RESEARCH ARTICLE

Prenylated chalcones 4-hydroxyderricin and xanthoangelol stimulate glucose uptake in skeletal muscle cells by inducing GLUT4 translocation

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Scope: Glucose uptake in skeletal muscle is crucial for glucose homeostasis.
Methods and results: Insulin and muscle contraction increase glucose uptake accompanied by the translocation of glucose transporter (GLUT) 4. In a search for promising foods, which can increase glucose uptake in skeletal muscle, we screened for active polyphenols by assaying for uptake of 2-deoxyglucose (2DG) in rat L6 muscle cells. Among 37 compounds, 4-hydroxyderricin and xanthoangelol, prenylated chalcones abundant in Ashitaba (*Angelica keiskei* Koidzumi, family Apiaceae), significantly increased 2DG uptake in L6 cells by 1.9-fold at 10 μM, compared with the level in DMSO-treated control cells. Next, we investigated the effect of these chalcones on the translocation of GLUT4 and its underlying mechanisms. The chalcones increased the GLUT4 level in the plasma membrane of L6 cells, but activated neither protein kinase C ζ/λ, Akt, nor adenosine monophosphate-activated protein kinase, all of which regulate the GLUT4 translocation. Interestingly, the oral administration of a titrated chalcone-enriched Ashitaba extract containing 150.6 mg/g (dry base) of 4-hydroxyderricin and 146.0 mg/g (dry base) of xanthoangelol suppressed acute hyperglycemia in oral glucose tolerance tests of mice.
Conclusions: Ashitaba is a promising functional food for the maintenance of the blood glucose level by inducing skeletal muscle-associated glucose uptake.

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Abbreviations: 2DG, 2-deoxyglucose; **2DG6P,** 2DG-6-phosphate; **AE,** Ashitaba extract; **AMPK,** adenosine monophosphate-activated protein kinase; **AUC,** area under the curve; **DCQA,** 3,5-di-*O*-caffeoylquinic acid; **ERK,** extracellular signal-regulated kinase; **FBS,** fetal bovine serum; **G6PDH,** glucose-6-phosphate dehydrogenase; **GLUT,** glucose transporter; **HRP,** horseradish peroxidase; **KRH,** Krebs-Ringer-HEPES; **MEM,** minimum essential medium; **NADP,** β-nicotinamide adenine dinucleotide phosphate; **OGTT,** oral glucose tolerance test; **PI3K,** phosphatidylinositol-3-kinase; **PKC,** protein kinase C; **PM,** plasma membrane; **RI,** radioisotope; **TEA,** triethanolamine

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1 Introduction

Insulin controls the amount of glucose level in blood by stimulating the uptake of glucose into skeletal muscle, the most important tissue for glucose homeostasis. The binding of insulin to its receptor induces activation of phosphatidylinositol-3-kinase (PI3K) and phosphorylation of downstream protein kinase C (PKC) ζ/λ and Akt leading to the translocation of glucose transporter (GLUT) 4 from intracellular pools to the plasma membrane (PM). In addition, muscle contraction and energy depletion induce the GLUT4 translocation through adenosine monophosphate-activated protein kinase (AMPK). Impairment of this translocation in skeletal muscle due to obesity results in glucose intolerance, and chronic hyperglycemia can cause several diseases including type 2 diabetes mellitus and cardiovascular diseases [1].

Several studies indicated the beneficial effects of dietary components on health [2]. There has been a growing interest in the maintenance of glucose homeostasis by dietary polyphenols. Resveratrol induced glucose uptake in muscle cells through a pathway involving AMPK [3]. Curcumin increased glucose uptake activity in skeletal muscle isolated from Wistar rats by activating a phospholipase C-PI3K pathway [4]. Both polyphenols induced the translocation of GLUT4. Caffeic acid phenethyl ester [5], kaempferol-3-neohesperidoside [6], and aspalathin (a *C*-glycosyl dihydrochalcone) [7] were found to regulate the metabolism of glucose in skeletal muscle *in vitro*, *ex vivo*, and *in vivo*, respectively. We also reported that (−)-epigallocatechin-3-gallate stimulated the uptake of glucose accompanied by the translocation of GLUT4 in L6 myotubes and increased GLUT4 levels in PM in skeletal muscle of rodents [8]. However, studies on glucose uptake-promoting polyphenols are limited.

Recently, Yamamoto N. *et al.* reported a simple high-throughput method using a non-radioactive 2-deoxyglucose (2DG) [9] for measuring glucose uptake activity, in which the accumulation of 2DG-6-phosphate (2DG6P) in muscle cells on 96-well microplate is measured as the fluorescence of resorufin derived from a glucose-6-phosphate dehydrogenase (G6PDH)/diaphorase/resazurin reaction. In the present study, we screened polyphenols, which stimulate glucose uptake in L6 myotubes, using this non-RI assay. Next, we investigated the effects of 4-hydroxyderricin and xanthoangelol, the most active of the 37 polyphenols tested and abundant in Ashitaba (*Angelica keiskei* Koidzumi, Apiaceae), on the translocation of GLUT4 in L6 myotubes. In addition, we evaluated whether an Ashitaba extract (AE) has modulating effects on acute hyperglycemia in ICR mice during the oral glucose tolerance test (OGTT).

