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Effects of an ethanolic extract of *Berberis vulgaris* fruits on hyperglycemia and related gene expression in streptozotocin-induced diabetic rats

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Abstract

Background: Finding a treatment for diabetes that does not have side effects has remained elusive. Among natural agents, much attention has been focused on phenolic compounds. For both their medicinal and nutritional value, barberry plants are of particular interest. *Berberis vulgaris* is known for its medicinal benefits in Iranian traditional medicine. In this study, we investigated the biological activities of *B.vulgaris* in a rodent model of experimentally induced diabetes.

Method: The fasting blood glucose (FBG), insulin, malondialdehyde (MDA), and total antioxidant levels were measured in experimental groups of rats, including normoglycemic control, diabetic control, and diabetics treated with *B.vulgaris*, and the gene expression of the stress proteins HSF-1, HSP27, and HSP70 as well as the enzymes glucokinase (GK) and glucose 6-phosphatase (G6P) were assayed by real-time PCR.

Results: The application of the alcoholic extract of *B.vulgaris* (25 and 100 mg/kg body mass) significantly increased the total antioxidant levels, decreased MDA and FBG levels, and also increased mRNA level of GK compared with the diabetic control group ($P \leq 0.05$). Our results also showed a reduction in HSP70 gene expression and a significant decrease in G6P gene expression in a dose-dependent manner ($P \leq 0.05$). The effect of alcoholic extract of barberry at a higher dose (100 mg/kg) was similar to glibenclamide.

Conclusions: *B.vulgaris* extract is beneficial in ameliorating oxidative stress and may be useful in the treatment of diabetes. The therapeutic potential of *B.vulgaris* may be through the modification of the gene expression of key enzymes or stress proteins and requires further investigation.

Keywords: *Berberis vulgaris*, Hyperglycemia, Oxidative stress, Heat shock protein, Glucokinase, Glucose 6-phosphatase

Background

Diabetes mellitus (DM) is a major public health concern with numerous clinical manifestations. Of these, hyperglycemia is a major cause of absolute or relative insulin deficiency. According to World Health Organization projections, the diabetic population may increase to ≥300 million by 2050 [1]. Free radical production is stimulated by hyperglycemia, leading to cell damage. In living organisms, oxidation is an essential process during the production of energy for use as fuel for biological processes. However, the increase in the formation of free radicals such as reactive oxygen species (ROS) plays a crucial rule in the pathophysiology of several diseases such as diabetes, cancer, and cardiovascular disease. Oxidative stress may also play a role in metabolic syndrome, which is indicated by the presence of several oxidative biomarkers [2]. In addition, the body's stress response may result in the progression or acceleration of numerous diseases related to oxidative stress. Heat shock proteins (HSPs) are a highly

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conserved and ubiquitously expressed family of proteins that respond to a wide variety of physical and metabolic stresses, including during protein refolding and degradation of damaged proteins; moreover, they also protect proteins against aggregation and maintain the overall integrity of cellular components. In summary, HSPs protect against chronically and acutely stressful conditions at the cellular level. Oxidative stress induces a protective HSP response in tissues; however, the protective role of HSP have less well-characterized as antioxidants [3, 4]. Antioxidants can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or progression of oxidizing chain reactions. Previous studies have demonstrated that antioxidants can reduce markers of oxidative stress in both experimental and clinical models of diabetes. The redox status is disrupted in both type 1 and type 2 DM due to hyperglycemia, resulting in stress-mediated cellular injury [5]. *Berberis vulgaris* (*B.vulgaris*) *Linn*, which is commonly known as *B.vulgaris*, belongs to the family Berberidaceae. *B.vulgaris L.*, a valuable native plant in Iran, has played a prominent role for researchers in herbal healing due to its medicinal benefits and nutritional value. The roots, stems, leaves, and fruits are used in medicine andin the food industry [6].

