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Comparative study of anti-diabetic effects of insulin, epigenin and *Salvia mirzayanii* extract in streptozotocin-induced diabetic male rats

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The use of hypoglycemic medications for diabetes has several limitations, such as adverse reactions and high rates of secondary failure. These adverse effects have forced patients with diabetes to use herbal medicines that have a similar degree of potency without side effects. So, there has been a growing interest in hypoglycemic agents from natural products, in particular, those derived from plant materials. In other words, searching for better agents from herbs or natural products to treat diabetes is duly needed. The purpose of this study was to compare the anti-diabetic effects of *Salvia mirzayanii* extract with insulin and epigenin in diabetic male rats.

The plant material was initially extracted and administered orally to the animals. After treatment of rats with insulin, epigenin or aqueous extract of *S. mirzayanii*, oral glucose tolerance test (OGTT), fasting blood glucose and animal weight changes were examined. To analyze the molecular function of insulin, epigeninand *S. mirzayanii*, expression of glucose transporter-4 (GLUT4), phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) genes in healthy and streptozotocin (STZ)-diabetic male rats were studied as well. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed for all groups and the data were normalized using the formula $2^{-\Delta\Delta Ct}$.Statistical analysis was performed by one-way analysis of variance.

In fasting blood glucose and OGTT, there was no significant difference between the normal control group and the diabetic groups treated with insulin, epigenin and *S. mirzayanii*. But there was a significant difference with the uncontrolled diabetic group ($P < 0.05$). Meanwhile, the uncontrolled diabetic group gained less weight compared to the other groups. RT-qPCR of beta-actin, GLUT4, G6Pase and PEPCK genes yielded products with lengths of 228, 140, 79 and 151 bp, respectively. In gene expression studies, there was a significant difference between the mRNA levels of GLUT4, G6Pase and PEPCK in control groups and the groups treated with insulin, epigenin and *S. mirzayanii*. The greatest effect on increasing the mRNA expression of GLUT4 was related to insulin, epigenin and *S. mirzayanii*, respectively. The greatest effect in reducing the mRNA expression of PEPCK was related to insulin, epigenin and *S. mirzayanii*, respectively. The greatest effect in reducing the mRNA expression of G6Pase was also related to insulin, but the effect of epigenin and *S. mirzayanii*was almost the same. Overall, obtained results show that *S. mirzayanii* can be utilized as a herbal medication with insulin and epigenin mimetic-activity.

Keywords: Diabetes, Epigenin, G6Pase, GLUT4, Insulin, PEPCK, *Salvia mirzayanii*

Diabetes is a chronic health condition with increasing incidence in human societies throughout the world. Diabetes mellitus is a metabolic syndrome disorder characterized by prolonged hyperglycemia and hyperlipidemia. It is associated with neuropathy, nephropathy, retinopathy, cardiovascular and cerebrovascular complications which are the main causes of morbidity and mortality. The increasing incidence of diabetes in children and young people, along with the associated morbidity, mortality, and growing racial and ethnic disparities, is a major public health challenge[1,2]. According to a recent report, new estimates predict that the number of people with diabetes will reach to nearly more than 1.3 billion

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people worldwide by the year 2050, up from about 529 million in 2021[3]. Patients with diabetes are generally treated with insulin and synthetic anti-diabetic agents, such as metformin. However, some of these drugs have limited efficacy and notable side effects, including drug tolerance, headache, megaloblastic anemia, dermatitis, acidosis, *etc*. Therefore, searching for safer and more effective hypoglycemic pharmaceuticals is of utmost interest[4-6].

In recent years, medicinal plants have provided valuable anti-diabetic therapeutics as complementary or alternative approaches to existing medications[7]. Herbal drugs with anti-diabetic potential act by their insulin-mimetic properties, inhibiting intestinal absorption of glucose, or insulin-dependent metabolic processes[8,9]. The hypoglycemic activity of a large number of medicinal plants has been evaluated and confirmed in different animal models[10-12]. For example, the members of the genus *Salvia* have attracted considerable interest due to the advantages, such as robust antioxidant, antiglycating and fewer side effects[13]. *Salvia* is the largest genera of the Lamiaceae family containing over 900 species throughout the world which demonstrate varied therapeutic activities to treat a variety of diseases[13,14]. Various species of *Salvia* family are used in conventional medicine to treat diabetes[15,16]. In folk Iranian medicine, *Salvia mirzayanii*, locally called Bitter Moor, is a perennial plant and grows in southern and central parts of Iran. The extract of *S. mirzayanii* has been used for diabetes and its antioxidant activity demonstrates this effect[13-15]. The hypoglycemic activity of *S. mirzayanii* in diabetic patients has also been reported by Moein and her colleagous[15]. Javid and his coworkers showed the significant inhibitory effect of *S. mirzayanii* extract on α-amylase as a diabetes marker enzyme[16]. The fact that *S. mirzayanii* is commonly used in folk medicine for the treatment of diabetes, and there are few reports of the anti-diabetic influences of this plant make it important that more detailed investigations through molecular function analysis are performed.