2 Materials and methods

2.1 Polyphenols

4-Hydroxyderricin and xanthoangelol were purified from ''Ashitaba Chalcone Powder'' distributed commercially by Japan Bio Science Laboratory (Osaka, Japan). The powder (2.0 g) was treated with ethyl acetate (100 mL × 3 times) at room temperature, and the solution was evaporated. The extract (309 mg) was separated by silica gel chromatography (64–210 μm, 3 × 20 cm; dichloromethane/acetone, 100:1) and the fractions monitored by TLC (silica gel 60; Merck, Darmstadt, Germany; dichloromethane/acetone 100:5, sulfuric acid). The fractions that showed yellow spots with different Rf values on TLC (0.65 or 0.37) were individually collected. Two fractions were evaporated and refined by reverse-phase medium-pressure liquid chromatography on a Wakosil 40C18 column (40 μm, 15 × 300 mm) eluted with methanol/water (95:5) at 5 mL/min. The chemicals obtained were crystallized in methanol and identified as 4-hydroxyderricin and xanthoangelol by measuring ^{1}H-NMR with an ECP-500 spectrometer (JEOL, Tokyo, Japan). Melting point was determined by a SSC/5200 DSC120 differential scanning calorimeter (Siko Instruments, Chiba, Japan). 4-Hydroxyderricin: 30.5 mg; Fine yellow needles; m.p. 143.6–144.1°C;^{1}H-NMR (500 MHz, $CDCl_3$, 22°C, TMS): δ = 1.68 (s, 3H; CH_3), 1.80 (s, 3H; CH_3), 3.38 (d, ^{3}J (H,H) = 7.1 Hz, 2H; CH_2), 3.9 (s, 3H; OCH_3), 5.22 (t, ^{3}J (H,H) = 7.1 Hz, 1H; CH), 6.49 (d, ^{3}J(H,H) = 9.2, 1H; aryl-H), 6.87 (d, ^{3}J (H,H) = 8.5 Hz, 2H; aryl-H), 7.47 (d, ^{3}J (H,H) = 15.6 Hz, 1H; CH), 7.56 (d, ^{3}J (H,H) = 8.5 Hz, 2H; aryl-H), 7.79 (d, ^{3}J (H,H) = 9.0 Hz, 1H; aryl-H), 7.83 (d, 3J (H,H) = 15.6 Hz, 1H; CH). Xanthoangelol: 37.5 mg; Fine yellow needles; m.p. 116.2–117.1°C;^{1}H-NMR (500 MHz, $CDCl_3$, 22°C, TMS): δ = 1.60 (s, 3H; CH_3), 1.68 (s, 3H; CH_3), 1.83 (s, 3H; CH_3), 2.10 (m, 4H; CH_2), 3.50 (d, ^{3}J (H,H) = 7.1 Hz, 2H; CH_2), 5.06 (t, ^{3}J (H,H) = 6.1 Hz, 1H; CH), 5.30 (dd, 3J (H,H) = 6.1, 7.2 Hz, 1H; CH), 6.42 (d, ^{3}J (H, H) = 8.7 Hz, 1H; aryl-H), 6.88 (d, ^{3}J (H,H) = 8.5 Hz, 2H; aryl-H), 7.47 (d, ^{3}J (H,H) = 15.4 Hz, 1H; CH), 7.57 (d, 3J (H,H) = 8.7 Hz, 2H; aryl-H), 7.73 (d, ^{3}J (H,H) = 8.9 Hz, 1H; aryl-H), 7.84 (d, ^{3}J (H,H) = 15.4 Hz, 1H; CH). The two isolates were identified as 4-hydroxyderricin and xanthoangelol by comparison of the recorded NMR data with those previously reported for the same compounds [10]. The purity of these two compounds was confirmed by an HPLC [column: TSK-gel ODS-80Ts, 4.6 × 150 mm (TOSOH); mobile phase: methanol/water (80:20, v/v); flow rate: 0.8 mL/min; detection: UV 280 and 320 nm, column temperature: 30°C]. For the animal study, an ethyl acetate extract of Ashitaba Chalcone Powder (Ashitaba extract, AE) was prepared as follows. In brief, the powder (10 g) was extracted by ethyl acetate (100 mL × 3 times) and the obtained solution was dried by an evaporator. The extraction yield of AE was 17.4% w/w. Two major chalcones in AE was analyzed by an HPLC and AE contained 150.6 mg/g (dry base) of 4-hydroxyderricin and 146.0 mg/g (dry base) of xanthoangelol. The dried AE was suspended in polysorbate 80 and used in animal test. Biochanin A, caffeic acid and chalcone were purchased from LKT Laboratories (St. Paul, MN). Xanthohumol and 3,5-di-*O*-caffeoylquinic acid (DCQA) came from Alexis Biochemicals (San Diego, CA).

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Chlorogenic acid and prunetin were purchased from Sigma (St. Louis, MO). Eupatilin was obtained from PhytoLab (Vestenbergsgreuth, Germany), and other polyphenols were purchased from Extrasyntese (Genay, France).

2.2 Chemicals

Minimum essential medium (MEM), 2DG, G6PDH, resazurin Nonidet P-40 and the anti-Pi-Akt antibody were purchased from Sigma; fetal bovine serum (FBS) from Biological Industries (Kibbutz Beit Haemek, Israel); BSA from Nakarai Tesque (Kyoto, Japan); Complete protease inhibitor, PhosSTOP phosphatase inhibitor, the cell proliferation reagent WST-1 and Lumi-Light Plus Western Blotting Substrate from Roche Diagnostics (Mannheim, Germany); wortmannin from BIOMOL International L. P. (Plymouth Meeting). The antibodies directed against GLUT4, Pi-PKCζ/λ, Pi-AMPK, and AMPK were purchased from Cell Signaling Technology (Beverly, MA), and the anti-PKCλ antibody from BD Biosciences (San Jose, CA). Diaphorase and β-nicotinamide adenine dinucleotide phosphate $(NADP)^+$ were provided from Oriental Yeast (Tokyo, Japan). The anti-GLUT1 and β-actin antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from WAKO Pure Chemicals, unless otherwise specified.

2.3 Cell culture

Rat skeletal muscle L6 cells (passage 27–37) were maintained in MEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For differentiation into myotubes, L6 cells were seeded on 96-well plates (4×10^3 cells in 0.2 mL) or 60-mm dishes (12×10^4 cells in 4 mL) in a culture medium. After 2 days, the medium was replaced with MEM containing 2% FBS and antibiotics and cultured for an additional 5 days. Fresh medium was added every 2 days. The differentiated cells were incubated in MEM containing 0.2% w/v BSA for 18 h and then used for each assay. Polyphenols were dissolved in DMSO and added to the cells at the concentration described below.

2.4 2DG uptake assay

Glucose uptake in L6 myotubes was measured using the enzymatic microplate 2DG uptake assay [9] with minor modification. L6 myotubes on 96-well plate were treated with DMSO, insulin (final concentration 0.1 μM), and polyphenols (3, 10 and 30 μM) in 0.2% w/v BSA/MEM for 4 h. The PI3K inhibitor wortmannin dissolved in DMSO was added to the myotubes 30 min before and during the treatment with the polyphenols. The cells were washed two times with Krebs-Ringer-HEPES (KRH) buffer (50 mM HEPES, pH 7.4, 137 mM sodium chloride, 4.8 mM potassium chloride, 1.85 mM calcium chloride, and 1.3 mM magnesium sulfate) containing 0.1% w/v BSA and incubated with 1 mM 2DG in 0.1% w/v BSA/KRH buffer for 20 min at 37°C in 5% CO_2. They were then washed two times with 0.1% w/v BSA/KRH buffer, lysed with 0.1 N sodium hydroxide, warmed at 60°C for 10 min, and dried at 85°C for 50 min. The dried cell lysate was solubilized with 0.1 N hydrochloric acid and 200 mM triethanolamine (TEA) (pH 8.1) and gently stirred using a microplate shaker. The lysate was mixed with an assay cocktail (50 mM TEA, pH 8.1, 50 mM KCl, 0.02% w/v BSA, 0.1 mM β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 2 units diaphorase, 150 units G6PDH, 2 μM resazurin) on 96-well plates and incubated at 37°C for 50 min. The fluorescence of resolufin was measured at 570 nm with excitation at 530 nm by using a Wallac 1420 ARVOsx Multi-label counter (Perkin-Elmer, Boston, MA). The 2DG concentration in a well was calculated based on a standard curve generated with a 2DG6P solution.

