Received: 9 November 2017 | Revised: 27 December 2017 | Accepted: 23 January 2018

DOI: 10.1002/ptr.6060

RESEARCH ARTICLE

WILEY

5-Hydroxyferulic acid methyl ester isolated from wasabi leaves inhibits 3T3-L1 adipocyte differentiation

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To investigate the compounds present in wasabi leaves (*Wasabia japonica* Matsumura) that inhibit the adipocyte differentiation, activity-guided fractionation was performed on these leaves. 5-Hydroxyferulic acid methyl ester (**1**: 5-HFA ester), one of the phenylpropanoids, was isolated from wasabi leaves as a compound that inhibits the adipocyte differentiation. Compound **1** suppressed the intracellular lipid accumulation of 3T3-L1 cells without significant cytotoxicity. Gene expression analysis revealed that **1** suppressed the mRNA expression of 2 master regulators of adipocyte differentiation, PPARγ and C/EBPα. Furthermore, **1** downregulated the expression of adipogenesis-related genes, GLUT4, LPL, SREBP-1c, ACC, and FAS. Protein expression analysis revealed that **1** suppressed PPARγ protein expression. Moreover, to investigate the relationship between the structure and activity of inhibiting the adipocyte differentiation, we synthesized 12 kinds of phenylpropanoid analog. Comparison of the activity among **1** and its analogs suggested that the compound containing the substructure that possess a common functional group at the ortho position such as a catechol group exhibits the activity of inhibiting the adipocyte differentiation. Taken together, our findings suggest that **1** from wasabi leaves inhibits adipocyte differentiation via the downregulation of PPARγ.

KEYWORDS

3T3-L1, 5-hydroxyferulic acid methyl ester, adipocyte differentiation, leaf, phenylpropanoid, *Wasabia japonica* Matsumura

1 | INTRODUCTION

Excessive accumulation of fat leads to obesity. Obesity has become a serious health problem mainly in developed countries as a risk factor of hypertension (Uno et al., 2012), dyslipidemia (Klop, Elte, & Cabezas, 2013), and Type II diabetes (Kahn, Hull, & Utzschneider, 2006). There is thus a need to prevent obesity to reduce the risk of these diseases. Against this background, in spite of the differentiation of preadipocytes into adipocytes eventually lead to the obesity, it was revealed that thiazolidine derivatives used for the treatment of diabetes promote the differentiation of preadipocytes into adipocytes (Bhattarai et al., 2010). For these reasons, adipocyte differentiation has attracted attention. 3T3-L1 preadipocytes have generally been used for studies of adipocyte differentiation, and numerous anti-differentiation compounds such as EGCg and soyasaponin have been found (Moon et al., 2007; Yang et al., 2015). Treatment of 3T3-L1 preadipocytes with dexamethasone, 3-isobutyl-1-methylxanthine, and insulin enhances the activities of peroxisome proliferator-activated receptor (PPAR) and CCAAT/enhancer binding protein (C/EBP) gene families (Cao, Umek, & McKnight, 1991; Wu, Bucher, & Farmer, 1996) and leads to their differentiation into adipocytes. PPARγ and C/EBPα are well-known as master regulators of adipocyte differentiation (Rosen & MacDougald, 2006). In particular, PPARγ is necessary for adipocyte differentiation (Rosen et al., 1999). Moreover, the expression of PPARγ and C/EBPα genes has been shown to lead to the expression of adipogenesis-related genes including glucose transporter type 4 (GLUT4), lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) (Auwerx, Schoonjans, Fruchart, & Staels, 1996; Rosen & Spiegelman, 2000; Wu, Xie, Morrison, Bucher, & Farmer, 1998). Therefore, the regulation of PPARγ expression is important in controlling adipocyte differentiation.

