong to polymethoxyflavonoids with a low polarity. Polymethoxyflavonoids have higher biological activities even at lower contents than hydroxyflavones [28].

3.2. Antioxidant Ability of Citrus sunki Peel Extract. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radical are by-products of normal oxygen metabolism. They participate in cellular functions and play a role in intracellular signaling [29]. However, excessive ROS production causes DNA damage, abnormal activation of cell growth regulators, and even apoptosis and necrosis [30–32]. Antioxidants play an important role in preventing oxidative stress in cells. Although synthetic antioxidants are effective, they have been reported to have some harmful effects [33]. Thus, natural antioxidants are preferred over synthetic antioxidants. Methods used to evaluate the efficacy of antioxidants include the use of butylated hydroxytoluene, propyl gallate, and butylated hydroxyanisole. The most common method is using DPPH radical [34]. There is also a method to confirm the oxidative stress protection effect by reacting H_2O_2 with cells treated with antioxidants [35, 36].

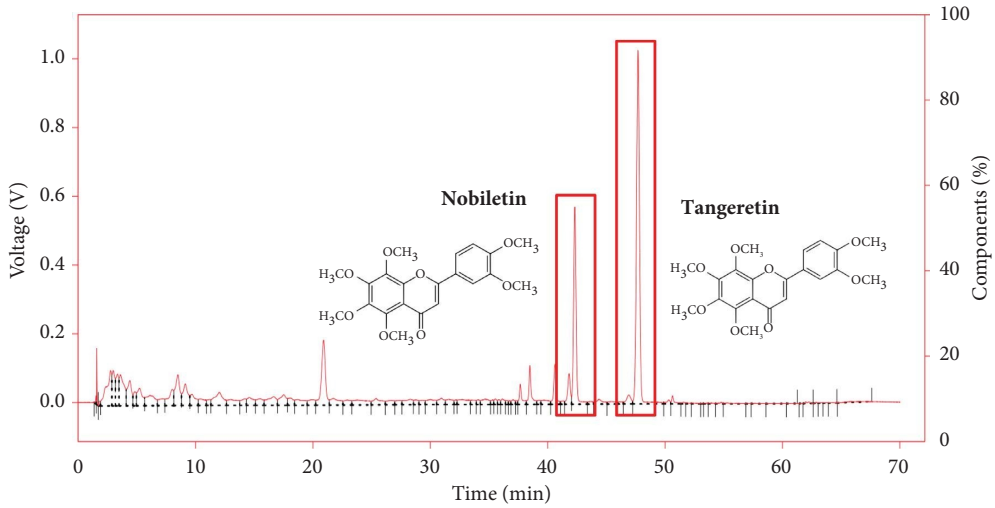


Figure 1: HPLC chromatograms of CPE. HPLC analysis showed a peak representing nobiletin at 42 min and a peak representing tangeretin at 47 min. The chemical structures of nobiletin and tangeretin are indicated next to each symbol.

We analyzed how well CPE could protect 3T3-L1 cells from ROS through DPPH radical scavenging activity and H_2O_2 reaction experiments. As a result of DPPH radical scavenging activity, more CPE resulted in higher DPPH radical scavenging activity (Figure 2(a)). DPPH radical scavenging activities of CPE50 and CPE100 were not significantly different from each other. However, CPE200 had a significantly higher DPPH radical scavenging activity than CPE50 and CPE100 ($P < 0.05$). DPPH radical scavenging ability of CPE was significantly and dose-dependently increased ($P < 0.05$). After treating 3T3-L1 cells with 700 μM H_2O_2, MTS assay was performed by replacing the culture medium containing MTS reagent without FBS. There was no significant difference between C and CD. CPE200, CPE300, and CPE400 had significantly higher absorbance than C (Figure 2(b)). CPE300 and CPE400 had significantly higher absorbance than CD ($P < 0.05$). Addition of CPE protected cells from H_2O_2 reaction. As a result of confirming the antioxidant ability of CPE through the DPPH radical scavenging ability experiment and H_2O_2 reaction experiment, the addition of CPE can suppress oxidative stress in cells.

Natural antioxidants contained in fruits and vegetables have the ability to inhibit lipid peroxidation and oxidation of decomposition products [37]. This ability can scavenge free radicals generated during the cell's oxidative metabolism and prevent excessive production of ROS [38], thus reducing oxidative stress and eliminating oxidative damage [39].

3.3. Effects of Citrus sunki Peel Extract on Proliferation Capacity of 3T3-L1 Cells. To measure the proliferation capacity, 3T3-L1 cells were proliferated in the general culture medium and culture medium containing 50, 100, 200, 300, and 400 μg/mL of CPE. CPE was dissolved in DMSO to mix with the culture medium. Since all treatment groups had 0.05% DMSO, a control group treated with only 0.05% DMSO was added (CDMSO). The toxicity of DMSO was confirmed by comparing C and CDMSO.

Total cell number and live cell number of 3T3-L1 cells proliferated for 4 days in T25 were measured through trypan blue staining (Figure 3(a)). Viability was measured based on the ratio of total cell count and live cell count (Figure 3(b)). There was no significant difference in live cell number between C and CDMSO. CPE100, CPE200, CPE300, and CPE400 had significantly more live cells than C ($P < 0.05$). CPE100, CPE300, and CPE400 had significantly higher live cell number than CDMSO ($P < 0.05$). Viabilities of all control and treatment groups were over 90%, showing no significant difference between them. As a result of the MTS experiment (Figure 3(c)), treatments containing CPE had significantly higher cell activity than C ($P < 0.05$). All treatments except for CPE400 had significantly higher cell activity than CDMSO ($P < 0.05$). Cell counting and MTS experiments showed that CPE was not cytotoxic. It did not have a negative effect on cell proliferation. The addition of 0.05% DMSO was not significantly cytotoxic either compared to C. The addition of CPE improved the proliferation of 3T3-L1 cells, and cell counting results are similar with MTS experiment results.

Through DPPH radical and H_2O_2 scavenging activity experiments, it was proven that CPE had an antioxidant activity. Various studies have been conducted to improve cell capacity by reducing oxidative stress, protecting cells against DNA damage [40], and enhancing differentiation of vestibular progenitor cells [41]. Additionally, antioxidant supplements can reduce oxidative stress in adipose derived stem cells, improving cell proliferation and increasing cell number [42]. Reducing oxidative stress using antioxidants can extend the lifespan of human mesenchymal stem cells and promote their differentiation into adipocytes, osteoblasts, and chondrocytes [43]. Citrus fruits are especially rich in natural antioxidants called flavonoids, which can scavenge ROS and protect cells from free radical damage [44–46]. It is expected that CPE, which has good antioxidant ability, can suppress the oxidative stress of 3T3-L1 caused by ROS, thus improving proliferation and increasing the number of cells.

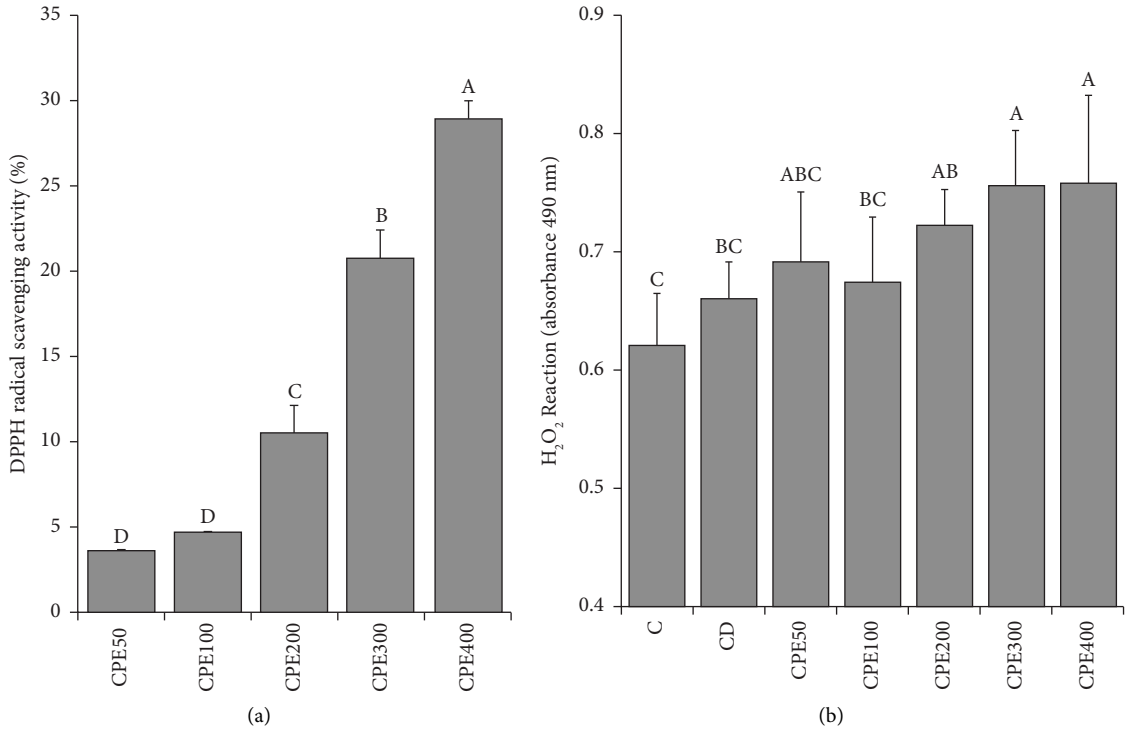


Figure 2: Result of (a) DPPH radical scavenging activity and (b) H_2O_2 reaction of various concentrations of *Citrus sunki* peel extract (50, 100, 200, 300, and 400 μg/mL). Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).

