Skeletal muscle plays a pivotal role in the regulation of glucose homeostasis. Approximately 70% of postprandial glucose is taken up by skeletal muscle (1, 2). Insulin and exercise induce glucose uptake by skeletal muscles by stimulating the translocation of glucose transporter (GLUT) 4 from intracellular pools to the plasma membrane (3). In obese humans, hypertrophic abdominal adipose tissue secretes high amounts of inflammatory cytokines and free fatty acids; the presence of these compounds causes a decline in the insulin sensitivity of skeletal muscle, leading in turn to a deficiency in its glucose uptake activity. Thus, obesity gives rise to hyperglycemia and type 2 diabetes mellitus.

Edible plants, including fruits, vegetables, and medicinal herbs, are attractive sources of promising chemicals for prevention of hyperglycemia and type 2 diabetes mellitus. Several types of polyphenols have been identified as either inhibitors of glucose transport into the small intestine (4) or inducers of glucose uptake into adipocytes (5). Additionally, recent studies have targeted skeletal muscle because of its crucial role in maintaining glucose homeostasis. We have also performed screening of glucose uptake inducers using rat L6 myotubes with a high-throughput system that enzymatically measures an incorporated glucose analog, 2-deoxyglucose (6). Among several active polyphenols identified in our study, the most potent enhancers—prenylated chalcones, 4-hydroxyderricin and xanthoangelol from Angelica keiskei (6)—induced GLUT4 translocation and suppressed acute hyperglycemia in mice (6).

Jujube (Ziziphus jujuba Mill.), a popular herbal medicine for analeptic and palliative purposes and a nutritional food in China and South Korea, is rich in natural compounds, including polyphenols, triterpenoids, and polysaccharides (7). Although this species has been reported to have bioactive properties, such as anti-cancer, anti-oxidative, and anti-obesity effects (7), its possible anti-hyperglycemic action targeting skeletal muscle has not been fully studied. In this study, we therefore investigated the anti-hyperglycemic action of jujube. We identified four active triterpenoids: betulonic, betulinic, oleanonic, and ursonic acids. These compounds enhanced glucose uptake in L6 myotubes in a GLUT4-dependent fashion. We also compared jujubes from three countries, namely, China, South Korea, and Japan, revealed that Japanese jujube has a higher content of active triterpenoids and is the most potent enhancer of glucose uptake.

Key Words Ziziphus jujuba, triterpenoids, skeletal muscle, glucose uptake, hyperglycemia

MATERIALS AND METHODS

Chemicals. Minimum essential medium (MEM, with Earle’s salts, L-glutamine, and sodium bicarbonate),
Kawabata K et al.

**Materials.** Dried fruits of *Z. jujuba* were purchased from commercial sources in China, South Korea, and Japan. In addition, jujube was harvested from Gyeongsan, South Korea, and from trees obtained from Miryang, South Korea, and planted in 2011 in Fukui, Japan.

**Extraction and isolation.** Dried fruits of Chinese jujube (6 kg) were extracted twice with methanol at room temperature for 2 wk. After concentration in vacuo, the combined methanol extracts were partitioned between ethyl acetate (EtOAc, 1 volume) and H$_2$O (2 volumes) to obtain an EtOAc-soluble fraction (124 g). This fraction was loaded onto a Wako Gel C-100 silica gel column and eluted using a stepwise gradient of -hexane-EtOAc [0–50%, 75%, and 100% EtOAc]. The 10% fractions, 18.8–75 mg/mL, were the fractions most strongly inducing glucose uptake of L6 myotubes, were combined; the mixture (9.7 g) was then separated into 14 fractions on a middle-pressure silica gel (Wako Gel B-0) column eluted using a stepwise gradient of n-hexane-EtOAc (fractions 1–13) and methanol (fraction 14). Fraction 7 was purified by preparative thin-layer chromatography (2:1 n-hexane:EtOAc) and preparative reversed-phase high-performance liquid chromatography (RP-HPLC) (80% acetonitrile) to yield compound 1 (66 mg) (Fig. 1). Fraction 9 was separated by preparative RP-HPLC (90% methanol) to yield compound 2 (23 mg). Fraction 10 was partitioned according to its solubility in toluene. The toluene-insoluble fraction was dissolved in DMSO or methanol and added to cells at the concentrations described below.