2.5 Cytotoxicity

The cytotoxicity of polyphenols was determined with the WST-1 assay and crystalviolet staining. Following the treatment of L6 myotubes on 96-well plate with DMSO or polyphenols (3, 10 and 30 μM) in 0.2% w/v BSA/MEM for 24 h, the cells were incubated in 0.2% w/v BSA/MEM containing WST-1 reagent for 4 h. The absorbance of the medium was measured at 450 nm with a reference wavelength of 630 nm using the Wallac 1420 ARVOsx. Then, the cells were fixed and stained with 2% ethanol containing 0.2% w/v crystal violet for 10 min. The wells were washed three times with tap water, and the stained cells were extracted with 50% ethanol containing 0.5% w/v SDS. The absorbance at 570 nm with a reference wavelength of 630 nm was measured using the Wallac 1420 ARVOsx.

2.6 Preparation of the PMplasma membrane fraction and whole protein

The plasma membrane (PM) fraction was prepared according to the method of Nishiumi and Ashida [11] with some modifications. After treatment with DMSO, insulin (0.1 μM), 4-hydroxyderricin (10 μM) and xanthoangelol (10 μM), the cells on 60-mm dishes were washed two times with KRH buffer and homogenized with buffer A (50 mM Tris, pH 8.0, 0.1% v/v Nonidet P-40, 0.5 mM DTT) containing a protease inhibitor and a phosphatase inhibitor using a microtube pestle (five strokes) and 27-gauge syringe needle (five passages). For the preparation of whole protein, part of the homogenate was mixed with RIPA buffer

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(50 mM Tris, pH 8.0, 150 mM sodium chloride, 0.5% w/v sodium deoxycholate, 1% w/v SDS, 1% v/v Nonidet P-40) supplemented with protease and phoshpatase inhibitors, and incubated on ice for 1 h with occasional mixing. After centrifugation at 16 000 × *g* for 20 min at 4°C, the supernatant was used as whole protein. For the preparation of the PM fraction, the remainder of the homogenate was centrifuged at 900 × *g* for 10 min at 4°C, and the pellet was suspended again in buffer A. After centrifugation at 900 × *g* for 10 min at 4°C, the pellet was washed with Nonidet P-40-free buffer A and spun at 900 × *g* for 10 min at 4°C. The precipitate obtained was resuspended with buffer A containing 1% v/v Nonidet P-40, and inhibitors and stood on ice for 1 h with occasional mixing. This suspension was centrifuged at 16 000 × *g* for 20 min at 4°C, and the supernatant was used as the PM fraction.

2.7 Western blotting

Aliquots of the PM fraction and whole protein were separated on 8% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Biotrace, Pall Corporation, Port Washington, NY). After blocking with Blocking one or Blocking one-P (for detection of phospho-proteins), the membranes were treated with appropriate specific primary antibodies overnight at 4°C, followed by the corresponding HRP-conjugated secondary antibody for 1 h at room temperature. The blots were developed using Lumi-Light Plus Western Blotting Substrate and detected with Light-Capture II (ATTO, Tokyo, Japan).

2.8 Oral glucose tolerance test (OGTT)

ICR mice (male, 6 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed in an air-conditioned room (25 ± 1°C) with a moisture of 55 ± 2% and a 12-h light/dark cycle (09:00–21:00). They had free access to a commercial rodent diet (Lab diet EQ; Japan SLC) and tap water. The mice were kept for 1 week before starting the experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations (the permission number: 21-07-02). After 18 h of fasting, the animals were randomly divided into three groups of three each and orally administered vehicle or AE (50 and 250 mg/kg BW: the latter is the maximum because of the difficulty in making the suspension of AE) 60 min before oral glucose loading (1 g/kg BW). Blood samples (20 μL) from a tail vein were collected in heparinized tubes at −60, 0, 15, 30, 60 and 120 min after the glucose administration and then centrifuged to separate plasma at 900 × *g* for 10 min at 4°C. Blood glucose levels were measured using Glucose CII-Test Wako, according to the recommendation of the manufacturer. The area under the curve (AUC) between 0 and 120 min was calculated by the trapezoidal rule.

2.9 Statistical analysis

Data are shown as the mean ± SD of results from three independent experiments. The statistical analysis was performed using the Dunnet test, except for the 2DG uptake inhibition assay (the Tukey-Kramer test). p-values < 0.05 were considered significant.

3 Results

3.1 Screening for polyphenols promoting glucose uptake in L6 myotubes

We initially searched for glucose uptake-inducing polyphenols in L6 myotubes with the enzymatic microplate 2DG uptake assay [9]. We tested 37 compounds (5 chalcones: 4-hydroxyderricin, xanthohumol, xanthoangelol, butein and chalcone; 6 flavanones: isosakuranetin, sakuranetin, eriodictyol, pinostrobin, flavanone and bavachinin; 7 flavonols: galangin, fisetin, kaempferol, quercetin, isorhamnetin, 3-hydroxyflavone and robinetin; 7 flavones: diosmetin, chrysin, luteolin, apigenin, baicalein, tangeretin and eupatilin; 6 isoflavones: *S*-equol, biochanin A, daidzin, prunetin, genistein and daidzein; 1 quinone: shikonin; 1 stilbene: resveratrol; 1 phenylpropanoid: caffeic acid; 2 quinic acid: 3, 5-di-*O*-caffeoylquinic acid, chlorogenic acid; and 1 tannin: theaflavin). The chalcones 4-hydroxyderricin (2.8-fold), xanthohumol (2.7-fold) and xanthoangelol (1.9-fold) and the flavanone isosakuranetin (2.1-fold) at 30 μM significantly stimulated glucose uptake in L6 myotubes, at levels much higher than or similar to those in insulin-treated cells (1.9-fold) (Fig. 1). In addition, 4-hydroxyderricin and xanthoangelol at 10 μM increased the glucose uptake activity to a similar extent as insulin. The chalcone butein, the flavonols galangin, fisetin, quercetin, kaempferol and isorhamnetin, the flavones diosmetin, chrysin and lutelin, the isoflavone daidzin and the naphthoquinone shikonin also significantly increased glucose uptake activity by 1.3- to 1.8-fold, while theaflavin showed slight but significant inhibition (0.8-fold).

