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Bioassay-guided Isolation of the Antidiabetic Active Principle from *Salvia miltiorrhiza* and its Stimulatory Effects on Glucose Uptake Using 3T3-L1 Adipocytes

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Abstract

Natural products, which reduce hyperglycemia by enhancing the glucose uptake in peripheral tissues, have been considered to be effective for treatment of Type-2 Diabetes Mellitus. *Salvia miltiorrhiza* (Labiatae), danshen, has been widely used traditional Chinese medicine for the treatment of various cardiovascular and cerebrovascular diseases. In the present study, different extracts of *Salvia miltiorrhiza* root were investigated for their ability to enhance glucose uptake in differentiated 3T3-L1 adipocytes. An *in vitro* bioassay guided fractionation approach was adapted to isolate the active principle of *Salvia miltiorrhiza* using extensive column chromatographic techniques. The structure of active compound was elucidated using various spectroscopic methods (ESI-MS, MALDI-ToF, ¹H-NMR, ¹³C-NMR, COSY, TOCSY, HETCOR) and determined to be magnesium salt of salvianolic acid B (SAB). SAB showed concentration dependent increase in glucose uptake in 3T3-L1 adipocytes. The efficacy of the active principle was also evaluated for its antidiabetic activity in streptozotocin-induced diabetic rats. SAB (25 mg/kg) significantly improved the glucose tolerance in diabetic rats (*p<0.05, ** p<0.01). The SAB treatment group showed significantly lower (*p<0.05) blood glucose levels over 120 min as compared to diabetic control group. Thus, these results suggested that SAB has the potential to be developed as a potential glucose-lowering agent by increasing glucose uptake in peripheral tissues in the treatment of diabetes mellitus.

Keywords: *Salvia miltiorrhiza*; Glucose uptake; 3T3-L1 adipocytes; ESI-MS; NMR; Diabetes mellitus; OGTT

List of Abbreviations: ODS: Octadecylsilyl; LC-MS/MS: Liquid Chromatography Coupled to Tandem Mass Spectrometry; MALDI-ToF: Matrix-Assisted Laser Desorption/Ionization Time of Flight; ESI: Electrospray Ionization; NMR: Nuclear Magnetic Resonance; COSY: Correlated Spectroscopy; TOCSY: Total Correlated Spectroscopy; HETCOR: Heteronuclear Correlation Spectroscopy; IR: Infrared; SAB: Salvianolic acid B

Introduction

Type 2 diabetes mellitus is a chronic metabolic disorder characterized by impaired insulin secretion and insulin sensitivity, which poses an imminent threat to become a worldwide epidemic according to world health organization [1-3]. Type 2 diabetes mellitus is associated with severe complications such as hypertension, dyslipidemia, microvascular and macrovascular cardiovascular diseases [4-6]. Insulin plays a regulatory role in this disease by stimulating the uptake of blood glucose into peripheral tissues through glucose transporters and the promotion of lipid biosynthesis in adipocytes [7]. Insulin resistance causes decrease in peripheral glucose disposal resulting in increased levels of blood glucose [7]. Some of the currently available antidiabetic drugs improve blood glucose levels by increasing the glucose uptake in peripheral tissues. However such drugs are associated with adverse side effects, which limit their use in diabetic patients. Therefore it is highly desirable to discover antidiabetic agents that improve blood glucose levels with minimal side effects.

Salvia miltiorrhiza (Labiatae), *danshen*, is an annual sage mainly found in china and neighboring countries. The dried root and its preparations are currently being used in china to treat patients with cardiovascular and cerebrovascular complications [8]. Many compounds have been isolated from *Salvia miltiorrhiza* roots, which could be classified as lipid soluble tanshinones and water-soluble phenolic acid compounds [9]. Among phenolic acids, salvianolic acid B is a major component of *S. miltiorrhiza* and extensive pharmacological