**2DG uptake assay.** Glucose uptake activity of L6 myotubes was measured using an enzymatic microplate 2DG uptake assay (6, 10, 11). L6 myotubes were treated with vehicle [DMSO, 0.5% (v/v) final concentration], insulin (0.1 μM), and samples (triterpenoids, 2–50 μM; fractions, 18.8–75 μg/mL) in 0.2% (v/v) BSA and antibiotics for 4 h at 37°C in a humidified atmosphere with 5% CO$_2$. The treated myotubes 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% (v/v) BSA. The cells were incubated in 0.1% (v/v) BSA/KRH buffer containing 1 mM 2DG for 20 min at 37°C in a humidified atmosphere with 5% CO$_2$. To check for the involvement of GLUT4, DMSO [0.1% (v/v) final concentration] and the GLUT4 inhibitor indinavir (100 μM) were added to the solution. The cells were then washed two times with penicillin, streptomycin, HEPES, insulin, 2-deoxyglucose (2DG), glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, and indinavir were purchased from Sigma (St. Louis, MO). Fetal bovine serum was obtained from Thermo Fisher Scientific (Waltham, MA). Resazurin and 2DG-6-phosphate (DG6P) were obtained from Thermo Fisher Scientific (Waltham, MA). Indinavir was obtained from Tokyo Chemical Industry (Tokyo, Japan) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. 

---

**Fig. 1.** Chemical structures of triterpenic acids.

- R$_1$ = R$_2$ = carbonyl
  - Betulonic acid (Compound 1)
- R$_1$ = OH, R$_2$ = H
  - Betulinic acid (Compound 2)
- Oleanonic acid (Compound 3)
- Ursolic acid

Yield compound 3 (41 mg). By comparing their masses and nuclear magnetic resonance spectra with authentic samples synthesized by the authors (see below) and as well as with published data, compounds 1–3 were respectively identified as betulonic acid (8), oleanonic acid (9), and betulinic acid (8).

**Synthesis of oleanonic, betulinic, and ursonic acids.** Oleanonic acid, betulinic acid, and ursolic acid were subjected to Jones oxidation (chromium trioxide/sulfuric acid in acetone) to respectively obtain oleanonic acid, betulinic acid, and ursolic acid with yields over 95%.

**Cell culture.** Rat skeletal muscle myoblast L6 cells (JCRB9081, Health Science Research Resources Bank, Osaka, Japan) were grown in MEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere with 5% CO$_2$. L6 cells (2×10$^4$ cells/mL, 0.2 mL) were added to 96-well plates. After 2 d, the cells were incubated in MEM containing 2% (v/v) FBS and antibiotics and allowed to differentiate into myotubes for 5 d. Fresh medium was added on day 3. The differentiated cells were then cultured in MEM containing 0.2% (v/v) BSA and antibiotics for 18 h and then used for a 2DG uptake assay and cell viability measurements. Samples were dissolved in DMSO or methanol and added to cells at the concentrations described below.

---

![Fig. 1. Chemical structures of triterpenic acids.](image-url)
increased muscle glucose uptake by jujube triterpenoids

0.1% (v/v) BSA/KRH buffer, lysed with 0.1 M sodium hydroxide (50 μL), warmed at 60°C for 10 min, and dried at 85°C for 60 min. The dried cell lysate was solubilized with 0.1 M hydrochloric acid (50 μL) and 200 mM triethanolamine (pH 8.1, 50 μL) and stirred using a microplate shaker. The lysate (10 μL) was mixed with an assay cocktail [50 mM triethanolamine (pH 8.1), 50 mM potassium chloride, 0.02% (w/v) BSA, 0.1 mM NADP+; 2 units diaphorase, and 150 units G6PDH].