3.2 Time-dependent increase in glucose uptake activity in 4-hydroxyderricin and xanthoangelol-treated L6 myotubes

Both 4-hydroxyderricin and xanthoangelol are *C*-prenylated chalcones (Fig. 2) abundant in the Japanese herb Ashitaba (*Angelica keiskei* Koidzumi) [12]. These two were found to induce glucose uptake in 3T3-L1 adipocytes [13]. As no such effect on skeletal muscle cells has been observed previously, we next investigated their glucose uptake-promoting activity

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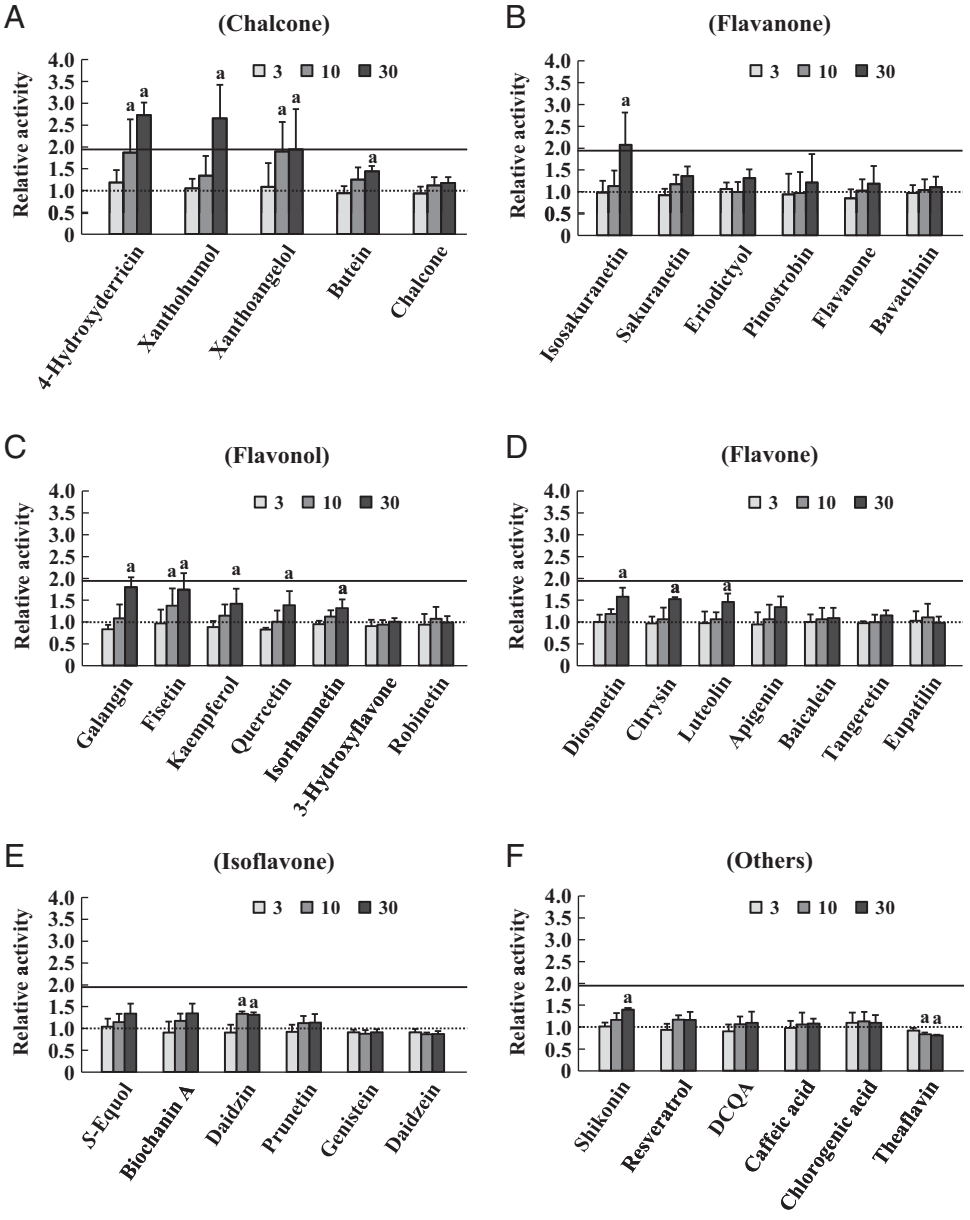

Figure 1. Effects of 37 polyphenols on glucose uptake in L6 myotubes. After incubation in 0.2% w/v BSA/MEM for 18 h, L6 myotubes were treated with DMSO (doted line), insulin (0.1 μM, full line) or polyphenols (3, 10 and 30 μM) for 4 h, and then incubated in 2DG-containing buffer for a further 20 min. The uptake of 2DG was determined as described in Section 2. (A) Chalcone; (B) flavanone; (C) flavonol; (D) flavones; (E) isoflavone; (F) others. Relative activity was calculated as the ratio of 2DG uptake in polyphenol-treated L6 myotubes to that in DMSO-treated cells. Data are shown as the mean ± SD from three independent experiments. A small capital letter indicates a statistically significant difference ($p < 0.05$) from the DMSO-treated cells.

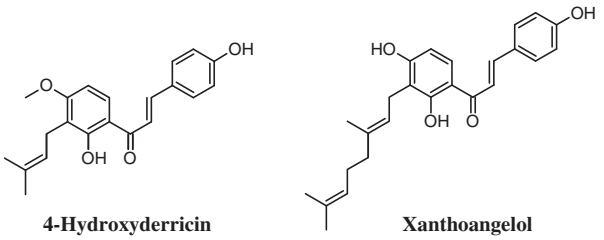

Figure 2. Chemical structure of 4-hydroxyderricin and xanthoangelol.

in L6 myotubes and the underlying mechanisms involved. Neither chalcone showed cytotoxicity at the concentration tested in the WST-1 assay, but rather increased WST-1 reducing activity without affecting cell density (Fig. 3). Also, because xanthoangelol at 30 μM decreased the density of L6 myotubes, we tested these compounds at 10 μM in other experiments *in vitro*.

