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Anti-Diabetic Action of *Cydonia oblonga* Seed Extract: Improvement of Glucose Metabolism via Activation of PI3K/AKT Signaling Pathway

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ABSTRACT

Context: Diabetes mellitus is one of the most common metabolic syndromes characterized by hyperglycemia. *Cydonia oblonga* Miller (Rosaceae) is used in traditional uyghur medicine and is proved to have antioxidant and antihypertensive properties, but the anti-diabetic activity of *C. oblonga* Mill. seeds extracts (CSE) is not yet explored.

Objective: The anti-diabetic ability of CSE and the underlying mechanism of action of CSE were investigated in L6 skeletal muscle cells.

Methods: CSE were evaluated for their inhibitory activity against protein tyrosine phosphatase 1B (PTP1B) *in vitro*. L6 myoblasts were differentiated to myotubes and treated with CSE (0 µg/ml to 100 µg/ml) for 1 h. Cell viability was assessed by MTT assay and cell cytotoxicity was detected by lactate dehydrogenase (LDH) release from L6 cells. The phosphorylation levels of four key proteins (IRS-1, AKT, GSK-3β and AMPK) involve in PI3K/AKT signaling pathway were examined by western blotting.

Results: CSE possess good anti-PTP1B activity with an IC₅₀ value of 3.5 µg/mL. Treatment of L6 skeletal muscle cells with CSE (0 µg/ml to 100 µg/ml) did not affect cell viability. CSE at 12.5 µg/ml increased glucose consumption and glycogen synthesis in L6 myotubes. Western blotting results showed that CSE increased the phosphorylation of IRS-1, AKT and GSK-3β protein in the present of insulin.

Discussion and Conclusion: These results suggested that CSE promoted glucose metabolism by activating PI3K/AKT signaling pathway in L6 cells. The study reported the anti-diabetic effect of CSE on *in vitro* cell model for the first time, which providing potential drug candidates for prevention and treatment of diabetes.

INTRODUCTION

Diabetes mellitus (DM), one of the most prevalent metabolic syndromes, is characterized by constant high levels of blood glucose (hyperglycemia). Type 1 diabetes known as insulin-dependent diabetes is characterized by a lack of insulin production^[1]. Type 2 diabetes is caused by the body's ineffective use of insulin and is the most common form of diabetes, which is closely related with insulin resistance^[2]. Thus; new therapeutic approaches to counter the increasing threat of diabetes mellitus are in

high demand. Chemotherapy is prevalent method for diabetes, such as sulfonylureas, biguanides, and thiazolidinediones, but these drugs are selectively limited and have toxic side effect to kidney and liver. In recent years, there has been an increase in research on the effects of natural plant-derived compounds in diabetes prevention and treatment.

Cydonia oblonga Miller (Rosaceae) is a small tree and its fruit is a pome with many seeds. They are used to produce juice, jam, or jelly, and are exploited to obtain valuable ingredients for the cosmetics and food industries^[3,4]. *C. oblonga* is a traditional Uighur medicine and has antioxidative and antithrombus properties. *C. oblonga* is used to treat or prevent cardiovascular disease in traditional Uyghur medicine^[5-7]. Its fruits and seeds have shown medicinal applications in the treatment of dyspepsia, diarrhea, anemia, hepatitis, gastric ulcers, and sore throat^[8]. *C. oblonga* ethanol leaf extracts significantly and dose-dependently reduced blood pressure which suggests its antihypertensive effect^[9]. Phenolics and organic acids composition of *C. oblonga* leaf was proved to have antioxidant and antiproliferative properties *in vitro* systems^[10]. *C. oblonga* leaf protected rabbit testes and spermatogenesis from damage induced by hypercholesterolemia^[11]. One study reports the phenolic profile and antiproliferative properties of *C. oblonga* leaf and fruit against human kidney and colon cancer cells^[12]. Most of research was performed on the chemical composition of *C. oblonga* fruit and leaf; however, the antidiabetic effect of *C. oblonga* seed is poorly studied.