Cell viability measurements. The viability of the L6 myotubes was measured via crystal violet staining. After treatment of L6 myotubes in a 96-well plate with DMSO or triterpenoids (2 and 50 μM) in 0.2% (w/v) BSA/MEM for 24 h, the cells were fixed and stained with crystal violet solution [0.2% (w/v) in 2% (v/v) ethanol] for 10 min. After washing the wells three times with tap water, the stained cells were extracted with sodium dodecyl sulfate solution [0.5% (w/v) in 50% (v/v) ethanol]. Optical density was measured at 570 nm, with a reference wavelength of 630 nm, using a SpectraMax M2 instrument. The optical density of the vehicle control (DMSO or methanol) was considered to represent 100% cell viability.

Preparation of plasma membrane fraction. Fractionation of plasma membrane proteins were performed by the method of Whitehead et al. (12) with some modifications. Briefly, the cells treated with insulin or triterpenoids for 3 h were rinsed twice in ice-cold 0.1% BSA/KRH buffer, scraped off with a scraper, and resuspended in 200 μL of HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail. Twenty microliters of the cell suspension was mixed in an equal amount of RIPA buffer to prepare the whole protein. The rest of the suspension was homogenized using a microtube pestle (three rotations/stroke, twenty strokes) and 27-gauge syringe (60 passages). Subcellular fractions were isolated by differential centrifugation. After 2,000 × g for 10 min at 4°C, supernatant was moved into a new tube. The pellet was homogenized as aforementioned in freshly added HES buffer supplemented with inhibitors, and its supernatant prepared by the centrifugation (2,000 × g for 10 min at 4°C) was collected into the tube. The plasma membrane fraction was precipitated by centrifugation at 18,000 × g for 20 min at 4°C. This pellet was resuspended in HES buffer supplemented with inhibitors, and centrifuged again at 2,000 × g for 10 min at 4°C to clean up contaminating material. The supernatant from this spin was centrifuged again at 18,000 × g for 20 min at 4°C, and then the obtained pellet was lysed with RIPA buffer.

Western blotting. Aliquots of the plasma membrane fraction and whole protein were separated on 8% polyacrylamide gels and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). After blocking with TBS-T (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween20) containing 5% (w/v) nonfat dry milk, the membranes were treated with anti-GLUT4 antibody (1,000 : 1, Novous Biologicals, Littleton, CO) or antipan-cadherin antibody (1,000 : 1, Cell Signaling Technologies, Beverly, MA) overnight at 4°C, followed by the HRP-conjugated secondary antibody for 1 h at room temperature. The blots were developed using Immunostar LD and detected with Fusion SL4 imaging system (Vilber Lumat, Marne-la-Vallée, France).

Gas chromatography-mass spectrometry (GC-MS) analysis. The ETOAc-soluble fraction (1 mg) dissolved in acetonitrile (200 μL) containing tridecanoic acid (20 μg) as an internal control was subjected to trimethylsilyl derivatization with trimethylsilyl imidazole (200 μL) at 60°C for 30 min. GC-MS analysis was performed using a GCMS-QP2010 instrument equipped with an AOC-20i autosampler (Shimadzu, Kyoto, Japan). Trimethylsilylated samples were injected in split mode (1/100) at 290°C and separated on a DB-5MS column (30 m length, 0.25 mm id, 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA) with helium as the carrier gas. The detection of triterpenoids was performed under the following conditions: initial oven temperature of 115°C (1 min hold), followed by 20°C/min up to 200°C, 5°C/min up to 290°C (2 min hold), 15°C/min up to 300°C (2 min hold), and 2°C/min up to 320°C (1.08 min hold) (13); interface temperature, 230°C; ion source temperature, 230°C; electron impact, 70 eV; acquisition mode, m/z 35–700 scan. Standard curves were created by serially diluting trimethylsilylated triterpenoids (3.13–200 μg/mL, R>0.993) and used to quantify the concentration of triterpenoids in the ETOAc-soluble fraction. Accuracy of triterpenoic acids...
in the standard curve were 92.2–101% (BNA), 92.0–103% (BLA), 88.6–101% (ONA), 98.1–114% (OLA), 88.6–101% (UNA), and 94.7–108% (ULA). Inter- and intra-assay precisions (n = 5) of triterpenoic acids were 3.3–8.7% and 1.8–6.2%, respectively.