studies have been reported for this compound. Salvianolic acid B inhibited the amyloid formation of human islet amyloid polypeptide and protects pancreatic beta cells against cytotoxicity [10]. Many authors have studied the effect of salvianolic acid B on different organ systems in animals as well as in humans. Salvianolic acid B showed beneficial and protective effect to brain from ischemia-reperfusion injury in animal studies [11]. In addition, salvianolic acid B has been shown to inhibit platelet aggregation [12] as well as cause oxidative modification of low-density lipoprotein (LDL) thereby, resulting into decreased uptake of LDL by cultured macrophages [13]. Furthermore, salvianolic acid B has been shown to stimulate the nitric oxide production of the endothelial cell [14] and inhibition of angiotensin II-induced hyperplasia [15]. It significantly inhibited the activity of stress-activated protein (SAP) kinase [16]. Magnesium salt of salvianolic acid B has been shown to possess potent hepatoprotective activity and shows an improved effect on uremic symptoms [17,18]. An *in vitro* study also revealed that salvianolic acid B is an excellent scavenger for free radicals, both cation radicals and anion radicals [19]. It has been reported that salvianolic acid B has potent inhibitory effects on lipid or biomembrane peroxidation in a superoxide anion generating system [20,21].

Recent studies focused on *S. miltiorrhiza* and/or salvianolic acid B linked their beneficial effects in treatment of diabetes mellitus and

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its associated manifestations. Chronic treatment with *S. miltiorrhiza* has been shown to inhibit the progression of diabetic nephropathy in streptozotocin induced diabetic animals along with down regulation of TSP-1 and TGF- β 1 in myocardial tissue, thereby improving the heart function of diabetic rats and protecting against cardiomyopathy [22]. Previous studies on natural plant extracts such as *M. charantia*, *L. speciosa*, *S. aqueum*, showed their ability to induce glucose uptake in 3T3-L1 adipocytes *in vitro* [6,23,24]. To date, there is no literature showing the direct effects of different extracts or isolated compounds of *S. miltiorrhiza* on glucose uptake in 3T3-L1 adipocytes. Henceforth the objective of present study was to elucidate the active principle of *S. miltiorrhiza* responsible for stimulating glucose uptake using bioassay-guided fractionation approach and access its emphasis *in vivo*.

Material and Methods

Materials

The dry decoction of salvia root was obtained and certified by Tong Ren Tang (Beijing, P.R. China) and New York Tong Ren Tang (Flushing, NY). The voucher specimen has been retained at the department of Pharmaceutical sciences at St. John's University, New York. Glucose, streptozotocin (STZ), insulin (IS), dexamethasone (DEX) and 3-Isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). 2-[1, 2- 3 H (N)-Deoxy-D-glucose (2-DG) was purchased from PerkinElmer (Waltham, MA). Male Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY). Salvianolic acid B standard was purchased from Ivy Fine Chemicals (Cherry Hill, NJ). 'Bakers' Silica gel (40 μ m flash chromatography packing) was purchased from VWR International (San Dimas, CA). White quartz sand, Davisil[®] silica gel (grade 62, pore size 150 Å, 60-200 mesh) and Sephadex[®] (LH-20) were purchased from Sigma-Aldrich (St. Louis, MO). Uniplate[®] silica gel GF plate and preparative silica gel GF plate were purchased from Analtech, Inc (Newark, DE). All other reagents were of analytical grade and used without further purification.

Other instruments

HPLC system (Shimadzu), equipped with Waters[™] 717 plus autosampler, Perkin-Elmer Spectrum One FT-IR Spectrometer, Shimadzu GCMS-QP5050A Gas Chromatography Mass Spectrometer, Bruker Daltonics[®] Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-ToF) Mass Spectrometer were used. 1 H NMR, 13 C NMR and APT (attached proton test) spectra were recorded on a 400 MHz Bruker instrument with trimethylsilane as an internal standard. Chemical shifts were recorded as ppm (δ). LC-MS/MS data was obtained from API 3000 LC-MS/MS system. Melting points were determined using a MEL-TEMP II melting point apparatus. Elemental analysis was performed by Atlantic Microlab Inc (Norcross, GA).