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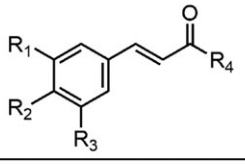
Wasabia japonica Matsumura is a plant of Japanese origin belonging to the Brassicaceae family. Wasabi rhizome is widely eaten as a garnish of sushi or sashimi in Japan, because it exhibits strong pungency derived from allyl isothiocyanates produced by the reaction of myrosinase and sinigrin. In addition, wasabi rhizome contains various isothiocyanates, which have been reported to exhibit many physiological functions, including antimicrobial (Inoue et al., 1983), antiplatelet (Morimitsu et al., 2000), anticancer (Morimitsu et al., 2000), and anti-allergic activities (Nagai & Okunishi, 2009; Yamada-Kato, Nagai, Ohnishi, & Yoshida, 2012). In contrast, wasabi leaves are mostly discarded because they contain little isothiocyanates. However, several studies on the components and functionalities of wasabi leaves have been conducted recently, showing that they contain flavonoids (Hosoya, Yun, & Kunugi, 2005), phenylpropanoids (Hosoya, Yun, & Kunugi, 2008), terpenoids (Yoshida, Hosoya, Inui, Masuda, & Kumazawa, 2015), and carotenoids (Yoshida et al., 2015). Meanwhile, the functional studies revealed that their effects include antioxidant (Hosoya et al., 2008) and anti-inflammatory activities (Yoshida et al., 2015). Interestingly, 5-hydroxyferulic acid methyl ester (**1**: 5-HFA ester; Figure 1), one of the phenylpropanoids isolated from wasabi leaves, but not isolated from other plants, showed both of these types of activity (Hosoya et al., 2008; Yoshida et al., 2015). Studies have also revealed that wasabi leaves exert various types of anti-obesity activity. For example, Ogawa et al. (2010) reported that a hot-water extract of wasabi leaves suppressed the differentiation of 3T3-L1 preadipocytes. Furthermore, Yamasaki et al. (2013) reported that hot-water extract of wasabi leaves exhibited an anti-obesity effect in a study using C57/BL mice. Additionally, Yamada-Kato et al. (2016) reported that 50% ethanol extract of wasabi leaves showed an anti-obesity effect via upregulation of the mRNA expression of β3-adrenergic receptor (β3AR) in interscapular brown adipose tissue. These studies have boosted interest in the potential beneficial effects of wasabi leaves, including those against obesity. Therefore, we investigated the compounds present in wasabi leaves that inhibit the adipocyte differentiation and the molecular mechanisms underlying such activity using 3T3-L1 preadipocytes.



	R_1	R_2	R_3	R_4	IC_{50} (μM)
1	OMe	OH	OH	OMe	63
2	OMe	OH	OMe	OH	>150
3	OMe	OH	OMe	OMe	>150
4	OMe	OH	OH	OH	81
5	OH	OH	H	OH	>150
6	OH	OH	H	OMe	46
7	OMe	OH	H	OH	>150
8	OH	OMe	H	OMe	>150
9	OH	OH	OH	OH	74
10	OH	OH	OH	OMe	145
11	OMe	OMe	H	OMe	111
12	OMe	OMe	OMe	OMe	91

FIGURE 1 Chemical structures of phenylpropanoid analogs and their IC_{50} values of lipid accumulation (%). Compounds **2**, **5**, and **7** were purchased from Sigma-Aldrich (Tokyo, Japan). Compounds **4** and **9** were obtained by the demethylation of **2** with BBr_3. Compound **1** was obtained by the methylation of **4** with trimethylsilyldiazomethane. Compounds **3**, **6**, **8**, **10**, **11**, and **12** were synthesized by methylation using trimethylsilyldiazomethane. The IC_{50} values of lipid accumulation were calculated as 100% cell viability and are presented as the mean (n = 3)

2 | MATERIALS AND METHODS

2.1 | Materials

The *W. japonica* Matsumura leaves used in this study were collected in Shizuoka, Japan, in April 2012.

2.2 | NMR spectroscopy

^1H (400 MHz) and ^{13}C nuclear magnetic resonance (NMR; 100 MHz) spectra, as well as all 2D NMR spectra, were recorded on a Bruker AVANCE III 400 spectrometer (Bruker BioSpin, Billerica, MA, USA). Standard pulse sequences and parameters were used for the experiments. The chemical shift values (δ) are reported in ppm, and the coupling constants (J) are reported in Hz. The chemical shifts in the ^1H and ^{13}C NMR spectra have been corrected using the residual solvent signals of methanol-d_4 (δ_H 3.31, δ_C 49.0).

