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NOTE

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Pomolic acid in persimmon peel suppresses the increase in glycerol-3 phosphate dehydrogenase activity in 3T3-L1 adipocytes

Ryoichi Izuchi[1,*] and Takahiro Katsuki[1]

[1]Toyo Institute of Food Technology, 23-2 4-chome, Minami-Hanayashiki, Kawanishi-shi, Hyogo, Japan

*Correspondence: Ryoichi Izuchi, ryouichi_izuchi@shokuken.or.jp

ABSTRACT

Persimmon peels, though usually discarded, are useful sources of nutraceuticals. In this study, persimmon peel–derived pomolic acid was found to suppress the increase in the activity of glycerol-3 phosphate dehydrogenase, a neutral fat synthesis-related enzyme, in 3T3-L1 adipocytes, whereas oleanolic and ursolic acids did not exert this effect. Therefore, persimmon peel may be an effective functional food material.

Keywords: 3T3-L1, peel, persimmon, pomolic acid, triterpenoid

When fresh persimmon (*Diospyros kaki* L.) fruit is processed into dried fruit, large quantities of fruit peels are discarded. However, the peels could be used as a functional food as they contain abundant polyphenols and carotenoids that help promote and maintain good health (Gorinstein *et al.* 2001). Tannins, the water-soluble components in persimmon, are reported to have antihypertensive (Uchida *et al.* 1990), antihyperglycemic (Lee, Cho and Yokozawa 2008), and antiviral (Ueda *et al.* 2013) properties. Persimmon is also rich in fat-soluble carotenoids, especially β-cryptoxanthin (Ebert and Gross 1985); however, fat-soluble components other than carotenoids have rarely been reported in persimmon, based on studies on food function. In this study, we explored the fat-soluble components in persimmon peel that affect adipocytes; if a component that suppresses lipid biosynthesis in adipose tissue can be identified, suitable applications of persimmon peels may be developed, including prevention of diabetes mellitus caused by obesity.

To study adipocytes, cells differentiated from mouse embryonic fibroblast-derived 3T3-L1 cells are often used (Green and Kehinde 1975). The cells differentiated into adipocytes show elevated activity of glycerol 3-phosphate dehydrogenase (GPDH), a triglyceride synthesis enzyme, along with increased triglyceride accumulation (Pairault and Green 1979; Wise and Green 1979). Increase in GPDH activity in the cells can serve as a differentiation marker of adipocytes and an indicator of triglyceride synthesis ability. The present study aimed to explore the constituents of persimmon peels that affect the GPDH activity in 3T3-L1 adipocytes.

Sun-dried 'Ichidagaki' persimmon peels from a farmer in Nagano Prefecture, and freeze-dried 'Hiratanenashi' persimmon peels and pulp from Wakayama Prefecture were pulverized in a food mill (Figure 1a). Thereafter, 25 g of each powdered sample was extracted with hexane (H_0 fraction) until no color was evident in the solvent. The residue was then extracted with acetone (A_0 fraction) as well as hexane. The extracts were then evaporated under vacuum at 40 °C. The concentration of each extract was set as the maximum amount that could be completely dissolved in dimethyl sulfoxide (DMSO).

Wakogel C-200 (FUJIFILM Wako Chemicals, Osaka, Japan) was soaked in benzene and packed to a length of approximately 150 mm into a glass column with an inner diameter of 45 mm. The packed column was then loaded with 83.2 mg of the A_0