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of insulin signaling, which dephosphorylates phosphotyrosine residues of insulin receptor (IR) or insulin receptor substrate-1 (IRS-1)^[13,14]. Therefore, it has been a potential drug target for the treatment of type 2 diabetes^[15]. Insulin receptor-substrate-1 (IRS-1) is a docking protein for several receptor tyrosine kinases^[16]. Once phosphorylated, IRS-1 binds to signaling proteins, including phosphatidylinositol 3-kinase (PI3K), that mediate the metabolic and growth functions of insulin^[17]. AKT is a key protein involved in PI3K pathway, and regulates downstream metabolic enzymes^[18]. Moreover, AKT activation promotes glycogenesis by phosphorylation and inactivation of glycogen synthase kinase-3 β (GSK-3 β , which down regulates glycogen synthase)^[19,20].

In our present study, *C. oblonga* seed extract was acquired using organic solvent extraction, macroporous resin for separation and purification, and were found to possess inhibition on PTP1B *in vitro*. With this background, cell-based biological assays were carried out to investigate the potential anti-diabetic effect of CSE *in vitro*. CSE was found to increase glucose consumption and glycogen synthesis in L6 myotubes. Further study is to probe its mechanism of action on glucose lowering. CSE was observed to up regulate phosphorylated IRS, AKT and GSK-3 β in L6 myotubes by western blot. These results suggested that CSE improves glucose metabolism through induction of glycogenesis. The increase of glycogen is likely a consequence of activation of insulin-stimulated PI3K/AKT signaling pathway. For all we know, this potential anti-diabetic bioactivity and action mechanism of CSE in L6 myotubes is reported for the first time.

MATERIALS AND METHODS

Reagents and chemicals

All cell culture supplements such as Fetal bovine serum (FBS), antibiotics, Dulbecco's modified Eagle's medium (DMEM), and Trizol reagent were purchased from Gibco® Life Technologies (Carlsbad, CA, USA). Specific antibodies for phospho-AKT, phospho-GSK-3 β , phospho-AMPK, and phospho-IRS-1, AKT, GSK-3 β and AMPK were obtained from Cell Signaling Technology (Danvers, MA, USA). Specific antibody for PTP1B was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and secondary antibodies were obtained from GE Healthcare Life Sciences. Human recombinant insulin was obtained from Sigma Chemical Corp. (St. Louis, MO, USA). Electrophoresis reagents, including Bis-Tris gels, running buffer, and poly (vinylidenedifluoride) (PVDF) membrane, were obtained from Invitrogen (Carlsbad, CA, USA). *C. oblonga* seeds were purchased from the China Xinjiang Uyghur Pharmaceutical Co., Ltd. in October 2011.

Preparation of *C. oblonga* seeds extract (CSE)

Dried *C. oblonga* seeds were purchased from Hetian region of Xinjiang in China and were identified by Prof. Shulman from the China Xinjiang Uyghur Pharmaceutical Co., Ltd. in October 2011. *C. oblonga* seeds were made powder and defatted using petroleum ether and then subjected to ethanolic extraction. About 460 g of seed powder was mixed with 70% ethanol (1:10) and extracted by rotating distillation at 60°C for 2.5 h. The reflux liquid was re-extracted three times under the same condition to ensure the complete extraction and then evaporated by rotary evaporator to remove residual alcohol. With macroporous absorption resin (AB-8) to absorb and purify, it was eluted using water and ethanol with different concentrations (10%, 30%, 50%, 70% and 95%). The extraction was combined and evaporated to dryness under reduced pressure. The residue was lyophilized and stored at -70°C and later this was used for *in vitro* and *in vivo* studies.

Propagation and maintenance of L6 cells

L6 rat skeletal myoblasts were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. L6 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified 5% CO₂ atmosphere. After 2 days of plating, the cells had reached 70% to 80% confluence (day 0). Differentiation was then induced by replacing the growth medium with DMEM supplemented with 2% horse serum and 20 nM insulin (differentiation medium I). After 3 days, the culture medium

was change to DMEM containing 2% horse serum (differentiation medium II). Myotube formation was achieved after 5-7 days of incubation, and the cells were used for subsequent experiments.