In this communication, we aimed to compare the anti-diabetic effects of *S. mirzayanii* extract with insulin and epigenin in diabetic rats. For this purpose, biochemical tests and expression analysis of glucose transporter-4 (GLUT4), phosphoenolpyruvate carboxykinase (EC 4.1.1.32, PEPCK) and glucose 6-phosphatase genes (G6Pase) in streptozotocin (STZ)-induced diabetes in rats were carried out.

Materials and Methods

Materials

RNA extraction kit and reverse transcription quantitative polymerase chain reaction (RT-qPCR) kits were procured from QIAGEN Company (Germany). Primers were synthesized by Bioneer Company (Daejon, Korea).

Preparation of *S. mirzayanii* aqueous extract

After providing the aerial parts and removing impurities, 100 g of the dried plant was initially boiled in 1 L of distilled water for 10 min and then maintained at 70°C for 24 h. The aqueous extract was filtered and dried at 50°C. The obtained extract (20 g per 100 g of crushed plant) was mixed and diluted with normal saline to achieve a specific concentration (600 mg/mL) and then filtered. The extract was stored at 4°C until used for experiments[13].

Animals

Male wistar rats at ages 6-9 months (200-250 g) and their foods were purchased from the Pasteur Institute of Iran, Iran. They were housed in an air-controlled room at a 12 h: 12 h light-dark cycle supplied with a pellet diet and tap water. The ingredients of pellet diet include; 23.0% crude protein, 10.0% moisture, 0.5% sodium chloride, 1.0% calcium, 0.65% phosphorus, 0.25% tryptophan, 0.33% methionine, 1.15% lysine, 0.7% threonine, 4.0% crude fat and 4.0% crude fiber. They were allowed to adapt to the laboratory conditions for one week before the beginning of the experiment. Also, they had free access to water and food before and after treatment. The rats were randomly divided into five groups (n=5), as follows: 1- Normal control (NC) group received a normal diet. 2- Diabetic control (DC) group received 1 mL of normal saline. 3- Diabetic rats treated orally with an aqueous extract of *S. Mirzayanii* (600 mg/kg) for two weeks. 4- Diabetic rats treated intraperitoneally with insulin (2.2 IU/kg). 5- Diabetic rats treated intraperitoneally with epigenin (10 mg/kg)[17]. The animals were fasted overnight (for about 12 hours) before the induction of anesthesia or the collection of blood samples. The exclusion criterion was the death of rats during the study. Finally, the animals were beheaded with a guillotine under complete anesthesia[13]. This study was approved by the ethics committee of Qazvin University of Medical Sciences (Ethical code# IR.QUMS.AEC.1402.006).

Induction of diabetes

Before the administration of STZ, animals were fasted but had free access to water. Diabetes was induced by a single intraperitoneal injection of STZ solution (50 mg/kg body weight)[11]. Control animals received an injection with normal saline. After 48 h of induction of diabetes, fasting blood sugar (FBS) levels were measured with a hand-held glucometer (Easy Gluco, Infopia Co., Ltd., South Korea). Rats with FBS levels above 220 mg/dl were considered as diabetic. Diabetic rats exhibited symptoms of frequent urination and polydipsia[11,18].

Evaluation of FBS, oral glucose tolerance test (OGTT) and animal weight changes

FBS levels were measured using Easy Gluco during the testing and the day before diabetes induction. The OGTT, in which the rats are challenged with a bolus of glucose, was performed across a two-hour time course according to the standard protocol[11,18]. Prior to the test, the rats were fasted for 16 hours and blood glucose level was monitored from the tail-tip using Easy Gluco. The rats then received 2.5 g/kg body weight of a 100 mg/mL glucose solution in sterile water delivered by oral gavage. At 10, 20, 30, 60, 90, and 120 min after the administration of glucose, blood was collected from the tail to measure the glucose concentration. The change of body weight of animals was regularly recorded during the experiment period.