2.3 | Cell culture and adipocyte differentiation

Murine preadipocyte 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% fetal bovine serum and penicillin (10 U/ml)/streptomycin (100 μg/ml). 3T3-L1 cells were seeded into a 96-well plate at 1×10^5 cells in 100 μl per well and preincubated for 1 day at 37 °C in a humidified atmosphere containing 5% CO_2. After the medium had been removed (Day 0), cells were cultured in the differentiation medium (containing 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine) with or without various concentrations of the test samples dissolved in dimethyl sulfoxide (DMSO) for 2 days. The concentration of DMSO in the medium was 0.1%. After 2 days (Day 2), the medium was removed and replaced with 100 μl of the maintenance medium (containing 10 μg/ml insulin) with or without the test samples. After incubating the cells for 3 days (Day 5), the medium was changed to fresh maintenance medium, followed by 3 days of incubation.

2.4 | Cell viability assay

Cell viability was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). After the culture medium had been removed, 100 μl of phosphate-buffered saline containing 2 μl of WST-8 solution was added to each well, and the cells were incubated

for 3 hr at 37 °C. The absorbance at 450 nm was measured using a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate for each sample. The proportion of living cells was determined from the difference in the absorbance values of the samples and the controls (0.1% DMSO).

2.5 | Oil Red O staining

After the measurement of cell viability, intracellular lipid droplets were stained with Oil Red O (Sigma-Aldrich, Tokyo, Japan), as described below. The cells were fixed with 100 µl of 4% formalin overnight at 4 °C. After removing the formalin, the cells were washed with 60% isopropanol and incubated with 50 µl of Oil Red O solution for 10 min at room temperature. Each well was washed with Milli-Q water 3 times and dried. Fifty microliters of isopropanol was added to each well, and the absorbance of each well was measured at 520 nm using a Spectra Max 190 microplate reader. All experiments were performed in triplicate for each sample. The level of lipid production was determined from the difference in the absorbance values of the samples and the controls (0.1% DMSO).

2.6 | Real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from 3T3-L1 cells using CellAmp Direct RNA Prep Kit for reverse transcription-polymerase chain reaction (Takara Bio, Kusatsu, Japan), in accordance with the manufacturer's instructions and was reverse-transcribed into cDNA using a PrimeScript® RT reagent kit (Takara Bio). The generated cDNA was subjected to PCR using SYBR® Premix DimerEraser (Takara Bio) and primers (Table 1). The PCR amplification was carried out in a Thermal Cycler Dice Real Time System II (Takara Bio) under the following conditions: 95 °C for 30 s followed by 45 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s. The relative levels of mRNA expression were normalized to β-actin in each sample and expressed as a proportion relative to that in uninduced cells (preadipocytes, Day 0), as calculated by the ΔΔCt method.

2.7 | Western blot analysis

Total cellular proteins from 3T3-L1 cells were extracted with RIPA buffer (Nacalai Tesque, Kyoto, Japan). Protein extracts were separated by 10% polyacrylamide gel and transferred to a polyvinylidine difluoride membrane. After blocking with TBST (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) that contained 5% skimmed milk for 1 hr at room temperature, the membrane was incubated with dilute solutions of primary antibodies against β-actin and PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. β-actin was used as an internal control. The membrane was then incubated with dilute solutions of horseradish-peroxidase-conjugated secondary antibodies for 1 hr at room temperature. The protein bands were visualized using a chemiluminescent detection reagent.