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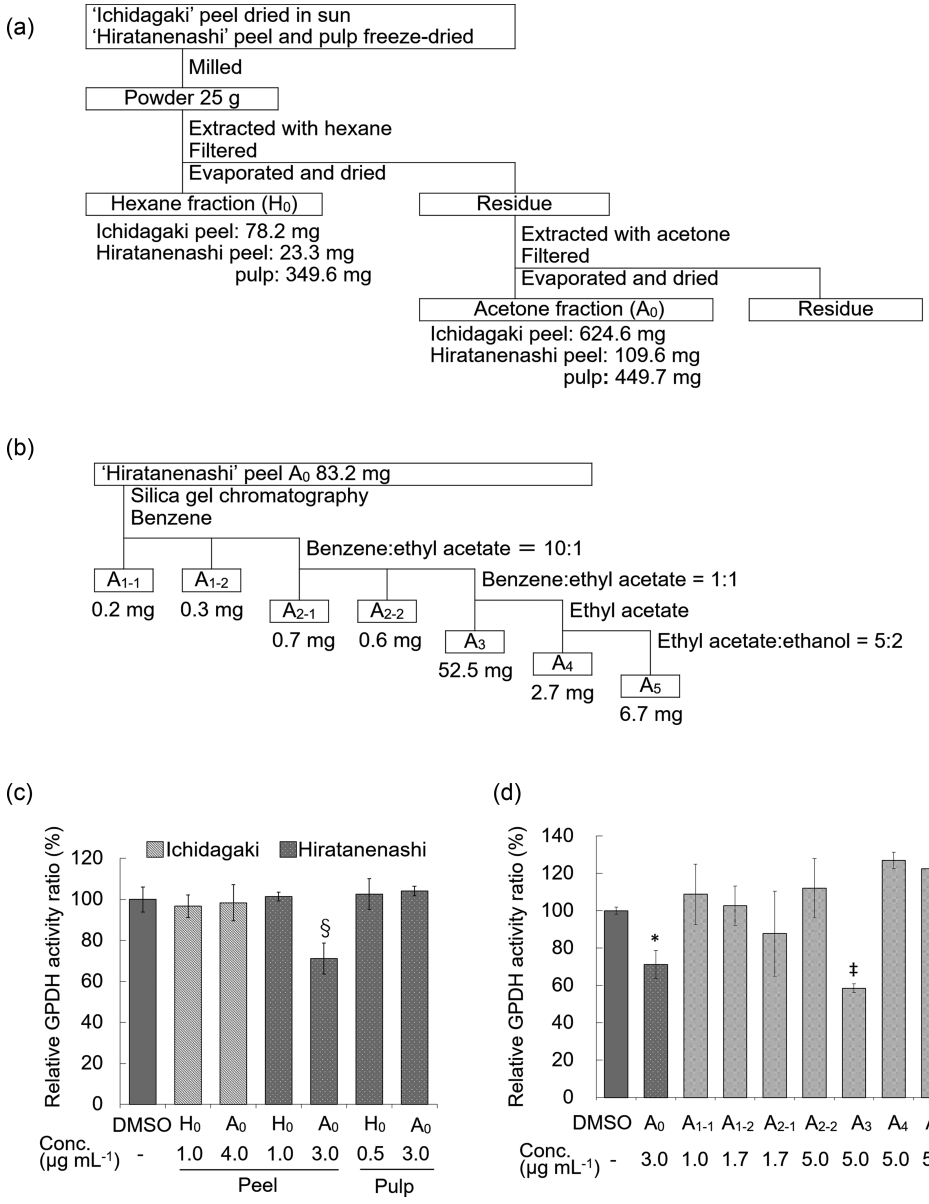

Figure 1. Preparation and yields of persimmon fruit peel fractions and effects of the fractions on GPDH activity in 3T3-L1 adipocytes. (a) Hexane and acetone fractions of 'Ichidagaki' fruit peel and 'Hiratanenashi' fruit peel and pulp. (b) Fractions from $A_{1\text{-}1}$ to A_5 were isolated from 'Hiratanenashi' fruit peel fraction A_0 using silica gel chromatography. The values shown below each fraction indicate the yield. (c) Effects of hexane and acetone fractions on GPDH activity in 3T3-L1 adipocytes. (d) Effects of fractions from $A_{1\text{-}1}$ to A_5 on GPDH activity in 3T3-L1 adipocytes. (c) and (d) After differentiation induction, the adipocytes were cultured in basic medium containing 5 μg mL^{-1} insulin and each fraction for 6 d. Data are expressed as mean ± SD ($n = 3$). $^{*}P < .05$, $^{\dagger}P < .01$, $^{\ddagger}P < .001$, and $^{\S}P < .0001$ vs DMSO-treated cells; Dunnett method.

fraction from the 'Hiratanenashi' peel that had been dissolved and separated as shown in Figure 1b. Each eluted fraction was evaporated under vacuum at 40 °C and subsequently dissolved in DMSO.

