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Original Article

Identification of baicalin from Bofutsushosan and Daisaikoto as a potent inducer of glucose uptake and modulator of insulin signaling-associated pathways

Yu-Ting Kuo [a], Chih-Chan Lin [b], Hsiao-Tzu Kuo [c], Jui-Hsiang Hung [d], Ching-Hsuan Liu [e,f], Alagie Jassey [g], Ming-Hong Yen [h], Shu-Jing Wu [c,**], Liang-Tzung Lin [e,i,*]

[a] Department of Medical Imaging, Chi Mei Medical Center, No. 901 Zhonghua Road, Yongkang District, Tainan, 710, Taiwan
[b] Department of Medical Research, Chi Mei Medical Center, No. 901 Zhonghua Road, Yongkang District, Tainan, 710, Taiwan
[c] Department of Nutritional Health, Chia-Nan University of Pharmacy and Science, 60 Erh-Jen 1st Road, Jen-Te Hsiang, Tainan, 717, Taiwan
[d] Department of Biotechnology, Chia-Nan University of Pharmacy and Science, 60 Erh-Jen 1st Road, Jen-Te Hsiang, Tainan, 717, Taiwan
[e] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, No. 250, Wu-Hsing Street, Taipei, 11031, Taiwan
[f] Department of Microbiology & Immunology, Dalhousie University, 5850 College Street, Halifax, Nova Scotia, B3H 4R2, Canada
[g] International Ph.D. Program in Medicine, College of Medicine, Taipei Medical University, No. 250, Wu-Hsing Street, Taipei, 11031, Taiwan
[h] Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, No. 100 Shih-Chuan 1st Road, Kaohsiung, 807, Taiwan
[i] Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, No. 250, Wu-Hsing Street, Taipei, 11031, Taiwan

ABSTRACT

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by hyperglycemia that can lead to long-term complications including heart diseases, stroke, retinopathy, and renal failure. Treatment strategies include stimulating glucose uptake and controlling blood glucose level. Bofutsushosan (BOF) and Daisaikoto (DAI) are two herb-based kampo medicines that have been demonstrated to improve metabolism-associated disorders including obesity, hyperlipidemia, and nonalcoholic fatty liver. Given their bioactivities against metabolic syndromes, we explored in this study the effect of BOF and DAI extracts on glucose absorption and used them as source to identify phytochemical stimulator of glucose absorption. Glucose uptake and mechanistic studies were evaluated in differentiated C2C12 skeletal muscle cells, and HPLC analysis was used to determine the molecular bioactive constituents. Our results indicated that the ethanolic extracts of BOF and DAI (BOFEE and DAIEE, respectively) enhanced the glucose uptake ratio in the differentiated C2C12 cells, and further analysis identified the flavone baicalin as a major constituent capable of efficiently stimulating glucose absorption. Mechanistic studies revealed that the effect from baicalin involved the activation of IRS-1 and GLUT-4, and implicated the AMPK, PI3K/Akt, and MAPK/ERK signaling cascades. Due to its potency, we suggest that baicalin merit further evaluation as a potential candidate anti-hyperglycemic agent for the treatment and management of T2DM.

1. Introduction

Chronic diseases such as type 2 diabetes mellitus (T2DM), obesity, hypertension, and dyslipidemia, exhibit impaired glucose and lipid metabolism. T2DM accounts for over 95% of all cases of diabetes globally [1,2], and it is a known major risk factor for cardiovascular diseases, metabolic syndromes, hyperlipidemia, renal failure, and retinopathy [3,4]. In treating diabetes mellitus, control of the blood glucose level is important. Various therapeutic strategies have been investigated to achieve this objective, such as insulin therapy to directly increase insulin concentration in the blood, insulin-sensitizing agents to enhance the response to endogenous insulin, sulfonylurea to enhance insulin secretion from pancreatic β cells, and α-glucosidase inhibitor to attenuate the rapid elevation of blood glucose after food intake by preventing the degradation of polysaccharides in the small intestine [5]. However, hypoglycemia and weight gain are the main side effects of conventional insulin secretagogues such as the sulfonylureas [6]. In addition, risks of the insulin sensitizers, the thiazolidinediones such as rosiglitazone and pioglitazone, include weight gain, anemia, edema, congestive heart failure, and pulmonary edema [6]. Developing alternative anti-diabetic agents and supportive treatments to alleviate the symptoms could help improve current therapeutic strategies against T2DM.