2.8 | Quantitative analysis of 5-HFA ester using LC–MS

The high-resolution electrospray ionization mass spectra were recorded using an Accela liquid chromatography (LC) system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a quadrupole mass spectrometer, Q-Exactive (Thermo Fisher Scientific). Xcalibur software was used for system control and data analysis. LC separation was performed on an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters, Milford, MA, USA). The mobile phase consisted of (a) 0.1% formic acid in H_2O and (b) 0.1% formic acid in acetonitrile. The gradient elution program was as follows: 0–1 min, 2% B; 1–16 min, 2–98% B. The column temperature was maintained at 40 °C. The flow rate was 0.4 ml/min, and the injection volume was 5 µl. MS detection was performed on a quadrupole mass spectrometer, Q-Exactive, equipped with an ESI interface. Compound **1** was monitored under negative ion mode and quantified in selected ion monitoring mode: *m/z* 223.0587–223.0631. Other parameters of the mass spectrometer were as follows: sheath gas, 40 Arb; auxiliary gas, 10 Arb; spray voltage, 2,000 V; vaporizer temperature, 300 °C; capillary temperature, 350 °C; and resolution, 70,000.

Dried wasabi leaves (10 mg) were extracted with 500 µl of MeOH by stirring for 30 s, sonication for 5 min, and centrifugation for 5 min twice. The supernatant was dried with nitrogen gas. The MeOH extract was redissolved with 1 ml of MeOH, filtered through a 0.20-µm membrane filter, and then injected into the LC–MS. To correct the obtained results by the rate of recovery of **1** from the wasabi leaves, **1** was spiked to the wasabi leaves, and the same preparation was carried out. The content of **1** was determined by an external standard method. Calibration curves in the 5- to 100-

TABLE 1 Sequences of primers used for reverse transcription-polymerase chain reaction

Gene name	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-actin	NM_007393	CCTGTGCTGCTCACCGAGGC	GACCCCGTCTCTCCGGAGTCCATC
PPARγ	NM_011146	TGTCGGTTTCAGAAGTGCCTTG	TTCAGCTGGTCGATATCACTGGAG
C/EBPα	NC_000073	CGCAAGAGCCGAGATAAAGC	CACGGCTCAGCTGTTCCA
GLUT4	NM_009204	ACGACGGACACTCCATCTGTTG	GGAGACATAGCTCATGGCTGGAA
LPL	M60845	TGGATGAGCGACTCCTACTTCA	CGGATCCTCTCGATGACGAA
SREBP-1c	NM_011481	CAGCACAGCAACCAGAAGC	CCTCCTCCACTGCCACAAG
ACC	NM_133360	GGGCACAGACCGTGGTAGTT	CAGGATCAGCTGGGATACTGAGT
FAS	NM_007989	GTGACCGCCATCTATATCG	CTGTCGTCTGTAGTCTTGAG

ng/ml range had good linearity (R^2 > 0.999) for a three-point plot. The lower limit of quantification was set as a signal-to-noise ratio of 10.

2.9 | Chemical synthesis of 5-HFA ester and its analogs

Sinapic acid (**2**, 900 mg, 4 mmol) was suspended in 30 ml of super-dehydrated CH_2Cl_2 (Wako Pure Chemicals) under argon atmosphere. Boron tribromide (BBr_3, 6.6 ml of 1 M) in CH_2Cl_2 solution (Wako Pure Chemicals) was then added dropwise with constant stirring at −78 °C. The solution was further stirred at room temperature for 24 hr. Then hydrochloric acid (6 ml of 1 M) was added and subjected to continuous stirring at room temperature for 2 hr. After the reaction, the production of a precipitate was observed. The biphasic solution and precipitate were divided by decantation to obtain the precipitate, which was then dissolved in H_2O. Next, the H_2O fraction, CH_2Cl_2 fraction, and precipitate solution were analysed by thin layer chromatography (TLC) to confirm which fraction contained 5-HFA (**4**). The results confirmed that the H_2O fraction and the precipitate solution contained **4**. Thus, the H_2O fraction and the precipitate solution were adjusted to pH 3 with 0.1 M sodium hydroxide, and then EtOAc was added to transfer **4** from H_2O to EtOAc. After confirmation by TLC analysis that **4** had been transferred to EtOAc, the EtOAc fraction was dehydrated using anhydrous sodium sulfate. High-performance liquid chromatography (HPLC) chromatograms of EtOAc fractions derived from the H_2O fraction and the precipitate solution were similar, and hence, these fractions were combined, and **4** was purified by HPLC. Compound **4** (87.2 mg, 0.42 mmol) was obtained in a 10.4% yield. Moreover, 3,4,5-trihydroxycinnamic acid (**9**, 85.6 mg, 0.44 mmol) was obtained as a byproduct in a 10.9% yield. Then **4** (35 mg, 0.17 mmol) was dissolved in 3.5 ml of MeOH. Trimethylsilyldiazomethane (TMSD; 2.2 ml of 10%) in hexane solution (Tokyo Chemical Industry, Tokyo, Japan) was then added dropwise with constant stirring at room temperature. After confirmation by TLC analysis that the spot of **4** had disappeared, the solution after the reaction had its solvent removed and was redissolved in MeOH for the purification of **1** by HPLC. Compound **1** (24.1 mg, 0.11 mmol) was obtained in a 64.6% yield. Sinapic acid (**2**), caffeic acid (**5**), and ferulic acid (**7**) were purchased from Sigma-Aldrich. Sinapic acid methyl ester (**3**) and trimethoxycinnamic acid methyl ester (**12**) were synthesized by methylation of **2** using TMSD. Caffeic acid methyl ester (**6**), isoferulic acid methyl ester (**8**), and dimethoxycinnamic acid methyl ester (**11**) were synthesized by methylation of **5** using TMSD. 3,4,5-Trihydroxycinnamic acid methyl ester (**10**) were synthesized by methylation of **9** using TMSD.