In Iran, two important *B.vulgaris* species are *B.Integerrima* (abi) and *B.vulgaris* (poloei). *B.vulgaris* has been shown to have anti-microbial and anti-fungal effects, to stimulate the immune system, and to have hypotensive properties that are attributable to berberine and other isoquinoline alkaloids, the main active ingredients of *B.vulgaris*. The secondary metabolites of *B.vulgaris*, which are used in the pharmaceutical industry, include berberin, berlambine, oxyberberine, oxycanthine, chlorumamine, anthocyanine, bervulcine, lambertine, and magniflorine [7]. Insulin and oral anti-diabetic agents such as sulfonylureas, biguanides, glycosidase inhibitors, and glinides are present available therapies for diabetes, which are used as mono-therapy or in combination to achieve better glycemic regulation. Many of these anti-diabetic agents have numerous serious side effects. Thus, finding a treatment for diabetes that does not have adverse side effects remains a challenge. Approximately 80 % of the world population use traditional medicines. More than 150 plant extracts are used to treat diabetes, and the potential uses of several plants are known at present. However, there is much we do not know about plants with hypoglycemic properties. Hypoglycemic activity could be related to the existence of saponins, polyphenols, or alkaloids. The root bark of *B.vulgaris* is recommended in the management of diabetes. However, the mechanisms action of the plant remains unknown, and in addition, the use of antioxidants for disease therapy remains debatable [1]. Some natural compounds such as antioxidants are effective in protecting the human body against damage caused by ROS. Among natural antioxidants, a great deal of research has focused on phenolic compounds because of their wide distribution. All phenolic classes are free radical scavengers due to their redox properties, which act as reducing agents, hydrogen donors, and single oxygen quencher agents. Phenols particularly flavonoids, can also have additional properties including anti-inflammatory, anti-bacterial, anti-fungal, and anti-cancer effects [2, 8]. *B.vulgaris* fruits contain phenolic compounds with beneficial antioxidant activity that can reduce damage due to free radicals and prevent chronic disease [2]. This study was conducted to investigate the effect of *B.vulgaris* against oxidative stress in experimentally induced diabetes in a rodent model. The most common substance inducing type 1diabetes in the rat is streptozocin (streptozotocin, STZ). STZ is taken up by pancreatic β-cells via glucose transporter GLUT2. The main cause of STZ-induced β-cell death is alkylation of DNA by the nitrosourea moiety of this compound. However, production of NO^{o} and ROS may also be involved in DNA fragmentation and other deleterious effects of STZ [9]. ROS plays an important role in pathogenesis of diabetes, so natural antioxidants could help us in managing diabetes.

Methods

Plant materials and preparation of ethanolic extracts

Fresh-ripened fruits of the *B.vulgaris* plant were collected from South Khorasan from August to October, 2013. The fresh fruits were washed, air dried, powdered, and extracted using 2 L of ethanol by refluxing for 48 h. The extract obtained was vacuum-evaporated to obtain the crude extract, which was dissolved in distilled water just prior to oral administration. Each extract was administered in two doses equal to 25 and 100 mg/kg [10]. A voucher specimen for *B.vulgaris* (H, No. 769) was preserved in the Herbarium of the School of Agriculture, Ferdowsi University of Mashhad, Iran.

Animal experiments

Wistar-derived male rats (6–8-week-old; 200 ± 20 g, purchased from the Pasteur Institute, Tehran, Iran) were raised at the university animal quarters and housed in a thermally controlled (25 ± 2 °C) room free from any sources of chemical contamination. Light was maintained on a 12 h dark–light cycle, and rats were fed a standard laboratory diet of rat chow (Pars Dam Co., Tehran, Iran) at the Animal House Lab research center, Birjand.

Study design

Thirty-five male Wistar rats, 6–8 months old and weighing 200–220 g, were obtained from the Birjand University of Medical Sciences animal facility. All animals were

allowed free access to standard chow diet (Pars Dam Co, Tehran, Iran) and tap water *ad libitum*. All of the rats were housed under standard conditions in groups of seven rats/cage at a temperature of 21–24 °C and a constant 12 h light/dark cycle. All of the experimental procedures were performed between 08:00 and 13:00. In each group, seven rats were rendered diabetic by the intraperitoneal injection of streptozotocin (60 mg/kg body weight) (Sigma Aldrich, USA) after fasting for 16 h. After 2 weeks, animals with plasma glucose levels >16.5 mM were considered diabetic. The 28 diabetic rats were divided into four groups in the order listed in Table 1, and seven healthy rats were as assigned to the healthy control group. All animal procedures were approved by the ethical committee of Birjand University of Medical Sciences in accordance with the Institutional Animal Ethics Committee.