Evaluation of protein tyrosine phosphates 1B (PTP1B) activity

CSE were evaluated for their inhibitory activity against PTP1B as described [21] *p*-Nitrophenyl phosphate (*p*-NPP) with a final concentration of 3.5 mM in the assay mixture (200 μ L) was used as substrate. The mixture of CSE (1.25 μ g/mL to 40 μ g/mL), PTP1B, and substrate was incubated at 30 °C for 30 min and immediately subjected to a 96-well microplate spectrophotometer under 405 nm. The PTP1B inhibitor sodium orthovanadate was used as positive control.

Assessment of cell viability and cytotoxicity

Cell viability was assayed by MTT analysis in 96-well tissue culture plates as described previously [22]. CSE treatment after 24 h, culture medium was removed from the wells, and MTT reagent at a concentration of 0.5 μ g/mL in DMEM was added to each well. After 4 h incubation at 37 °C, MTT reagent in DMEM was removed and then the blue-colored formazan product was solubilized in DMSO for 20 min. The absorbance of converted dye was measured at a wavelength of 570 nm. For the cytotoxicity assay, the amount of lactate dehydrogenase (LDH) that leaked from damaged cells into culture medium was measured using LDH reagent kit (Nanjing Jiancheng Bioengineering Institute). Absorbance was measured at a wavelength of 450 nm.

Glucose consumption and Lactic acid assays

Glucose consumption was conducted as described previously [23]. L6 skeletal muscle cells were cultured in 96-well tissue culture plates for 8 h in 37 °C. Then culture medium was replaced by serum-free DMEM supplemented with 0.25% BSA and treated with 12.5 μ g/mL CSE and/or insulin (final concentration 100 nmol/L) for 24 h. The glucose concentration in the culture medium was determined using Glucose Oxidase Assay Kit (Applygen Technologies Inc., Beijing, PR China), The amount of glucose consumption was calculated by subtracting the glucose from the control (blank well). To measure lactic acid production, cells were treated using the same culturing procedure as described above, and lactate concentration was detected using Lactic Acid Detection Kit (Nanjing Jiancheng Bioengineering Institute, China)

Glycogen content and hexokinase activity assays

Glycogen content in the presence or absence of insulin was assessed in L6 cells grown in 6 well plates using Muscle Glycogen Assay Kit (Nanjing Jiancheng Bioengineering Institute, China). Results were normalized by protein concentration measured by Thermo Scientific Pierce BCA Protein Assay Kit, and glycogen content was presented as micrograms glucose equivalent per well and expressed as mg/g of the cells. Hexokinase activity was detected by Hexokinase Assay Kit (Nanjing Jiancheng Bioengineering Institute, China).

Western blotting

The whole cells were lysed with lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% igepal CA-630 [Sigma-Aldrich], 10 mM MgCl₂, 1 mM EDTA, 25 mM NaF, 1 mM Na₃VO₄, and EDTA-free protease inhibitor mix [Sigma-Aldrich]). Protein concentration was measured using Pierce® BCA Protein Assay Kit (Thermo Scientific). Equal amounts of pre-denatured protein (50 μ g/lane) were separated by SDS-PAGE on 10% polyacrylamide gels and electro transferred onto PVDF membranes. After incubating with blocking buffer (5% BSA in Tris-buffered saline with 0.1% Tween-20) for 1 h, the membranes were reacted with primary antibodies (1:1000 dilution) against phospho-AKT, phospho-GSK-3 β , phospho-AMPK, phospho-IRS-1, AKT, GSK-3 β , AMPK, Glut4 and β -actin with shaking overnight at 4°C. After three times washes with TBST buffer, the membranes were further incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. The immune reactive bands were detected with ECL Western Blotting Detection Reagent (GE Healthcare, UK).