2.10 | Statistical analysis

Experimental values are expressed as the mean ± standard deviation. Statistical analysis was performed using Student's *t* test. Values of $p < .05$ were considered to represent statistical significance.

3 | RESULTS

3.1 | Identification and quantification of compounds in wasabi leaves that inhibit the adipocyte differentiation

Activity-guided fractionation from the MeOH extract of wasabi leaves obtained from the study by Yoshida et al. (2015) was performed. Compound **1** was isolated from the EtOAc extract. To determine the cytotoxicity of **1** and its activity of inhibiting the adipocyte differentiation, 3T3-L1 cells were treated with it at a concentration of 25–100 μM. As shown in Figure 2, **1** decreased lipid accumulation in a dose-dependent manner at concentrations of 25–100 μM with no cytotoxicity. The IC_{50} value of **1** was determined to be 63 μM. Quantitative analysis using LC–MS revealed that 2.2 ± 0.1 μg of **1** was present in 1 g of dried wasabi leaves.

3.2 | Effect of 5-HFA ester (1) on the expression of adipocyte differentiation- and adipogenesis-related genes in 3T3-L1 cells

To identify the mechanism involved in the activity of inhibiting the adipocyte differentiation of **1**, gene expression was analysed by reverse transcription-polymerase chain reaction. As shown in Figure 3a, the mRNA expression of PPARγ and C/EBPα, two transcription factors that are important in the regulation of adipocyte differentiation, was suppressed significantly by treatment with 100 μM **1**. Next, we examined the protein expression level of PPARγ using western blot analysis. Treatment with 100 μM **1** suppressed the protein expression of PPARγ (Figure 3b). We also evaluated the mRNA expression of adipogenesis-related genes, including GLUT4, LPL, SREBP-1c, ACC, and FAS. The expression of all of these genes was suppressed by treatment with 100 μM **1** (Figure 4).

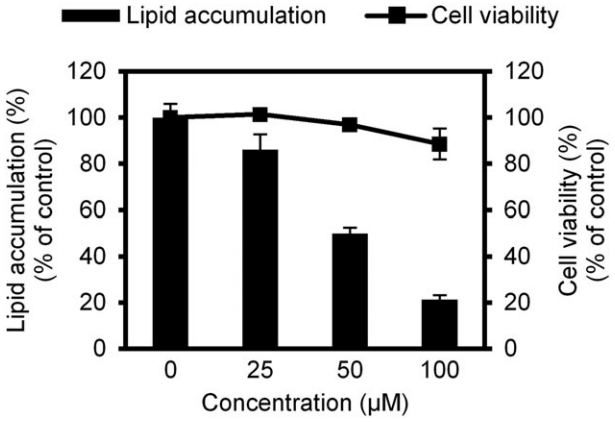


FIGURE 2 Effect of **1** on lipid accumulation and cell viability in 3T3-L1 cells. 3T3-L1 cells were incubated with 25–100 μM **1** for 8 days. Cell viability was evaluated using a Cell Counting Kit-8 on Day 8. The intracellular lipid accumulation was measured by Oil Red O staining on Day 9. The results are expressed as a percentage relative to that of control cells and are presented as mean ± standard deviation (n = 3)