Skeletal muscle plays an important role in maintaining glucose homeostasis. About 75% of postprandial blood glucose clearance takes place in this tissue and two molecular mechanisms are responsible for the glucose transport, including the insulin signaling pathway and the activation of AMP-activated protein kinase (AMPK) pathway [7]. In insulin signaling, receptor activation leads to receptor tyrosine kinase activity and phosphorylation of insulin receptor substrate (IRS) proteins including IRS-1. This in turn promotes the downstream PI3-kinase (PI3K)/Akt and the MAP kinase (MAPK)/ERK cascades that are responsible for insulin's metabolic and mitogenic actions on the cell, respectively. Through the metabolic PI3K/Akt arm, insulin accelerates glucose uptake through phosphorylation and activation of Akt, leading to glucose transporter-4 (GLUT-4) translocation from intracellular storage to the plasma membrane [8]. T2DM is characterized by defects in insulin signaling, including decrease in insulin-stimulated receptor tyrosine kinase activity and the receptor-mediated IRS phosphorylation, which are considered as mechanisms for insulin resistance [9]. In addition, muscle contraction/exercise through the activation of AMPK also leads to GLUT-4 translocation from the cytosol to the plasma membrane [10]. Activation of AMPK leads to the phosphorylation and regulation of a number of downstream targets, which promote insulin sensitivity, suppress the production of hepatic glucose, induce glucose uptake in muscle tissue, block fatty acid synthesis, and stimulate lipid oxidation [11]. The latter is caused by AMPK regulation of acetyl-coenzyme A carboxylase (ACC), which in its active form (dephosphorylated) can catalyze the conversion of acetyl-CoA to malonyl-CoA in the lipid synthesis pathway. Phosphorylation of AMPK results in phospho-ACC, the inactive form, leading to inhibition of the acetyl-CoA to malonyl-CoA conversion, and promotes the transportation of fatty acids into the mitochondria for oxidation [11]. Enhancing glucose uptake through modulation of the signaling molecules in the insulin and AMPK cascades could be beneficial for the treatment of T2DM.

Currently there is an important need to develop novel anti-hyperglycemic agents that are safe and effective. Traditional

herbal medicines including *kampo* medicines are a potential source for the development of such drugs. These include the *kampo* medicines Bofutsushosan (BOF) and Daisaikoto (DAI), which have been documented to exert effects against metabolic syndromes including obesity, hyperlipidemia, and nonalcoholic fatty liver disease [12—15]. In addition, several components in BOF and DAI such as baicalin and paeoniflorin [12,16,17] are also known to improve diabetes- or obesity-related metabolic dysfunctions and inflammatory responses including glucose intolerance, insulin resistance, fatty liver, and neuropathies *in vitro* and in animal models [18—24]. Given their potential activity against obesity and metabolic conditions, in this study, we examined the treatment effect of ethanolic extracts from BOF and DAI on glucose uptake and further identified baicalin as a major molecular constituent that is at least partially responsible for this effect. The underlying mechanistic impact of baicalin on T2DM-related pathways were subsequently investigated.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), insulin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT), penicillin, streptomycin, emodin, baicalin, sennoside A, paeoniflorin, and chrysophanol were purchased from Sigma—Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and horse serum (HS) were obtained from GIBCO BRL (Gaithersburg, MD, USA). 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) was obtained from Molecular Probes (Eugene, OR, USA). Anti-IRS-1 (Cat#2390), anti-phospho-IRS-1 (Ser612; Cat#3203), anti-ACC (Cat#3676), anti-phospho-ACC (Ser79; Cat#3661), anti-Akt (Cat#9272), anti-phospho-Akt (Ser473; Cat#4058), anti-AMPK (Cat#5832), anti-phospho-AMPK (Thr172; Cat#2531), anti-PI3K (Cat#4257), anti-p38 MAPK (Cat#9212), anti-phospho-p38 MAPK (Thr180/Tyr182; Cat#9216), anti-ERK1/2 MAPK (Cat#9102), anti-phospho-ERK1/2 MAPK (Thr202/Tyr204; Cat#9101), anti-GLUT-4 (Cat#2213), HRP-linked anti-mouse IgG (Cat#7076), and HRP-linked anti-rabbit IgG (Cat#7074) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin antibody (Cat#A5441) was purchased from Sigma—Aldrich. AMPK-specific siRNA (Cat#SC-45313) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other chemicals are of analytical grade.

2.2. Cell culture

The C2C12 myoblast cell line (ATCC CRL-1772) derived from mouse skeletal muscle was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. To induce differentiation, the cells were incubated with DMEM supplemented with 2% HS at confluence and replaced every 2 days for the next 4 days. Differentiation was determined by microscopic observation of myotube formation and assessment of creatine kinase activity as previously described [25]. All experiments were performed on differentiated C2C12 cells.

