Antidiabetic Effects of *Scrophularia Striata* Ethanolic Extract Via Suppression of *Pdx1* And *Ins1* Expression In Pancreatic Tissues of Diabetic Rats

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Abstract

Background: Diabetes mellitus (DM) is a pathological disease that results in severe metabolic imbalances and abnormal changes in many tissues, especially in the pancreas, where oxidative stress plays an important role in etiology. Therefore, the therapeutic effects of *Scrophularia striata* were investigated using an animal model in the control of diabetic injury and pancreatic complications caused by diabetes.

Methods: A total of 66 rats (weight 220-250 g) were randomly divided into: Healthy Control group; Diabetic control group; 3 experimental healthy groups; 3 treatment groups; and 3 pretreatment groups. Diabetes was induced in rats by intraperitoneal STZ (60 mg/kg bw). FBS, HbA1c and insulin were measured after four weeks. *Pdx1* and *Ins1* gene expression was assessed by RT-PCR. The histological evaluation was also performed with H&E staining. The data were analyzed by SPSS ver20 using ANOVA and Tukey tests.

Results: By treatment with *S. striata* ethanolic extract, these factors were close to the normal range. The expression of the *Pdx1* and *Ins1* genes increased in the treated rats after treatment with *S. striata* extract.

Conclusions: Therefore, by improving serum indexes and *Pdx1* and *Ins1* gene expression, *S. striata* may be used as adjuvant therapy for complications of diabetes.

Introduction

One of the oldest known disorders is diabetes mellitus (DM) in humans. It is assumed that DM due to the inherent stresses of modern lifestyles and the increasing prevalence of diabetes is becoming a major public health problem affecting millions of people worldwide. According to the World Health Organization (WHO), by 2025, 300 million people will have diabetes. Diabetes mellitus is a pathological disease, and as a result causes severe metabolic imbalances and abnormal changes in many tissues, especially in the pancreas, where an important role in etiology is played by oxidative stress. High oxidative stress because of persistent and chronic high blood sugar is tested in diabetic and experimental animal models, thus destroying the activity of the antioxidant defense system and thereby producing free radicals [1–3]. In diabetes mellitus, changes in the defense mechanisms of endogenous free radical scavenging may lead to ineffective inhibition of oxygen-reactive species, leading to oxidative damage and tissue damage. Tissue damage has been suggested and streptozotocin acts as a diabetic agent due to its ability to destroy pancreatic β -cells, possibly by the mechanism of free radicals [4, 5].

The pancreas is the synthesis, storage and secretion site of insulin. In the pancreatic islets, there is a complex signaling cascade for the secretion of glucose-stimulating insulin that includes ATP-sensitive potassium channels (K_{ATP}). In the presence of glucose, an increase in the intracellular ATP/ADP ratio causes the K_{ATP} channels to close, resulting in the plasma membrane depolarization, extracellular calcium influx, and exocytosis activation. The cell plasma membrane islet has K_{ATP} channels, although

most K_{ATP} channels are located on secretory granule membranes. The K_{ATP} channels of pancreas have four sulfonylurea-regulating receptor subunits (SUR1) and four potassium pore-formation subunits ($K_{ir6.2}$) [6]. One of the chronic autoimmune diseases is type 1 diabetes (T1D) in which insulin-producing beta-cells in the pancreas are destroyed, resulting in chronic high blood sugar. Pancreatic exocrine abnormalities have been described in recent decades in terms of anatomy and function. It is not clear whether exogenous changes of in T1D are relevant to identical genetic, immunological, and environmental events that lead to the destruction of beta-cells and are secondary to functional β cell loss. Therefore, insulin acts as a trophic agent for the exocrine compartment [7].

The main goal of caring for diabetic patients is to minimize the risk of microvascular and macrovascular complications by returning blood pressure, and lipid and glycemic profiles to normal. The particular aim for glycemic control is to reach glycated hemoglobin (HbA_{1c}) to a normal range because good glycemic control is important to decrease the long-term microvascular complication risk in both type 1 and 2 diabetes [8]. HbA_{1c} can be measured at any time of the day, regardless of the duration of the fast or the content of the previous meal. It can also be analyzed using a portable device with a small blood sample [9]. Glycated hemoglobin (HbA_{1c}) analysis in blood enables evidence of a person's average blood glucose level over the past two to three months, which is the red blood cell (RBC) half-life [10].

