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Original Article

# Effect of *Ferula Assafoetida* on Cytoplasmic Membrane Glucose Transporter Isotype-4 of C2C12 Cell Line

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**A B S T R A C T**

**Background and Aims:** *Ferula Assafoetida* is an antioxidant plant which has long been used in Iranian traditional medicine. Recently, it has been reported to have hypoglycemic and hyperinsulinemic effects, but the molecular mechanism of this effect have not been sufficiently described. This study was a step to evaluate the molecular mechanism of *Ferula assafoetida* action as an antihyperglycemic agent. For this purpose, some signling pathways of the hypoglycemic effect of its extract using $C_2C_{12}$ mouse cell line were examined.

**Materials and Methods:** $C_2C_{12}$ cells were differentiated in DMEM medium supplemented with 2% heat inactivated horse serum, and treated with 10 µg/ml extract of assafoetida in presence or absence of phosphoinositide 3-kinase (PI-3K) inhibitor. The concentration of Glucose transporter type 4 (GLUT4) in cytosol and cytoplasmic membrane were determined using SDS–polyacrylamide gel electrophoresis and western blotting analyses.

**Results:** Data indicated that assafoetida treatment increases translocation of the GLUT4 to the cell membrane in C2C12 cell line via PI3K/Akt signaling pathway activation.

**Conclusion:** our finding indicated that assafoetida has a potential antidiabetic effect and may be considered as antidiabetic drug.

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## Introduction

The effect of free radicals on diabetes is well established. Diabetes generates reactive oxygen species capable of causing oxidative damage to macromolecules. The role of antioxidant drugs in patients with type II diabetes mellitus is vitally important [1, 2]. Some studies have indicated the role of cell signaling with antioxidants [3,4]. Muscle insulin resistance is the initial metabolic defect in type 2 diabetes [5]. Skeletal muscle is more importantly responsible for glucose uptake [6]. Glucose transporter translocation plays an important role in the stimulation of glucose transport. Glucose transporter isotype 4 (GLUT4) is a member of glucose transporters that exists in insulin-responsive tissues such as skeletal muscle, adipose tissue, and heart [7]. Principal mechanisms for insulin glucose uptake through the translocation of GLUT4 are mediated by phosphatidylinositol 3-kinase (PI3K). Another essential mechanism is stimulated by AMP-activated protein kinase (AMPK) [8]. In a number of traditional medicinal plants, their properties are useful in the treatment of diabetes mellitus [9, 10].

Sustainable research on medicinal plants is necessary. Therefore, investigating these mechanisms in muscles and offering new natural products which increase glucose transporter translocation can give an excellent approach for phytotherapy of diabetes. Asafoetida has been found to be a rich source of gum-resin obtained from the roots of *Ferula assafoetida* [11] and it is a medicinal plant native of Iran [12]. Some parts of the plant, such as roots and leaves are edible and are used by the people [13].

In many studies, antioxidant potential of various components of *Ferula assafoetida* like 2, 2-diphenyl-1-picrylhydrazyl [DPPH] free radical scavenging activity, hydroxyl radical scavenging activity, nitric oxide scavenging activity and superoxide radical scavenging activity has been established. The results obtained in those study show that *Ferula assafoetida* has enough ability for use as a natural antioxidant agent [10, 13]. Study on the antidiabetic effect of Asafoetida extract is performed [10] but the mechanism of glucose reduction is not clear.

The oleo-gum resin of this plant contains sedative, expectorant, analgesic, carminative, stimulant antiepileptic, antistomachache, antidiabetic, anti-inflammatory, antimicrobial, anti-angiogenic and anti-cancer effects [12].

## Materials and Methods

### Preparation of the extract

The *Ferula assafoetida* extract was obtained from the herbal Medicine Research Center of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The powdered plant (5g) was maintained in distilled water (50 ml) at room temperature, and after filtration it was stored at 4°C for analysis.

### Cell Culture and differentiation

C2C12 cell line was purchased from Pasture Institute of Iran. About $2\times10^4$ C2C12 myoblast cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich,

USA) containing 12% fetal bovine serum (FBS) (Gibco,USA), 50 µg⁄ml streptomycin,and 2.5 units/ml penicillin G (Sigma–Aldrich,USA). The cell line was maintained in a 5% CO2 atmosphere at 37°C and cell viability was assessed by 0.4% trypan blue (Sigma-Aldrich,USA). Cell culture medium was exchanged each day until reaching near 80% confluent conditions. Myoblast differentiation to myotubes was induced by the addition of DMEM supplemented with 2% heat-inactivated horse serum (Invitrogen, Cat No.:26050-088,USA) and polynucleated myotube formation monitored microscopically until day 6 of differentiation induction.