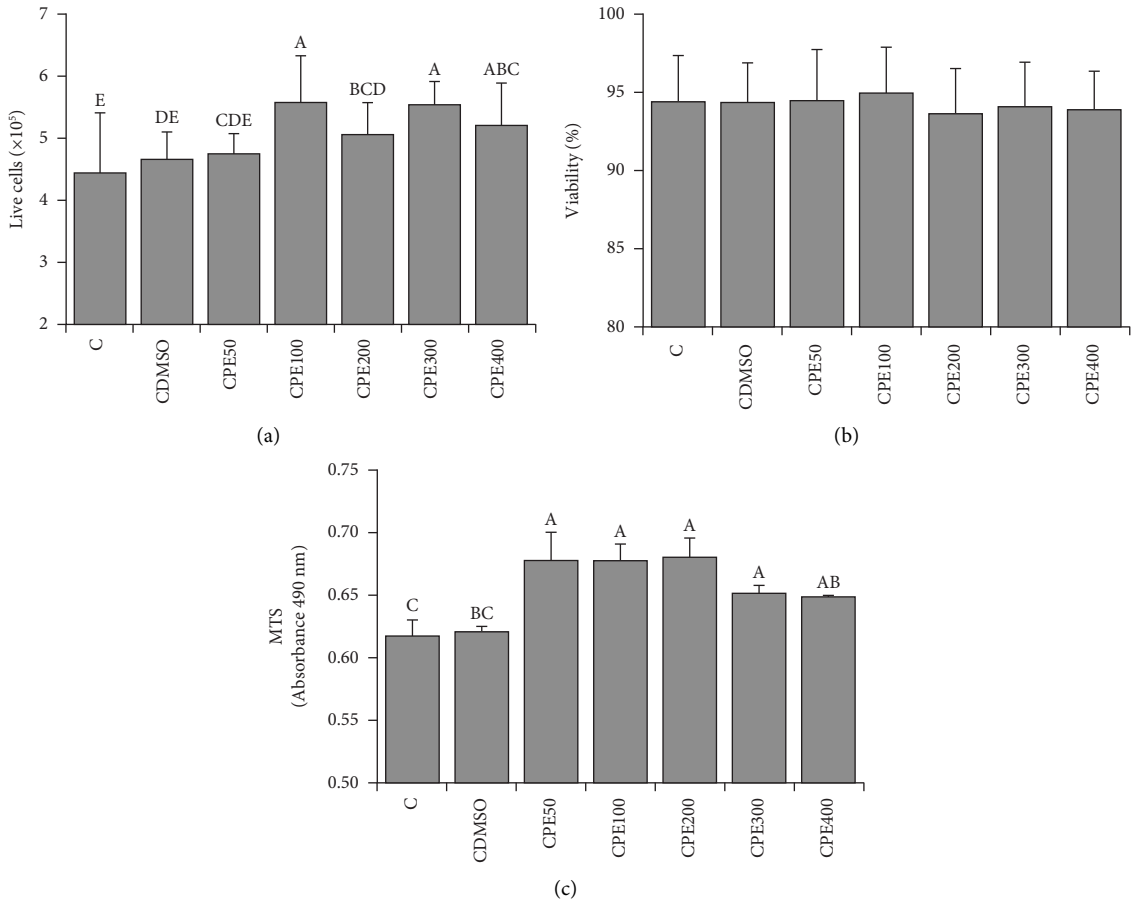


Figure 3: Proliferation capacity of 3T3-L1 cells treated with *Citrus sunki* peel extract. (a) Live cell count, (b) viability were measured, and (c) MTS assay was performed. Viability was calculated as the ratio of total cell number and live cell number. Absorbance was measured at 490 nm. Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).

3.4. Effects of Citrus sunki Peel Extract on Differentiation Capacity of 3T3-L1 Cells. To measure the differentiation capacity, 3T3-L1 cells were grown in the general culture medium and then differentiated in the differentiation medium containing different components. Then, 3T3-L1 were differentiated in the general differentiation medium (C), differentiation medium with only 0.05% DMSO (CDMSO), and medium containing 50, 100, 200, 300, and 400 μg/mL of CPE (CPE50, CPE100, CPE200, CPE300, and CPE400, respectively).

To measure differentiation capacity, 3T3-L1 cells were differentiated for 9 days. Differentiation of 3T3-L1 cells cultured in media supplemented with CPE and DMSO as well as normal medium was confirmed by Oil Red O staining (Figure 4(a)) and Nile Red staining (Figure 4(b)).

Oil Red O, a dye that strongly stains lipids, has been used in adipocyte analysis for a long time because it is inexpensive, not harmful, and quick for identification by staining [47, 48]. Oil Red O is a diazo dye whose chemical formula is three aromatic rings with two azo groups [49]. Using Oil Red O oil-soluble fluorescent dye, neutral lipids, cholesterol esters, and lipoproteins (mainly triglycerides) can be dyed red [49, 50]. Using isopropanol, the dye can be eluted from cells and quantitatively measured by measuring absorbance [51–54]. Nile Red dye, 9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, is also used to stain lipid droplets with red fluorescence (excitation, 515-560; emission, greater than 590 nm) under a fluorescence microscope [55]. It binds to neutral lipids inside cells. It has lipophilic properties. Thorpe [56] has discovered that when Nile Blue and phenoxazine dyes, which bind to polar lipids, are boiled in dilute acid, they become red phenoxazone dye, which can bind to neutral lipids.

After Oil Red O staining, absorbance was measured at 492 nm to analyze the differentiation ability of 3T3-L1 cells (Figure 4(c)). There was no significant difference absorbance between C and CDMSO. Absorbance values of CPE200, CPE300, and CPE400 were significantly higher than those of C and CDMSO ($P < 0.05$). In particular, CPE300 had the highest absorbance ($P < 0.05$). Lipid droplet images were confirmed through Nile Red fluorescence staining.

To analyze the effect of CPE on the differentiation ability of 3T3-L1 cells, relative gene and protein expression levels of Cebpb, Cebpa, and FASN related to adipogenesis were measured by RT-qPCR and Western blot, respectively. During the differentiation of preadipocytes, CEBP family members play diverse roles in adipogenesis and differentiation. CEBPB and CEBPG are the first transcription factors induced in preadipocytes after exposure to differentiation factors. They directly mediate differentiation [57]. During adipocyte differentiation, CEBPB and CEBPD can induce C/EBPA expression [58]. As reported by Lin and Lane [59], when CEBPA expression is blocked in preadipocytes, no triacylglycerol accumulation occurs and no fat-specific genes are expressed, indicating that CEBPA is essential for differentiation of preadipocytes. During adipogenesis, CEBPB not only induces CEBPA but also acts as a transcriptional regulator of PPARG [60]. CEBPA and PPARG can enhance mutual expression. These factors can promote adipogenesis and increase lipid accumulation [61, 62]. Before inducing adipogenic differentiation, the expression of CEBPA is not altered. Their expression indicates a high adipogenic potential [63]. Consequently, CEBPA, a transcriptional factor in preadipocytes, is characterized as a critical regulator of adipogenic differentiation [64, 65]. Fatty acid synthase (FASN) is a delayed adipogenic marker. It is a key enzyme in fatty acid metabolism [66, 67].

RT-qPCR was performed to compare gene expression levels of *Cebpb* (Figure 5(a)), *Cebpa* (Figure 5(b)), and *FASN* (Figure 5(c)). *Actb* was used as a housekeeping gene. There was no significant difference in the expression of these genes between C and CDMSO. However, *Cebpb* gene expression levels of CPE200, CPE300, and CPE400 were significantly higher than those of C ($P < 0.05$). In particular, CPE300 and CPE400 had the highest *Cebpb* gene expression levels ($P < 0.05$). *Cebpa* gene expression levels of CPE100, CPE200, CPE300, and CPE400 were significantly higher than those of C ($P < 0.05$). In particular, *Cebpa* gene expression levels in CPE300 and CPE400 were significantly higher than other treatment groups ($P < 0.05$). All CPE treatment groups had significantly higher *FASN* gene expression than C ($P < 0.05$). In particular, CPE100, CPE200, CPE300, and CPE400 groups had the highest *FASN* gene expression levels ($P < 0.05$). Regarding gene expression levels of *Cebpb*, *Cebpa*, and *FASN*, CPE300 and CPE400 groups had higher gene expression levels overall.