There is ample evidence that β -cell regeneration and function in the adult pancreas are mediated partly by pancreatic and duodenal homeobox 1 (*Pdx1*), and that changes in expression are associated with the alterations in the expression of target genes, including insulin 1 (*Ins1*) [3]. *Pdx1* is an important transcription factor needed for pancreatic development and maintains the distinctive function of β -cells, especially the regulation of normal glucose-insulin secretion [11, 12]. The first transcription factor produced in the growing pancreas is *Pdx1*, and lack of this factor causes pancreatic agenesis because of the inability to generate a variety of duct, exocrine or endocrine cells. Conditional elimination of *Pdx1* from β cell formation by insulin Cre lines leads to hyperglycemia [13, 14]. Previous studies have shown that *Pdx1* plays a major role in diabetes and that reducing the expression of *Pdx1* exacerbates diabetes [15, 16].

Scrophularia striata, known as “Teshnedari” from the Scrophulariaceae family, is one of the most important herbal medicines that is widely used in western Iran. This herb is an annual or perennial plant that has a zygomorphic flower and 5 petals flower, and the calyx has lobes and its fruit is a capsule with many seeds. It has been reported that *Scrophularia striata* has some medicinal effects including analgesic, antimicrobial, nephrotic, nitric oxide suppressant, antitumor, hepatic protection and anti-inflammatory effects [17–20]. Due to the medicinal effects of *Scrophularia striata* plants and their side effects, they have not been studied thus far. In this study, using an animal model, the effects of this plant were investigated in preventing diabetes-induced damage to the pancreas and its functions.

Material And Methods

Ethics statement

All animal maintenance and procedures were in accordance with recommendations established by the Animal Ethics Committee of the Islamic Azad University, North Tehran Branch, as well as the United States NIH guidelines (publication no. 85–23). The Ethics Committee approved the protocols of The Islamic Azad University, North Tehran Branch. All surgical procedures were carried out beneath deep anesthesia, and all efforts had been made to minimize suffering. (ethical code: IR.IAU.TNB.REC.1400.552.). The study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

Animals

In this study, 66 Wistar rats were purchased in the weight range of 180-330 grams from Pasteur Institute, Tehran, Iran. They were transferred to the Laboratory Animal Breeding Center of North Tehran Azad University. The animals were kept in a laboratory animal breeding center with fiberglass cages (15×30×35 cm) and a 12 h light/12 h dark cycle and temperature (22 ± 2 °C), light with a relative humidity range of 40-60%. The rats were fed particular food obtained from Behparvar Company, Tehran, Iran. Tap water was used to maintain hydration, and the experimental tubes were used in each cage as their water supply with sufficient water and food during the experiment. All research and laboratory animal care were performed by responding to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2012). When their weight reached 220-250 g, the experiment was started.

Selection of Diabetic Rats by inducing Streptozotocin

Streptozotocin (STZ; Sigma Aldrich, USA) was used to induce diabetes. STZ was dissolved at a dose of 60 mg/kg body weight and injected intraperitoneally into rats. Blood glucose was monitored 48 h after STZ injection. To measure the inactive serum glucose level glucometer tapes were used on the rat tail vein. If the measured blood glucose was higher than 300 mg/dl, we considered diabetic rats [21].

Preparation of *Scrophularia striata* ethanolic extract

Scrophularia striata (Teshnedari) is a regional plant that was collected from highlands of the Zagros mountain chain, Ilam, Iran. Considering that the floral part of the plant is used to obtain the extraction, this plant is collected in spring. It was identified at the Traditional and Complementary Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran. Dehydration is the primary step of all the steps of plant preparation for extraction. After collection, the plants needed to be cleaned with water even before the dehydration step. Then, the soaking method was used to extract the plant material. The soaking method was performed twice to ensure efficient extraction of the material. Every 200 grams of dried *Scrophularia striata* received 400 ml of ethyl alcohol (Merck, Germany), and their mixture was placed in darkness for 72 hours. The mixture was passed through filter paper three times. After the last filtering stage, it was placed in a dark place to slowly evaporate the alcohol. The dried extract was stored at 0-4 °C. Finally, the dried extract was dissolved in the normal saline to obtain the required doses (100-400 mg/kg) [20].

Experimental Design

The rats were randomly divided into 11 groups (n= 6: healthy control group (receiving propylene glycol which is the solvent of the extracted medicine as solvent); diabetic control group (induced streptozotocin); three experimental healthy groups (receiving the extract at 100, 200 and 400 mg/kg bw/day), three experimental treatment groups (receiving the extract at 100, 200 and 400 mg/kg bw/day), and three pretreatment groups (receiving the extract at 100, 200 and 400 mg/kg bw/day). In this experiment, the pretreatment groups were healthy at the beginning of the experiment. In the pretreatment groups, daily gavage was performed with solvent for 2 weeks before and after STZ injection at 3 specified doses. The other groups also underwent daily gavage with the extract solution and solvent for 4 weeks.