C2C12 cells, after differentiation, were incubated in 4 flasks for glucose uptake. This was designed to investigate the effect of f.assafeotida extract on C2C12 myotubes and its role in the regulation of cell signaling. Analyses were then initiated on the day 8 after myotube differentiation. One flask was treated with 10 µg/ml the extract for one hour without PI3K inhibitor (LY294002, Sigma-Aldrich). The second flask was maintained without the extract and inhibitor, and the third flask was treated without the extract but with PI3K (20 µMolar) inhibitor. Then the remaining flask was treated with both extract and PI3K inhibitor.

**Cytotoxicity assay**

To determine the maximal non-toxic dose of f.assafoetida, extract the cytotoxicity of it on cells was measured using phase-contrast microscope counting assay.

**Preparation of the membrane and cytosolic fractions**

C2C12 cells were collected and centrifuged. Then deposits were collected and mixed with 0.3 ml lysis buffer [5 mM Tris-Hcl,150 mM, 1 mM NaF, 1% anti protease, 1 mM PMSF (phenylmethylsulfonyl fluoride), Sodiumdeoxycholate] and were left to lyse for 30 min on ice with periodic vortexing. The supernatant was centrifuged in HES buffer (225 mM sucrose, 4 mM $Na_2EDTA$, 20 mM HEPES) at 20000 RPM for 45 min. Plasma membrane and cytosolic protein fractions were collected and microtubes were placed at -70°C before analysis. The GLUT4 protein concentration was measured for both fractions by Bradford method.

**Western blotting**

The GLUT4 protein concentration in supernatants collected for Immunoblot analysis was measured for both fractions by Bradford method (Coomassie brilliant blue was obtained from Serva electrophoresis). Then it was separated on 10% SDS–polyacrylamide gel (Merck Company, Germany), transferred to nitrocellulose membrane (Millipor,USA), and then immunoblot analysis was performed using primary antibodies. After overnight incubation at 4°C with the primary antibody, the nitrocellulose membrane was washed with PBS buffer for 4 times, and the membrane was agitated 5 minutes each time for washing so as to remove other unbound primary antibodies Santa Cruz Biotechnology, USA. incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz

Biotechnology, USA. was performed for 2 hours at room temperature with agitation (ECL, Amersham Biosciences, Buckinghamshire, UK).

After this step, the membrane was washed with PBS buffer for 4 times, and the membrane was agitated 5 minutes each time so as to remove other unbound secondary antibodies.

For observing nitrocellulose membrane blots, chemiluminescence method was used.

The Ethics Committee of Shahid Sadoughi University of Medical Sciences approved this research.

**Statistical analysis**

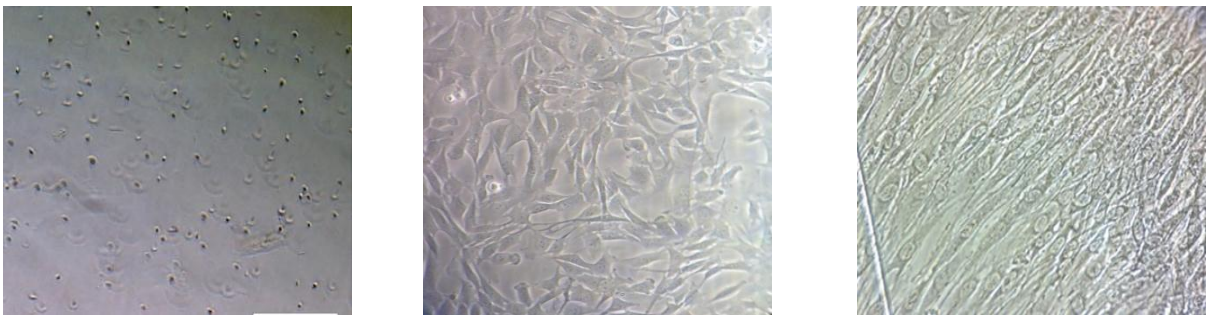
The results were analyzed using SPSS v.16.0 software (SPSS Inc, Chicago, IL, USA). Student's t-test was applied for comparing the means of the two samples. The P-values less than 0.05 were considered as statistically significant.

# Results

Microscopic images of undifferentiated and differentiated C2C12 cell line culture are shown in Fig.1. The concentration of 10 µg/ml of the extract had no effect on C2C12 Cell line proliferation, differentiation, or viability.

The cytosolic and membrane GLUT4 bands in the control, C2C12 cell line treated with the extract, and C2C12 cell line treated with the extract and PI3k inhibitor are shown in Fig.2 and Fig.3.