Western blotting analysis was performed to compare protein expression levels of Cebpb (Figure 5(d)), Cebpa (Figure 5(e)), and FASN (Figure 5(f)). Actb was used as a housekeeping protein. Western blot band intensity was used to measure protein expression (Figure 5(g)). There were no significant differences in protein expression levels between C and CDMSO. All treatments with CPE had significantly higher Cebpb protein expression than C ($P < 0.05$). All treatments with CPE also had significantly higher Cebpa protein expression than C ($P < 0.05$). In particular, CPE300 and CPE400 had higher Cebpa protein expression levels than other groups ($P < 0.05$). CPE300 and CPE400 had significantly higher FASN protein expression than C ($P < 0.05$). Regarding protein expression levels of Cebpb, Cebpa, and FASN, CPE300 and CPE400 had higher protein expression levels overall.

There is a study that addition of dried extract of *Citrus aurantium*, the same citrus fruit, improved adipocyte differentiation by promoting CEBPB expression in 3T3-L1 cells [68]. In addition to nobiletin, various citrus flavonoids enhance and positively affect adipogenesis [69–71]. Hesperetin, a flavonoid in citrus fruit, inhibited ROS production during the adipogenesis of 3T3-L1 [72], and naringenin and hesperetin extracted from *Citrus aurantium* enhanced the transcription of adiponectin and enhanced the expression of PPARG [73]. In fact, many studies have shown that citrus peel and juice extracts can inhibit adipogenesis and reduced differentiation of preadipocytes. Our experimental results revealed that CPE could promote adipogenesis. This might be due to diverse compositions and nutritional products in citrus fruits studied [68, 74].

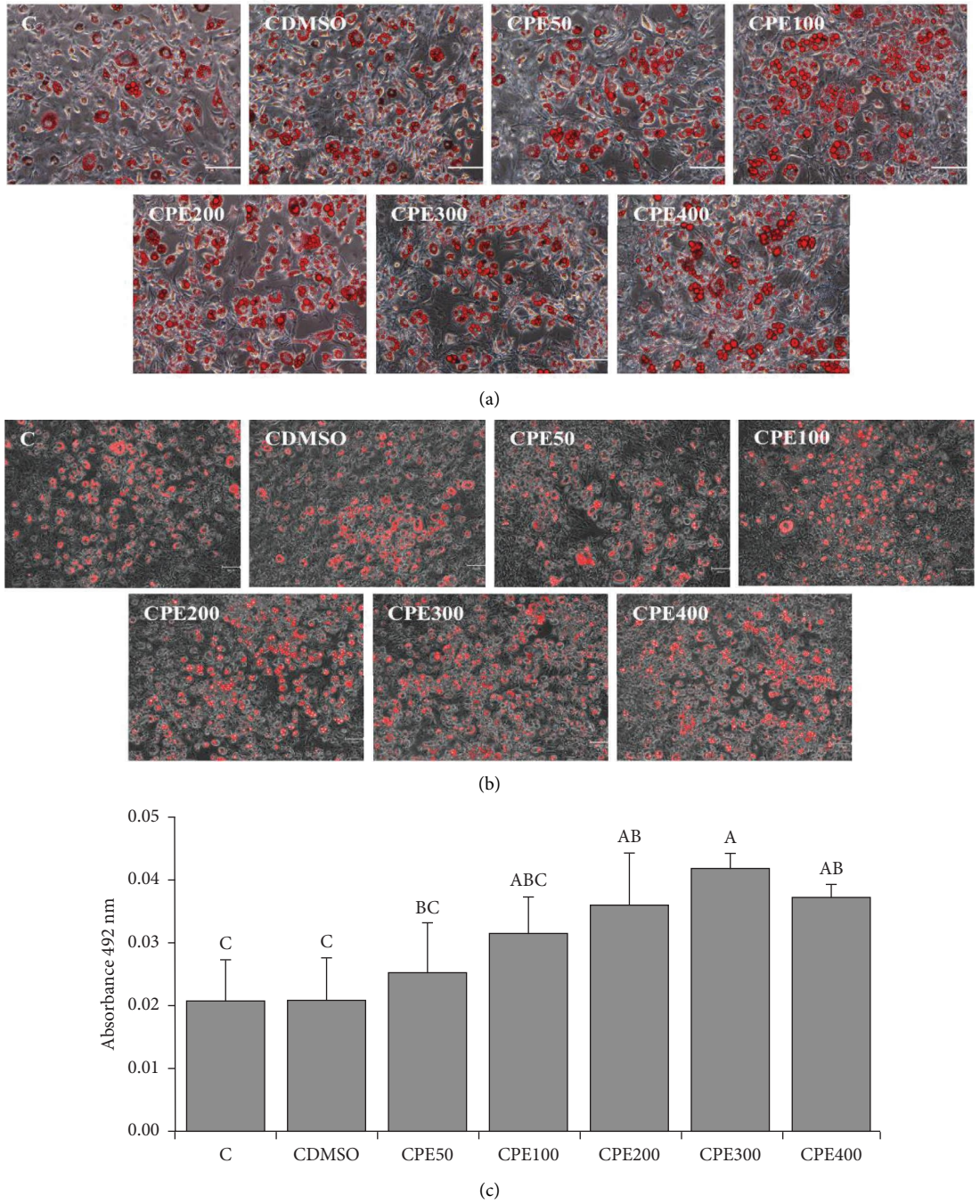


Figure 4: Oil Red O staining, Nile Red staining, and quantification of Oil Red O staining in 3T3-L1 cells treated with *Citrus sunki* peel extract. (a) 3T3-L1 cells, stained with Oil Red O, were visualized in optical microscopy (×200). (b) 3T3-L1 cells, stained with Nile Red, were visualized in optical microscopy (×100). (c) In 3T3-L1 cells stained with Oil Red O lipid was quantified by measuring absorbance. Absorbance was measured at 490 nm. Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).

As a result of analyzing the differentiation ability of 3T3-L1 cells through fat staining, RT-qPCR, and Western blot experiments, genes and proteins related to adipogenic differentiation were found to be highly expressed in 3T3-L1 cells treated with CPE300 and CPE400. Thus, it is believed that CPE300 and CPE400 could lead to more adipogenic differentiation.

3.5. Effects of Nobiletin and Tangeretin on Proliferation Capacity of 3T3-L1 Cells. To measure the proliferation capacity, 3T3-L1 cells were proliferated in a general culture medium as control (C), a culture medium with 25, 50, or 100 μM of nobiletin (N25, N50, or N100), a culture medium with 25, 50, or 100 μM of tangeretin (T25, T50, or T100), and a culture medium with 25 or 50 μM of both nobiletin and