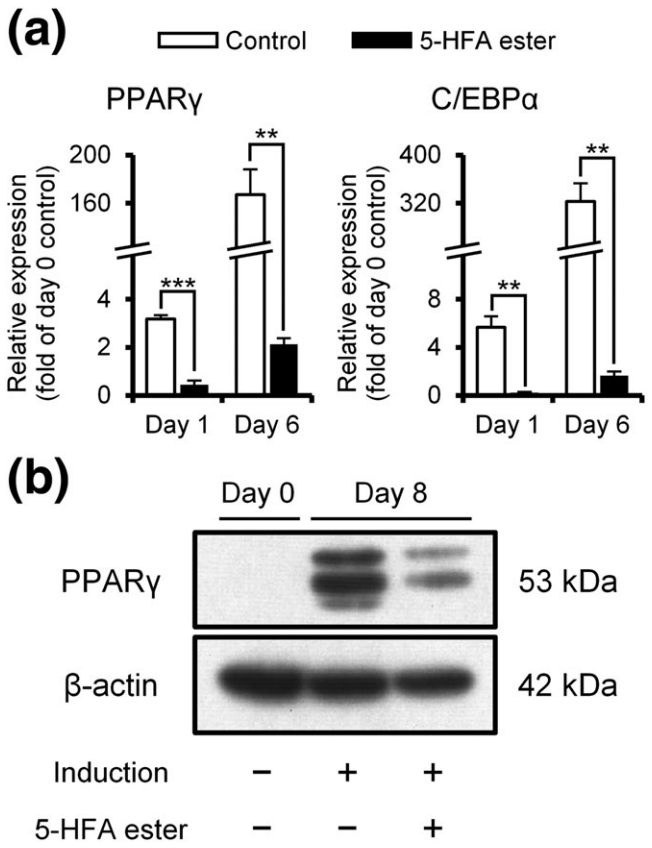


FIGURE 3 Effect of **1** on the expression of adipocyte differentiation-related genes in 3T3-L1 cells. 3T3-L1 cells were incubated with 100 μM **1** for 8 days. (a) Total RNA was extracted from 3T3-L1 cells on Days 1 and 6. The mRNA expression levels of adipocyte differentiation-related genes, PPARγ and C/EBPα, were analysed by reverse transcription-polymerase chain reaction and normalized to β-actin. The results are expressed relative to the levels of uninduced cells (Day 0 control) and are presented as mean ± standard deviation (n = 3). $^{**}p < .01$ and $^{***}p < .001$, compared with the control. (b) Total cellular proteins were extracted from 3T3-L1 cells on Day 8. The level of PPARγ protein expression was analysed by western blotting