Isolation of S4LH06 from salvia root

The dry decoction of crushed salvia root were powdered and passed through a 40-mesh sieve. The powder (~40 g) was sonicated with 70% ethanol (EtOH) for 6 hrs at room temperature. The ethanolic extract was filtered and concentrated under reduced pressure below 40°C and lyophilized to yield dry residue [4,25]. The crude EtOH extract was suspended in distilled water and extracted with chloroform in a separatory funnel. The chloroform fraction then concentrated under reduced pressure below 30°C, and designated as the chloroform fraction. The remaining aqueous layer was subjected to extraction with ethyl acetate (EtOAc) in similar manner as chloroform. The EtOAc layer then concentrated under reduced pressure below 30°C and designated as the ethyl acetate fraction. The remaining aqueous layer was extracted with n-butanol in similar manner discussed above

and collected as n-butanol fraction and aqueous fraction. The aqueous fraction from n-butanol extraction was precipitated by 100% ethanol, yielding water precipitate (WP) and water supernatant (WS). The n-butanol fraction was subjected to a silica gel (40 μ m) column and eluted with a 20% stepwise gradient with EtOH in chloroform. The fraction eluted with 100% EtOH was then separated by using silica gel (grade 62, pore size 150 Å, 60-200 mesh) flash chromatography and eluted with a stepwise gradient of ethanol in chloroform. Seven fractions (S0-S6) were collected and concentrated under reduced pressure below 30°C. The fraction eluted with 100% ethanol (S4 fraction) was further passed through a sephadex LH-20 column and eluted with MeOH-H₂O (40:60/v:v). Six different fractions (S4LH01-S4LH06) were collected and lyophilized to dry residues. S4LH06 was further purified using preparative thin layer chromatography (TLC) plate (mobile phase; chloroform: ethyl acetate: formic acid; 1:1:0.1/v:v) and sephadex LH-20 column to yield purified light yellow amorphous powder of S4LH06. The final yield of S4LH06 was 0.03 to 0.06% with a purity >98% based on TLC, HPLC, elemental analysis and ESI-MS.

Structure elucidation and characterization of purified S4LH06

The structure of purified S4LH06 was determined to be magnesium salt of salvianolic acid B [9,26,27]: C₃₆H₂₈O₁₆Mg. It was identified and confirmed by comparative analysis of its ESI-MS, 1 H-NMR, 13 C-NMR, Correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), heteronuclear correlation spectroscopy (HETCOR), IR spectrum, HPLC chromatogram and mass spectral data with authentic sample data and/or with literature data.

Cell culture

3T3-L1 fibroblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10% bovine serum (Invitrogen Corp, Carlsbad, CA) in 5% CO₂ at 37°C. Differentiation was induced by treating the cells with DMEM containing 3-Isobutyl-1-methylxanthine (IBMX, 0.5 mM), dexamethasone (DEX, 0.25 μ M), insulin (IS, 1 mg/l), and 10% BS for 72 hours. The cells were refed with DMEM containing 10% BS and 1 mg/L IS for the following 48 hours after which cells were kept in the maintenance media (DMEM+10% BS). More than 90% of the cells expressed adipocyte phenotype between 11 and 14 days after the initiation of differentiation and were used for the glucose uptake experiments.

2-Deoxyglucose uptake assay

Glucose uptake was determined as the rate of 2-[1, 2- 3 H(N)]-Deoxy-D- glucose uptake, using a modification of a previous method with a few modifications [28,29]. Confluent and differentiated 3T3-L1 adipocytes were washed (thrice) and incubated with serum free DMEM for 2 hours. After 2 hrs, cells were washed thrice with DPBS and incubated with DPBS (glucose free incubation) for 30 minutes at 37°C. After glucose free incubation, the cells were incubated with DPBS containing different fractions of salvia root, purified compounds or control for 10 minutes at 37°C. Glucose uptake was initiated by addition of 2-[1, 2- 3 H(N)]-Deoxy-D- glucose (1 μ Ci/ml) in each well. After 10 min, cells were rinsed three times with cold PBS to stop the transport then lysed by incubating the samples with 1% Triton X100 for 30 min at room temperature. Cell lysates of each well were mixed with aqueous scintillation fluid and the amount of radioactivity incorporated into cells were measured in a liquid scintillation counter and normalized with its protein content. Protein concentration of each sample was quantified using a BCA protein assay kit. Final data were expressed as the percentage of control. Insulin concentration response

curve was performed (data not shown) and insulin (1 μ M) was used as a positive control in each glucose uptake experiment.