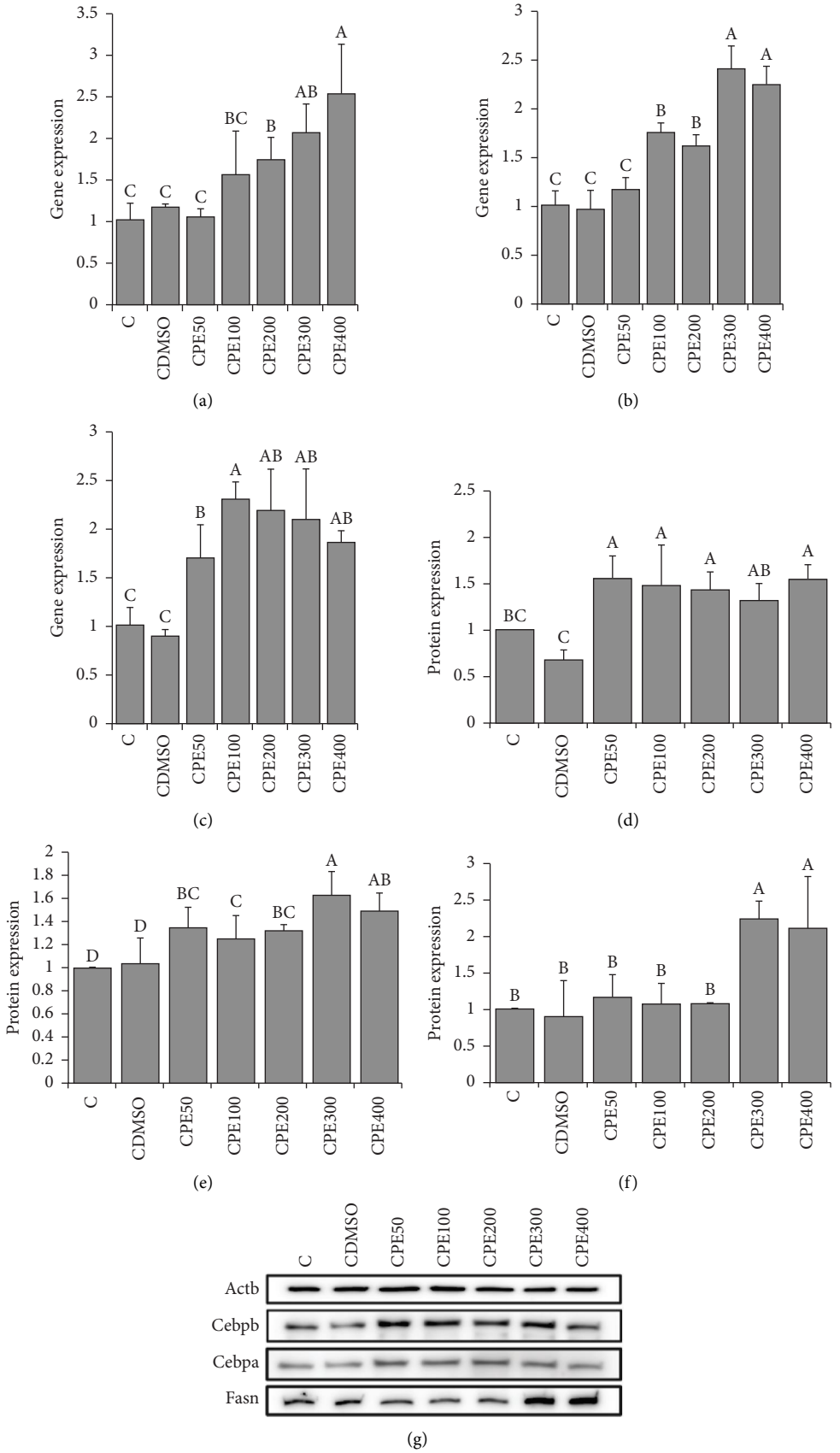


Figure 5: Differentiation capacity of 3T3-L1 cells, treated with *Citrus sunki* peel extract, analyzed using Western blot and RT-qPCR. The relative gene expression of (a) *Cebpb*, (b) *Cebpa*, and (c) *FASN*, adipogenesis-related protein. *Actb* was used as a housekeeping gene. The relative protein expression of (d) Cebpb, (e) Cebpa, and (f) FASN, adipogenesis-related protein. Actb was used as a housekeeping protein. (g) Western blot band intensity was used to measure protein expression. Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).

tangeretin (NT50 or NT100). Nobiletin and tangeretin were dissolved in DMSO to treat the culture medium. Since all treatment groups were treated with 0.05% DMSO, a group treated with only 0.05% DMSO (CDMSO) was set as the control. The toxicity of DMSO was confirmed by comparing C and CDMSO.

Total cell number and live cell number of 3T3-L1 cells proliferated for 4 days in T25 were measured through trypan blue staining (Figure 6(a)). Viability was measured based on the ratio of total cell number to live cell number (Figure 6(b)). There was no significant difference in viable cell count between C and CDMSO. There was no significant difference in cell viability between C and all treatments except for N100, with N100 having significantly lower cell viability than C. After treatment with nobiletin, the number of live cells was significantly decreased in a dose-dependent manner ($P < 0.05$). After treatment with tangeretin, the number of live cells was also significantly decreased in a dose-dependent manner ($P < 0.05$). NT100 had significantly fewer viable cells than NT50 ($P < 0.05$). As a result of the MTS assay (Figure 6(c)), the number of active cells in N100 was significantly lower than that in N25 ($P < 0.05$). T100 had significantly fewer active cells than T25 and T50 ($P < 0.05$). NT100 also had significantly fewer active cells than NT50 ($P<0.05$). As a result of cell counting and MTS experiments, nobiletin and tangeretin contents had negative correlations with the proliferative capacity of 3T3-L1 cells. Nobiletin inhibited cell proliferation and induced G2/M cell cycle arrest and apoptosis in hepatocellular carcinoma (HCC) cells of rats and humans, MH1C1 and HepG2 [75]. Additionally, nobiletin inhibited the proliferation of glioma cells, which are mostly considered cancer, and the cell cycle was arrested in the G0/G1 phase. Cycle-related genes cyclin E, cyclin B, and BCL-2 were suppressed by nobiletin [76]. In addition, the cell proliferation of glioma cells was also inhibited by tangeretin. Cells treated with tangeretin had reduced expression of cell cycle regulatory genes, including *cyclin D* and *cdc-2* [77]. The effects of nobiletin and tangeretin on the proliferative capacity of 3T3-L1 cells spontaneously immortalized have not been studied much. However, it is expected that proliferation is reduced for similar reasons as proliferation is inhibited in other cells.

3.6. Effects of Nobiletin and Tangeretin on Differentiation Capacity of 3T3-L1 Cells. To measure the differentiation capacity, 3T3-L1 cells were grown in the general culture medium and then differentiated in the differentiation medium containing different components. The 3T3-L1 cells were differentiated in the general differentiation medium as control (C), differentiation medium with only 0.05% DMSO (CDMSO), culture medium with 25, 50, or 100 μM of nobiletin (N25, N50, N100), culture medium with 25, 50, or 100 μM of tangeretin (T25, T50, T100), or culture medium with 25 or 50 μM of both nobiletin and tangeretin (NT50 or NT100).

To measure differentiation capacity, 3T3-L1 cells were differentiated for 9 days. Differentiation of 3T3-L1 cells cultured in media supplemented with nobiletin, tangeretin, and DMSO as well as normal medium was confirmed by Oil Red O staining (Figure 7(a)) and Nile Red staining (Figure 7(b)). After Oil Red O staining, absorbance was measured at 492 nm to analyze the differentiation ability of 3T3-L1 cells (Figure 7(c)). Absorbance values of all treatments added with nobiletin or tangeretin or both were significantly higher than those of C ($P < 0.05$). In particular, the absorbance was significantly higher in T25 and T50 than other treatment groups ($P < 0.05$). Differentiation images of 3T3-L1 cells were confirmed through Nile Red fluorescent staining.

To analyze the effects of nobiletin and tangeretin on the differentiation capacity of 3T3-L1 cells, relative gene and protein expression levels of Cebpb, Cebpa, and FASN related to adipogenesis were measured using RT-qPCR and Western blot. RT-qPCR was used to compare gene expression levels of *Cebpb* (Figure 8(a)), *Cebpa* (Figure 8(b)), and *FASN* (Figure 8(c)). Actb was used as a housekeeping gene. There was no significant difference in the expression level of *Cebpb*, *Cepba*, or *FASN* between C and CDMSO. Expression levels of *Cebpb, Cebpa,* and *FASN* genes were significantly higher in groups treated with nobiletin, tangeretin, or both nobiletin and tangeretin than in C and CDMSO groups ($P < 0.05$). Gene expression levels of *Cebpb* were significantly higher in N50 and N100 than in N25 ($P < 0.05$). T25, T50, and T100 groups showed no significant difference in the *Cebpb* gene expression level. There is no significant difference in the *Cebpb* gene expression level between NT50 and NT100. The gene expression level of *Cebpa* was significantly higher in N50 than in N25 ($P < 0.05$). N100 had a significantly lower gene expression level of *Cebpa* than other treatments with nobiletin ($P < 0.05$). There was no significant difference in the *Cebpa* gene expression level between T25 and T100. The *Cebpa* gene expression level in T50 was significantly higher than other treatments with tangeretin ($P < 0.05$). NT100 had a higher *Cebpa* gene expression level than NT50 ($P < 0.05$). N25 and N100 showed no significant difference in the *FASN* gene expression level. N50 had significantly lower *FASN* gene expression ($P < 0.05$). T25 had a significantly higher *FASN* gene expression level than T50 ($P < 0.05$). However, the *FASN* gene expression level in T25 was not significantly different from that in T100. NT50 had a significantly higher *FASN* gene expression level than NT100 ($P < 0.05$). These RT-qPCR results showed that the addition of nobiletin or tangeretin significantly improved the expression of genes related to adipogenic differentiation compared to the control ($P < 0.05$).

Western blotting analysis was performed to compare protein expression levels of Cebpb (Figure 8(d)), Cebpa (Figure 8(e)), and FASN (Figure 8(f)). Actb was used as a housekeeping protein. Western blot band intensity was used to measure protein expression (Figure 8(g)). There was no significant difference in the protein expression level of Cebpb, Cebpa, or FASN between C and CDMSO. The Cebpb protein expression level was not significantly different