To estimate the time course of the glucose uptake-promoting activity of 4-hydroxyderricin and xanthoangelol, we measured 2DG uptake in L6 myotubes treated with these chalcones (10 μM) for 1–4 h. Insulin stimulated 2DG uptake in a time-dependent manner (1.6- to 2.0-fold) (Fig. 4). Both chalcones also induced a significant increase in the intracellular 2DG level during 1–2 h to a similar extent as insulin (4-hydroxyderricin: 1.7-fold at 2 h, xanthoangelol: 1.6-fold at 1 h and 1.8-fold at 2 h), while at 4 h showed a slight decrease (4-hydroxyderricin: 1.6-fold, xanthoangelol: 1.5-fold) ($p < 0.05$).

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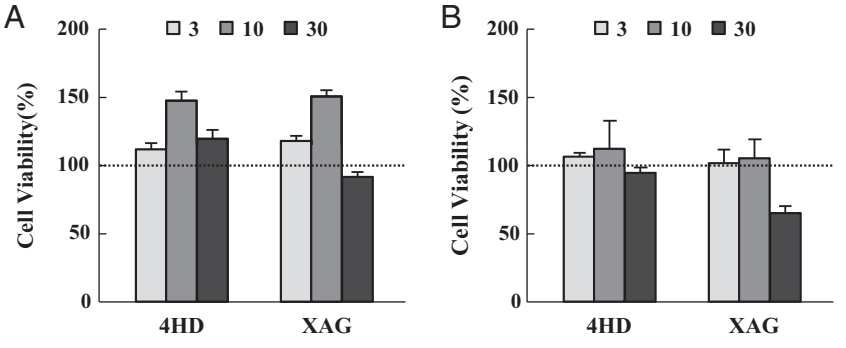

Figure 3. Cytotoxicity of 4-hydroxyderricin and xanthoangelol in L6 myotubes. L6 myotubes were treated with DMSO (dot line) or polyphenols (10 and 30 μM) for 24 h, and cell viability was evaluated by (A) WST-1 assay and (B) crystal violet staining as described in Section 2. Cell viability was normalized to the value for DMSO-treated cells. Data are shown as the mean ± SD from three independent experiments. 4HD, 4-hydroxyderricin; XAG, xanthoangelol.

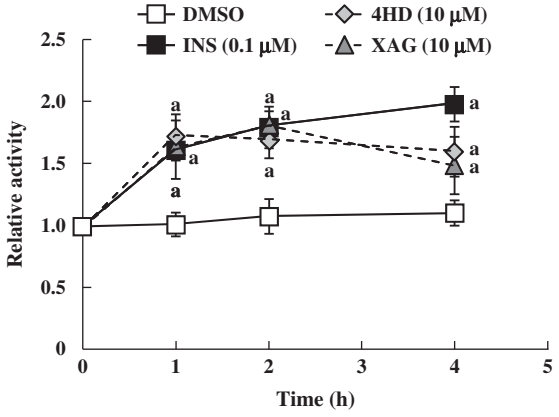

Figure 4. 4-Hydroxyderricin and xanthoangelol stimulated glucose uptake in L6 myotubes in a time-dependent manner. After incubation in 0.2% w/v BSA/MEM for 18 h, L6 myotubes were treated with DMSO, insulin (INS, 0.1 μM), 4-hydroxyderricin (4HD, 10 μM), or xanthoangelol (XAG, 10 μM) for the periods indicated, and then incubated in 2DG-containing buffer for further 20 min. The uptake of 2DG was determined as described in Section 2. The uptake was normalized to that in untreated control cells at 0 h. Data are shown as the mean ± SD from three independent experiments. A small capital letter indicates a statistically significant difference ($p < 0.05$) from the DMSO-treated cells at each time point.

3.3 Induction of GLUT4 translocation in L6 myotubes by 4-hydroxyderricin and xanthoangelol and its underlying mechanisms

GLUT4 is a major transporter of glucose and its translocation from the cytosol to PM is induced by insulin under postprandial conditions. We therefore investigated whether 4-hydroxyderricin and xanthoangelol induce the translocation of GLUT4 in L6 myotubes. In insulin-treated cells, the GLUT4 level in the PM fraction increased at 1–4 h as compared with that in control cells (Fig. 5). 4-Hydroxyderricin highly induced the translocation of GLUT4 at 1 h and sustained the same level until 4 h, while xanthoangelol stimulated it in a time-dependent manner. PKCζ/λ, Akt and AMPK are known to have key roles in the GLUT4 translocation. To elucidate the mechanisms of action of 4-hydroxyderricin and xanthoangelol, we examined the activation of PKCζ/λ, Akt and AMPK. However, neither chalcone affected the phosphorylation of these kinases in L6 myotubes (Fig. 6A). Moreover, the PI3K inhibitor wortmannin failed to suppress the chalcones-induced 2DG uptake (Fig. 6B).

3.4 Suppression of acute hyperglycemia in ICR mice administered the Ashitaba extract

We performed OGTT using AE-administered ICR mice to evaluate the blood glucose-regulating effect of 4-hydroxyderricin and xanthoangelol. In the vehicle-treated mice, the blood glucose level after glucose loading showed a time-dependent increase from 0 to 30 min (106 ± 15 mg/dL at 0 min; 223 ± 20 mg/dL at 30 min) and then gradually decreased (134 ± 10 mg/dL at 120 min) (Fig. 7). Interestingly, acute hyperglycemia was significantly inhibited in the AE-treated group. The peak blood glucose level in the mice administered AE at 50 and 250 mg/kg BW was 196 ± 16 and 175 ± 8 mg/dL, respectively. AE at 250 mg/kg BW significantly suppressed the increase in the blood glucose level at 15–60 min, and at 50 mg/kg, did so at 60 min ($p < 0.05$).

4 Discussion

In the present study, we found 15 active compounds influencing the uptake of glucose in L6 myotubes (Fig. 1). Our findings suggest chalcones, flavonols and flavones to be important targets for glucose uptake-promoting polyphenols. In addition, among the active compounds, 4-hydroxyderricin, xanthohumol and xanthoangelol, all of which are *C*-prenylated chalcones, showed much higher levels of activity (Fig. 1A). A reactive α,β-unsaturated carbonyl group in chalcones is known to regulate cell functions, such as the NF-E2-related factor 2/Kelch-like ECH-associated protein 1 system [14], and may influence the glucose uptake activity in muscle cells, while the activity of butein and chalcone were lower than those of *C*-prenylated chalcones. We have found that the glucose uptake-promoting activity of epicatechin derivative with 3-*O*-acyl chain (C14 and C16) was much higher than that of epicatechin (Ueda M., Fuse N., Kawabata K., Mizushina Y.