Glucose tolerance test

The Animal Care Committee of St. John's University, NY approved all animal protocols. Male Sprague-dawley (SD) rats (body weight 175~200 g) were fasted overnight and injected with streptozotocin (STZ, i.p.) dissolved in citrate buffer (pH 4.5) at a dose of 45 mg/kg. Two days after STZ injection, rats with blood glucose concentration higher than 300 mg/dl (at fasting state) were considered as diabetic and included in the present study. They were randomized in control (diabetic control) and test groups. In an oral glucose tolerance test, 16 hours fasted animals were intraperitoneally dosed with test compound (S4LH06, 25 mg/kg) or saline control at -15 min followed by oral gavage of glucose (2.0 g/kg). Blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min using glucose meter [25,30]. The relative glucose percentage change was determined using the following equation: glucose level at particular time point (mg/dl)/glucose level at zero time point (mg/dl) * 100.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM), and *p*-values less than 0.05 were considered significant. One-way analysis of variance (ANOVA) was performed in compound treatment. Prism Graph-Pad (Version 5.0) software was used for statistics and plotting.

Results

Glucose uptake activity of salvia miltiorrhiza fractions

The decoction pieces of salvia root were extracted with 70% aqueous EtOH. The crude extract was then extracted with chloroform, ethyl acetate and n-butanol using a separatory funnel. We initially tested all the fractions of salvia root (70% EtOH, chloroform, ethyl acetate, n-butanol, water supernatant and water precipitate) at 0.2 mg/ml to determine their effect on glucose uptake in differentiated 3T3-L1 adipocytes. All fractions (except WS, and WP) showed significant potentiation of glucose uptake (ranging between 120% and 187%) as compared to DPBS control (100%, *p*<0.05, Figure 1). The n-butanol (nBu) fraction was the most active with respect to glucose uptake (Figure 1). This fraction was chromatographed over a silica gel column (40 μ m) and separated into ten fractions. All ten fractions (Tested concentration: 0.2 mg/ml) were examined for their effect on glucose uptake. The most active subfraction, 100% EtOH (Figure 2, *p*<0.001), showed significant increase in glucose uptake, which then rechromatographed on a second silica gel column (Grade 62, pore size 150 \AA , 60-200 mesh) and separated into seven fractions (S0-S6). Fractions S3, S4 and S5 (Tested concentration: 0.1 mg/ml) showed significant increase in glucose uptake (data not shown).

The most active subfraction, S4, was subjected to sephadex LH-20 column and separated into six fractions (S4LH01-S4LH06). Further purification of the most active fraction, S4LH06 (Figure 3), by preparative thin layer chromatography and sephadex LH-20 column resulted in the identification of the magnesium salt of salvianolic acid B.

Effect of purified S4LH06 on glucose uptake in 3T3-L1 adipocytes

Treatment of fully differentiated 3T3-L1 adipocytes with increasing doses of purified compound S4LH06 resulted in significant enhancement in glucose uptake (Figure 4). S4LH06 increased glucose