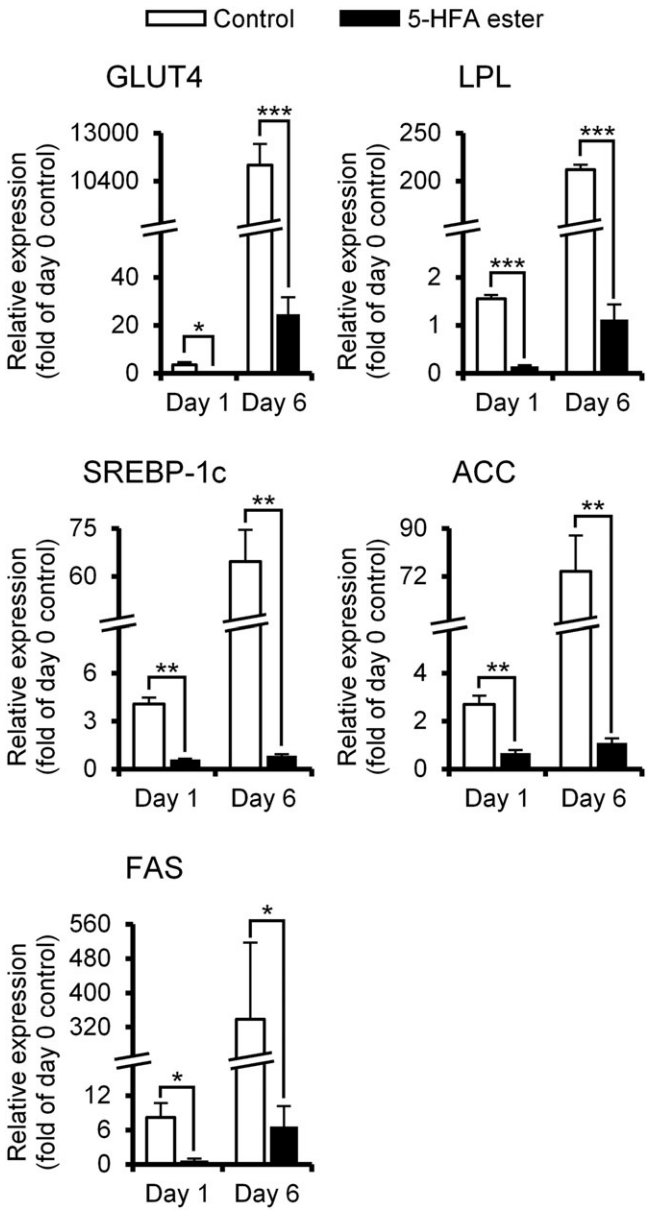


FIGURE 4 Effect of **1** on the expression of adipogenesis-related genes in 3T3-L1 cells. 3T3-L1 cells were incubated with 100 μM **1** for 6 days. Total RNA was extracted from 3T3-L1 cells on Days 1 and 6. The mRNA expression levels of adipogenesis-related genes, GLUT4, LPL, SREBP-1c, ACC, and FAS, were analysed by reverse transcription-polymerase chain reaction and normalized to β-actin. The results are expressed relative to the levels of uninduced cells (Day 0 control) and are presented as mean ± standard deviation (n = 3). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, compared with the control

3.3 | Activities of phenylpropanoid analogs against the adipocyte differentiation

To investigate the relationship between the structure and activity of inhibiting the adipocyte differentiation, a cell viability assay and Oil Red O staining using 3T3-L1 cells treated with 25–100 μM of the 11 different analogs of **1** were performed (Figure 1). Among these analogs, the IC_{50} values of **4**, **6**, **9**, and **12** were determined to be less than 100 μM. Compound **6** (IC_{50} = 46 μM) showed the highest activity of inhibiting the adipocyte differentiation among the 12 kinds of phenylpropanoid. Slight effects were observed in the treatment with **10** and **11**. Compounds **2**, **3**, **5**, **7**, and **8** had little effect on the activity of inhibiting the adipocyte differentiation.

4 | DISCUSSION

In the present study, we found that **1** is one of the compounds in wasabi leaves that inhibit the adipocyte differentiation. Compound **1** suppressed two transcription factors that are important in the regulation of adipocyte differentiation, namely, PPARγ and C/EBPα, as shown in Figure 3. Moreover, **1** suppressed expression of the PPARγ protein. These results suggested that **1** exerts its anti-differentiation effects by regulating PPARγ expression. However, it is not clear whether **1** directly acts on PPARγ. It is thus necessary to clarify the target molecule of **1** by evaluating the expression of genes located upstream of PPARγ, such as C/EBPβ, C/EBPδ, the KLF family, and the GATA family (Matsuo, Kondo, Kawasaki, Tokuyama, & Imamura, 2015). Incidentally, Ilavenil et al. (2016) reported that coumaric acid, an analog of **1**, might downregulate