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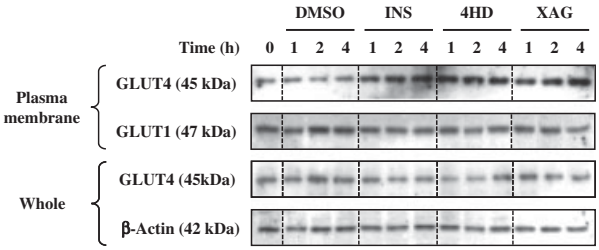

Figure 5. 4-Hydroxyderricin and xanthoangelol induced the GLUT4 translocation in L6 myotubes. Following incubation in 0.2% w/v BSA/MEM for 18 h, L6 myotubes were treated with DMSO, insulin (INS, 0.1 μM), 4-hydroxyderricin (4HD, 10 μM), or xanthoangelol (XAG, 10 μM) for 1–4 h. Thereafter, the PM fraction and whole protein were subjected to Western blotting as described in Section 2. Results shown are representatives of three independent experiments.

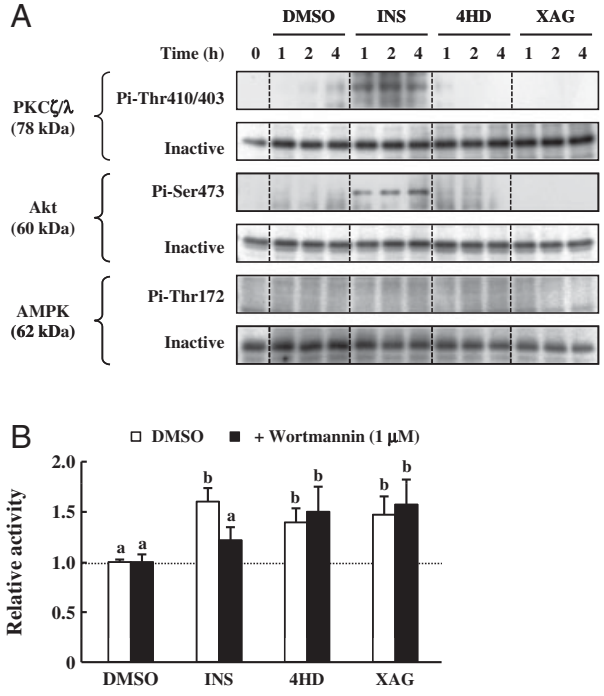

Figure 6. 4-Hydroxyderricin and xanthoangelol had no effect on the activation of PKCζ/λ, Akt, and AMPK in L6 myotubes. (A) After incubation of L6 myotubes with DMSO, insulin (INS, 0.1 μM), 4-hydroxyderricin (4HD, 10 μM), or xanthoangelol (XAG, 10 μM) for 1–4 h, whole protein was analyzed by Western blotting using non-phospho-specific and phospho-specific antibodies as described in Section 2. Results shown are representative of three independent experiments. (B) After a 30-min incubation of L6 myotubes with 1 μM wortmannin, DMSO, insulin (INS, 0.1 μM), 4-hydroxyderricin (4HD, 10 μM), or xanthoangelol (XAG, 10 μM) was added and incubated for a further 4 h, and then the cells were subjected to a 2DG uptake assay as described in Section 2. Data are shown as the mean ± SD from three independent experiments. Different small capital letters indicate a statistically significant difference ($p < 0.05$).

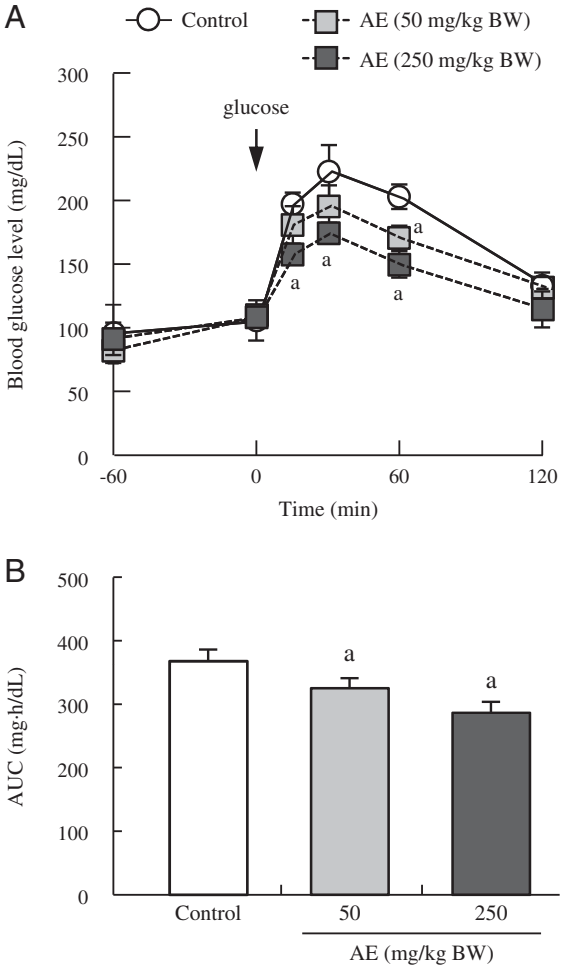

Figure 7. The AE suppressed increases in the blood glucose level in the mice. At 60 min after the oral administration of vehicle (control) or AE (50 and 250 mg/kg BW), the animals were subjected to an OGTT with 1 g/kg BW glucose. Blood samples were collected from a tail vein before (−60 and 0 min) and at 15, 30, 60 and 120 min after glucose feeding. The blood glucose level was measured as described in Section 2. The OGTT results were exhibited as (A) time course of the blood glucose level and (B) its AUC. Data are shown as the mean ± SD from three independent experiments consisting of three mice in each group. A small capital letter indicates a significant difference ($p < 0.05$) from (A) the control mice at each time point and (B) the AUC value of the control group, respectively.

and Ashida H. *et al.*, unpublished data). These findings suggest that highly hydrophobic moieties, such as the prenyl group and the acyl group, may allow acting effectively in or on the myotubes and bringing out the potential of compounds. A catechol structure is also a reactive and characteristic group of polyphenols, but quercetin, luteolin and the *O*-methylated compounds, isorhamnetin and diosmetin increased the glucose uptake activity to a similar extent. Furthermore, galangin and chrysin with no phenolic hydroxyl group in the B ring highly promoted glucose uptake in L6 myotubes. To get insights into the mechanism of action underlying the

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observed effects, we need to examine the activity of a wide range of polyphenols with our screening model.