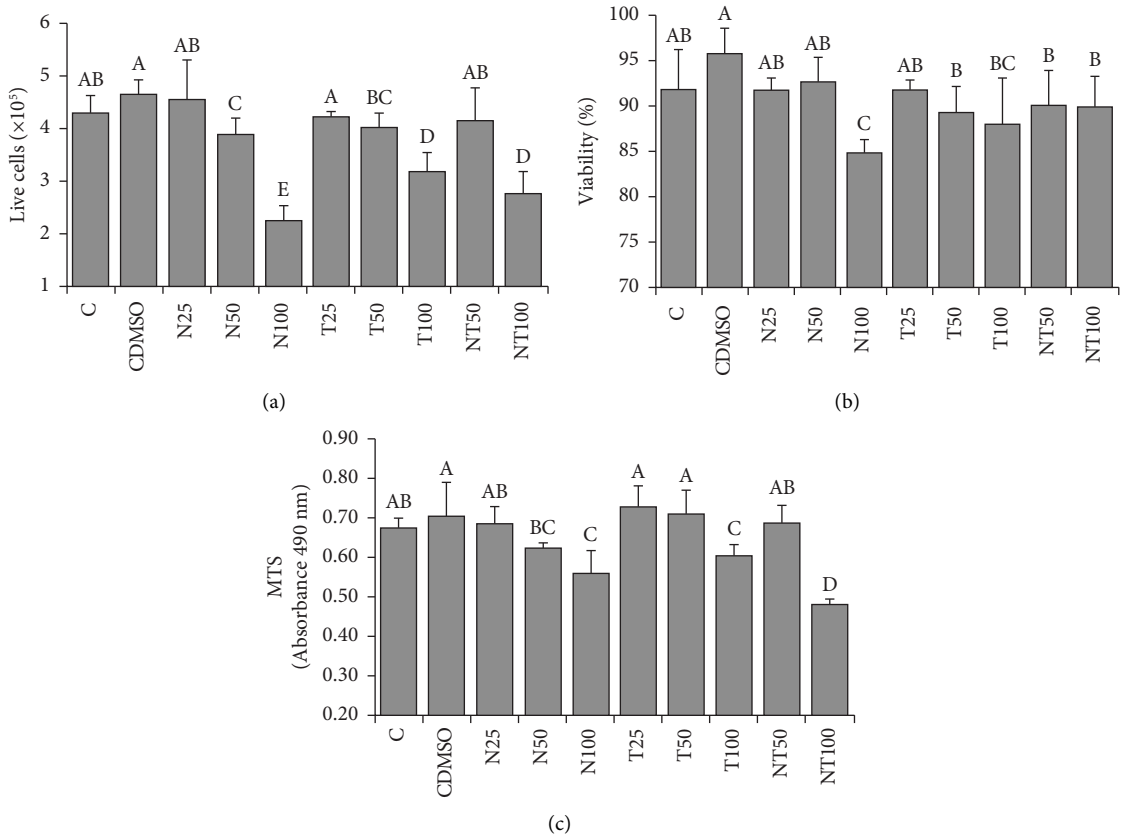
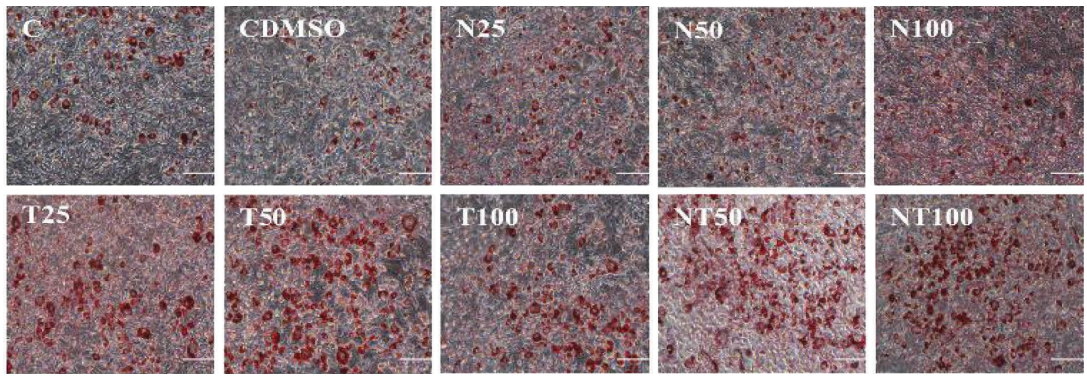


Figure 6: Proliferation capacity of 3T3-L1 cells treated with nobiletin and tangeretin. (a) Live cell count and (b) viability were measured, and (c) MTS assay was performed. Viability was calculated as the ratio of total cell number and live cell number. Absorbance was measured at 490 nm. Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).



(a)

Figure 7: Continued.

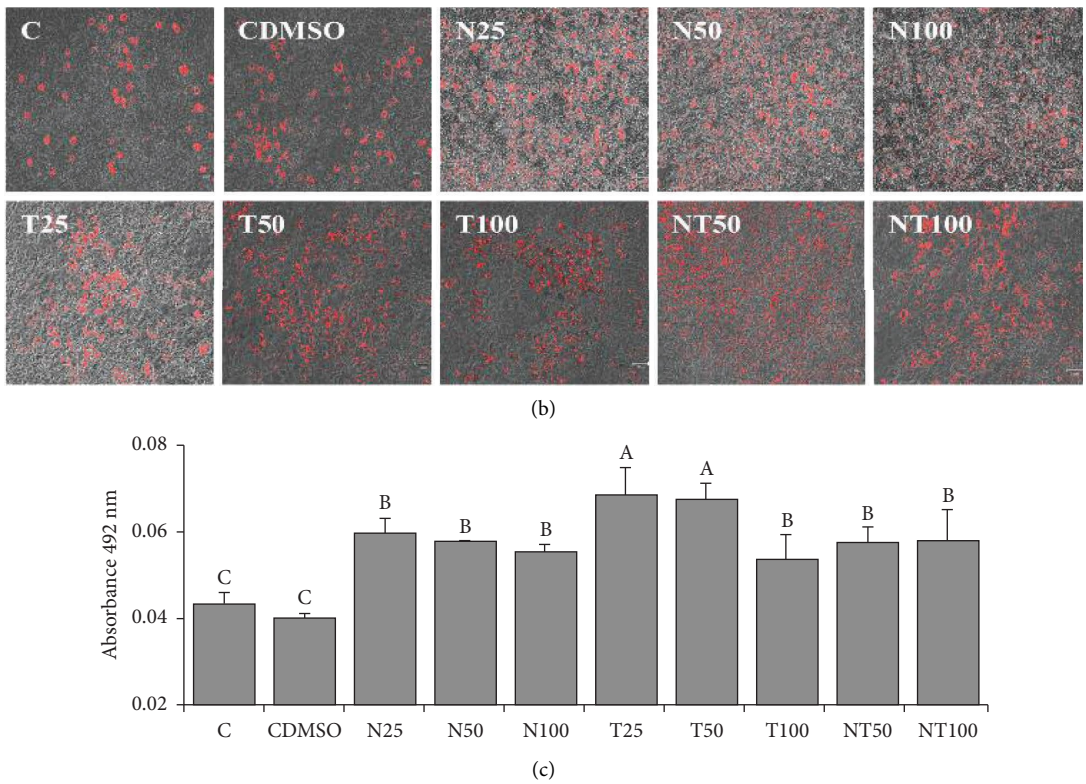


Figure 7: Oil Red O staining, Nile Red staining, and quantification of Oil Red O staining in 3T3-L1 cells treated with nobiletin and tangeretin. (a) 3T3-L1 cells, stained with Oil Red O, were visualized in optical microscopy (×200). (b) 3T3-L1 cells, stained with Nile Red, were visualized in optical microscopy (×100). (c) In 3T3-L1 cells stained with Oil Red O lipid was quantified by measuring absorbance. Absorbance was measured at 490 nm. Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).

between N25 and C. However, Cebpb protein expression levels in N50 and N100 were significantly higher than those in the control group ($P < 0.05$). Cebpb protein expression levels in groups treated with tangeretin had no significant differences from each other. However, they were significantly higher than those in the control group ($P < 0.05$). Although Cebpb protein expression levels of NT50 and NT100 were also not significantly different from each other, they were significantly higher than those in the control group ($P < 0.05$). The Cebpa protein expression level in N100 was not significantly different from that in the control group. However, Cebpb protein expression levels of N25 and N50 were significantly higher than those of the control groups ($P < 0.05$). The Cebpa protein expression levels of the treatment groups with Tangeretin were all significantly higher than the control group ($P < 0.05$). In particular, the Cebpa protein expression level of T50 was significantly higher than T25 and T100 ($P < 0.05$). Although Cebpa protein expression levels of NT50 and NT100 were not significantly different from each other, they were significantly higher than those of the control groups ($P < 0.05$). The FASN protein expression level in N100 was not significantly different from that in the control group. However, FASN protein expression levels in N25 and N50 were significantly higher than those in the control group ($P < 0.05$). FASN protein expression levels in groups treated with tangeretin were significantly higher than those in the control groups ($P < 0.05$). FASN protein expression levels of NT50 and NT100 were also significantly higher than those in the control groups ($P < 0.05$). Western blot results showed that adding 100 μM nobiletin to 3T3-L1 did not result in a clear difference in protein expression compared to the control. Lone et al. [78] also showed similar results in which treatment of 3T3-L1 cells with nobiletin resulted in the downregulation of adipogenic proteins such as FASN. Treatment with nobiletin also improved the synthesis of lipolytic proteins such as p-HSL and p-ACC, which are involved in the hydrolysis of triglycerides, and is expected to be the cause of the inhibition of lipolysis [78]. However, adding 25 or 50 μM of nobiletin, 25–100 μM tangeretin, or 25–50 μM of both substances significantly improved the expression levels of adipogenic differentiation-related proteins compared to the control ($P < 0.05$).