The translocation of GLUT4 from cytoplasm to the plasma membrane of C2C12 cell line increased at a concentration of 10 µg/ml of F. assafoetida extract (Fig.4a) ($p<0.05$). Also, The translocation of GLUT4 from cytoplasm to the plasma membrane of C2C12 cell line was decreased in the presence of PI3K inhibitor compared at concentration of 10 µg/ml of the extract (Fig.4b) ($p<0.05$). Cytosolic GLUT4 in the C2C12 cell line treated with the extract was higher than the control group (Fig.5a) ($p<0.05$). There was no significant difference in the cytosolic GLUT4 present in C2C12 cell line treated with the extract and also in the extract PI3K inhibitor (Fig. 5b).



A B C

**Fig. 1.** Microscopic images from C2C12 cell line culture; A: immediately after culture B: 48 hr after culture, and C: 6-7 days after culture.

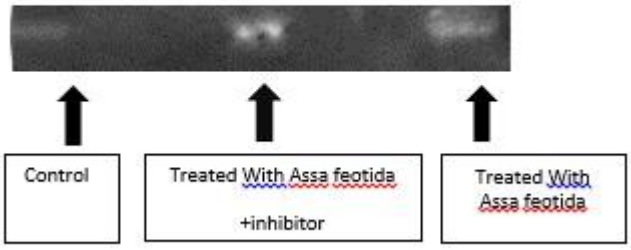



**Fig. 2.** Cytosolic GLUT4 bands


**Fig. 3.** Membrane GLUT4 bands

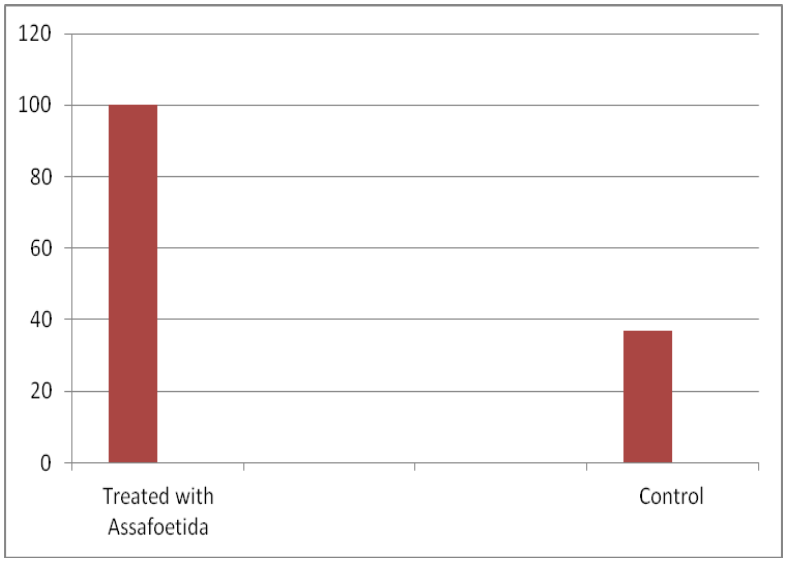



**Fig. 4a.** Comparison of GLUT4 percent in the two membrane fractions.

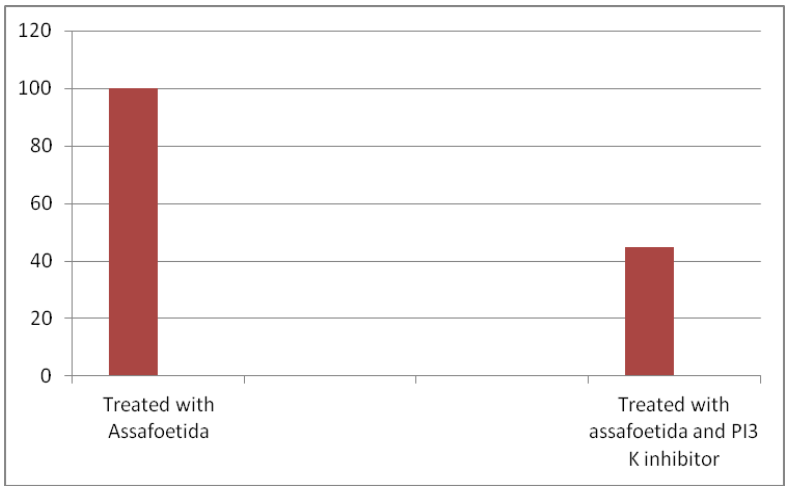



**Fig. 4b.** Comparison of GLUT4 percent in the two membrane fractions.

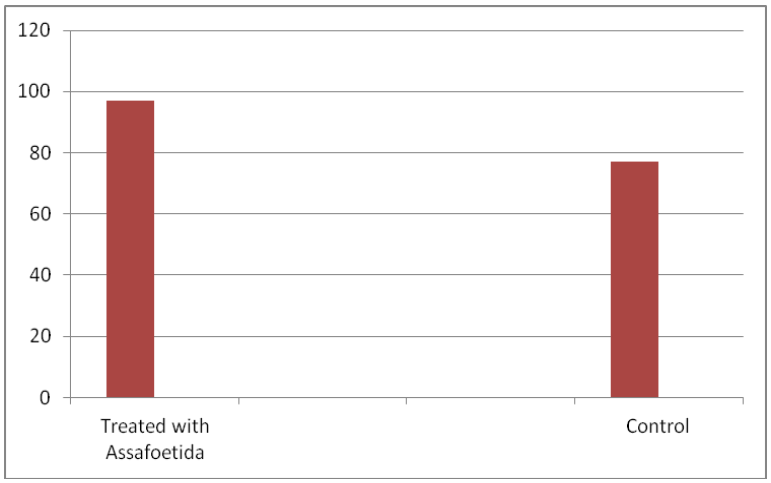



**Fig. 5a.** Comparison of GLUT4 percent in cytosolic fractions

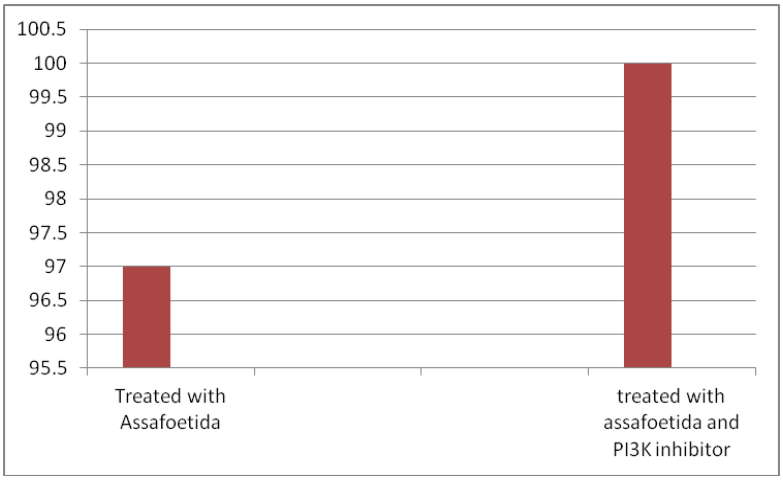



**Fig. 5b.** Comparison of GLUT4 percent in cytosolic fractions

## Discussion

The insulin receptor is a transmembrane receptor tyrosine kinase that regulates glucose and lipid metabolism. Insulin stimulates glucose uptake and GLUT4 translocation in the skeletal muscles [14]. In recent years, the health advantages of *Ferula assafoetida* gum in type 2 diabetic rats and humans have been studied extensively regarding a variety of actions, including anti-diabetic, anti-angiogenic, anti-obesity, and anti-cancer effects [12]. Previously, different path ways have been suggested for damage of cells in diabetic patients. Most of them have involved oxidative stress signaling for glucose metabolism in diabetes [11]. Some anti-diabetic effects of asafoetida on diabetes have been related to its antioxidant effect. The potentials of the extract are DPPH free