2.3. Plant materials and extracts preparation

Bofutsushosan (BOF) is composed of 18 herbal ingredients: *Atractylodes macrocephala* (1 g), *Schizonepeta tenuifolia* (1 g), *Forsythia suspensa* (1 g), *Ephedra sinica* (1 g), *Mentha spicata* (1 g), *Saposhinkoviae Radix* (1 g), *Angelica sinensis* (1 g), *Rheum palmatum* (1 g), *Ligusticum chuanxiong* (1 g), *Scutellaria baicalensis* (2 g), *Gypsum Fibrosum* (2 g), *Platycodon grandiflorum* (2 g), *Glycyrrhiza uralensis* (4 g), *Talcum Crystallinum* (6 g), *Gardenia jasminoides* (1 g), *Natrium Sulfuricum* (1 g), *Paeonia lactiflora* (1 g), and *Zingiber officinale* (2 g). Daisaikoto (DAI) is composed of 8 medicinal herbs: *Bupleurum falcatum* (8 g), *Pinellia ternate* (5 g), *S. baicalensis* (3 g), *P. lactiflora* (3 g), *Zizyphus jujube* (2 g), *Citrus aurantium* (2 g), *Z. officinale* (5 g), and *R. palmatum* (2 g). These herbs were purchased from a local herbal medicine store and anatomically authenticated. To prepare the water extracts, each prescription (50 g) was decocted three times with 500 ml of boiling distilled water for 1 h. After removing the supernatant, the residue was further extracted with 500 ml of distilled water under the same condition. The decoction obtained from the two separate extractions were mixed, concentrated, and lyophilized. The dried aqueous extracts of Bofutsushosan (BOFWE) and Daisaikoto (DAIWE) were collected and stored at 4 °C until use. To prepare the ethanolic extracts, 100 g of each BOF or DAI was soaked with 1 L of ethanol (95%) at room temperature for 6 days. After filtering the extracts with filter paper (Advantec, Tokyo, Japan), the filtrate collected was concentrated and lyophilized. The dried ethanolic extracts of Bofutsushosan (BOFEE) and Daisaikoto (DAIEE) were stored at 4 °C until use. The following yields were obtained: BOFWE = 13.10%, DAIWE = 10.50%, BOFEE = 19.00%, and DAIEE = 15.30%. The stock solutions of the extracts were prepared in DMSO and stored at −20 °C until use. Diluted samples were freshly prepared for each experiment with a final DMSO concentration of 0.1%. Negative control samples were always treated with the same amount of DMSO (0.1% v/v) as used in the corresponding experiments. Insulin was included for comparison.

2.4. HPLC assay and analysis

All extracts were performed on a Hitachi chromatograph system (HITACHI Co. Inc., Japan) equipped with a quaternary pump (L-7100), an autosampler (L-7200), and a UV-VIS detector (L-7420). The chromatographic data were recorded and processed with a D-7000 Multi-HSM Manager software. A Mightysil RP-18 GP column (5 μm, 4.6 × 250 mm; Kanto Chemical Co. Inc., Tokyo, Japan) was used at room temperature. The mobile phase was composed of (A) aqueous phosphoric acid (0.1%, v/v) and (B) acetonitrile using a gradient elution of: 100% A at 0—3 min, 100-80% A at 3—15 min, 80-65% A at 15—40 min, 65-40% A at 40—80 min, 40-30% A at 80—100 min, and 100% B at 100—120 min. The sample injection volume was 20 μl and the flow rate was 1.0 ml/min. The detection wavelength was set at 272 nm. To process the samples, 50.0 mg of BOFEE or DAIEE

was combined with 1 ml methanol and then extracted under sonication for 30 min. All the test samples were filtered through a 0.45 μm membrane filter before chromatographic analysis. The HPLC-UV fingerprint standard of extracts were recorded and compared to five standard reference compounds (baicalin, chrysophanol, emodin, paeoniflorin, and sennoside A) for quantitative assessment.

2.5. Cell viability assay

Cell viability was measured by MTT colorimetric assay according to the manufacturer's instructions. Briefly, C2C12 cells were treated with control alone (0.1% DMSO) or various concentrations of the test samples for 24 h. Cells were then washed with PBS before adding 50 μl of FBS-free medium containing MTT (5 mg/ml). After 4 h of incubation at 37 °C, the medium was discarded and the resulting formazan blue crystals in the cells were dissolved in DMSO. The optical density was measured at 550 nm using a plate reader.

2.6. Glucose uptake analysis

Glucose uptake was measured according to a previously described method [26]. C2C12 cells were treated with control alone (0.1% DMSO), insulin only (150 nM; positive control), or different concentrations of the test samples for 24 h. The cells were then starved in low-glucose serum-free DMEM for 12 h and then treated with or without 100 μM of the fluorescent glucose analog 2-NBDG for 30 min. Cellular uptake of 2-NBDG fluorescence was measured using a fluorometer at excitation and emission wavelengths of 465 and 540 nm, respectively.