Fraction A_3 was dissolved in ethanol and analyzed with high-performance liquid chromatography (HPLC). For HPLC, an LC-20ADXR (Shimadzu, Kyoto, Japan), autosampler SIL-20AC, and a photodiode array SPD-M20A were used. A C_{30} carotenoid column (250 mm × 4.6 mm; particle size, 5 μm; YMC, Kyoto, Japan) was used, and the operating conditions were as follows: column temperature, 37 °C; mobile phase, water:acetonitrile = 1:4 (containing 0.1% [v/v] acetic acid); flow rate, 0.6 mL min^{-1}; sample injection volume, 5 μL. The chromatogram peaks corresponding to 200 nm absorbance were designated as $A_{3\text{-}a}$, $A_{3\text{-}b}$, and $A_{3\text{-}c}$ and compared against the oleanolic and ursolic acid standards.

Fraction A_3 was dissolved in ethanol to a final concentration of 11.3 mg mL^{-1}. Compound $A_{3\text{-}a}$ was separated with a preparative LC Delta 600E multisolvent delivery system (Waters, Milford, MA, USA). The LC operating conditions were as follows: Aqua C_{18} column (250 mm × 10 mm; particle size 5 µm; Phenomenex, Torrance, CA, USA); column temperature, 37 °C; mobile phase A, water; mobile phase B, acetonitrile; gradient condition, 0 min (B: 80%) → 12.5 min (100%) → 30 min (100%); flow rate, 4.44 mL min^{-1}. A 135 µL ethanolic solution of the A_3 fraction was injected into the LC and the $A_{3\text{-}a}$ peak, with a retention time of approximately 4 min at 200 nm UV absorbance, was collected. The $A_{3\text{-}a}$ fraction was dried and 1.5 mg of it dissolved in 0.6 mL of methanol-d4. The solution was then used for NMR spectroscopy (AVANCE II 800 MHz NMR apparatus fitted with a TCI CryoProbe; Bruker).

3T3-L1 cells (JCRB9014; the Health Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (No. 1059010; Thermo Fisher Scientific, Waltham, MA, USA) containing 100 U mL^{-1} penicillin G, 100 µg mL^{-1} streptomycin, and 10% (v/v) newborn calf serum, as the basic medium, at 37 °C, with 5% CO_2 and 95 ± 5% relative humidity. The cells were seeded at a density of 8×10^4 cells $well^{-1}$ onto a 6-well plate (AGC Techno Glass, Shizuoka, Japan), incubated for 3 d till confluence and subsequently cultivated for another 2 d. The medium was then replaced with basic medium supplemented with 10 µg mL^{-1} insulin, 500 µM 3-isobutyl-1-methylxanthine, and 250 nM dexamethasone, and the cells were cultured for 3 d to induce their differentiation into adipocytes. The medium was then replaced with basic medium containing 5 µg mL^{-1} insulin; it was replaced with fresh medium every 2 d. Each fraction and triterpenoid were dissolved in DMSO and then added to culture medium during or after induction of differentiation.

GPDH activity was measured with a kit (Cosmo Bio, Tokyo, Japan). The culture medium was removed first, and the cells were washed twice with phosphate-buffered saline (PBS). To each well, 1 mL of enzyme extracting solution was added. The cells were stripped from the culture plate with a pipette and transferred to a tube. They were then homogenized using an ultrasonic homogenizer (TAITEC Corporation, Saitama, Japan) and then centrifuged at 4 °C and 12 800 × *g*, for 5 min. Each supernatant sample was mixed with the substrate solution in a 96-well plate, and absorbance values of the mixtures were recorded using a microplate reader (BioTek Instrument, Winooski, VT, USA) at 340 nm, at 30 s intervals for 7 min. GPDH activity was calculated from the differential absorbance values between 30 and 90 s. DNA concentration of the supernatant samples was measured with a Picogreen DNA quantification kit (Thermo Fisher Scientific, Waltham, MA, USA). Relative difference in the number of cells was corrected based on the measured DNA concentration, and GPDH activity was calculated using the following equation: GPDH activity = $[((A_{340(90s)} - A_{340(30s)})/6.22) \times$ (total volume/sample volume) × (dilution rate/path length)]/DNA conc. The relative GPDH activity in each group was compared to that in the DMSO-treated group.

The 3T3-L1 adipocytes treated with 5 µM pomolic acid for 6 d were fixed with 10% (v/v) formalin/phosphate buffer (pH 7.4) for 10 min. The fixed cells were washed with PBS, dried, and stained for 20 min with Oil Red O solution (0.5% [v/v] Oil Red O in 2-propanol solution:1% [w/v] dextrin aqueous solution = 6:4). The stained cells were washed once with 60% (v/v) 2-propanol and then with PBS, until no excess pigment came out, and were finally dried. The pigment was extracted with 2-propanol. Absorbance at 510 nm was used as an index of lipid accumulation.