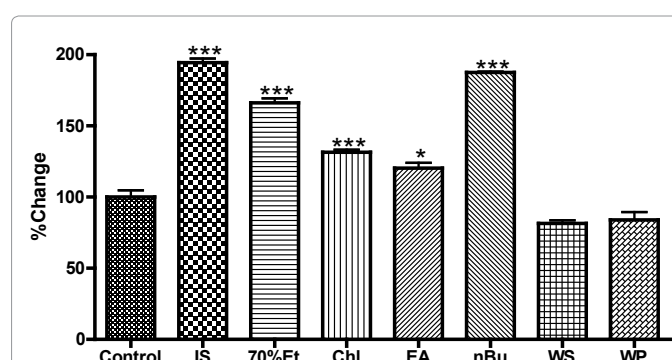


Figure 1: Glucose Uptake Activity of Salvia Root Fractions resulted from Liquid-Liquid Extraction, Test concentration 0.2 mg/ml. *vs. control *p*<0.05, *** vs. control *p*<0.001. (IS:Insulin; ET:Ethanol; Chl:Chloroform; EA:Ethylacetate; nBu: Normal Butanol; WS: Water Supernatant; WP: Water Precipitate).

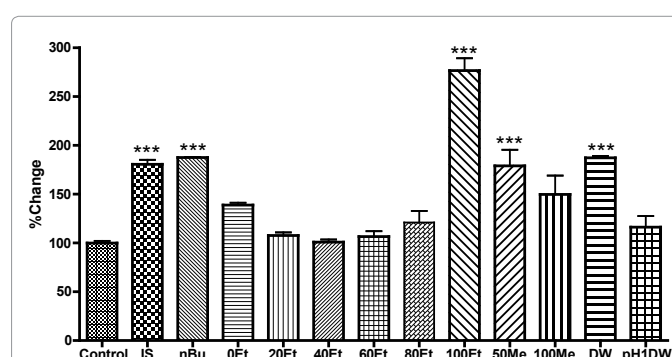


Figure 2: Glucose Uptake Activity of Fractions derived from nBu fraction by Silica gel (40 μ m) Flash Chromatography, Test concentration: 0.2 mg/ml, *** vs. control *p*<0.001 (IS:Insulin; nBu:Butanol; Et: Ethanol; Me:Methanol; DW:Distilled Water).

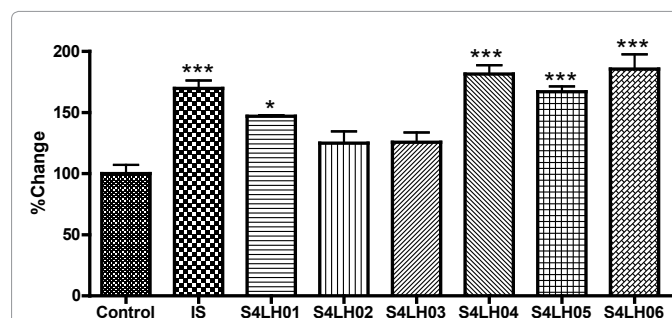
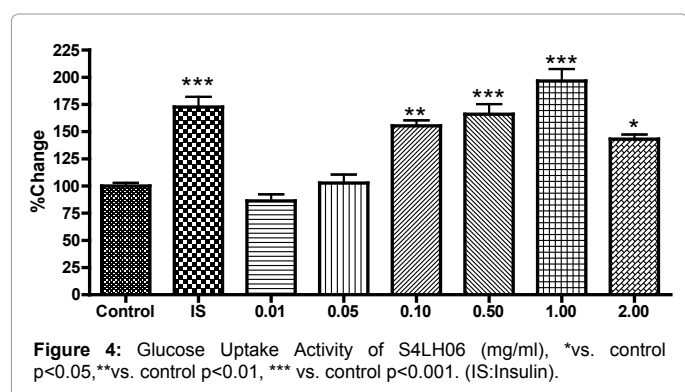


Figure 3: Glucose Uptake Activity of Fractions derived from S4 fraction by Sephadex LH-20 Liquid Chromatography, Test concentration: 0.1 mg/ml, *vs. control *p*<0.05, *** vs. control *p*<0.001 (IS: Insulin).

uptake in a concentration dependent manner from ranging from 102% to 196% as compared control (100%) over the concentration range of 0.01 mg/ml-2 mg/ml. S4LH06 (1 mg/ml) showed highest enhancement of glucose uptake activity of 196% as compared to control. Higher concentration of S4LH06 (2 mg/ml) did not further increase glucose uptake activity in 3T3-L1 adipocytes.