2.7. Preparation of plasma membrane proteins from skeletal muscle cells

Plasma membrane proteins were disrupted by sonication to release caveolae and then prepared according to an earlier reported method with modifications [27]. Cells were lysed on ice in 10 ml lysis buffer A (0.32 mol/l sucrose, 5 mmol/l Tris-HCl pH 7.5, 120 mmol/l KCl, 1 mmol/l EDTA, 0.2 mmol/l PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Cell debris and nuclei were removed by centrifugation at 1000 × g for 10 min. A plasma membrane-containing pellet was then obtained by centrifugation of the supernatant at 15,000 × g for 30 min. The pellet was subsequently resuspended in buffer B (20 mmol/l HEPES, 10% glycerol, 2% Triton X-100, 1 mmol/l EDTA, 0.2 mmol/l PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin). Finally, the lysates were solubilized for 2 h at 4 °C and then centrifuged for 30 min at 10,000 × g to yield the purified plasma membrane fraction in the collected supernatant.

2.8. AMPK siRNA transfection

Cells (2 × 10^5) were plated in 60 mm plates overnight before transfection with AMPK siRNA (100 nM) using commercial transfection reagent (Qiagen, Hilden, Germany) for 48 h according to the manufacturer's instructions. The efficacy of the AMPK siRNA knockdown was confirmed by Western blotting analysis.

2.9. Western blot analysis

Total protein was extracted from cells and processed by standard Western blotting technique using the following specific primary antibodies: anti-IRS-1 (1:1000), anti-phospho-IRS-1 (1:1000), anti-PI3K (1:1000), anti-Akt (1:1000), anti-phospho-Akt (1:1000), anti-ACC (1:1000), anti-phospho-ACC (1:1000), anti-AMPK (1:1000), anti-phospho-AMPK (1:1000), anti-p38 (1:1000), anti-phospho-p38 (1:2000), anti-ERK (1:1000), anti-phospho-ERK (1:1000), anti-GLUT-4 (1:1000), and anti-β-actin (1:5000). Primary antibody recognition was detected with the respective secondary antibody, either anti-mouse IgG or anti-rabbit IgG antibodies linked to horseradish peroxidase, and analyzed with the ECL Western blotting analysis system (Amersham, Aylesbury, UK). The expression of β-actin was used as loading control.

2.10. Statistical analysis

Data were presented as means ± standard deviation (SD) from three independent experiments. Values were evaluated by one-way ANOVA, followed by Duncan's or Dunnett's multiple range test using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and GraphPad Prism 7 software (San Diego, CA, USA), respectively.
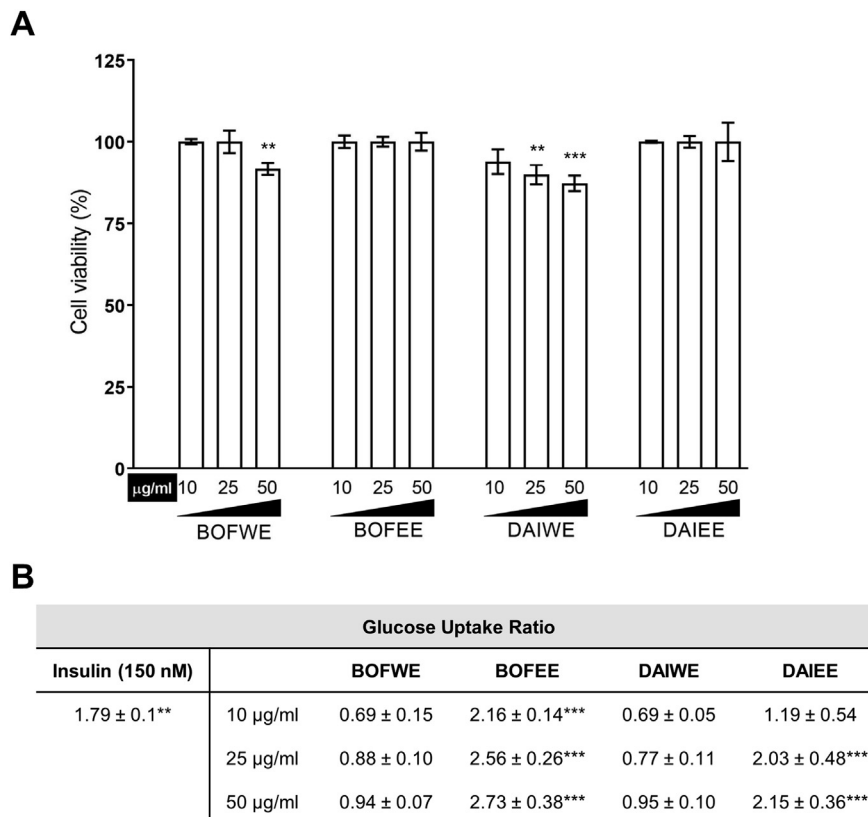
3. Results

3.1. The ethanolic extracts of BOF and DAI induce glucose absorption in C2C12 cells

To test the cytotoxicity of BOFWE, DAIWE, BOFEE, and DAIEE, various concentrations (0, 10, 25, and 50 μg/ml) of the extracts were used to treat the differentiated C2C12 cells. Results demonstrated that BOFEE and DAIEE did not affect C2C12 cell viability compared to BOFWE (91.72 ± 1.83%) and DAIWE (87.25 ± 2.39%) at the highest concentration of 50 μg/ml used (Fig. 1A). Examination of glucose uptake induced by these extracts via absorption analysis of the fluorescent glucose analog 2-NBDG showed that BOFEE and DAIEE have significantly increased C2C12 uptake of glucose ratio in a dose-dependent manner (P < 0.05), whereas BOFWE and DAIWE exerted a limited effect (Fig. 1B). BOFEE also appeared to have a stronger stimulatory activity compared to DAIEE. These results suggest that both BOFEE and DAIEE can significantly promote glucose absorption in C2C12 cells, therefore possessing potential anti-hyperglycemic effects.