The Japanese herb Ashitaba, an umbelliferous perennial plant found along the Pacific coast from the Bousou peninsula to the Kii peninsula and on the Izu islands of Japan, is drunk as a tea and used as a vegetable as well as the folk medicine as a diuretic, laxative, analeptic and galactagogue. Several of the compounds in this plant have been identified including coumarins, flavanones and chalcones [12, 15–19]. Among them, 4-hydroxyderricin and xanthoangelol are considered to be major active compounds, reported to inhibit gastric acid secretion [20], show anti-tumor promoting activity [21] and anti-bacterial activity [22], have vasorelaxant effect [23], exhibit anti-angiogenic activity [24, 25], reduce an increase in blood pressure [26] and induce apoptosis [27]. Also, Enoki T. *et al.*, demonstrated an anti-diabetic effect of these chalcones [13]. According to their report, 4-hydroxyderricin and xanthoangelol showed glucose uptake-promoting, but not differentiation-inducing activity in 3T3-L1 adipocytes and a suppressive effect on increases in the blood glucose level in KK-A^y mice after 3 weeks on a 4-hydroxyderricin-containing diet. In the present study, we have revealed the induction of GLUT4-dependent glucose uptake in L6 myotubes (Figs. 1, 4 and 5) and the attenuation of acute hyperglycemia in the mice following a single oral administration of AE (Fig. 7). These results strongly support that Ashitaba and its extract abundant in 4-hydroxyderricin and xanthoangelol are a promising foods for preventing the development of metabolic syndrome.

Ashitaba cultivated in Japan were reported to contain around 0.4 g of chalcones (4-hydroxyderricin and xanthoangelol) *per* 100 g dried leaves [28], while AE in our study has about 150 mg/g dry weight each of 4-hydroxyderricin and xanthoangelol. According to our preliminary data, plasma levels of 4-hydroxyderricin and xanthoangelol in mouse were approximately 10 and 1.0 μM, respectively, after oral administration of AE at 200 mg/kg BW (equivalent to 60 mg chalcones/kg BW) (data not shown), suggesting the association between *in vitro* (Figs. 4 and 5) and *in vivo* (Fig. 7) studies. However, because we administered a single and high dose of AE orally to mouse, future research should assess the beneficial effect of AE after long-term intake within a normal dietary range.

GLUT4 is the primary mediator of glucose uptake into insulin-stimulated skeletal muscle cells. The binding of insulin to its receptor activates PI3K, which, in turn, induces the activation of PKCζ/λ and Akt through the downstream kinase phosphoinositide-dependent protein kinase-1. Then, both PKCζ/λ and Akt mediate the translocation of GLUT4 from the cytosol to PM *via* actin remodeling and activation of the exocytosis regulator Rab, respectively [29]. In addition, the activation of AMPK by energy depletion and elevated intracellular Ca^{2+} levels lead to induction of the GLUT4 translocation [30]. Because 4-hydroxyderricin and xanthoangelol stimulated the translocation of GLUT4 in L6 myotubes (Fig. 5), it appeared to be due to the activation of either the insulin signaling pathway or the AMPK pathway. Unexpectedly, however, these compounds had no effect on the phosphorylation of PKCζ/λ, Akt, or AMPK, and their glucose uptake-promoting activities were not lowered by treatment with a PI3K inhibitor (Fig. 6), suggesting that 4-hydroxyderricin and xanthoangelol affect factors further downstream. Also, recent papers reported the contribution of conventional/novel PKC [31], sirtuin [32], extracellular signal-regulated kinase (ERK) 1/2 and p38 [33] to the GLUT4 translocation, although there was no change in the phosphorylation of ERK1/2 and p38 in chalcone-treated L6 myotubes (data not shown) To uncover the mechanisms of action, it is necessary to perform a comprehensive analysis of the signaling components affected by 4-hydroxyderricin and xanthoangelol.

In conclusion, we screened for polyphenols promoting the uptake of glucose in L6 myotubes, and found several highly active compounds. Among them, the chalcones 4-hydroxyderricin and xanthoangelol, which are abundant in Ashitaba, caused the translocation of GLUT4 in L6 myotubes, though they exhibited neither insulin-like nor AMPK-inducing effects. AE containing 4-hydroxyderricin and xanthoangelol suppressed acute hyperglycemia, suggesting that these compounds stimulate glucose uptake in skeletal muscle in ICR mice in a GLUT4-dependent manner. The mechanism by which prenylated chalcones from Ashitaba induce the GLUT4 translocation in muscle cells will be addressed further in the near future.

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5 References

[1] Schenk, S., Saberi, M., Olefsky, J. M., Insulin sensitivity: modulation by nutrients and inflammation. *J. Clin. Invest.* 2008, *118*, 2992–3002.

[2] Williamson, G., Manach, C., Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* 2005, *81*, 243S–255S.

[3] Penumathsa, S. V., Thirunavukkarasu, M., Zhan, L., Maulik, G. *et al.*, Resveratrol enhances GLUT-4 translocation to the caveolar lipid raft fractions through AMPK/Akt/eNOS signalling pathway in diabetic myocardium. *J. Cell. Mol. Med.* 2008, *12*, 2350–2361.

[4] Cheng, T. C., Lin, C. S., Hsu, C. C., Chen, L. J. *et al.*, Activation of muscarinic M-1 cholinoceptors by curcumin to increase glucose uptake into skeletal muscle isolated from Wistar rats. *Neurosci. Lett.* 2009, *465*, 238–241.

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[5] Lee, E. S., Uhm, K. O., Lee, Y. M., Han, M. S. *et al.*, CAPE (caffeic acid phenethyl ester) stimulates glucose uptake through AMPK (AMP-activated protein kinase) activation in skeletal muscle cells. *Biochem. Biophys. Res. Commun.* 2007, *361*, 854–858.

[6] Zanatta, L., Rosso, A., Folador, P., Figueiredo, M. S. *et al.*, Insulinomimetic effect of kaempferol 3-neohesperidoside on the rat soleus muscle. *J. Nat. Prod.* 2008, *71*, 532–535.

[7] Kawano, A., Nakamura, H., Hata, S., Minakawa, M. *et al.*, Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. *Phytomedicine* 2009, *16*, 437–443.