Sample preparation

Normal and diabetic rats were anesthetized with ether. Blood was collected from the rats in order to measure glucose, insulin, and other biochemical factors. For gene expression analysis, liver biopsies of normal and diabetic rats were performed, and the samples were placed in liquid nitrogen.

Ferric reducing antioxidant power (FRAP) assay

Total antioxidant power in the serum of the five groups mentioned above was evaluated by the FRAP assay according to the procedures of Benzie & Strain [11]. Ferric iron was reduced to its ferrous state due to the presence of antioxidant activity and low pH levels in the samples, which led to the formation of an intensive blue ferrous-tripyridyltriazine complex. This complex could be monitored at a maximum absorption of 593 nm. In total, 2 ml of FRAP reagent consisting of 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ) solution in HCl, and 20 mM $FeCl_3.6H_2O$ solution in a proportion of 10:1:1 (v/v) was added to 50 μl of sample. After 15 min, the absorbance was measured at 593 nm [11].

Table 1 List of animal groups with different treatments

Groups	Status and treatment
1	Healthy, treated with saline in equivalent volume to the test treatments
2	Diabetic, treated with saline in equivalent volume to the test treatments
3	Diabetic, treated with ethanolic extract of *B.vulgaris* (25 mg/kg)
4	Diabetic, treated with ethanolic extract of *B.vulgaris* (100 mg/kg)
5	Diabetic, treated with glibenclamide (0. 6 mg/kg)

Measurement of thiobarbituric acid reactive substances (TBARS)

The TBARS assay measures the amount of malondialdehyde (MDA) present in the sample. At the end of the treatment period, blood was collected from the hearts of the rats in order to measure the MDA levels. Plasma samples (300 μl) were added to 3 ml TBARS reagent (7.5 g trichloroacetic acid, 187 mg TBA, and 6.25 ml chloridric acid). Next, the mixture was warmed for 20 min in a boiling water bath. Finally, the absorbance of the samples was determined at 532 nm [12].

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Trizol extraction reagent (Bioneer, Korea) was used for the isolation of total RNA from the excised liver tissue of diabetic and normal rats. Total RNA was isolated according to the manufacturer's recommendations. The integrity of the mRNA was confirmed by electrophoresis in a denaturing 1 % agarose gel. The Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas, USA) was used to reverse-transcribe 1 μg of RNA in a final volume of 20 μl. qRT-PCR of β-actin (a reference gene), Hsp70, and HSF1 was performed using the specific primers listed in Table 2. The reaction mixture consisted of 2× ABI SYBR Green PCR Master Mix, 2 μl cDNA, and 0.2 μL of each primer. Amplifications were performed on the ABI Step One™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with 40 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 30s, and data collection at 80 °C for 20 s. The intensities of the mRNA levels were normalized to those of the β-actin product. Gene expression ($2^{-\Delta\Delta CT}$) was compared between the groups treated with *B.vulgaris* and the diabetic controls using the C_T method [13].

Histological studies

For the evaluation of pancreatic β cell regeneration in response to *B.vulgaris*, the number of pancreatic islets

Table 2 Real-time primer sequences

Gene	Sequences
HSPA4 (hsp70)	5'-TGGCATTTTCAGTGTGTCCAG-3' (forward) 5'-CACCTGCATCTTCTCTTCTTCCT-3' (reverse)
HSF1	5'-CTGGCCAGCATTCAGGAACTT-3' (forward) 5'-GTAGTGCACCAGCTGCTTTC-3' (reverse)
HSPB1 (hsp27)	5'-CCCTGGACGTCAACCACTTC-3' (forward) 5'-AGCCATGTTCATCCTGCCTT-3' (reverse)
GK (glucokinase)	5'-TGGTGCTTTTGAGACCCGTT-3'(forward) 5'-GAAGCCCCAGAGTGCTTAGG-3'(reverse)
G6P (glucose 6-phosphatase)	5'-CGTCACCTGTGAGACTGGAC-3'(forward) 5'-ACGACATTCAAGCACCGGAA-3'(reverse)
β-actin	5'-GTCCACCCGCGAGTACAAC-3' (forward) 5'-GACGACGAGCGCAGCGATA-3' (reverse)

was calculated. For this analysis, the pancreatic tissue was removed and immediately fixed in 10 % formalin. After fixation, paraffin blocks were cut into 5 μm thick sections and stained with hematoxylin and eosin. Next, the stained sections of pancreatic tissue were examined under a light microscope. The total number of islet cells in each section was counted, the total area of each stained section was characterized, and the average number of pancreatic islets per cm^2 was calculated.