Preparation of tissue samples

On the day of surgery, all rats were anesthetized with ketamine HCL (50 mg/kg). Following complete anesthesia, a piece of gastrocnemius muscular and hepatic tissues was isolated. Afterwards, the prepared samples were washed with cold normal saline and placed at −80°C in an RNA buffer solution for further experiments. Finally, the animals were beheaded with a guillotine under complete anesthesia[11,19].

Expression analysis of GLUT4, PEPCK andG6Pase

To investigate the effects of *S. mirzayanii* extract, mRNA expression measurements of GLUT4, PEPCK andG6Pase was performed by RT-qPCR. The primers of GLUT4، PEPCK, G6Pase and β-actin were designed using DNASIS MAX 3.0 software (DNASIS version 3.0, Hitachi Software Engineering Co., Ltd., Tokyo, Japan) (Table 1). β-actin gene was used as an internal standard (housekeeping gene). Frozen liver and muscle samples of each group were thawed and used for RNA extraction. Total RNA was extracted via RNeasy Plus Universal Mini Kit (QIAGEN, Germany). The purity of RNA at 260/280 OD ratio and RNA integrity was evaluated and only samples of high purity (OD 260/280 > 1.8) were subjected to further manipulation. RT-qPCR was performed using QuantiNova SYBR Green RT-qPCR Kit (QIAGEN, Germany). The PCR products were electrophoresed in 4.0% agarose gel, stained with Safe-Green dye and visualized under a UV lamp. The results were normalized with the levels achieved for β-actin and then calculated relative to the control group.

Statistical analysis

All statistical tests were carried out by GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA). Data were presented as mean ± standard deviation, and statistically analyzed using one-way ANOVA.

Results and Discussion

Diabetes is a chronic metabolic syndrome that affects the population worldwide. Knowledge and awareness of diabetes, its risk factors, complications and management are important aspects for better control and improving the quality of life. The use of hypoglycemic medications for diabetes has several limitations, such as adverse reactions and high rates of secondary failure. These adverse effects have forced patients with diabetes to

Table 1 — Primer sequences and expected ampliocn size of the genes for RT-qPCR analysis

Gene	Sequence (5′→3′)	Length (mer)	Tm (°C)	GC%	Product Size (Base pair)
GLUT4	Forward: ACAATGTCTTGGCTGTGCTG	20	55.2	50	140
	Reverse: TCCCACATACATAGGCACCA	20	55.4	50	
PEPCK	Forward: CATTACCCAAGAGCAGAGAG	20	51.2	50	151
	Reverse: GAATGGGATGACATACATGG	20	51.4	45	
G6Pase	Forward: TTCCGGTGCTTGAATGTCGT	20	60.0	50	79
	Reverse: GCAAGGTAGATCCGGGACAG	20	58.4	60	
β-actin	Forward: ACGGTCAGGTCATCACTATC	20	50.1	50	228
	Reverse: AGAGGTCTTTACGGATGTCA	20	50.6	45	

utilize herbal medicines that have a similar degree of potency without side effects. For these reasons, there has been a growing interest in alternative hypoglycemic agents from natural products, in particular, those derived from plant materials. Thus, searching for better agents from herbs or natural products that can be used to treat diabetes is duly needed[20-22].

In recent years, numerous plant extracts and plant formulations have been found to regulate the expression of genes in metabolic pathways of diabetic animal models[23]. The change in gene expression is an important component of the pathogenesis of diabetes. Different species of *Salvia* have been used in traditional medicine to treat diabetes[24-26]. Many of these species and their compounds have considerable antioxidant properties acting by enzymatic and non-enzymatic pathways[27,28]. Previous studies have reported the hypoglycemic effect and anti-hyperlipidemic potency of *S. mirzayanii* in diabetes in rats, while the action mode of this plant has not been reported yet. In this communication we evaluated the influence of *S. mirzayanii* on the GLUT4 as a glucose transporter and two key enzymes of carbohydrate metabolism at the mRNA levels of diabetic rats using RT-qPCR, and compared with the obtained results for insulin and epigenin.