Dissection and pancreas sample collection

After completing four weeks of medication for all the groups, the rats in each group were dissected with a heart under anesthesia, blood was taken directly from their hearts and then centrifuged. Blood samples were collected with a cardiac puncture, to measure some biochemical factors including fasting blood sugar (FBS), hemoglobin A1c (HbA1c), and insulin; the serum was separated quickly, and then were aliquoted and stored at -70 °C. Additionally, the pancreatic tissues were fixed in 10% formalin for histopathological examination.

Histological assessment of pancreas tissue

On the dissection day, the rat was fasted for 12 hours to anesthetize with ether. Cross-sections of all pancreas lobes were collected, fixed in coded jars containing formaldehyde buffer solution, and then embedded in paraffin. Sections 5 µm in size were stained with hematoxylin and eosin (H&E) and examined by light microscopy. The status of histological damage was considered for atrophy, fibrosis and regeneration status as negative, mild, moderate and severe [22].

Determination of serum FBS, HbA1c and insulin in the studied rats

After the experimental period, all animals were weighed by on a digital scale, and then anesthetized with ketamine (622 mg/kg) and xylazine (62 mg/kg), and blood samples were taken from their hearts. The serum was separated by centrifugation at 10000 × g for 10 minutes and hemoglobin A1c (HbA1c) was measured by latex enhanced immunoturbidimetric assay with an HbA1c quantitative detection kit (Pars Azmoon, Tehran, Iran) using a photometric method. Fasting blood sugar (FBS) was measured by a photometric method with a glucose quantity detection kit (GOD-PAP) (Pars Azmoon, Tehran, Iran), and serum insulin was measured by enzyme-linked immunosorbent assay using a Pars Azmoon test kit according to the manufacturer's instructions [23–25].

Assessment of PDX1 and Ins1 gene expression by quantitative real-time PCR

Total RNA was extracted from the samples immediately after sampling according to standard protocols using an RNA purification kit (GeneJET™ RNA Purification Kit # K0732, Thermo Scientific - Fermentas, Latvia). Total RNA was treated with DNase to remove contaminated genomic DNA using DNase deletion reagents (DNase I, RNase-free (# EN0521) Fermentas, Latvia), according to the manufacturers' protocol. RNA quality was assessed by agarose gel electrophoresis and UV spectroscopy. The cDNA was synthesized using a first strand transcription cDNA synthesis kit (Thermo Scientific - Fermentas, Latvia), according to the manufacturers' protocol. After sequencing both *Pdx1* and *Ins1* gene types and finding specific sequences of the species, the primers were designed by oligo7 software and blasted on the NCBI website. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used for normalization as an endogenous reference gene. The primers were *Pdx1* forward 5'CCTTTCCCGAATGGAACCGA3', *Pdx1* reverse 5'TTTTCCACGCGTGAGCTTTG3' and *Ins1* forward 5'GTCAAACAGCACCTTTGTGGT3' and *Ins1* reverse 5'AGAAACCACGTTCCCCACAC3'. Agarose gel electrophoresis was used to evaluate the predicted size of the PCR amplitudes of genes. Standard curves for each gene were prepared using serial dilutions (1: 4) of cDNA fused from total RNA extracted from the samples. In each experiment, the value of R^2 was the standard curve 0.99, and no-template control experiments resulted in the absence of a detectable signal. Quantitative RT-PCR was performed using SYBR Green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2 ×) # K0221, Thermo Scientific - Fermentas, Latvia). A triplicate method was performed for quantitative real-time PCR using a 7900HT Fast Real-Time PCR System with a Fast 96-well Block Module (Applied Biosystems, Foster City, CA, USA). PCR data were obtained by Sequence Detector Software (SDS version 2.3 Rev C Patch, Applied Biosystems) and quantified by the standard curve method. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold versus extracted RNA quantity. Samples were measured in one plate for one target gene and their C_t values were in the linear range of the standard curve. In the qPCR test, outliers or sample failures were repeated for each gene. The Pfaffle formula is used to calculate the ratio. Quantitative real-time PCR and analysis of expression data were performed based on a previous study [26].

Statistical Analysis

All data were statistically analyzed by SPSS-20 software. After confirming the normality of the data, one-way analysis of variance (ANOVA-one way) and Tukey's test were performed. The results are presented as the mean $\pm$ S. E. M. Statistical inference was used.