STATISTICAL ANALYSIS

Data are represented as the means \pm SD. All experiments in cells were performed at least in triplicate. When multiple comparisons were performed, the significance was analyzed by one-way ANOVA (SPSS 12.0). **P* < 0.05 and ***P* < 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of CSE on PTP1B activity *in vitro*

Protein tyrosine phosphatase 1B (PTP1B), a major negative regulator of insulin signaling, has emerged as an attractive drug target for the treatment of type 2 diabetes [24]. We found that CSE inhibited PTP1B activities up to 40% and 60% at concentrations of 2.5 and 5 μ g/mL, respectively (**Figure 1**). The *in vitro* assay results show that CSE have significant inhibitory activity against PTP1B with an IC₅₀ value of 3.5 μ g/mL \pm 0.21 μ g/mL compared with the positive control sodium orthovanadate (IC₅₀ = 2.15 \pm 0.47). it is suggested that CSE is a good PTP1B inhibitor and has potential anti-diabetic activity *in vitro*.

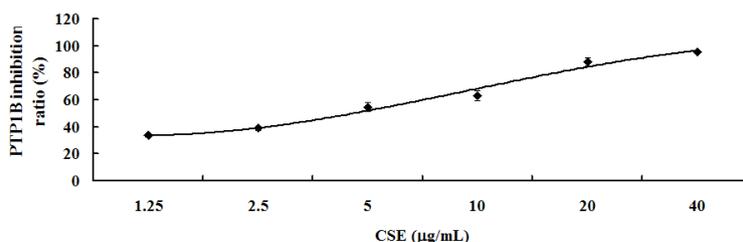
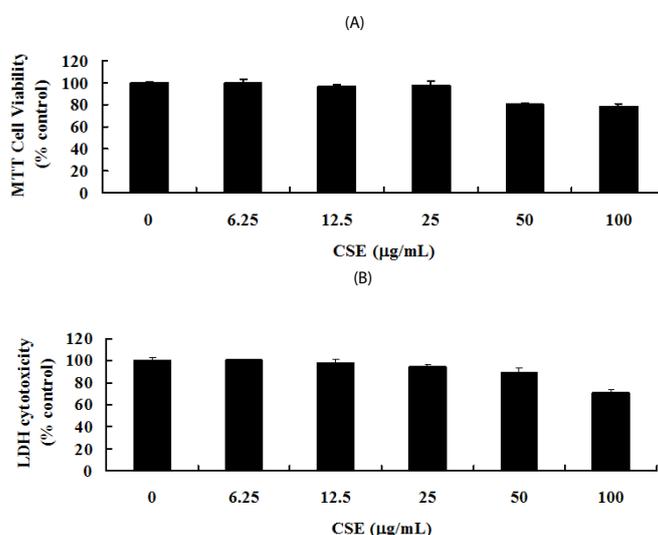


Figure 1. Effects of CSE extracts on PTP1B inhibitory activity *in vitro*. The values are means \pm SD of three independent experiments. CSE concentration from 1.25 $\mu\text{g}/\text{mL}$ to 40 $\mu\text{g}/\text{mL}$.

Effect of CSE on cell viability and cytotoxicity

Cell viability was assessed by MTT analysis and cell cytotoxicity was detected by lactate dehydrogenase (LDH) release from L6 cells. Neither cell viability (**Figure 2A**) nor LDH cytotoxicity (**Figure 2B**) were changed at 0 $\mu\text{g}/\text{mL}$ to 25 $\mu\text{g}/\text{mL}$ concentration range. The data appeared to reduce slightly at the concentration of 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ but was not statistically significant. Treatment of L6 skeletal muscle cells with CSE (0 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$) did not affect cell viability. The results revealed that CSE concentration at 12.5 $\mu\text{g}/\text{mL}$ was non-toxic on L6 cell growth.



* $P < 0.05$, ** $P < 0.01$ vs. control.