3.2. Baicalin is a major bioactive constituent of BOFEE and DAIEE that efficiently stimulates glucose uptake induction in C2C12 skeletal muscle cells

Isolating bioactive compounds from BOFEE and DAIEE could yield potential candidates for development as therapeutics against T2DM. For this purpose, we performed an HPLC analysis to identify the major constituents in the two ethanolic extracts. We used five bioactive compounds (baicalin, chrysophanol, emodin, paeoniflorin, and sennoside A) that are most typically found in the herbal contents of BOF and DAI

A

B

Glucose Uptake Ratio				
Insulin (150 nM)		**BOFWE**	**BOFEE**	**DAIWE**	**DAIEE**
1.79 ± 0.1**	10 µg/ml	0.69 ± 0.15	2.16 ± 0.14***	0.69 ± 0.05	1.19 ± 0.54
	25 µg/ml	0.88 ± 0.10	2.56 ± 0.26***	0.77 ± 0.11	2.03 ± 0.48***
	50 µg/ml	0.94 ± 0.07	2.73 ± 0.38***	0.95 ± 0.10	2.15 ± 0.36***

Fig. 1 – **Effects of water and ethanol extracts of Bofutsushosan (BOFWE, BOFEE) and Daisaikoto (DAIWE, DAIEE) on C2C12 cell viability (A) and glucose uptake ratio (B). Values are means ± SD from three independent experiments; statistical significance compared to 0.1% DMSO control analyzed by one-way ANOVA and Dunnett's multiple comparison test is indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.**
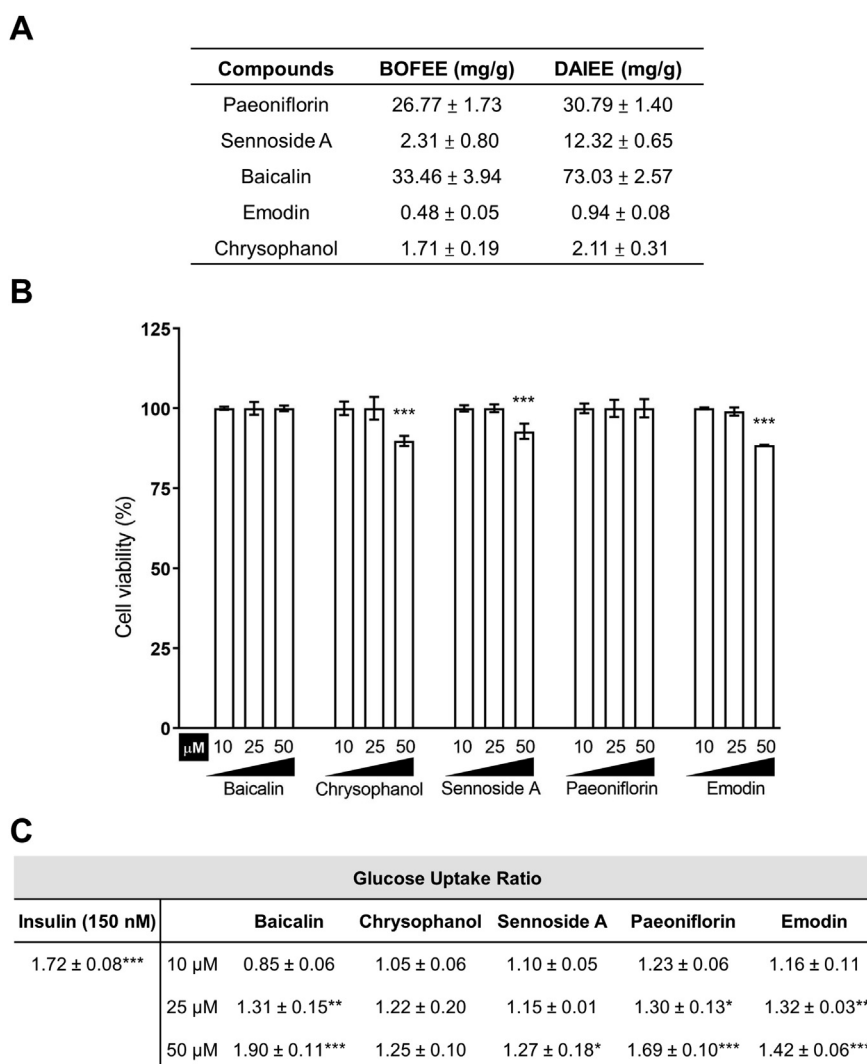
[12,16,17] as reference standards and compared their chromatogram with that from BOFEE and DAIEE. Results revealed that all five small molecules were present in BOFEE and DAIEE with varying concentrations, with the lipophilic flavonoid glycoside baicalin having the highest content level in DAIEE (73.03 ± 2.57 mg/g) and BOFEE (33.46 ± 3.94 mg/g), followed by paeoniflorin (30.79 ± 1.40 in DAIEE and 26.77 ± 1.73 mg/g in BOFEE, respectively) (Fig. 2A).