Identification of active compound S4LH06

Based on the spectroscopic analysis of the purified active compound (S4LH06), obtained from the n-butanol fraction of the ethanolic extract of *Salvia Miltiorrhiza*, was identified as magnesium salt of Salvianolic acid B. The purity of S4LH06 determined by TLC and



HPLC analysis. The HPLC chromatogram of S4LH06 showed a single peak at 1.6 min (Column, HILIC; Mobile phase, acetonitrile: water, 9:1/v:v) and at 6 min (Column, HILIC; Mobile phase, chloroform: ethyl acetate, 1:1/v:v). TLC of S4LH06 showed a single blue spot under UV light after being sprayed with 5% FeCl₃-MeOH. The matrix-assisted laser desorption/ionization-time of fly (MALDI-ToF) and Liquid chromatography coupled to negative electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) were used to determine the molecular weight of S4LH06. MALDI-MS analysis of S4LH06 in positive mode showed 3 mass peaks [M+1]⁺, which correspond to m/z 521, 741, and 908. ESI-MS data of S4LH06 in negative mode showed molecular ion peaks [M-1]⁻ of m/z 519 and 739 along with highest intense peak m/z 717. The elemental analysis of S4LH06 showed only C, 56.28; H, 4.44; O, 36.31. Based on the mass spectrum analysis and elemental analysis of S4LH06, the molecular weight of S4LH06 was determined to be 740 [9,26,27]. The molecular formula of S4LH06 was assigned as C₃₆H₂₈O₁₆Mg. The presence of magnesium was determined by flame atomic absorption analysis.

Magnesium salt of salvianolic acid B : light yellow amorphous powder; ¹H-NMR (DMSO-d₆, 400 MHz) δ10.2-8.5 (7H, m, Ar-OH), 7.5 (1H, d, J=15.9Hz, CH=CH), 7.3-6.2 (11H, m, ArHx8), 6.3 (1H, d, J=15.9Hz, CH=CH), 5.3 (1H, d, J=4.9Hz, Ar-CH(O)-C), 5.0 (2H, m, CH-COOx2), 4.4 (1H, d, J=4.9Hz, Ar-C(O)-CH-), 3.0-2.8 (4H, m, -CH₂x2); ¹³C-NMR (MeOH-d₄, 400 MHz) δ 176.5, 175.7, 171.5, 167.5, 147.7, 145.1, 145.0, 144.5, 144.3, 143.4, 143.2, 141.1, 132.7, 129.8, 129.3, 125.4, 123.3, 120.7, 120.6, 119.9, 117.0, 116.6, 116.2, 115.84, 115.78, 115.2, 114.99, 114.94, 112.2, 86.9, 77.6, 76.7, 57.3, 37.4, 36.9; ESI-MS (Q1 mass, negative mode) 827.2, 815.2, 743.9, 739.2, 717.5, 537.9, MS2 of peak (m/z 717): 537.1, 518.5, 339.0, 320.5, 294.9. The two-dimension ¹H NMR of S4LH06 in MeOH-d₄, COSY, was performed to determine the connectivity of protons by their spin-spin coupling. A detailed coupling between protons was determined by performing selective 1D-TOCSY. APT was performed to determine the number of protons attached to each carbon and the carbon-proton connections were confirmed by 2-D HETCOR. S4LH06 showed IR absorption bands at 3367 cm⁻¹ and 1719 cm⁻¹, suggesting the presence of hydroxyl (OH) and carbonyl (C=O) groups in the molecule.