Effect of *S. mirzayanii* on FBS, OGTT and body weight

To study the effect of *S. mirzayanii* extract on diabetic rats, FBS levels, OGTT and body weight changes were measured. During 12 weeks of extract administering, *S. mirzayanii*, insulin and epigenin suppressed the increase in FBS level in the treated diabetic group compared to the diabetic control group (Fig. 1). The results of OGTT in the rats treated with *S. mirzayanii*, insulin and epigenin showed a reduction in glucose levels compared to the normal control and diabetic control groups (Fig. 2). Results from body weight measurements also revealed less changes in the diabetic groups treated with *S. mirzayanii*, insulin and epigenin than in the diabetic control group. (Fig. 3). As preliminary data, these obtained results mean that *S. mirzayanii* has an anti-diabetic effect. These data were in agreement with the findings about *S. mirzayanii*[13,15,29]. The hypoglycemic effects for the other species of *salvia* genus like *S. hydrangea*[30], *S. officinalis*[31],

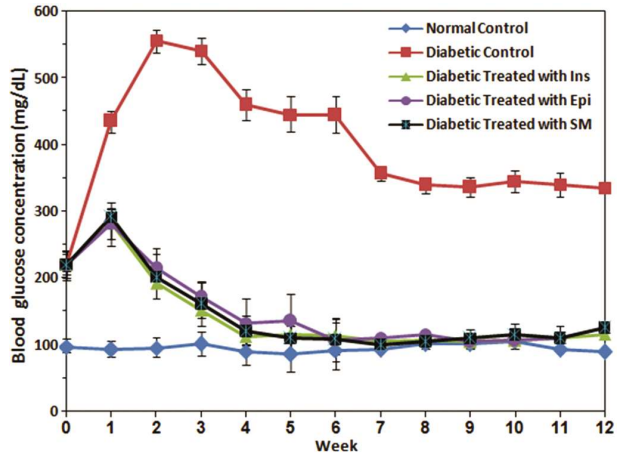

Fig. 1 — Variation of blood glucose concentration during 12 weeks in normal control group, diabetic control group, diabetic group treated with S. mirzayanii (SM) aqueous extract, diabetic group treated with insulin (Ins) and diabetic group treated with epigenin (Epi)

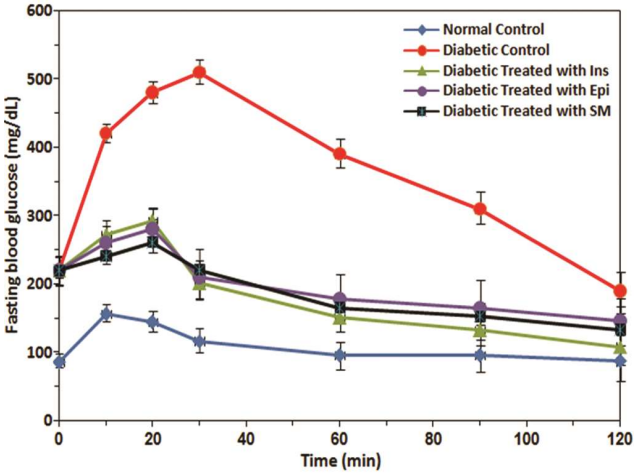

Fig. 2 — Variation of fasting blood glucose in OGTT after 120 min in normal control group, diabetic control group, diabetic group treated with *S. mirzayanii* (SM) aqueous extract, diabetic group treated with insulin (Ins) and diabetic group treated with epigenin (Epi)

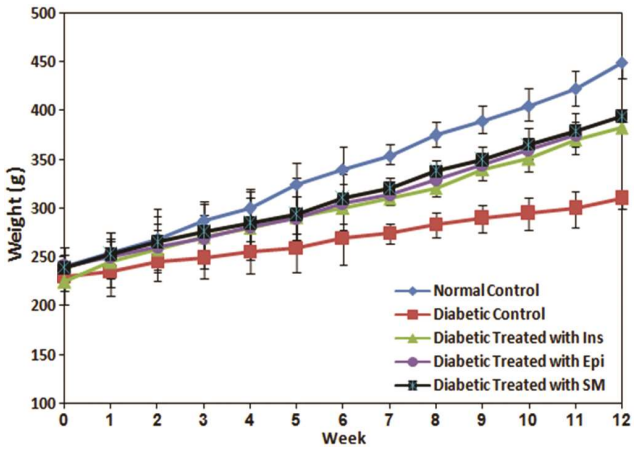

Fig. 3 — Variation of body weight after 12 weeks in normal control group, diabetic control group, diabetic group treated with *S. mirzayanii* (SM) aqueous extract, diabetic group treated with insulin (Ins) and diabetic group treated with epigenin (Epi)