Statistical analysis. All data are represented as means±SD from at least three independent experiments. Comparisons between groups were evaluated by Dunnett’s test, Tukey-Kramer’s test, or Student’s t-test using SPSS 16.0J (SPSS Inc., Chicago, IL). Significance was reached at values of p < 0.05.

RESULTS

Identification of triterpenic acids as glucose uptake stimulators in L6 myotubes

An enzymatic 2DG uptake assay is a convenient technique for high-throughput measurement of glucose uptake activity in cells and tissues (10, 11). By bioassay-guided separation of a Z. jujuba EtOAc extract, three triterpenic acids, namely, betulonic acid (1), oleanonic acid (2), and betulinic acid (3), were isolated and identified as active compounds stimulating glucose uptake in L6 myotubes (Fig. 1). First, four of seven fractions obtained by the open column chromatography enhanced glucose uptake in L6 myotubes (Fig. 2A). In these fractions, 10% and 20% EtOAc fractions were high yield and showed similar components by TLC analysis (data not shown).

Hence, the mixture of 10% and 20% EtOAc fractions was separated to 14 subfractions by open column chromatography, which were then estimated their activity. Subfractions 6 to 11 induced glucose uptake by over 1.5 fold compared with the vehicle control (Fig. 2B). Among them, high yield subfractions (7, 9, and 10) were subjected to further purification by preparative TLC and/or HPLC, and finally, three triterpenic acids, betulonic acid (7), oleanonic acid (9), and betulinic acid (10) were isolated as the active compounds. Insulin (0.1 μM), the positive control, stimulated glucose uptake in L6 myotubes by 1.7 fold relative to the negative control, while the three triterpenic acids similarly caused a 1.5- to 2.2-fold increase in this activity in a dose-dependent manner (Fig. 3A). Semi-

Fig. 3. Glucose uptake activity and cytotoxicity in triterpenoid-treated L6 myotubes. L6 myotubes were treated with insulin (0.1 μM), DMSO (vehicle), and triterpenoids (2–50 μM) for 4 h at 37˚C. Glucose uptake activity (A) was then measured as described in “Materials and Methods.” For the estimation of cytotoxicity, crystal violet staining of cells was performed after 4 and 24 h incubation with DMSO and triterpenoids (50 μM) (B). *p<0.05 vs. the DMSO group. BLA, betulinic acid; BNA, betulonic acid; OLA, oleanolic acid; ONA, oleanonic acid; I, insulin; ULA, ursolic acid; UNA, ursonic acid; V, vehicle.

by the open column chromatography enhanced glucose uptake in L6 myotubes (Fig. 2A). In these fractions, 10% and 20% EtOAc fractions were high yield and showed similar components by TLC analysis (data not shown). Hence, the mixture of 10% and 20% EtOAc fractions was separated to 14 subfractions by open column chromatography, which were then estimated their activity. Subfractions 6 to 11 induced glucose uptake by over 1.5 fold compared with the vehicle control (Fig. 2B). Among them, high yield subfractions (7, 9, and 10) were subjected to further purification by preparative TLC and/or HPLC, and finally, three triterpenic acids, betulonic acid (7), oleanonic acid (9), and betulinic acid (10) were isolated as the active compounds. Insulin (0.1 μM), the positive control, stimulated glucose uptake in L6 myotubes by 1.7 fold relative to the negative control, while the three triterpenic acids similarly caused a 1.5- to 2.2-fold increase in this activity in a dose-dependent manner (Fig. 3A). Semi-