Statistical analysis

Using SPSS version 16, the data were statistically computed by one-way ANOVA, and the means were compared using Student's *t* test. $P < 0.05$ was considered statistically significant. The data are expressed as mean ± SD.

Results

Effects of *B.vulgaris* on biochemical parameters and oxidative stress markers

There was a significant difference in the total antioxidant and MDA levels between diabetic rats and normal rats (Table 3). The total antioxidant levels were significantly lower in diabetic rats and exhibited markedly increased MDA levels and had significantly decreased total antioxidant power ($P \leq 0.05$) compared to the control rats. The results show that treatment with *B.vulgaris* significantly increased the total antioxidant fresh mass levels, decreased elevated MDA levels, and increased the pancreatic islets ($P \leq 0.05$). The insulin level was drastically decreased in the diabetic rats, and treatment with *B.vulgaris* dose-dependently improved insulin secretion as well as reduction in FBG (Table 3).

Effect of *B.vulgaris* on the gene expression of HSP70, HSP27, and HSF1

A significant increase in the expression of HSP70, HSP27, and HSF1 genes ($P < 0.05$) in diabetic rats compared to the normoglycemic control group, assessed by mRNA level, is shown in Fig. 1. Treatment with *B.vulgaris* reduced the expression level of HSP70, HSP27, and HSF1 in a dose-dependent manner.

Effects of *B.vulgaris* on the gene expression of glucokinase (GK) and glucose 6-phosphatase (G6P)

GK was downregulated in samples from diabetic rats compared with normal group. *B.vulgaris* treatment increased the gene expression of GK in diabetic rats compared with the diabetic control group (Fig. 2). These relationships were statistically significant ($P < 0.05$). G6P gene was upregulated in samples from diabetic rats compared with normal tissue (Fig. 2). The gene expression of G6P levels in diabetic rats decreased in the presence of *B.vulgaris* compared to the diabetic control group ($P < 0.05$).

Discussion

The data presented here shows that *B.vulgaris* hydroalcoholic extract ameliorates the hyperglycemia-mediated oxidative stress and related gene expression. DM is a major illness characterized by hyperglycemia due to absolute or relative deficiency of insulin [1]. In this study, we have demonstrated that an alcoholic extract of the *B.vulgaris* plant is able to normalize blood glucose levels and reduce stress protein expression in rats with streptozotocin-induced diabetes.

The alcoholic extract from *B.vulgaris* is recommended for the management of diabetes. Considering the wide use of this plant, in this study, we investigated the plasma glucose and insulin levels as well as oxidative stress markers under treatment with *B.vulgaris* in diabetic rats. The MDA and FBG levels were increased, in contrast to insulin and total antioxidants, which were decreased in rats with streptozotocin-induced diabetes (Table 3). The results shown in Table 3 clearly indicate that *B.vulgaris* significantly increase insulin, pancreatic β cell numbers and total antioxidants and spontaneously reduce blood FBG and MDA levels. The findings of the current study are in close agreement with the results of other studies [14, 15]. Several studies have demonstrated the beneficial effects of *B.vulgaris* on oxidative stress parameters in diabetic rats, which are consistent with our results [6, 16]. In diabetes, the pancreas is considered to be the principal target organ susceptible to oxidative stress and damage produced by free radicals. Therefore,

Table 3 Effects of *B.Vulgaris* on biochemical parameters of normoglycemic and STZ-induced diabetic rats