radical scavenging, hydroxyl radical scavenging, nitric oxide scavenging and superoxide radical scavenging activities [12]. For the present study, our primary aim was to investigate if *Ferula assafoetida* modulates glucose uptake in skeletal muscle cells. Here, the effects of *Ferula assafoetida* on glucose uptake and improved insulin sensitivity were demonstrated by activating PI3K/AKT and GLUT4 in C2C12 muscle cells.

Some studies have further demonstrated that oxidative stress such as ROS-mediated signaling leads to activation of PI3K/AKT pathway [15]. Garlic attenuates cardiac oxidative stress via activation of PI3K/AKT/Nrf2-Keap1 pathway in diabetic rat [16]. Furthermore, Berberine is an antioxidant plant that is used in traditional medicine in some countries, and harbors antidiabetic effects through activation of AMPK [17]. Considering these results, potent antioxidant effect of *Ferula assafoetida* may well be involved in the activation of PI3K/AKT Pathway. The PI3K/Akt has a protective role under oxidative stress [18]. Asafoetida glucose signaling pathways has not been studied before, this study has been performed but in some other plants. For example, curcumin is able to activate AMPK but not PI3K/Akt signaling pathways [19]. In this study, we investigated the effect of different concentrations of F.assafoetida extract on glucose uptake signaling pathways in the C2C12 muscle cells. The concentration of 10 µg/ml the extract increased the translocation of GLUT4 from cytoplasm to the plasma membrane and glucose uptake. The effects of asafetida on PI3-kinase/Akt and AMPK signaling pathways were examined in C2C12 myotubes by treating the cells with 10 µg/ml of the extracct for 1 h. At this point, the glucose uptake pathways of it on skeletal muscle cells were demonstrated by augmenting PI3-kinase signaling pathways in this cell line.

## Conclusion

Our findings suggest that F.assafoetida extract has a positive effect on glucose uptake by activating the insulin signaling pathway.

### Conflict of Interest

The authors declare that they have no conflict of interest.

## Acknowledgement

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