[8] Ueda, M., Nishiumi, S., Nagayasu, H., Fukuda, I. *et al.*, Epigallocatechin gallate promotes GLUT4 translocation in skeletal muscle. *Biochem. Biophys. Res. Commun.* 2008, *377*, 286–290.

[9] Yamamoto, N., Sato, T., Kawasaki, K., Murosaki, S. *et al.*, A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. *Anal. Biochem.* 2006, *351*, 139–145.

[10] Baba, K., Nakata, K., Taniguchi, M., Kido, T., Kozawa, M., Chalcones from *Angelica keiskei*. *Phytochemistry* 1990, *29*, 3907–3910.

[11] Nishiumi, S., Ashida, H., Rapid preparation of a plasma membrane fraction from adipocytes and muscle cells: application to detection of translocated glucose transporter 4 on the plasma membrane. *Biosci. Biotechnol. Biochem.* 2007, *71*, 2343–2346.

[12] Kozawa, M., Morita, N., Baba, K., Hata, K., The structure of xanthoangelol, a new chalcone from the roots of *Angelica keiskei* KOIDZUMI (Umbelliferae). *Chem. Pharm. Bull.* 1977, *25*, 515–516.

[13] Enoki, T., Ohnogi, H., Nagamine, K., Kudo, Y. *et al.*, Antidiabetic activities of chalcones isolated from a Japanese Herb, *Angelica keiskei*. *J. Agric. Food Chem.* 2007, *55*, 6013–6017.

[14] Kim, E. H., Surh, Y. J., 15-deoxy-Delta12,14-prostaglandin J2 as a potential endogenous regulator of redox-sensitive transcription factors. *Biochem. Pharmacol.* 2006, *72*, 1516–1528.

[15] Nishimura, R., Tabata, K., Arakawa, M., Ito, Y. *et al.*, Isobavachalcone, a chalcone constituent of *Angelica keiskei*, induces apoptosis in neuroblastoma. *Biol. Pharm. Bull.* 2007, *30*, 1878–1883.

[16] Ogawa, H., Nakamura, R., Baba, K., Beneficial effect of laserpitin, a coumarin compound from *Angelica keiskei*, on lipid metabolism in stroke-prone spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* 2005, *32*, 1104–1109.

[17] Akihisa, T., Tokuda, H., Hasegawa, D., Ukiya, M. *et al.*, Chalcones and other compounds from the exudates of *Angelica keiskei* and their cancer chemopreventive effects. *J. Nat. Prod.* 2006, *69*, 38–42.

[18] Akihisa, T., Tokuda, H., Ukiya, M., Iizuka, M. *et al.*, Chalcones, coumarins, and flavanones from the exudate of *Angelica keiskei* and their chemopreventive effects. *Cancer Lett.* 2003, *201*, 133–137.

[19] Sugii, M., Ohkita, M., Taniguchi, M., Baba, K. *et al.*, Xanthoangelol D isolated from the roots of *Angelica keiskei* inhibits endothelin-1 production through the suppression of nuclear factor-kappaB. *Biol. Pharm. Bull.* 2005, *28*, 607–610.

[20] Murakami, S., Kijima, H., Isobe, Y., Muramatsu, M. *et al.*, Inhibition of gastric H+, K(+)-ATPase by chalcone derivatives, xanthoangelol and 4-hydroxyderricin, from *Angelica keiskei* Koidzumi. *J. Pharm. Pharmacol.* 1990, *42*, 723–726.

[21] Okuyama, T., Takata, M., Takayasu, J., Hasegawa, T. *et al.*, Anti-tumor-promotion by principles obtained from *Angelica keiskei*. *Planta Med.* 1991, *57*, 242–246.

[22] Inamori, Y., Baba, K., Tsujibo, H., Taniguchi, M. *et al.*, Antibacterial activity of two chalcones, xanthoangelol and 4-hydroxyderricin, isolated from the root of *Angelica keiskei* KOIDZUMI. *Chem. Pharm. Bull. (Tokyo)* 1991, *39*, 1604–1605.

[23] Matsuura, M., Kimura, Y., Nakata, K., Baba, K. *et al.*, Artery relaxation by chalcones isolated from the roots of *Angelica keiskei*. *Planta. Med.* 2001, *67*, 230–235.

[24] Kimura, Y., Taniguchi, M., Baba, K., Antitumor and antimetastatic activities of 4-hydroxyderricin isolated from *Angelica keiskei* roots. *Planta. Med.* 2004, *70*, 211–219.

[25] Kimura, Y., Baba, K., Antitumor and antimetastatic activities of *Angelica keiskei* roots, part 1: Isolation isolation of an active substance, xanthoangelol. *Int. J. Cancer.* 2003, *106*, 429–437.

[26] Ogawa, H., Ohno, M., Baba, K., Hypotensive and lipid regulatory actions of 4-hydroxyderricin, a chalcone from *Angelica keiskei*, in stroke-prone spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* 2005, *32*, 19–23.

[27] Tabata, K., Motani, K., Takayanagi, N., Nishimura, R. *et al.*, Xanthoangelol, a major chalcone constituent of *Angelica keiskei*, induces apoptosis in neuroblastoma and leukemia cells. *Biol. Pharm. Bull.* 2005, *28*, 1404–1407.

[28] Baba, K., Taniguchi, M., Shibano, M., Minami, H., The components and line breeding of *Angelica keiskei* KOIDZUMI. *Bunseki Kagaku* 2009, *58*, 999–1009.

[29] Zaid, H., Antonescu, C. N., Randhawa, V. K., Klip, A., Insulin action on glucose transporters through molecular switches, tracks and tethers. *Biochem. J.* 2008, *413*, 201–215.

[30] Huang, S., Czech, M. P., The GLUT4 glucose transporter. *Cell Metab.* 2007, *5*, 237–252.

[31] Wijesekara, N., Tung, A., Thong, F., Klip, A., Muscle cell depolarization induces a gain in surface GLUT4 *via* reduced endocytosis independently of AMPK. *Am. J. Physiol. Endocrinol. Metab.* 2006, *290*, E1276–E1286.

[32] Breen, D. M., Sanli, T., Giacca, A., Tsiani, E., Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK. *Biochem. Biophys. Res. Commun.* 2008, *374*, 117–122.

[33] Deng, J. Y., Hsieh, P. S., Huang, J. P., Lu, L. S. *et al.*, Activation of estrogen receptor is crucial for resveratrol-stimulating muscular glucose uptake *via* both insulin-dependent and -independent pathways. *Diabetes* 2008, *57*, 1814–1823.

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