S. sahendicahas[32], *S. miltiorrhiza*[33,34] have also been reported in previous studies.

Molecular function analysis of *S. mirzayanii*

In order to better analyse the molecular function of *S. mirzayanii* and compare with the activity of insulin and epigenin, the effect of this herb as well as insulin and epigenin on the expression of GLUT4, PEPCK and G6Pase was studied. The expression levels of GLUT4, PEPCK andG6Pase genes were assessed in normal control rats, diabetic control rats and diabetic rats treated with *S. mirzayanii* aqueous extract, insulin and epigenin.

Effect of *S. mirzayanii* on mRNA and protein expression of GLUT4

GLUT4 is a transmembrane carrier protein that allows glucose to passively move through cell membranes. It is the main transporter for the transfer of glucose between muscle and blood and for the reabsorption of glucose in the kidney. The mRNA levels of GLUT4 in the muscle tissue of all groups were analyzed. The expression level of GLUT4 mRNA in the normal control group was considered as a reference to calculate the other groups. Treatment of diabetic rats with *S. mirzayanii*, insulin and epigenin aqueous extract elevated GLUT4 mRNA expression when compared with the normal control and diabetic control groups (Fig. 4). RT-qPCR results showed that *S. mirzayanii*, insulin and epigenin administration in diabetic rats induces a significant increase in GLUT4 expression. This finding means that *S. mirzayanii* increases GLUT4 expression to induce effects like insulin and epigenin. As seen (Fig. 4), the greatest effect on increasing the mRNA expression of GLUT4 was achieved with insulin, *S. mirzayanii* and epigenin, respectively.

Effect of *S. mirzayanii*, insulin and epigenin on mRNA expression of PEPCK and G6Pase

The expression of PEPCK and G6Pase mRNAs were recorded when the diabetic rats treated with *S. mirzayanii* aqueous extract, insulin and epigenin. After treatment with *S. mirzayanii*, insulin and epigenin, the expression of PEPCK (Fig. 5) and G6Pase (Fig. 6) mRNAs were significantly decreased compared to the normal control and diabetic control rats. These findings suggest that the anti-hyperglycemic action of *S. mirzayanii* is likely to be associated with up-regulation of GLUT4 in muscle and down-regulation of PEPCK and G6Pase expressions in liver as documented by RT-qPCR. The greatest effect in reducing the mRNA

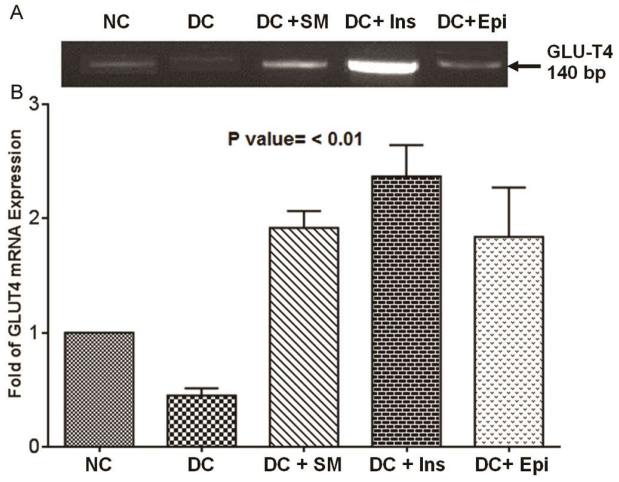

Fig. 4 — The densitometry (A) and RT-PCR analysis (B) of GLUT4 mRNA expression in normal control (NC), diabetic control (DC), diabetic control treated with SM (DC + SM), diabetic control treated with insulin (DC + Ins) and diabetic control treated with epigenin (DC + Epi). Data were normalized with that of β-actin and then calculated as relative to the NC group. Each value represents the mean ± SEM (n = 10 per group) in two independent experiments. $P< 0.01$: Significant difference *vs*. control group