To examine whether the molecular components identified in BOFEE and DAIEE exert bioactivity on glucose transport, we explored their effect on C2C12 cell viability and glucose uptake induction at various concentrations (0, 10, 25, and 50 µM) using the earlier described methods. Both baicalin and paeoniflorin did not exert observable cytotoxicity at the maximum concentration used (50 µM), whereas chrysophanol, sennoside A, and emodin decreased cell viability by about 10% (Fig. 2B). When tested for their ability to stimulate absorption of 2-NBDG, baicalin induced the highest ratio of glucose uptake in a dose-dependent fashion, followed by paeoniflorin, emodin, sennoside A, and chrysophanol (Fig. 2C). Given its high content in the extracts and also exhibiting the highest glucose uptake activity, these results suggested that baicalin is a major component of BOFEE and DAIEE that efficiently induced glucose absorption in C2C12 cells. Baicalin was therefore chosen for the subsequent experiments.

3.3. The IRS-1 phosphorylation, metabolic PI3K/Akt signaling, and GLUT-4 translocation are involved in baicalin's stimulation of glucose absorption

Due its ability to induce glucose uptake, we next investigated the molecular mechanism associated with baicalin's effect by treating the differentiated C2C12 cells with the natural agent for 24 h and harvesting total protein for Western blot analysis. Lysates were probed for signaling cascade molecules associated with insulin's action and regulation of glucose absorption. As shown in Fig. 3A, treatment with baicalin dose-dependently enhanced IRS-1 phosphorylation (phospho-IRS-1), indicating its activation. Furthermore, at the highest concentration baicalin also enhanced levels of PI3K and phospho-Akt, both of which are also upregulated by insulin treatment (Fig. 3A). Since the PI3K/Akt-mediated metabolic arm of insulin action is also known to regulate the translocation of the glucose transporter, GLUT-4, we also probed for this protein in the plasma membrane of C2C12 cells following baicalin treatment. Interestingly, baicalin dose-dependently increased

A

Compounds	BOFEE (mg/g)	DAIEE (mg/g)
Paeoniflorin	26.77 ± 1.73	30.79 ± 1.40
Sennoside A	2.31 ± 0.80	12.32 ± 0.65
Baicalin	33.46 ± 3.94	73.03 ± 2.57
Emodin	0.48 ± 0.05	0.94 ± 0.08
Chrysophanol	1.71 ± 0.19	2.11 ± 0.31

B

C

Insulin (150 nM)		Baicalin	Chrysophanol	Sennoside A	Paeoniflorin	Emodin
1.72 ± 0.08***	10 µM	0.85 ± 0.06	1.05 ± 0.06	1.10 ± 0.05	1.23 ± 0.06	1.16 ± 0.11
	25 µM	1.31 ± 0.15**	1.22 ± 0.20	1.15 ± 0.01	1.30 ± 0.13*	1.32 ± 0.03**
	50 µM	1.90 ± 0.11***	1.25 ± 0.10	1.27 ± 0.18*	1.69 ± 0.10***	1.42 ± 0.06***

(Glucose Uptake Ratio)

Fig. 2 − Molecular constituents of Bofutsushosan and Daisaikoto ethanol extracts (BOFEE and DAIEE). (A) HPLC analysis-derived quantitation of the molecular constituents (paeoniflorin, sennoside A, baicalin, emodin, and chrysophanol) in BOFEE and DAIEE. (B) Cytotoxicity of the different molecular constituents in C2C12 cells. (C) Effects of the different molecular constituents on C2C12 glucose uptake ratio. Values are means ± SD from three independent experiments; statistical significance compared to 0.1% DMSO control analyzed by one-way ANOVA and Dunnett's multiple comparison test is indicated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

transport of GLUT-4 to the plasma membrane (Fig. 3B), suggesting that the flavonoid's stimulation of glucose uptake also involves this transporter.