Citrus fruits contain polymethoxyflavonoids such as nobiletin and tangeretin, and these flavonoids improved lipid and glucose homeostasis and increased adiponectin expression in insulin-resistant hamsters [79]. Tangeretin, which constitutes a large portion of citrus peel together with nobiletin, is also a potential insulin sensitizer [80]. Both nobiletin and tangeretin improved the expression of adiponectin, an insulin sensitivity factor, and suppressed the expression of resistin, an insulin resistance factor [81]. Some natural products act as ligands for PPARG and enhance adipogenic differentiation [82]. However, Saito et al. [83]

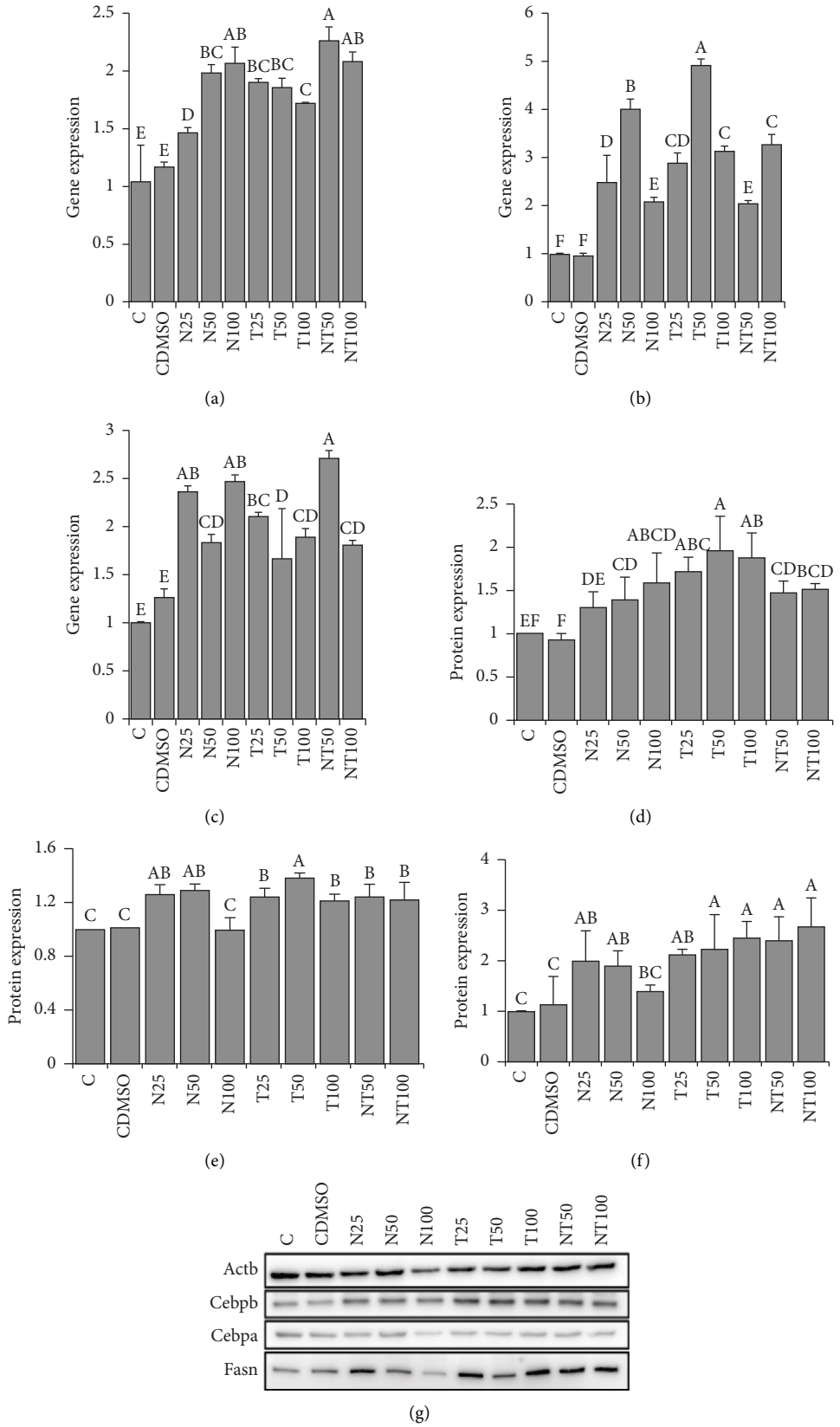


Figure 8: Differentiation capacity of 3T3-L1 cells, treated with nobiletin and tangeretin, analyzed using Western blot and RT-qPCR. The relative gene expression of (a) *Cebpb*, (b) *Cebpa*, and (c) *FASN*, adipogenesis-related protein. *Actb* was used as a housekeeping gene. The relative protein expression of (d) Cebpb, (e) Cebpa, and (f) FASN, adipogenesis-related protein. Actb was used as a housekeeping protein. (g) Western blot band intensity was used to measure protein expression. Values are means ± SD ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$).

have shown that nobiletin, a major flavonoid in CPE, can increase the CEBPB expression of 3T3-L1 cells but did not directly act as a ligand for PPARG. Expression of CEBPB requires activation of CREB and phosphorylation of extracellular signal-regulated kinase (ERK) in the early stages of adipogenesis. Nobiletin increased the phosphorylation of CREB and ERK in neurons [84] and increased the phosphorylation of CREB and ERK in 3T3-L1 cells [83]. These results indicate that nobiletin promotes the activation of CEBPB in the early stages of adipogenesis, thereby enhancing subsequent adipogenic differentiation-related genes and promoting adipogenic differentiation.

4. Conclusions

As interest in cultured meat increases worldwide, research on natural products and chemicals for efficient cell proliferation and differentiation is rapidly increasing. In particular, because the cells must be able to be ingested, it is considered important to use natural products rather than artificial materials. Therefore, we treated 3T3-L1 preadipocyte cells with *Citrus sunki* peel extract and its main ingredients, nobiletin and tangeretin. In conclusion, CPE can improve the antioxidant capacity of cells. This effect might have contributed to the improvement of proliferation capacity of 3T3-L1 cells. In fact, the proliferative capacity of 3T3-L1 cells treated with 100~400 μg/mL CPE was significantly higher than that of C. Additionally, 300–400 μg/mL CPE significantly improved the expression levels of genes and proteins related to the adipogenic differentiation of 3T3-L1 cells with more production of fatty acids. Nobiletin or tangeretin can reduce the proliferation capacity of 3T3-L1 preadipocytes. However, nobiletin, tangeretin, or both nobiletin and tangeretin can improve the expression levels of genes and proteins related to the adipogenic differentiation of 3T3-L1 preadipocytes to produce more fatty acids and lipid droplets. A positive effect of CPE by improving the differentiation ability of 3T3-L1 might be due to the influence of nobiletin and tangeretin present in CPE. More detailed studies are needed to claim that CPE improves the cellular activity of adipocytes.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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References

[1] R. G. J. Dohmen, S. Hubalek, J. Melke et al., "Muscle-derived fibro-adipogenic progenitor cells for production of cultured bovine adipose tissue," *NPJ Sci Food*, vol. 6, no. 1, p. 6, 2022.

[2] M. J. Post, "Cultured meat from stem cells: challenges and prospects," *Meat Science*, vol. 92, no. 3, pp. 297–301, 2012.

[3] N. Stephens, L. Di Silvio, I. Dunsford, M. Ellis, A. Glencross, and A. Sexton, "Bringing cultured meat to market: technical, socio-political, and regulatory challenges in cellular agriculture," *Trends in Food Science & Technology*, vol. 78, pp. 155–166, 2018.

[4] M. Meat, "Tasting cultivated meat in The Netherlands," 2023, https://mosameat.com/blog/tasting-cultivated-meat-in-the-netherlands.

[5] Sfa, "Safety of alternative protein," 2023, https://www.sfa.gov.sg/food-information/risk-at-a-glance/safety-of-alternative-protein.

[6] Usda, "Human food made with cultured animal cells," 2023, https://www.fsis.usda.gov/inspection/compliance-guidance/labeling/labeling-policies/human-food-made-cultured-animal-cells.

[7] G. J. Todaro and H. Green, "Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines," *The Journal of Cell Biology*, vol. 17, no. 2, pp. 299–313, 1963.

[8] M. D. Lane and Q. Q. Tang, "From multipotent stem cell to adipocyte," *Birth Defects Research Part A: Clinical and Molecular Teratology*, vol. 73, no. 7, pp. 476-477, 2005.

[9] B. Satari and K. Karimi, "Citrus processing wastes: environmental impacts, recent advances, and future perspectives in total valorization," *Resources, Conservation and Recycling*, vol. 129, pp. 153–167, 2018.

[10] S.-H. Kang, Y.-J. Lee, C.-H. Lee et al., "Physiological activities of peel of jeju-indigenous citrus sunki hort. Tanaka," *Korean Journal of Food Science and Technology*, vol. 37, no. 6, pp. 983–988, 2005.

[11] S. T. Rahideh, M. Keramatipour, M. Nourbakhsh et al., "Comparison of the effects of nobiletin and letrozole on the activity and expression of aromatase in the MCF-7 breast cancer cell line," *Biochemistry and Cell Biology*, vol. 95, no. 4, pp. 468–473, 2017.

[12] E. Galati, M. T. Monforte, S. Kirjavainen, A. Forestieri, A. Trovato, and M. M. Tripodo, "Biological effects of hesperidin, a citrus flavonoid.(Note I): antiinflammatory and analgesic activity," *Farmaco*, vol. 40, no. 11, pp. 709–712, 1994.

[13] H.-C. Ko, M.-G. Jang, C.-H. Kang et al., "Preparation of a polymethoxyflavone-rich fraction (PRF) of Citrus sunki Hort. ex Tanaka and its antiproliferative effects," *Food Chemistry*, vol. 123, no. 2, pp. 484–488, 2010.