Data are presented as mean ± SD. Dunnett's method, for comparing more than 3 test groups, and Student's *t*-tests, for comparing 2 test groups, were used to identify significant differences between treatment means. $P < .05$ was considered to denote significant difference from the control (DMSO treatment). All data were processed using GraphPad Prism v. 7 (GraphPad Software, La Jolla, CA, USA).

The hexane-extracted H_0 and acetone-extracted A_0 fractions were prepared from 'Ichidagaki' peel and 'Hiratanenashi' peel and pulp (Figure 1a), and their effects on GPDH activity in 3T3-L1 adipocytes were determined subsequently. In all cases, the yield of A_0 fraction was higher than that of H_0 fraction. Each fraction at the indicated concentrations was evaluated for its effects on GPDH activity in 3T3-L1 adipocytes (Figure 1c). GPDH activity in the cells treated with 'Hiratanenashi' peel A_0 fraction was significantly lower than that in control cells.

Focusing on the 'Hiratanenashi' peel A_0 fraction, silica gel chromatography was used for further fractionation (Figure 1b). Effects of each fraction at the indicated concentrations on GPDH activity were evaluated in 3T3-L1 cells (Figure 1d). GPDH activity in the cells treated with A_3 fraction, the fraction with the highest yield, was significantly lower than in the cells treated with DMSO (Figure 1d).

The A_3 fraction was analyzed with LC and 3 peaks were identified. The retention time and MS/MS spectra for $A_{3\text{-}b}$ and $A_{3\text{-}c}$ were the same as those for ursolic acid and oleanolic acid, respectively (Figure 2a and Figure S1). $A_{3\text{-}a}$ was analyzed with NMR (Figure 2b, and Table S1 and Figure S2), and was identified as pomolic acid.

Effects of a range of concentration of pomolic, oleanolic, and ursolic acids on GPDH activity in 3T3-L1 adipocytes were investigated next (Figure 3a). GPDH activity was lower in the cells treated with 5 µM pomolic acid, and was significantly lower ($P < .05$) in the cells treated with >10 µM pomolic acid than in those exposed to DMSO. Oleanolic and ursolic acids had no apparent effect on GPDH activity. Further, the amount of lipid accumulation in the cells treated with 5 µM pomolic acid was lower than that in the DMSO-treated group (Figure 3b). Conversely, GPDH activity in 3T3-L1 cells treated with pomolic acid only during differentiation induction was higher than in those treated with DMSO, whereas oleanolic and ursolic acids had no effect on the activity (Figure 3c).

In this study, we specifically focused on the fat-soluble components present in persimmon peel, which were extracted stepwise using hexane and acetone. Our results indicated pomolic acid to be a candidate antiobesity component. Pomolic acid had earlier been found in Osmanthus flower (Yoo *et al.* 2013) and sea buckthorn leaf (Yang *et al.* 2013). It also occurs in the persimmon calyx (Matsuura and Iinuma 1977) and leaf (Chen *et al.* 2002). However, its presence in persimmon fruit peel has not been reported. Rather, earlier studies indicated oleanolic and ursolic acids to be present in raw persimmon fruit (Zhou *et al.* 2010).

Pomolic acid treatment, after cell differentiation, suppressed the increase in GPDH activity in 3T3-L1 adipocytes, whereas ursolic and oleanolic acids treatment had no such effect. The GPDH enzyme is involved in triglyceride synthesis; 3T3-L1 cells treated with pomolic acid indeed suppressed the increase in lipid amounts. Throughout differentiation and lipogenesis, lipid accumulation is reduced and GPDH activity is inhibited in 3T3-L1 cells treated with pomolic or ursolic acid (He *et al.* 2013; Yang *et al.* 2013). Although these triterpenoids have been reported to affect preadipocytes, whether they affect cells during culture in the differentiation medium or in the insulin-containing medium after differentiation is induced is unclear.