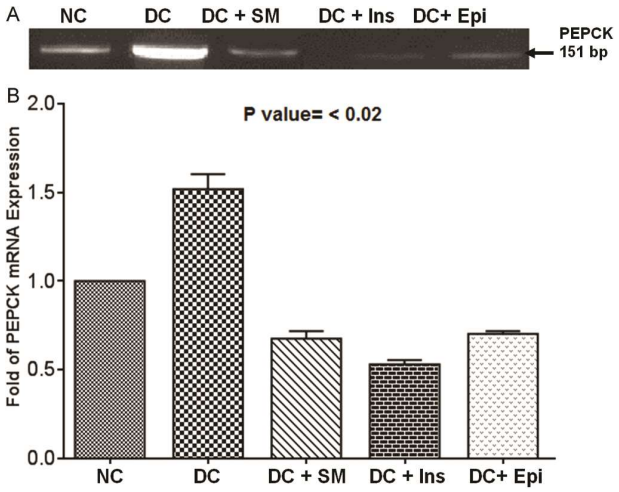

Fig. 5 — The densitometry (A) and RT-PCR analysis (B) of PEPCK mRNA expression in normal control (NC), diabetic control (DC), diabetic control treated with SM (DC + SM), diabetic control treated with insulin (DC + Ins) and diabetic control treated with epigenin (DC + Epi). Data were normalized with that of β-actin and then calculated as relative to the NC group. Each value represents the mean ± SEM (n = 10 per group) in two independent experiments. $P< 0.02$: Significant difference *vs*. control group

expression of PEPCK was related to insulin, *S. mirzayanii* and epigenin, respectively. Additionally, the greatest effect in reducing the mRNA expression of G6Pase was related to insulin, but the effect of epigenin and *S. Mirzayanii* was almost the same.

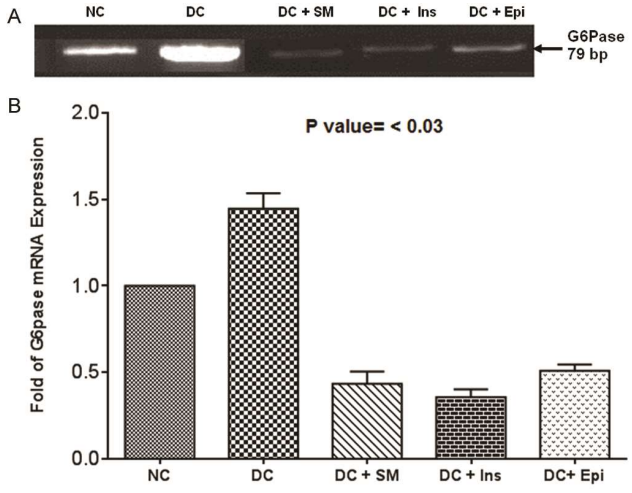

Fig. 6 — The densitometry and RT-PCR analysis of G6Pase mRNA expression in normal control (NC), diabetic control (DC), diabetic control treated with SM (DC-Treated with SM), diabetic control treated with insulin (DC-Treated with Ins) and diabetic control treated with epigenin (DC-Treated with Epi). Data were normalized with that of β-actin and then calculated as relative to the NC group. Each value represents the mean ± SEM (n = 10 per group) in two independent experiments. *P*< 0.03: Significant difference *vs*. control group

Conclusion

In this work, *S. mirzayanii* aqueous extract significantly influenced the biochemical parameters, e.g., FBS level and OGTT. In fasting blood glucose and glucose tolerance tests, there was no significant difference between the control group and the diabetic groups treated with insulin, epigenin and *S. mirzayanii* extract. In gene expression analysis, the greatest effect on increasing the mRNA expression of GLUT4 was related to insulin, epigenin and *S. mirzayanii*, respectively. The greatest effect in reducing the mRNA expression of PEPCK was related to insulin, epigenin and *S. mirzayanii*, respectively. The greatest effect in reducing the mRNA expression of G6Pase was related to insulin, but the effect of epigenin and *S. mirzayanii* was nearly the same. These findings indicate that *S. mirzayani* can improve hyperglycemia via the increment of GLUT4 mRNA level, and prohibition of gluconeogenesis pathway in the liver. We can conclude that *S. mirzayanii* extract affects the genes related to glucose metabolism via up-regulation of GLUT4 and down-regulation of PEPCK and G6Pase expressions. The potential *S. mirzayanii* in decreasing hyperglycemia was lower than insulin, but was nearly similar to the epigenin. In other words, *S. mirzayanii* can be a good herbal medication with insulin and epigenin mimetic-activity.

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Conflict of interest

All authors declare no conflict of interest.

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