Fig. 4. Glucose transporter 4 (GLUT4)-dependent glucose uptake in L6 myotubes treated with triterpenoids. (A) L6 myotubes were treated with insulin (0.1 μM), DMSO (vehicle), and triterpenoids (50 μM) for 4 h at 37˚C. Glucose uptake activity in DMSO (closed bar) or indinavir (open bar, 100 μM)-containing Krebs-Ringer-HEPES buffer was then measured as described in “Materials and Methods.” (B) Following the treatment with insulin (0.1 μM), DMSO (vehicle), and triterpenoids (50 μM) for 4 h at 37˚C, the cells were homogenized to prepare the plasma membrane fraction and whole protein, which were then subjected to Western blotting. The GLUT4 band intensity was normalized to that of cadherin. Results shown are representative of three independent experiments. *p<0.05 vs. the DMSO group. BLA, betulinic acid; BNA, betulonic acid; OLA, oleanolic acid; ONA, oleanonic acid; I, insulin; ULA, ursolic acid; UNA, ursonic acid; V, vehicle.
synthesized ursonic acid, which is a positional isomer of oleanonic acid and known to be contained in *Z. jujuba* fruits (14), also up-regulated glucose uptake activity. At a concentration of 50 μM, the three triterpenoids had no effect on cell viability up to 24 h (Fig. 3B). In contrast, oleanolic acid and ursolic acid, which are also known *Z. jujuba* components, failed to stimulate glucose uptake in spite of the absence of cytotoxicity and reduced cell viability at 24 h (Fig. 3B). Insulin-stimulated glucose uptake in L6 myotubes was affected by the GLUT4 inhibitor indinavir, with 75% suppression (Fig. 4A). In addition, the effects of triterpenoids (50 μM) were attenuated by this inhibitor by 63 to 83%, a difference that was statistically significant. Furthermore, triterpenoids increased the GLUT4 level in the plasma membrane without affecting its total level in whole proteins (Fig. 4B).

**Comparison of glucose uptake stimulation by *Z. jujuba* extracts from China, South Korea, and Japan**

*Jujube* fruits are popular as traditional medicinal foods in China and South Korea. In Japan, jujube cultivation commenced in Fukui in 1998, with processed forms such as extracts, confectionaries, and tea bags now commercially available. We therefore compared the glucose uptake potency of *Z. jujuba* from South Korea and Japan with that of Chinese jujube, the source of the identified triterpenoids. GC-MS spectra of jujube EtOAc extracts differed among the three countries, with variations in triterpenic acid content also detected (Fig. 5). Chinese jujube had the lowest betulonic and betulinic acid contents but the highest ursonic and ursolic acid levels (Fig. 6A). Jujube samples from South Korea and Japan had similar triterpenic acid compositions, while oleanonic and oleanolic acids were more abundant in Japanese than South Korean jujube. Total amounts of glucose uptake-promoting triterpenoids were 1.8, 3.2, and 3.5 mg/g dry weight in Chinese, South Korean, and Japanese jujube, respectively. In parallel with these amounts, glucose uptake activity in L6 myotubes exposed to jujube extracts increased in the following order relative to non-treated controls: China (1.2 to 1.6 fold), South Korea (1.4 to 1.8 fold), and Japan (1.4 to 2.0 fold) (Fig. 5B).

**DISCUSSION**

In this study, we observed that a jujube methanol
extract had a glucose uptake-promoting effect on rat L6 myotubes, with betulonic, betulinic, oleanonic, and ursonic acids identified as the active compounds (Figs. 1 and 3). Intriguingly, oleanonic acid and ursolic acid did not promote glucose uptake and were highly toxic to myotubes compared with oleanonic and ursonic acids. Considering that betulonic acid was found to be more potent than betulinic acid, the keto group at the C3 position may be an important factor in glucose-uptake promotion and reduction of cytotoxicity.