PPARγ2 expression through hydrogen and hydrophobic interactions with the PPARγ2 receptor domain. Thus, among the active compounds found in this study, there may be compounds showing a similar mechanism of action to exert their activity of inhibiting the adipocyte differentiation. Compound **1** also inhibited the mRNA expression of adipogenesis-related genes, including GLUT4, LPL, SREBP-1c, ACC, and FAS. Ji, Doumit, and Hill (2015) reported that increased expression of SREBP-1c leads to the activation of PPARγ. Therefore, it is possible that **1** acts on SREBP-1c. In addition, SREBP-1c is regulated by insulin signaling (Czech, Tencerova, Pedersen, & Aouadi, 2013), so examining the effects of **1** on SREBP-1c regulatory factors may reveal the mechanism behind the activity of inhibiting the adipocyte differentiation of **1**.

Comparison of the activity between **1** and its analogs revealed that almost all active compounds contain the substructure that possesses a common functional group at the ortho position such as catechol and veratrole groups. However, Nishina et al. (2015) reported that among the flavonoids containing the catechol group, luteolin suppressed lipid accumulation of 3T3-L1 cells, whereas tricetin promoted lipid accumulation. Therefore, we think that further investigation is necessary to elucidate the structure–activity relationship applicable to compounds of several skeletons. In phenylpropanoids, as with **6** and **10**, the intensity of activity changed depending on the number of functional groups. The intensity of activity also changed in association with structural differences of the carboxylic acid moiety, as in the case of **5** and **6**. Thus, it was suggested that the activity depends not only on the catechol or veratrole groups but also on the polarity of the whole structure. Juman et al. (2010) reported that caffeic acid 2-phenylethyl ester significantly inhibited lipid accumulation of 3T3-L1 cells at 50 μM. Furthermore, Imai et al. (2015) reported that caffeic acid 6-phenylhexyl ester and caffeic acid decyl ester, both at a concentration of 4 μM, suppressed lipid accumulation by 46% and 75%, respectively. Additionally, 3,4,5-trihydroxycinnamic acid decyl ester at a concentration of 4 μM also suppressed lipid accumulation by 84%. Therefore, it is considered that the activity is strengthened by replacing the carboxylic acid moiety with a low-polarity alkyl group. Caffeic acid derivatives are present in wasabi leaves as glycosides (Yoshida et al., 2015); thus, there may be a need to perform further investigation of wasabi leaves, including of the extraction conditions and the sample treatment, to discover compounds that are more effective at inhibiting adipocyte differentiation.

Regarding studies on the anti-obesity effect of wasabi leaves, Ogawa et al. (2010) reported that a hot-water extract of wasabi leaves suppressed the differentiation of 3T3-L1 preadipocytes. Moreover, Yamasaki et al. (2013) reported that an anti-obesity effect was observed in C57/BL mice fed a high-fat diet containing a hot-water extract of wasabi leaves, possibly due to the suppression of lipid accumulation in liver and white adipose tissue. Although **1** may exert these effects, its content was determined to be 2.2 ± 0.1 μg/g of dried wasabi leaves in this study. Therefore, it is unable to explain the whole activity of crude extract of wasabi leaves with **1** only. We consider that **1** is just only one of the active compounds present in wasabi leaves. However, we confirmed that several other fractions showed the activity of inhibiting the adipocyte differentiation. Studies of the elucidation of other active compounds present in these fractions are in progress.

In conclusion, **1** present in wasabi leaves was revealed to inhibit adipocyte differentiation. Future investigations of wasabi leaves and **1** as anti-obesity agents are anticipated.

ACKNOWLEDGEMENTS

We thank Dr. Toshiro Ohta (University of Shizuoka) for technical assistance with the experiments. We also thank Dr. Yuko Shimamura and Prof. Shuichi Masuda (University of Shizuoka) for providing PCR primers.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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How to cite this article: Misawa N, Hosoya T, Yoshida S, Sugimoto O, Yamada-Kato T, Kumazawa S. 5-Hydroxyferulic acid methyl ester isolated from wasabi leaves inhibits 3T3-L1 adipocyte differentiation. *Phytotherapy Research*. 2018;1–7. https://doi.org/10.1002/ptr.6060