



The Effect of Rattle Plant Fruit's Hydroalcoholic Extract on the Phosphofructokinase-1 Gene Expression Level in Type-1 Diabetic Rats

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Abstract

Diabetes is one of the most common metabolic disorders and is currently considered one of the most important causes of death in human societies. For many years, natural medicines, especially medicinal plants, served as the basis for treatment, with their raw materials used in the pharmaceutical industry. In the present study, the effect of the hydroalcoholic extract of the fruit of the rattle plant on blood sugar and phosphofructokinase-1 (PFK-1) expression was investigated. In this study, 45 male rats (150-300 g) were randomly divided into 3 groups (n= 15) (the healthy group, the diabetic group without treatment, and the diabetic group under the treatment of rattle fruit extract). Type 1 diabetes was induced by the injection of streptozotocin. The diabetic group received 300 mg/kg of rattle fruit extract through gavage for 30 days. The blood glucose level and the expression level of the PFK-1 gene in liver tissue were measured. The results of the present study show that blood glucose was significantly reduced in treated diabetic rats compared to untreated diabetic rats. The extract of the rattle plant fruit does not affect the expression of the phosphofructokinase gene. The present study shows that the consumption of hydroalcoholic extract from the fruit of the rattle plant had an effect on glucose reduction, but it did not affect the level of gene expression.

Keywords: Rat, Diabetes (type 1), Rattle plant, phosphofructokinase-1

Background

Many plants are studied in the traditional medicine of different nations for the treatment of diabetes. The hypoglycemic effects of many of these plants have been investigated and confirmed [1, 2]. Among the medicinal plants, garlic, onion, dill, green tea, sage, cumin, and cinnamon are known as herbal medicines effective in controlling the blood glucose levels of patients. However, the antidiabetic effects of many other medicinal plants remain undiscovered [3, 4]. Among these, the rattle plant (*Prosopis farcta*) belongs to the Leguminosea family and Mimosoideae subfamily, which is native to the dry and barren areas of Western America, Africa, and South Asia; e.g., Iran, India, Afghanistan, etc (Figure 1) [5]. It is cultivated in Sistan and Baluchistan provinces and is reported to have healing pharmacological effects for stomach ulcers, dysentery, rheumatism, laryngitis, chest pain, and shortness of breath [2]. Moreover, the anti-diabetic [2, 3], anti-spasm, and anti-inflammatory benefits of the rattle plant have been mentioned in other studies [2, 6]. Alkaloid compounds and sesquiterpenes in this plant show antimicrobial activity [3]. Many antidiabetic agents affect glucose metabolism in the liver. Various enzymes, including phosphofructokinase-1 (PFK-1), are actively involved in glucose metabolism.

In physiological conditions, this reaction can be irreversible. The enzyme, together with ATP, produces fructose-1-6-bisphosphate by phosphorylating fructose-6-phosphate for the second time. Also, in abundant glucose

conditions, the concentration of fructose-2-6-bisphosphate increases and strengthens the glycolysis pathway by activating phosphofructokinase-1 and inhibiting fructose 1-6 bisphosphatase. However, in glucose deficiency conditions, the concentration of fructose 2-6-phosphate decreases, causing the inactivation of phosphofructokinase-1 on one side, and the activation of fructose 1-6 bisphosphatase on the other, thereby strengthening the glucose pathway regeneration. The mentioned regulatory process causes glucagon's effect in strengthening glucose regeneration to lead to the release of glucose in the liver rather than increasing the rate of glycolysis [7]. Any disturbance in the process of this cycle causes diabetes. Diabetes is characterized by high blood glucose levels and a disorder in the metabolism of carbohydrates, fats, and proteins, which is accompanied by an absolute or relative lack of insulin.