Results

Effects of *Scrophularia striata* ethanolic extract on FBS, HbA1c and insulin in serum

According to Graph 1, FBS and HbA1c increased in the sham group compared to the control group. In addition, FBS was significantly reduced in the 400-treatment group compared to the sham and control groups ($P \leq 0.05$). On the other hand, HbA1c index reduced in all the treatment, experimental healthy and pretreatment groups compared to the sham and control groups, which was significant in 200 healthy

experimental and 100 pretreatment experimental groups ($P \leq 0.05$). However, insulin decreased significantly in the sham group compared to the control group, and all the treatment and experimental healthy groups increased the insulin index significantly in a dose-dependent manner.

Effects of *Scrophularia striata* ethanolic extract on *Pdx1* and *Ins1* gene expression

Gene expression was compared between groups based on $\Delta\Delta$ ct and fold change. According to an analysis of $\Delta\Delta$ ct, when the $\Delta\Delta$ ct value in a group is low gene expression will be higher. In addition to fold change, whatever fold change value in a group is high, therefore gene expression will also be high. As a result, in the sham group, *Pdx1* and *Ins1* gene expression-compared to that in the control group. In all of the other groups, *Ins1* gene expression was increased compared to that in the sham group. Otherwise, *Pdx1* gene expression just increased in the treatment 400 group compared to the sham group. The effect of *S. striata* was dose-dependent (Table 1).

Table 1. Comparison of PDX1 and Ins1 gene expression in the studied rats

Group	Fold Change	
	Ins1	PDX1
<i>Sham Vs Control</i>	0.19	5.35
<i>Treatment 100 Vs Sham</i>	2.85	1.27
<i>Treatment 200 Vs Sham</i>	3.97	2.00
<i>Treatment 400 Vs Sham</i>	6.37	6.36
<i>Experimental Healthy 100 Vs Sham</i>	1.85	1.01
<i>Experimental Healthy 200 Vs Sham</i>	2.16	1.34
<i>Experimental Healthy 400 Vs Sham</i>	3.41	0.02
<i>Pretreatment 100 Vs Sham</i>	0.54	1.85
<i>Pretreatment 200 Vs Sham</i>	0.46	2.16
<i>Pretreatment 400 Vs Sham</i>	0.29	3.41

Effects of *S. striata* ethanolic extract on histopathology of the pancreas

Pancreas histopathologic results are shown in Table 2. Histological study showed that the pancreas tissues of the control group had negative atrophy and fibrosis status and regeneration status. The pancreatic tissues in the sham group showed moderate atrophy and fibrosis status and negative regeneration status.

As shown in Fig 1, no significant tissue changes were observed in the sham group (Fig 1. A), which were healthy and experimental healthy groups treated with 400 mg/kg *S. striata* extract (Fig 1. C). whereas

degeneration of Langerhans islet cells was observed the diabetic control group, and some degree of atrophy and fibrosis was seen in the structure of the pancreas (Fig 1. B). In the pretreatment group (400 mg/kg *S. striata* extract), normal ascites conditions were observed Fig 1. D). Finally, in the treatment group (400 mg/kg *S. striata* extract), the islets of Langerhans in cellularity improved compared to the control group and were somewhat structurally closer to the sham group, which indicates the effectiveness of the treatment (Fig 1. E).

Table 2. Histopathological results in the studied rats

Group	Atrophy & fibrosis status	Regeneration status
<i>Treatment 100</i>	Negative	Negative
<i>Treatment 200</i>	Negative	Negative
<i>Treatment 400</i>	Negative	Negative
<i>Experimental healthy 100</i>	Negative	Suspicious positive
<i>Experimental healthy 200</i>	Negative	Suspicious positive
<i>Experimental healthy 400</i>	Negative	Suspicious positive
<i>Pretreatment 100</i>	Negative	Negative
<i>Pretreatment 200</i>	Mild	Negative
<i>Pretreatment 400</i>	Negative	Negative
<i>Sham</i>	Moderate	Negative
<i>Control</i>	Negative	Negative

Discussion

Persistent and chronic hyperglycemia reduces antioxidant defense system activity and therefore causes the production of free radicals. Increased levels of free radicals along with the failure of the natural antioxidant system generally cause cellular dysfunction and death. Oxidative stress is generated in diabetic situations and may be involved in the development of beta-cell dysfunction in the pancreas [27].