The active triterpenoids identified in jujube may enhance glucose uptake in L6 myotubes through GLUT4 translocation (Fig. 4). It is possible that triterpenoids activate the insulin signaling, but the underlying mechanism remains to be clarified. Corosolic acid (2α-hydroxysalicylic acid) from banana leaves acts as a hypoglycemic compound in KK-Ay type 2 diabetic mice (15). This compound induces the translocation of GLUT4 in hindlimb muscle of KK-Ay mice (15), probably via inhibition of protein tyrosine phosphatases (PTPs) (16). Ursolic acid and its synthetic derivative inhibit PTP1B activity, with the latter exhibiting 10-fold higher inhibition than the parent compound and enhancing glucose uptake in L6 myotubes (17). Oleanolic acid also has effects on PTP1B inhibition and anti-diabetic activity of streptozotocin-induced diabetic rats (18). Furthermore, TGR5, a G protein-coupled bile acid receptor expressed in heart and skeletal muscle (19, 20), is known to recognize betulinic and oleanolic acids (21, 22). In our study, however, oleanolic and ursolic acids had no effect on glucose uptake in L6 myotubes, which suggests that the active triterpenoids may induce GLUT4 translocation in a PTP1B- and TGR5-independent manner. We plan to investigate the action mechanisms of these triterpenoids in the near future.

Japanese jujube had a stronger promotional effect on glucose uptake than jujube from the other two studied countries (Fig. 6B), presumably because Japanese jujube had higher amounts of active triterpenoids (3.5 mg/g total dry weight vs. 3.2 and 1.8 for South Korean and Chinese jujube, respectively). We observed additional peaks in the chromatogram of the Japanese jujube extract (Fig. 5C) that may correspond to β-sitosterols and, probably, triterpenic acids according to the GC-MS database. β-Sitosterol has been shown to enhance glucose uptake in L6 myotubes (23), suggesting that Japanese jujube extract may contain a large amount of unidentified compounds acting as the glucose uptake enhancer, and thus showed the highest activity among the three countries. The Japanese jujube extract at the tested concentration (75 μg/mL) may have around 0.6 μM of the active triterpenoids (Fig. 6A) and caused a 2-fold increase of glucose uptake in L6 myotubes (Fig. 6B), while the activity of each triterpenoid at 2 μM was weak (about a 1.5-fold increase) or inactive (Fig. 3A). These results also indicate the contribution of unidentified active compounds, such as other types of triterpenic acids (14) and plant sterols (23), in the Japanese jujube extracts as well as the possibility of the synergistic activation of a cell signaling pathway leading to the GLUT4 translocation by the active triterpenoids. In this study, we focused on the highly active EtOAc fractions (10% and 20%) obtained by the first fractionation, while two other fractions (0% and 40%) also indicated potent activity (Fig. 2A). These fractions may contain other types of active compounds which act synergistically with and/or are more potent than the active triterpenic acids identified in this study. We would expect to see a similar triterpenic acid composition for jujube from South Korea and Japan, as Japanese jujube originated from young trees from Miryang, South Korea. Contrary to this prediction, however, the oleanonic acid content of Japanese jujube was higher than that of South Korea, which suggests that an aspect of the cultivation environment, such as soil composition, affects the amount of a given secondary product.

**CONCLUSION**

In conclusion, we identified four triterpenoids in dried
jujube fruits that act as glucose uptake-promoting compounds, namely, betulonic, betulinic, oleanonic, and ursonic acids. In addition, our findings suggest that Japanese jujube is a promising functional food for preventing and improving hyperglycemia. The anti-hyperglycemic activity of the identified triterpenoids and Japanese jujube extracts will be investigated in vivo in the near future.

**Funding**

This study was supported in part by a grant from the Hokuriku Industrial Advancement Center.

**Acknowledgments**

We gratefully acknowledge the GC-MS technical assistance of Mr. Yoshibu Takagi and Dr. Takao Hibi of Fukui Prefectural University (Fukui, Japan). We also thank Dr. Dong Chan Yoo of Kokando Co., Ltd. (Toyama, Japan) for help in obtaining information about tested Z. jujuba fruits from South Korea.

**REFERENCES**