Experimental groups[a]	Fasting Blood Glucose (FBG) (mmol/L)	Insulin (μU/ml)	Malondialdehyde (MDA) (μmol/L)	Total antioxidant (FRAP) (μmol/L)	Number of pancreatic islets (cm^{-2})
Normoglycemic Control	7.6 ± 0.4[b*]	15.2 ± 0.7*	1.27 ± 0.5*	780 ± 14*	43 ± 3.5*
Diabetic Control	25 ± 1.2	7.4 ± 0.3	4.3 ± 0.7	550 ± 21	13 ± 1.8
Diabetic + *B.vulgaris* (25 mg/kg body mass)	12.9 ± 0.7*	9.2 ± 0.5	2.9 ± 0.3*	655 ± 18*	18 ± 1.9*
Diabetic + *B.vulgaris* (100 mg/kg body mass)	8.2 ± 0.4*	11.9 ± 0.6*	2.1 ± 0.2*	760 ± 16*	29 ± 2.6*
Diabetic + Glibenclamide (0.6 mg/kg body mass)	7.8 ± 0.8*	13.4 ± 0.4*	1.85 ± 0.1*	810 ± 15*	34 ± 0.4*

[a]For details of experimental conditions see the text
[b]Data are expressed as mean ± SD of 8 rats in each group. In each column
*was considered significant at $P < 0.05$ when compared with the diabetic control group

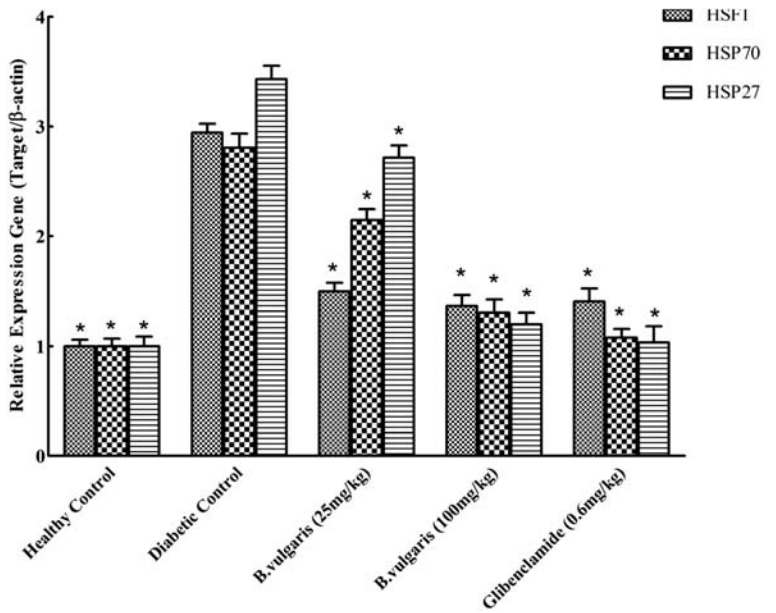

Fig. 1 The effect of *B.vulgaris* on the gene expression of HSF1, HSP70, and HSP27 mRNA levels. *B.vulgaris* decreased the expression of these genes in the pancreas tissue of diabetic rats. Data represents relative gene expression (target gene/β-actin) and are expressed as the mean ± SEM of three replicates. *$P < 0.05$ was considered a significant difference between diabetic and control groups

agents with antioxidant properties are quite useful and reasonable in the treatment of pancreatic oxidative stress [15, 17]. The antioxidant properties of different varieties of *B.vulgaris* from various countries have been demonstrated [18]. Furthermore, *B.vulgaris* contains phenolic compounds with beneficial antioxidant activities that can prevent chronic diseases and cancers [5].

In the present study, we found that treatment with *B.vulgaris* led to a significant decrease in the blood glucose level by decreasing the gene expression of G6P