Based on ¹H NMR, COSY, TOCSY, ¹³CNMR, APT, HETCOR, IR spectrum data, it was concluded that S4LH06 has four aromatic rings, three ethyl linkers, one trans-alkene, four carbonyl groups and seven-eight hydroxyl groups (thirteen aromatic protons, eight aliphatic protons, four carbonyl carbons, and twenty six aromatic carbons). Salvianolic acid B [31] contains all the proposed moieties of S4LH06. The flame atomic absorption analysis revealed that S4LH06 (C₃₆H₂₈O₁₆Mg, MW=740) is a magnesium salt of salvianolic acid B. Thin layer chromatographic analysis of both S4LH06 and standard salvianolic acid B showed a single blue spot after being sprayed with

5% FeCl₃-MeOH. ESI-MS analysis of S4LH06 and standard salvianolic acid B showed the identical mass peaks with a molecular ion of m/z [M-1]⁻ at 519, 739 and 717 (highest intensity). ¹H and ¹³CNMR spectrum data of S4LH06 were mostly superimposable to the standard salvianolic acid B.

Oral glucose tolerance test (OGTT)

To investigate whether S4LH06 could improve glucose tolerance *in vivo* upon single dose administration, we tested S4LH06 in a streptozotocin induced diabetic rat model. Based on oral glucose tolerance test analyses, S4LH06 (25 mg/kg, i.p) improved glucose tolerance as compared to vehicle control (Saline, i.p) (Figure 5). At time points of 30, 45, and 60 min after glucose challenge, S4LH06 reduced % relative glucose compared to control from an average of 187% to an average of 106% with p<0.01 (30-min time point), from 184% to 104% (p<0.01, 45 min time point) and from 176% to 110% with p<0.05 (60-min time point), respectively. Integrated area under the glucose curve over 120 min (AUC_{glucose}) of S4LH06 treatment group was significantly lower (p<0.05) as compared to diabetic control group (Figure 5). Taken together, the results from this experiment demonstrated that S4LH06 may possess a hypoglycemic activity *in vivo*.

Discussion

The present study is the first to report the bioassay-guided fractionation of a crude extract (70% EtOH in H₂O) of *Salvia miltiorrhiza* roots, which led to the isolation, characterization, and identification of phenolic acid, magnesium salt of salvianolic acid B (SAB) and its effect on glucose uptake *in vitro* using 3T3-L1 adipocytes. Salvianolic acid B, a tetramer of caffeic acid, is a major phenolic product found in *S. miltiorrhiza* [32-34]. It consists of three danshensu units and one caffeic acid unit bound by an ester linkage (Zhao et al.,). There is an increasing awareness and interest in the antioxidant properties and other positive effects of phenolic acids in prevention of various human diseases. Phenolic acids present in *Salvia miltiorrhiza* have been shown to possess protective properties against amyloid β-induced cytotoxicity, indicative of beneficial effects of this herb in Alzheimer's disease (AD) treatment [33]. The root of *S. miltiorrhiza* is widely used in Asian countries for the treatment of coronary heart disease, cerebrovascular disease, hypertension, chronic renal failure, and dysmenorrhea and as cytotoxic against human tumor cell lines [34-37].

In present study, magnesium salt of salvianolic acid B was isolated, purified and characterized from *S. miltiorrhiza* roots by column chromatography using silica gel, sephadex LH-20 followed by spectroscopic analysis. Several methods have been reported for

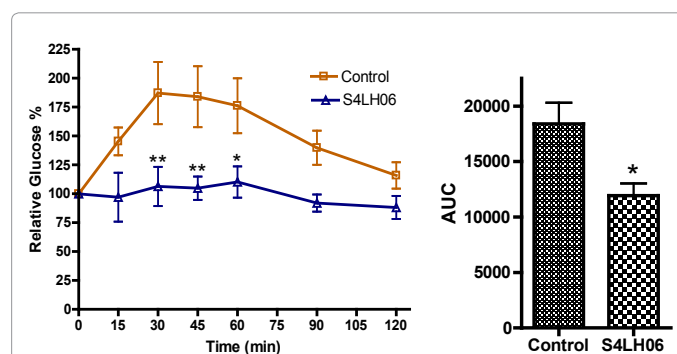


Figure 5: Glucose Tolerance Test in STZ-Rats. Control group, saline with IP; test group, S4LH06 in saline 25 mg/kg with IP. Left, relative glucose change after glucose load (2 g/kg); right, area under the curve (AUC_{glucose}) of control and test group. *vs. control p<0.05, **vs. control p<0.01.