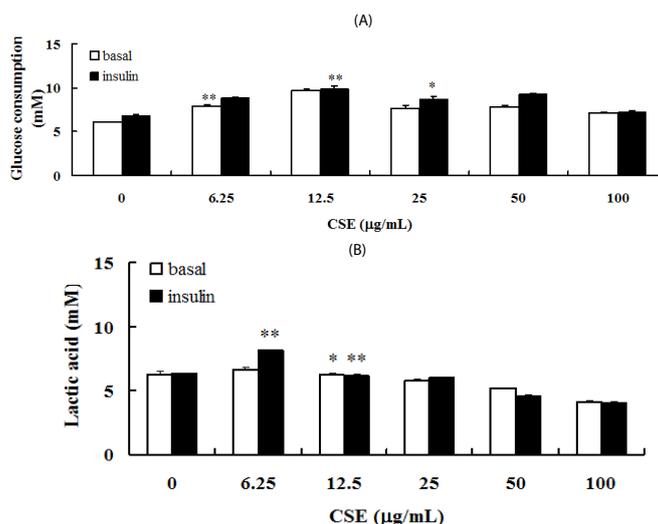
Figure 2. Effects of CSE on cell viability (A) and cytotoxicity (B). Cell viability was assayed by MTT analysis in 96-well tissue culture plates. Lactate dehydrogenase (LDH) that leaked from damaged cells into culture medium was measured using LDH reagent kit. Data represent mean \pm SD of three independent experiments.

CSE increased the glucose consumption and lactic acid production

To investigate metabolic activity of CSE, we first examined them in the regulation of glucose consumption and lactic acid production in rat L6 myotubes cell. L6 myotube cells were treated with **A-C** at increasing concentrations (0 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$). In the absence of insulin, CSE give an increase of glucose consumption with varying concentration from 0 $\mu\text{g}/\text{mL}$ to 12.5 $\mu\text{g}/\text{mL}$, but higher concentrations (12.5 $\mu\text{g}/\text{mL}$) failed to further increase glucose consumption (**Figure 3A**). With the insulin addition, glucose consumption increased more obviously from 6.25 $\mu\text{g}/\text{mL}$ to 12.5 $\mu\text{g}/\text{mL}$, this indicates CSE act in an insulin-dependent manner to stimulate glucose utilization. Glucose is utilized to produce ATP through aerobic respiration in mitochondria or anaerobic respiration (glycolysis) outside mitochondria. Activation of glycolysis leads to the production of lactic acid. Thus, we examined the effect of CSE on lactic acid production. There was slight change in the basal lactic acid production, while the insulin-induced lactic acid production increased significantly at concentration of 6.25 $\mu\text{g}/\text{mL}$ (**Figure 3B**). The results indicated that CSE played a positive role in promoting the glucose consumption and lactic acid production in insulin-dependent manner.

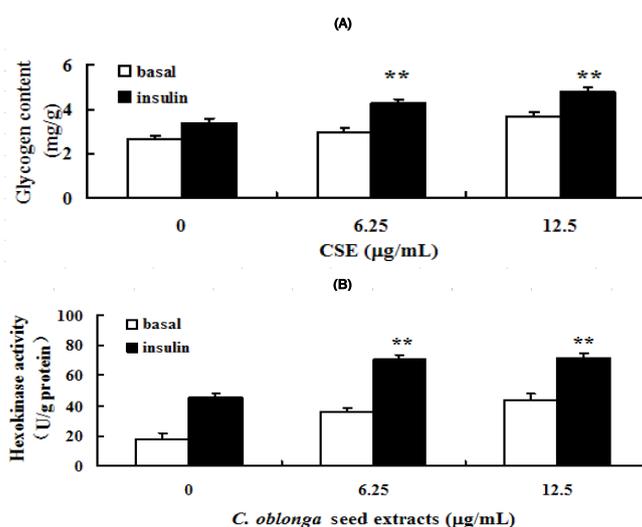
Positive effect of CSE on glycogen synthesis and hexokinase activity