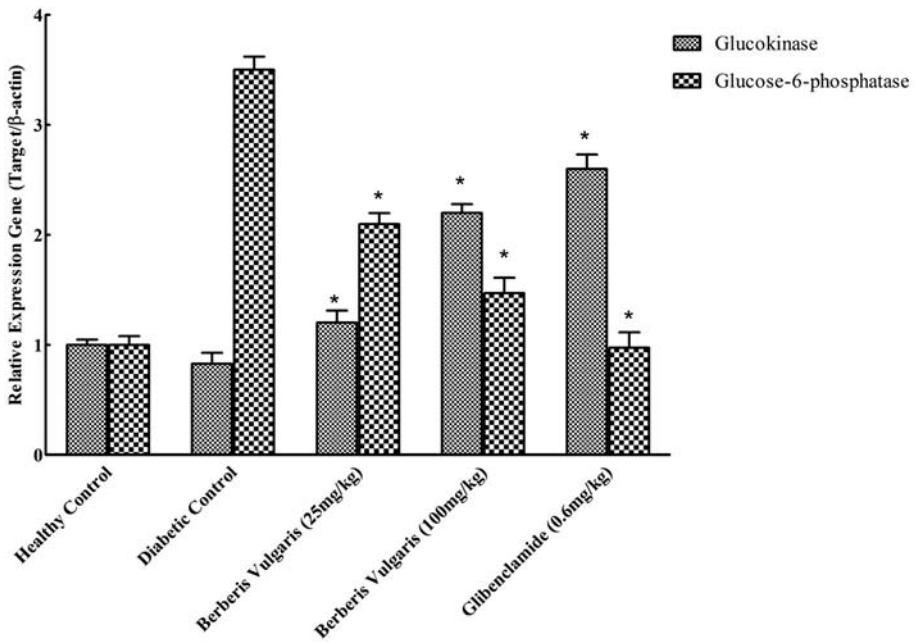

Fig. 2 The effect of *B.vulgaris* on the gene expression of GK and G6P as assessed by mRNA levels. *B.vulgaris* altered the GK and G6P expression in the liver tissue from diabetic rats. The data represents relative gene expression (target gene/β-actin) and are expressed as the mean ± SEM of three replicates. *$P < 0.05$ was considered significant

(Fig. 2). These results suggest that *B.vulgaris* downregulates the gluconeogenesis pathway in diabetic rats. There was also a significant decrease in GK mRNA level in diabetic rats when compared with the normal group. Treating diabetic rats with *B.vulgaris* resulted in a significant improvement in the gene expression of GK compared with the diabetic control group ($P < 0.05$; Fig. 2). Furthermore, it was established that the expression of stress genes such as HSP70, HSP27, and HSF1 increased significantly in the pancreas tissue of diabetic rats. These results support the hypothesis that there is a significant correlation between oxidative stress and the gene expression of HSPs. We also demonstrated that *B.vulgaris* ameliorated oxidative stress due to a reduction in HSP70 and HSP27 gene expression (Fig. 1). β Cell regeneration observed in *B.vulgaris* treating groups confirms protective effects of *B.vulgaris* in pancreas (Table 3). These observations revealed that *B.vulgaris* in a dose-dependent manner and tissue specifically improved hyperglycemia-mediated oxidative stress in diabetic rats. *B.vulgaris* in high doses showed the same effect as glibenclamide that can be considered for use in pharmaceutical industry. In the present study, the biochemical, gene expression, and histological results collectively revealed that *B.vulgaris* has protective effects against oxidative stress in diabetic rats. Moreover, the hypoglycemic effect of *B.vulgaris* could be through an increase in insulin level, resulting in the regulation of the glycolysis and gluconeogenesis pathways, and, in this case, the effect on glycolysis is notable. To better understand the exact mechanism of the effects of *B.vulgaris* and to develop its application in clinical trials, additional genes involved in glucose and lipid metabolism and related signaling pathways should be investigated.

Conclusions

B.vulgaris ameliorated oxidative stress in diabetic rats by increasing total antioxidant capacity accompanied by a decrease in lipid peroxidation. Reduced level of stress genes in treated diabetic groups confirm the ability of *B.vulgaris* to decrease pancreatic oxidative stress and resulting regeneration in β cells. Moreover, the mechanism of the hypoglycemic effect of this plant may be through modification in the gene expression of hepatic GK and G6P. To uncover exact mechanism action of *B.vulgaris*, its needed to understand the specific role of stress proteins in pancreatic β cells regeneration.

Abbreviations

FBG: fasting blood glucose; FRAP: Ferric reducing antioxidant power assay; G6P: Glucose 6-phosphatase; GK: Glucokinase; HSF: Heat shock factor; HSP: Heat shock protein; MDA: Malondialdehyde; ROS: Reactive oxygen species.

Competing interests

The authors declare that there is no competing interest.

Authors' contributions

MH designed the study and participated in data analysis. ES participated in study design and helped in manuscript preparation. M Gh and RH participated in data analysis and manuscript preparation. All authors read and approved the final manuscript.

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