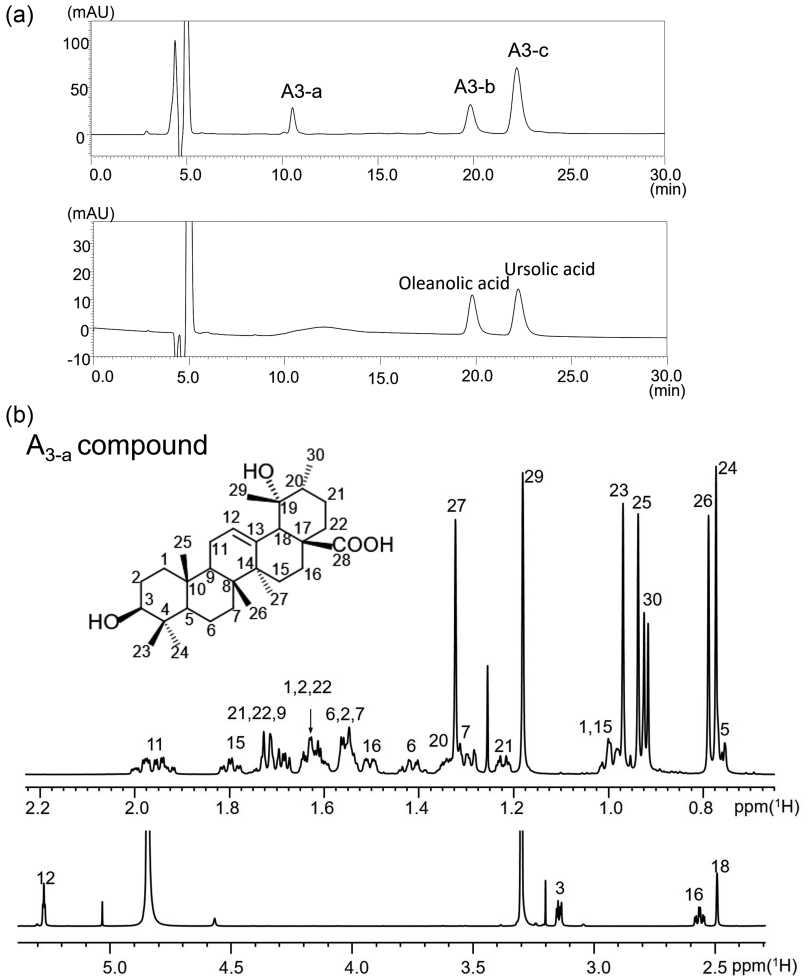

Figure 2. HPLC chromatogram of fraction A_3 and ^{1}H-NMR spectrum of compound $A_{3\text{-a}}$. (a) HPLC chromatogram peaks of the A_3 fraction and oleanolic and ursolic acids detected at 200 nm. (b) ^{1}H-NMR spectrum (methanol-d_4, 800 MHz) of $A_{3\text{-a}}$. Number labels on each ^{1}H-NMR spectrum signal indicate protons combined with the same numbered carbon in pomolic acid. $\delta_H = 3.3$ ppm of methanol-d_4 was the basis of the chemical shift.

In this study, pomolic acid suppressed the increase in GPDH activity in adipocytes only when 3T3-L1 cells were treated with pomolic acid during culture in an insulin-containing medium after induction of differentiation; in contrast, it promoted an increase in GPDH activity in differentiated 3T3-L1 adipocytes only when these cells were treated with pomolic acid during culture in the differentiation medium.

The 3T3-L1 cells treated with pomolic acid only during differentiation medium showed enhanced differentiation, evidenced by the increase in GPDH activity. Therefore, suppression of the increase in GPDH activity due to pomolic acid treatment of 3T3-L1 cells only during culture in an insulin-containing medium after induction of differentiation is suggested to affect differentiated adipocytes and not preadipocytes.

Pomolic, oleanolic, and ursolic acids have similar molecular targets as they structurally resemble each other. However, the structural difference between pomolic acid and ursolic acid lies in the hydroxyl group at the C-19α position, and the structural difference between pomolic acid and oleanolic acid lies in the hydroxyl group at the C-19α position and the methyl group at the C-19β position. The hydroxyl group at C-19α may contribute to the inhibitory activity of pomolic acid during osteoclastogenesis (Tan *et al.* 2015). This structure could also be predicted to play an important role in suppressing the increase in GPDH activity in 3T3-L1 adipocytes. If the difference in activity could be investigated in detail, based on the structure at the 19α position, the mechanism of action may be elucidated, and a component with stronger activity could be subsequently developed.