isolation of salvianolic acid from *S. miltiorrhiza* such as preparative high-speed counter-current chromatography (HSCCC) [9,26] and microwave-assisted extraction method coupled reverse-phase high-performance liquid chromatographic (RP-HPLC) method with UV detection [38]. The *in vitro* bioassay data demonstrated that different fractions of *S. miltiorrhiza* such as ethyl acetate, chloroform, n-butanol, and water fractions significantly increased the glucose uptake in 3T3-L1 adipocytes. (Figures 1-3) Further purification of n-butanol fraction of *S. miltiorrhiza* led us to isolation of magnesium salt of salvianolic acid B (SAB) that shows concentration dependent potentiation of glucose uptake activity in 3T3-L1 adipocytes. Maximal response achieved by isolated SAB (concentration: 1 mg/ml) was comparable to glucose uptake activity of insulin alone (Figure 4). However, other studies have established that plant extracts and its compounds have the ability to stimulate glucose uptake far better in the presence of insulin than in the absence of insulin [39,40]. In contrast, our results demonstrate that, in the absence of insulin, magnesium salt of salvianolic acid B is equally efficacious in stimulating the glucose uptake in 3T3-L1 adipocytes, which suggests that SAB can increase glucose uptake through insulin independent mechanisms. Cellular uptake of glucose in adipocytes is a tightly controlled process, mediated by a facilitated glucose transporter GLUT4 in adipocytes [41,42]. Understanding the regulation of GLUT4 and glucose transport are found to be extremely challenging, primarily because it involves several signal-transduction pathways that are superimposed on a complex series of vesicle transport process. Insulin binds to a surface receptor on muscle and fat cells and triggers a cascade of signaling events that culminates in GLUT4 translocation [41]. It has been also suggested that many phenolic compounds such as quercetin and its glycosides can stimulate AMPK (AMP activated protein kinase), which ultimately stimulate glucose uptake by activation of GLUT4 [43]. However, further experiments need to be performed to elaborate the complex mechanisms of salvianolic acid B at cellular and molecular levels.

In vivo studies have shown that increase glucose uptake in muscle and fat cells result in amelioration of diabetes mellitus by decreasing the blood glucose levels in diabetic animals [44-46]. Hence, to determine the effect of magnesium salt of SAB (glucose uptake inducer) on hyperglycemia, we performed oral glucose tolerance test (OGTT) in streptozotocin induced diabetic rats. The SAB treated diabetic rats showed a significant decrease in blood glucose levels as compared to the controls over a period of 120 min (Figure 5). We speculate that the improved glucose tolerance in diabetic rats is due to the stimulatory effects of SAB on glucose uptake in peripheral tissues. However, methanolic root extracts of salvia species have reportedly shown potent inhibitory action against the enzyme protein tyrosine phosphatase 1B (PTP1B) [47,48], which further inhibits insulin signaling; and selective inhibition of PTP1B is a potential drug target for the treatment of type 2 diabetes. Our studies show that purified SAB from *S. miltiorrhiza* improves glucose tolerance supporting to the evidence that *S. miltiorrhiza* can be exploited further for its anti-diabetic properties. Based on our data and the existing literature, we acknowledge that multiple pathways for SAB action might be involved for its overall antihyperglycemic activity *in vivo* and there is evidence that SAB may reduce diabetes through its protective effect on pancreatic beta-cells [10].

Conclusion

The present study demonstrated the isolation, characterization, and identification of magnesium salt of salvianolic acid B (SAB) from *Salvia miltiorrhiza* root. SAB significantly increased the glucose uptake in differentiated 3T3-L1 adipocytes. It also improved glucose tolerance in streptozotocin induced diabetic rats. It therefore provides an

important lead for development of salvianolic acid B as hypoglycemic agent for the treatment of diabetes mellitus. Further studies have to be performed to elaborate the complex mechanism of salvianolic acid B *in vivo*.

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