Glycogen is the principal storage form of glucose in animal cells [25]. Both muscle and liver glycogen reserves are important for whole body glucose metabolism. To probe glycogenesis, the formation of glycogen from glucose, we detected glycogen content in L6 myotubes according to anthrone-sulfuric acid colorimetric method. CSE resulted in enhanced glycogen content at both 6.25 $\mu\text{g}/\text{mL}$ and 12.5 $\mu\text{g}/\text{mL}$ concentration after insulin stimulation (**Figure 4A**). Increase of glycogen content in skeletal muscle by CSE further proved that they might improve glucose utilization through promoting glycogen synthesis. Glucose is utilized to produce ATP through aerobic respiration or anaerobic respiration (glycolysis) in L6 cells. The first step in glycolysis is the phosphorylation of glucose by hexokinase [26,27]. CSE effectively enhance hexokinase activity in both basal and insulin group (**Figure 4B**), indicating they might improve glucose metabolism through AMPK-mediated glycolysis. The results revealed that CSE might promote glucose metabolism by glycogen synthesis or glycolysis.



* $P < 0.05$, ** $P < 0.01$ vs. control.

Figure 3. Effects of CSE on glucose consumption (A) and lactic acid production (B). L6 skeletal muscle cells were starved with 0.25% bovine serum albumin (BSA) overnight. Cells were then further incubated for 1 h in serum-free medium containing CSE (0 µg/mL to 12.5 µg/mL) and/or insulin (100 nM). The glucose concentration in medium was determined by glucose oxidase method. Lactic acid production was detected using kit. Data represent mean \pm SD of three independent experiments.



* $P < 0.05$, ** $P < 0.01$ compared with the control.

Figure 4. Effect of CSE on glycogen content (A) and hexokinase activity (B). Differentiated L6 skeletal muscle cells were starved overnight. L6 Cells were treated by CSE at 6.25 µg/mL and 12.5 µg/mL in the presence or absence of insulin (100 nM). The glycogen content was determined according to anthrone-sulfuric acid colorimetric method. Hexokinase activity was detected by hexokinase kit. The results are the mean \pm SD of independent experiments performed in triplicate.

Western blotting analysis

Activation of PI3K-AKT insulin signaling pathway could promote glucose metabolism. The protein levels of the key factors in AKT pathway in L6 myotube were examined by western blotting. We firstly assessed whether IRS-1 and AKT phosphorylation was altered. The total protein level of IRS-1 and AKT had no significant changes compared with the control group (**Figure 5A**). In the absence of insulin, 6.25 µg/mL and 12.5 µg/mL CSE exhibited positive effect on phosphorylation of AKT, while IRS treated by CSE was slightly lower than that of the control group. CSE increased phosphorylation level of GSK-3 β at 6.25 µg/mL concentration. More over phosphorylation level of AMPK group is much higher than that of the control group in the absence of insulin. Based on the results above, the phosphorylation level of IRS-1 and AKT was not enhanced by CSE without insulin synergy, while phosphorylated levels of AMPK and GSK-3 β proteins was facilitated by CSE only.

In the insulin group, the IRS, AKT, and GSK-3 β phosphorylation were significantly enhanced by CSE (**Figure 5B**). However, CSE treatment has almost no effect to phospho-AMPK expression. CSE could improve the phosphorylation of AKT and GSK-3 β , and the effect is much better after L6 cells were treated with the combination of CSE and insulin. The results indicated that CSE increased phosphorylation of IRS, AKT, and GSK-3 β mainly through an insulin-sensitive PI3K/AKT signaling pathway.

The process of glycogen synthesis is triggered by the sequential phosphorylation activation of IRS, AKT and GSK-3 β . Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle cells [28,29]. In this experiment, with

increase of phosphor-IRS-1, the phosphorylation level of AKT which is a downstream protein of IRS-1 was also increased. After activation of AKT, phosphorylated GSK-3 β which is a critical downstream molecule in PI3K/AKT signaling was increased too. Thus it can be seen that CSE promoted glucose metabolism is mainly in glycogen synthesis process which was because of activation of insulin-stimulated PI3K/AKT signaling pathway.