[14] Y.-X. Lu, Q. Zhang, J. Li et al., "Antidiabetic effects of total flavonoids from Litsea Coreana leve on fat-fed, streptozotocin-induced type 2 diabetic rats," *The American Journal of Chinese Medicine*, vol. 38, no. 04, pp. 713–725, 2010.

[15] S. I. Kang, H. S. Shin, H. M. Kim et al., "Immature citrus sunki peel extract exhibits antiobesity effects by.BETA.-Oxidation and lipolysis in high-fat diet-induced obese mice," *Biological & Pharmaceutical Bulletin*, vol. 35, no. 2, pp. 223–230, 2012.

[16] H.-S. Shin, S.-I. Kang, H.-C. Ko et al., "Anti-inflammatory effect of the immature peel extract of Jinkyool (Citrus sunki Hort. ex Tanaka)," *Food Science and Biotechnology*, vol. 20, no. 5, pp. 1235–1241, 2011.

[17] Y. Nogata, K. Sakamoto, H. Shiratsuchi, T. Ishii, M. Yano, and H. Ohta, "Flavonoid composition of fruit tissues of Citrus species," *Bioscience Biotechnology and Biochemistry*, vol. 70, no. 1, pp. 178–192, 2006.

[18] S. Chung, S. Kim, Y. Choi, E. Song, and S. Kim, "Status of citrus fruit production and view of utilization in Cheju," *Food Industry and Nutrition*, vol. 5, no. 2, pp. 42–52, 2000.

[19] J. J. Wu, Y. Cui, Y. S. Yang et al., "Mild mitochondrial depolarization is involved in a neuroprotective mechanism of citrus sunki peel extract," *Phytotherapy Research*, vol. 27, no. 4, pp. 564–571, 2013.

[20] E. Yoshigai, T. Machida, T. Okuyama et al., "Citrus nobiletin suppresses inducible nitric oxide synthase gene expression in interleukin-1β-treated hepatocytes," *Biochemical and Biophysical Research Communications*, vol. 439, no. 1, pp. 54–59, 2013.

[21] K. Amarsanaa, H. J. Kim, E. A. Ko, J. Jo, and S. C. Jung, "Nobiletin exhibits neuroprotective effects against mitochondrial complex I inhibition via regulating apoptotic signaling," *Experimental Neurobiology*, vol. 30, no. 1, pp. 73–86, 2021.

[22] N. Braidy, S. Behzad, S. Habtemariam et al., "Neuroprotective effects of citrus fruit-derived flavonoids, nobiletin and tangeretin in alzheimer's and Parkinson's disease," *Cns & Neurological Disorders-Drug Targets*, vol. 16, no. 4, pp. 387–397, 2017.

[23] S.-Y. Choi, J.-H. Hwang, H.-C. Ko, J.-G. Park, and S.-J. Kim, "Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-κB and ROS production in LPS-activated RAW 264.7 cells," *Journal of Ethnopharmacology*, vol. 113, no. 1, pp. 149–155, 2007.

[24] O. Gulsen and M. L. Roose, "Chloroplast and nuclear genome analysis of the parentage of lemons," *Journal of the American Society for Horticultural Science*, vol. 126, no. 2, pp. 210–215, 2001.

[25] V. Negro, G. Mancini, B. Ruggeri, and D. Fino, "Citrus waste as feedstock for bio-based products recovery: review on limonene case study and energy valorization," *Bioresource Technology*, vol. 214, pp. 806–815, 2016.

[26] R. Casquete, S. M. Castro, A. Martín et al., "Evaluation of the effect of high pressure on total phenolic content, antioxidant and antimicrobial activity of citrus peels," *Innovative Food Science & Emerging Technologies*, vol. 31, pp. 37–44, 2015.

[27] Y. G. Li, X. Y. Wang, H. F. Chen, J. B. Yuan, Y. Meng, and W. L. Yang, "Comparison of the chemical constituents of raw Fructus Aurantii and Fructus Aurantii stir-baked with bran, and the biological effects of auraptene," *Journal of Ethnopharmacology*, vol. 269, Article ID 113721, 2021.

[28] Nihhs, *Study of Setting up the Database on the Functional Molecule of the Newly Promising Citrus Varieties and the Utilization*, National Institute of Horticultural and Herbal Science (NIHHS), Bisan-ri Soi-myeon, Republic of korea, 2016.

[29] J. Hancock, R. Desikan, and S. Neill, "Role of reactive oxygen species in cell signalling pathways," *Biochemical Society Transactions*, vol. 29, no. 2, pp. 345–349, 2001.

[30] J. M. Griscavage, A. J. Hobbs, and L. J. Ignarro, "Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds," *Advances in Pharmacology*, vol. 34, pp. 215–234, 1995.

[31] İ. Gulcin, "Antioxidants and antioxidant methods: an updated overview," *Archives of Toxicology*, vol. 94, no. 3, pp. 651–715, 2020.

[32] J. M. Mates, J. A. Segura, F. J. Alonso, and J. Marquez, "Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis," *Archiv für Toxikologie*, vol. 82, no. 5, pp. 273–299, 2008.

[33] F. N. Ekinci Akdemir, İ. Gülçin, B. Karagöz, R. Soslu, and S. H. Alwasel, "A comparative study on the antioxidant effects of hesperidin and ellagic acid against skeletal muscle ischemia/reperfusion injury," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 31, no. sup4, pp. 114–118, 2016.

[34] İ. Gulcin and S. H. Alwasel, "DPPH radical scavenging assay," *Processes*, vol. 11, no. 8, p. 2248, 2023.

[35] R. Apak, A. Calokerinos, S. Gorinstein et al., "Methods to evaluate the scavenging activity of antioxidants toward reactive oxygen and nitrogen species (IUPAC Technical Report)," *Pure and Applied Chemistry*, vol. 94, no. 1, pp. 87–144, 2022.

[36] E. Seong, H. Choe, H. Heo et al., "Protective effect of andrographis paniculata against oxidative damage in C2C12 skeletal muscle cells," *Journal of the Korean Society of Food Science and Nutrition*, vol. 51, no. 12, pp. 1259–1265, 2022.

[37] A. Yadav, R. Kumari, A. Yadav, J. Mishra, S. Srivatva, and S. Prabha, "Antioxidants and its functions in human body-A Review," *Research in Environment and Life Sciences*, vol. 9, no. 11, pp. 1328–1331, 2016.

[38] L. Zuo, Z. Ren, J. Zhang et al., "Role of ROS and nutritional antioxidants in human diseases," *Frontiers in Physiology*, vol. 9, p. 477, 2018.

[39] C. Boydens, B. Pauwels, K. Decaluwé, P. Brouckaert, and J. Van de Voorde, "Relaxant and antioxidant capacity of the red wine polyphenols, resveratrol and quercetin, on isolated mice corpora cavernosa," *The Journal of Sexual Medicine*, vol. 12, no. 2, pp. 303–312, 2015.

[40] J. Ji, V. Sharma, S. Qi et al., "Antioxidant supplementation reduces genomic aberrations in human induced pluripotent stem cells," *Stem Cell Reports*, vol. 2, no. 1, pp. 44–51, 2014.

[41] S.-H. Song, K. Kim, J. J. Park, K. H. Min, and W. Suh, "Reactive oxygen species regulate the quiescence of CD34-positive cells derived from human embryonic stem cells," *Cardiovascular Research*, vol. 103, no. 1, pp. 147–155, 2014.

[42] L.-Y. Sun, C.-Y. Pang, D.-K. Li et al., "Antioxidants cause rapid expansion of human adipose-derived mesenchymal stem cells via CDK and CDK inhibitor regulation," *Journal of Biomedical Science*, vol. 20, pp. 53–11, 2013.

[43] T.-M. Lin, J.-L. Tsai, S.-D. Lin, C.-S. Lai, and C.-C. Chang, "Accelerated growth and prolonged lifespan of adipose tissue-derived human mesenchymal stem cells in a medium using reduced calcium and antioxidants," *Stem Cells and Development*, vol. 14, no. 1, pp. 92–102, 2005.

[44] H. Ashraf, M. S. Butt, M. J. Iqbal, and H. A. R. Suleria, "Citrus peel extract and powder attenuate hypercholesterolemia and hyperglycemia using rodent experimental modeling," *Asian Pacific Journal of Tropical Biomedicine*, vol. 7, no. 10, pp. 870–880, 2017.

[45] I. Erlund, "Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology," *Nutrition Research*, vol. 24, no. 10, pp. 851–874, 2004.

[46] S. Shafi, H. R. Ansari, W. Bahitham, and S. Aouabdi, "The impact of natural antioxidants on the regenerative potential of vascular cells," *Frontiers in cardiovascular medicine*, vol. 6, p. 28, 2019.

[47] H. Green and O. Kehinde, "Sublines of mouse 3t3 cells that accumulate lipid," *Cell*, vol. 1, no. 3, pp. 113–116, 1974.

[48] W. Kuriharcuch and H. Green, "Adipose conversion of 3t3 cells depends on a serum factor," *Proceedings of the National Academy of Sciences*, vol. 75, no. 12, pp. 6107–6109, 1978.

[49] S. Bharati, K. Anjaly, S. Thoidingjam, and A. B. Tiku, "Oil Red O based method for exosome labelling and detection," *Biochemical and Biophysical Research Communications*, vol. 611, pp. 179–182, 2022.