The pomolic acid in persimmon fruit peel may, thus, affect lipid metabolism. It could have antiobesity effects, considering that it strongly suppressed the increase in GPDH activity in 3T3-L1 adipocytes. Persimmons are produced in large quantities in East Asian countries, including China, South Korea, and Japan. However, vast amounts of the fruit peels are discarded during the production of dried persimmon fruits, despite the medicinal and therapeutic values of the triterpenoids. Our study outcomes suggest that they should be conserved and used as a source of functional food materials, rather than being rejected as

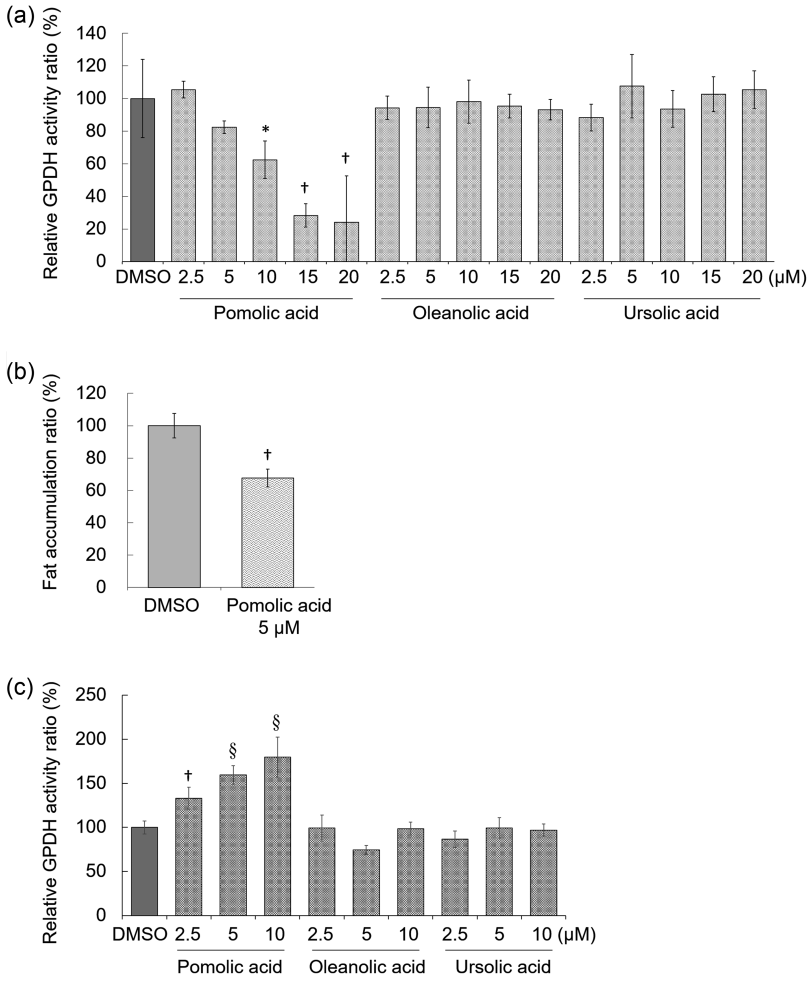

Figure 3. Effect of each triterpenoid on GPDH activity in 3T3-L1 cells, and lipid accumulation due to pomolic acid. (a) GPDH activity in 3T3-L1 adipocytes treated with each triterpenoid after differentiation induction. (b) Lipid accumulation was determined by Oil Red O staining. (a) and (b) After differentiation induction, the adipocytes were cultured in basic medium containing 5 μg mL^{-1} insulin and each triterpenoid for 6 d. (c) GPDH activity in 3T3-L1 adipocytes treated with triterpenoids during differentiation induction. 3T3-L1 preadipocytes were treated with each triterpenoid for 3 d during differentiation induction. After differentiation, the adipocytes were cultured in basic medium containing 5 μg mL^{-1} insulin for 3 d. Data are expressed as mean ± SD ($n = 3$). $^{*}P < .05$, $^{\dagger}P < .01$ and $^{\S}P < .0001$ vs DMSO-treated cells. (a) and (c) Dunnett method and (b) Student's unpaired *t*-test.

processing waste. Therefore, it would be important to determine the effective doses of the active constituents, via *in vivo* testing, and consider the effective usage of persimmon peel in future studies.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

R.I. designed the experiments. All authors performed the experiments, analyzed the data, and have read and approved the final manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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