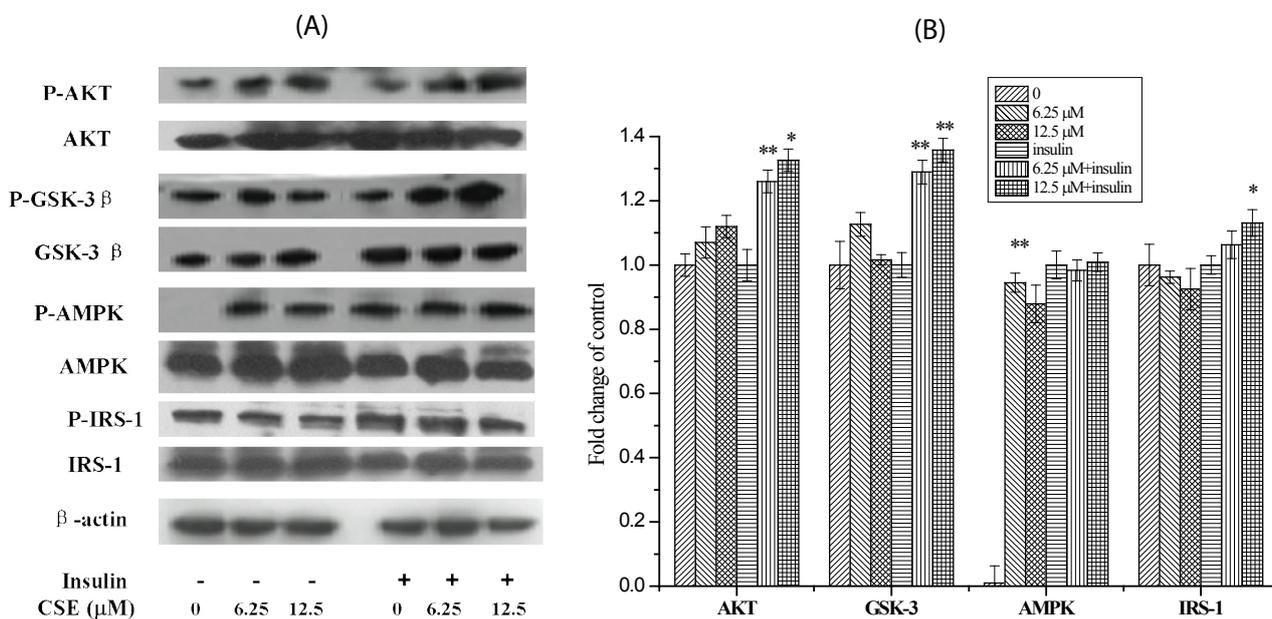


Figure 5. Effect of CSE on phosphorylations of IRS-1, Akt, GSK-3 β and AMPK protein. Differentiated L6 skeletal muscle cells were treated by CSE at 6.25 μ g/mL and 12.5 μ g/mL in the presence or absence of insulin (100 nM). Protein levels were examined by western blotting.

CONCLUSION

The incidence of type 2 diabetes mellitus (T2DM) is increasing worldwide, and it is characterized by insulin resistance [30]. Therapeutic strategies for the intervention of T2DM should center on the improvement of insulin resistance and glucose metabolism [31]. In present study, CSE were found to have positive effect on insulin-stimulated glucose consumption, lactic acid production and glycogen synthesis in differentiated L6 myotubes. The results demonstrated that CSE promoted glucose metabolism and have hypoglycemic effect *in vitro*. To probe the underlying action mechanisms of CSE, we investigated alteration of phosphorylated IRS-1, AKT, and GSK-3 β which involves in Insulin signal transduction pathway. The western blotting results indicated that CSE improved glucose metabolism by activating PI3K/AKT insulin signaling pathway in L6 myotubes. In conclusion, the present study demonstrated for the first time that *C. oblonga* seeds present hypoglycemic effect of CSE and elaborated the mechanism of action using a skeletal muscle model of insulin resistance. Our results suggest *C. oblonga* seeds as a promising agent for diabetes prevention and/or treatment and will certainly encourage future studies to increase our knowledge on the anti-diabetes potential of this plant.

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