[50] A. Mehlem, C. E. Hagberg, L. Muhl, U. Eriksson, and A. Falkevall, "Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease," *Nature Protocols*, vol. 8, no. 6, pp. 1149–1154, 2013.

[51] V. Arsenescu, R. I. Arsenescu, V. King, H. Swanson, and L. A. Cassis, "Polychlorinated biphenyl-77 induces adipocyte differentiation and proinflammatory adipokines and promotes obesity and atherosclerosis," *Environmental Health Perspectives*, vol. 116, no. 6, pp. 761–768, 2008.

[52] L. Janderová, M. McNeil, A. N. Murrell, R. L. Mynatt, and S. R. Smith, "Human mesenchymal stem cells as an in vitro model for human adipogenesis," *Obesity Research*, vol. 11, no. 1, pp. 65–74, 2003.

[53] G. Y. Kim, H. H. Jeong, S. J. Yeom, C. S. Lee, and C. Roh, "Efficient and reliable screening of anti-obesity agents on a micro-cell pattern chip," *Journal of Chemical Technology & Biotechnology*, vol. 91, no. 10, pp. 2688–2692, 2016.

[54] W. Wang, Y. Zhang, W. Lu, and K. Liu, "Mitochondrial reactive oxygen species regulate adipocyte differentiation of mesenchymal stem cells in hematopoietic stress induced by arabinosylcytosine," *PLoS One*, vol. 10, no. 3, Article ID e0120629, 2015.

[55] P. Greenspan, E. P. Mayer, and S. D. Fowler, "Nile red: a selective fluorescent stain for intracellular lipid droplets," *The Journal of Cell Biology*, vol. 100, no. 3, pp. 965–973, 1985.

[56] J. F. Thorpe, "XXXVI.—a reaction of certain colouring matters of the oxazine series," *Journal of the Chemical Society Transactions*, vol. 91, no. 0, pp. 324–336, 1907.

[57] G. J. Darlington, S. E. Ross, and O. A. MacDougald, "The role of C/EBP genes in adipocyte differentiation," *Journal of Biological Chemistry*, vol. 273, no. 46, pp. 30057–30060, 1998.

[58] E. D. Rosen and O. A. MacDougald, "Adipocyte differentiation from the inside out," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 12, pp. 885–896, 2006.

[59] F. T. Lin and M. D. Lane, "Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes," *Genes & Development*, vol. 6, no. 4, pp. 533–544, 1992.

[60] J. K. Hamm, B. H. Park, and S. R. Farmer, "A role for C/EBPβ in regulating peroxisome proliferator-activated receptor γ activity during adipogenesis in 3T3-L1 preadipocytes," *Journal of Biological Chemistry*, vol. 276, no. 21, pp. 18464–18471, 2001.

[61] E. D. Rosen, C. H. Hsu, X. Wang et al., "C/EBPα induces adipogenesis through PPARγ: a unified pathway," *Genes & Development*, vol. 16, no. 1, pp. 22–26, 2002.

[62] T. Tanaka, N. Yoshida, T. Kishimoto, and S. Akira, "Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene," *The EMBO Journal*, vol. 16, no. 24, pp. 7432–7443, 1997.

[63] Y. Huang, A. K. Das, Q. Y. Yang, M. J. Zhu, and M. Du, "Zfp423 promotes adipogenic differentiation of bovine stromal vascular cells," *PLoS One*, vol. 7, no. 10, Article ID e47496, 2012.

[64] F. T. Lin and M. D. Lane, "Ccaat/enhancer binding-protein-alpha is sufficient to initiate the 3t3-L1 adipocyte differentiation program," *Proceedings of the National Academy of Sciences*, vol. 91, no. 19, pp. 8757–8761, 1994.

[65] P. Tontonoz, E. Hu, and B. M. Spiegelman, "Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor," *Cell*, vol. 79, no. 7, pp. 1147–1156, 1994.

[66] S. A. Habinowski and L. A. Witters, "The effects of AICAR on adipocyte differentiation of 3T3-L1 cells," *Biochemical and Biophysical Research Communications*, vol. 286, no. 5, pp. 852–856, 2001.

[67] J. B. Kim and B. M. Spiegelman, "ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism," *Genes & Development*, vol. 10, no. 9, pp. 1096–1107, 1996.

[68] G. A. Raciti, F. Fiory, M. Campitelli et al., "Citrus aurantium L. dry extracts promote C/ebpβ expression and improve adipocyte differentiation in 3T3-L1 cells," *PLoS One*, vol. 13, no. 3, Article ID e0193704, 2018.

[69] J. Bae, Y. Yang, X. Xu et al., "Naringenin, a citrus flavanone, enhances browning and brown adipogenesis: role of peroxisome proliferator-activated receptor gamma," *Frontiers in Nutrition*, vol. 9, Article ID 1036655, 2022.

[70] K. Kuroyanagi, M. S. Kang, T. Goto et al., "Citrus auraptene acts as an agonist for PPARs and enhances adiponectin production and MCP-1 reduction in 3T3-L1 adipocytes," *Biochemical and Biophysical Research Communications*, vol. 366, no. 1, pp. 219–225, 2008.

[71] K. Onda, N. Horike, T. Suzuki, and T. Hirano, "Polymethoxyflavonoids tangeretin and nobiletin increase glucose uptake in murine adipocytes," *Phytotherapy Research*, vol. 27, no. 2, pp. 312–316, 2013.

[72] Y. J. Lee, M. J. Seo, O. H. Lee, K. J. Kim, and B. Y. Lee, "Hesperetin inhibits lipid accumulation and ROS production during adipocyte differentiation in 3T3-L1 cells," *Journal of Food Biochemistry*, vol. 41, no. 3, Article ID e12348, 2017.

[73] Z. Liu, Z. Ren, J. Zhang, C. C. Chuang, E. Kandaswamy, and T. Zhou, "Naringenin and hesperetin, two flavonoids derived from up-regulate transcription of adiponectin," *Phytotherapy Research*, vol. 22, no. 10, pp. 1400–1403, 2008.

[74] M. Campitelli, A. Desiderio, G. Cacace et al., "Citrus aurantium L. Dry extracts ameliorate adipocyte differentiation of 3T3-L1 cells exposed to TNFα by down-regulating miR-155 expression," *Nutrients*, vol. 12, no. 6, p. 1587, 2020.

[75] H. Ohnishi, M. Asamoto, K. Tujimura et al., "Inhibition of cell proliferation by nobiletin, a dietary phytochemical, associated with apoptosis and characteristic gene expression, but lack of effect on early rat hepatocarcinogenesis *in vivo*," *Cancer Science*, vol. 95, no. 12, pp. 936–942, 2004.

[76] L. M. Lien, M. J. Wang, R. J. Chen et al., "Nobiletin, a polymethoxylated flavone, inhibits glioma cell growth and migration via arresting cell cycle and suppressing MAPK and akt pathways," *Phytotherapy Research*, vol. 30, no. 2, pp. 214–221, 2016.

[77] L. L. Ma, D. W. Wang, X. D. Yu, and Y. L. Zhou, "Tangeretin induces cell cycle arrest and apoptosis through upregulation of PTEN expression in glioma cells," *Biomedicine & Pharmacotherapy*, vol. 81, pp. 491–496, 2016.

[78] J. Lone, H. A. Parray, and J. W. Yun, "Nobiletin induces brown adipocyte-like phenotype and ameliorates stress in 3T3-L1 adipocytes," *Biochimie*, vol. 146, pp. 97–104, 2018.

[79] R. W. Li, A. G. Theriault, K. Au et al., "Citrus polymethoxylated flavones improve lipid and glucose homeostasis and modulate adipocytokines in fructose-induced insulin

resistant hamsters," *Life Sciences*, vol. 79, no. 4, pp. 365–373, 2006.

[80] J. Guo, J. Chen, W. Ren et al., "Citrus flavone tangeretin is a potential insulin sensitizer targeting hepatocytes through suppressing MEK-ERK1/2 pathway," *Biochemical and Biophysical Research Communications*, vol. 529, no. 2, pp. 277–282, 2020.

[81] Y. Miyata, H. Tanaka, A. Shimada et al., "Regulation of adipocytokine secretion and adipocyte hypertrophy by polymethoxyflavonoids, nobiletin and tangeretin," *Life Sciences*, vol. 88, no. 13-14, pp. 613–618, 2011.

[82] K. L. Han, M. H. Jung, J. H. Sohn, and J. K. Hwang, "Ginsenoside 20(S)-Protopanaxatriol (PPT) activates peroxisome proliferator-activated receptor $\gamma\gamma$," *Biological & Pharmaceutical Bulletin*, vol. 29, no. 1, pp. 110–113, 2006.

[83] T. Saito, D. Abe, and K. Sekiya, "Nobiletin enhances differentiation and lipolysis of 3T3-L1 adipocytes," *Biochemical and Biophysical Research Communications*, vol. 357, no. 2, pp. 371–376, 2007.

[84] H. Nagase, N. Omae, A. Omori et al., "Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities," *Biochemical and Biophysical Research Communications*, vol. 337, no. 4, pp. 1330–1336, 2005.