3.4. Baicalin's treatment induces activation of MAPK/ ERK and AMPK cascades

To further clarify the impact baicalin on the T2DM-related pathways, we also probed for signaling molecules in the MAPK/ERK and AMPK cascades in C2C12 cells treated with the natural agent for 24 h. As shown in Fig. 3C, a dose-dependent enhancement in phospho-p38 and phospho-ERK is observed with baicalin's treatment, indicating that the mitogenic arm of insulin action also plays a role in mediating baicalin's bioactivity. In addition, baicalin appears to also modestly enhance AMPK phosphorylation at increasing

concentrations, which is accompanied by a significantly upregulated expression of ACC and phospho-ACC (Fig. 3D). These results therefore suggest that baicalin's bioactivity also implicates AMPK and the inactivation of ACC (phosphorylated ACC form).

To better establish the AMPK-glucose uptake relationship in baicalin's treatment, we suppressed AMPK expression by siRNA transfection and treated the C2C12 cells with or without baicalin. RNA interference (RNAi)-targeting with AMPK-specific siRNA efficiently diminished AMPK expression with or without baicalin treatment (Fig. 4A). More importantly, knockdown of AMPK substantially reduced the up-regulated absorption of glucose by baicalin treatment (Fig. 4B), highlighting AMPK's contribution to baicalin's effect in enhancing glucose absorption. Similar result was observed with insulin stimulation of glucose uptake (Fig. 4B).
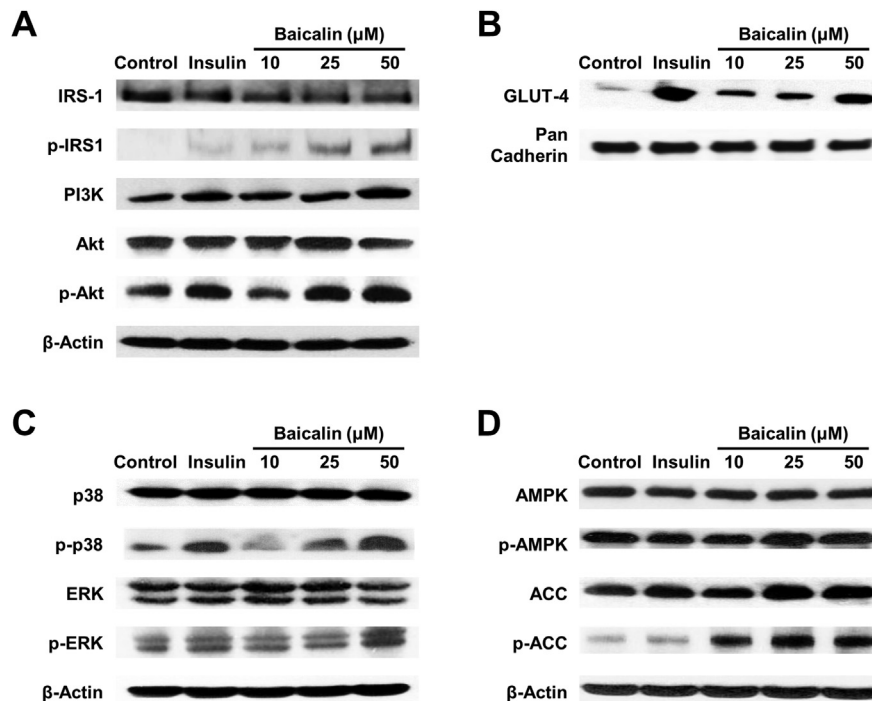
Fig. 3 – Effects of baicalin on the modulation of the antidiabetic pathways in C2C12 cells. (A) The IRS-1 and PIK/Akt-based insulin signaling pathway. (B) The GLUT-4 translocation pathway. (C) The p38/ERK pathway. (D) The AMPK/ACC pathway. Insulin (150 nM) was included for comparison. The cell lysates were subjected to Western blot analysis with antibodies against the indicated proteins. β-actin served as a loading control. Representative blots from three independent experiments are shown.
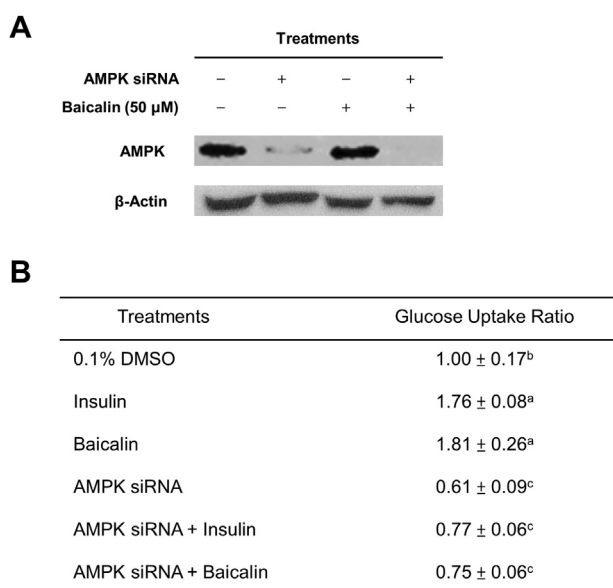
Collectively, the above results suggest that baicalin's ability to trigger glucose uptake implicates various cascades including activation of IRS-1 phosphorylation, the metabolic PI3K/Akt signaling, GLUT-4 translocation, MAPK/ERK signaling, and the AMPK-associated pathway.