Despite numerous studies on the pathogenesis of diabetes mellitus (DM) and new therapeutic strategies in recent years, the molecular mechanisms of DM pathogenesis and the antidiabetic action of drugs remain largely unknown. In the present investigation, STZ diabetic rats fed *Scrophularia striata* ethanolic extract (at doses of 100, 200 and 400 mg/kg bw) per day for four consecutive weeks exhibited significant blood glucose and hemoglobin A_{1c} (HbA_{1c}) reductions and increases in blood insulin levels. These observations are in agreement with a study that showed increased blood glucose concentrations in STZ-hyperglycemic rats compared to control rats [28]. This hypoglycemic potential, in addition to confirming the traditional use of this plant as a blood glucose-lowering agent has been reported in the literature [29].

Phytochemical studies on *Scrophularia striata* have shown the presence of compounds such as flavonoids, cinamic acid, phenylpropanoid, neptrin, flavonoid glycoside, acteoside1, quercetin and phenylpropanoid glycoside [30–32]. However, to date, the biological activity of these compounds has not been reported to have a hypoglycemic effect. Flavonoids are a group of phenolics of secondary plants with strong antioxidant properties. Independent studies have shown that flavonoids have antidiabetic effects [33]. Therefore, the same group of compounds (e.g., flavonoids) is predicted to respond to the antidiabetic and antioxidant properties of *S. striata*. Our results indicated that *Scrophularia striata* ethanolic extract can produce a dose-dependent stimulation of insulin release in rats.

The effect of hypoglycemia in plants may be because of the presence of insulin-like substances in plants, the stimulation of B cells to produce more insulin, the fiber found in plants at high levels to interfere with the absorption of carbohydrates or the effect of plants regenerative on pancreatic tissue [34–36]. Theoretically, hypoglycemic plants work through various mechanisms, such as modifying insulin sensitivity, increasing glucose-dependent insulin secretion, and stimulating islet regeneration of Langerhans in the pancreas of STZ-induced diabetic rats. In addition, the role of antioxidant compounds in the protection and treatment of diabetes has been remarked upon in various scientific studies. For instance, treating diabetic animals injected with STZ with N-acetyl-L-cysteine (NAC), a known antioxidant, prohibits high blood sugar by reducing oxidative stress and restoring beta-cell function [33]. On the other hand, STZ has been shown to cause selective damage to pancreatic beta-cells [37]. Hence, beta cell insulin content decreased in untreated diabetic control rats; however, in the *Scrophularia striata* treatment groups, this pancreatic insulin content was augmented.

There is ample evidence that β -cell regeneration and function in the adult pancreas are mediated partly by pancreatic and duodenal homobox 1 (*Pdx1*), and the changes in its expression are associated with alterations in the expression of target genes, including insulin 1 (*Ins-1*) [3]. In two previous studies, *Pdx1* and a subset of other major islet-enriched transcription factors were found to be significantly decreased in human diabetes mellitus type 2 (T2DM) islet β -cells [38, 39]. In this study, we also indicated that the *Scrophularia striata* ethanolic extract increased *Ins-1* gene expression. The increased expression levels of the *Pdx1* and *Ins1* genes may be related to the methylation effect of *Scrophularia striata* in streptozotocin-induced diabetic rats. However, further investigations are needed to evaluate the methylated genes involved in the regeneration of the pancreas by *Scrophularia striata* administration.

Conclusion

According to the results of the present study, the antidiabetic effects of the *S. striata* ethanolic extract were confirmed. In addition, it observed that the *S. striata* extract has the effect of reduction in FBS and HbA1c, in addition it has an increasing effect on serum insulin. Moreover, it was observed that the studied extract was effective in the pancreas by increasing *Pdx1* and *Ins1* gene expression. Generally, *S. striata* ethanolic extract, because of its antioxidant properties, can be considered a beneficial medicine in treatment of diabetes and pancreatic tissue complications due to diabetes. Further study of the detailed

mechanisms is needed. This study has confirmed the other studies about the antidiabetic and protective properties of *S. striata* in diabetes. Therefore, additional studies can confirm these findings.

Declarations

Acknowledgment

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Figures

Figure 1

Data are expressed as the mean $\pm$ SD; n=6 for each group. 100, *S. striata* 100 mg/kg bw; 200, *S. striata* 200 mg/kg bw; 400, *S. striata* 400 mg/kg bw. Significant differences compared to the Sham and Control groups are shown $P \leq 0.05^*$, and $P \leq 0.01^{**}$, respectively. ANOVA and Tukey's were applied to evaluate the data.

Figure 2

Legend not included with this version.