4. Discussion

Various natural products and extracts have been demonstrated to stimulate glucose uptake in muscle cells. These include *Astragalus* polysaccharide, saffron (*Crocus sativus* L.), propolis extract, berberine, resveratrol, and tangeretin [28–33]. In this study, we demonstrated that the ethanolic extracts BOFEE and DAIEE exhibited potent activity in enhancing glucose absorption in C2C12 cells. More importantly, we showed that baicalin is a major bioactive constituent of these two herbal extracts that contributes to their effect. Our identification of BOFEE, DAIEE, and their bioactive component baicalin adds to the expanding array of candidate agents that could be further explored as potential anti-hyperglycemic treatments against T2DM.

Induction of GLUT-4 translocation via modulation of the insulin signaling and the activation of AMPK is a potential therapeutic strategy to enhance glucose absorption in T2DM [34,35]. Our results demonstrated that baicalin markedly induced GLUT-4 translocation (Fig. 3B) by up-regulating the phosphorylation of IRS-1 and AMPK (Fig. 3A,D), resulting in

enhanced glucose uptake in the C2C12 skeletal muscle cells (Fig. 2C). Therefore, baicalin may serve as an activator of IRS-1 and AMPK to enhance GLUT-4 translocation and could be useful for application to improve T2DM insulin resistance. Our data is consistent with recent studies that showed baicalin as an inducer of GLUT-4 plasma membrane expression and glucose uptake through the activation of Akt and p38 signaling pathways in the rat L6 myotubes [36] and skeletal muscles of high fat diet-induced obese mice [21]. While both Akt and p38 pathways were involved in GLUT-4 translocation in these studies, the Akt pathway seemed to play the major role for this effect [36]. On the other hand, we also observed the activation of AMPK and downstream inhibition of ACC in the baicalin-treated C2C12 cells (Fig. 3D). A similar effect has been reported in the adipose tissue of normal mice [20] and the hepatic tissue in obese rats and mice [18,19] that were systemically administered with baicalin, resulting in enhanced glucose uptake in the adipocytes and attenuated liver steatosis. In skeletal muscles, AMPK is crucial in regulating glucose uptake and fatty acid oxidation. Current evidence suggests that, upon muscle contraction, exercising, or the stimulation of AMPK activators, AMPK primes the Akt substrate AS160 or TBC1D4 for insulin signaling [37]. In other words, the activation of AMPK promotes Akt signaling pathway and therefore triggers more GLUT-4 translocation for glucose uptake. This may explain why the glucose uptake was significantly decreased when AMPK was suppressed by siRNA knockdown in baicalin-treated cells (Fig. 4B).

A

B

Treatments	Glucose Uptake Ratio
0.1% DMSO	1.00 ± 0.17[b]
Insulin	1.76 ± 0.08[a]
Baicalin	1.81 ± 0.26[a]
AMPK siRNA	0.61 ± 0.09[c]
AMPK siRNA + Insulin	0.77 ± 0.06[c]
AMPK siRNA + Baicalin	0.75 ± 0.06[c]

Fig. 4 – Effects of baicalin on the AMPK signaling pathway. (A) Cells were transfected with or without AMPK siRNA before treated with 50 μM baicalin or 0.1% DMSO control for 24 h. Insulin (150 nM) was included for comparison. The cell lysates were subjected to Western blot analysis with antibodies against the indicated proteins. β-actin served as a loading control. Representative blots from three independent experiments are shown. (B) Effect of AMPK siRNA on the baicalin-stimulated glucose uptake. Values are means ± SD from three independent experiments. Means within the same column but with different superscript are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range tests.

In this study, we identified baicalin as a major bioactive molecule that at least partially contributes to the stimulatory effect of BOFEE and DAIEE on glucose absorption. Although baicalin most significantly enhanced the glucose uptake ratio compared to the other molecular constituents assessed, we could not rule out that additional components in BOFEE and DAIEE could also exert an influence on the glucose transport. Further examination on the molecular contents of BOFEE and DAIEE, and comparative analysis to establish activity correlation, may help better characterize the full function of these extracts in influencing glucose homeostasis and deduce additional phytochemical stimulators of glucose absorption.

In conclusion, this is the first report identifying baicalin as a major component in BOFEE and DAIEE in stimulating glucose uptake in differentiated C2C12 skeletal muscle cells. Owing to its apparent anti-hyperglycemic effect, we suggest that baicalin could be further explored as candidate agent for the treatment of T2DM.

Conflicts of interest

The authors declared that no competing interests exist.

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