Any alterations in PFK-1 activity can impact the balance of glucose utilization and energy production in the body. Studies have shown that abnormalities in PFK-1 activity may contribute to insulin resistance, a hallmark of type 2 diabetes, as well as impaired glucose uptake and utilization by cells. In the context of diabetes, particularly type 2 diabetes, there is evidence to suggest that dysregulation of PFK-1 activity may be linked to the development and progression of the disease [8]. The increasing glycolytic rate at the PFK step in nondiabetic cells leads to losses in cell proliferation and mitochondrial activity redolent of diabetic CPCs [9].



Figure 1. The fruit of the rattle plant used in the study

Furthermore, dysregulation of PFK-1 can also affect the production of other metabolites and signaling molecules that play a role in glucose homeostasis and insulin sensitivity. Research has indicated that PFK-1 activity may be influenced by various factors, including insulin levels, glucose concentrations, and hormonal signals, all of which are dysregulated in diabetes [10]. Overall, the relationship between PFK-1 and diabetes is complex and multifaceted, and further research is needed to fully understand the molecular mechanisms underlying this connection. Targeting PFK-1 activity may hold potential therapeutic implications for the management of diabetes and related metabolic disorders.

Methods

This experimental study was conducted on 2-3-month-old male Wistar rats with an average weight of 150-300 grams. 45 rats were randomly divided into 3 groups of 15: group 1-healthy (non-diabetic control), group 2-diabetic without treatment (diabetic control), and group 3- diabetic with treatment (diabetic treated with extract). To evaluate the changes in gene expression studied in this research, samples extracted from rat liver tissue were used. The fasting blood glucose level and weight of the rat were measured at three time points.

Hydroalcoholic extract preparation method

To prepare the hydroalcoholic extract, the fruit of the rattle plant was first collected from the surface of the desert lands of eastern Iran. Then the dried fruit is powdered. 500 grams of the obtained powder were poured into a 5000 mL beaker, and 96% ethyl alcohol, which was converted to 70% alcohol with distilled water, was added until it covered the powder. It was stirred with a Heidolph homogenizer for one hour at a speed of 2. Then it was kept in the dark for 48 hours. The resulting solution was passed through filter paper and then filtered once more with a vacuum pump to ensure the purity of the extract. The solution obtained from the last stage was dried in an oven under sterile conditions at 45 degrees for 24 hours. The dried powder was ultimately weighed and stored at

4°C. Then, by adding physiological serum to a certain weight of the extract, the concentration of 300 mg/kg extract was prepared and used to treat diabetic rats.

Type-1 diabetes induction method

The experimental model of type-1 diabetes mellitus (insulin-dependent diabetes) was established in male rats by intraperitoneal injection of streptozotocin. A one-gram vial of streptozotocin powder, purchased from Sigma with the code S0130, was dissolved in 0.1 M normal saline buffer with a pH range of 2.4-5.3. The prepared streptozotocin solution reached a volume of 60 cc. The net amount of streptozotocin injected into each animal was 60 mg per kilogram of body weight. The animals were considered diabetic if their blood glucose values were above 140 mg/dl on the third day after injection. The injection procedure had to be repeated for rats with fasting blood glucose levels below 140 mg/dl [11]. Blood glucose levels were also measured on days 15 and 30 after injection with streptozotocin. The weight of the rats was measured simultaneously with their blood glucose level using a digital scale.

Treatment with the hydroalcoholic extract of rattle fruit

The diabetic group was treated for 30 days using 300 mg/kg of fruit hydroalcoholic extract per kg of body weight, and was given the treatment in the form of tube feeding through gavage. During this period, the healthy group and the diabetic group without treatment also received physiological serum, which was prepared by Daropaksh Company, Tehran-Iran.

Measurement of blood glucose level and weight of rats

Evaluation of blood glucose level and weight was measured 2 days after streptozotocin injection, before the administration of the extract, and then on the 15th and 30th days of the study. Blood samples were taken from the rat's lateral tail vein, and the fasting blood glucose level was measured using a glucometer device. During the study, the weight of the rats was measured and recorded using a digital scale. At the end of the

study (day 30), the rats were killed after anesthesia with ether, and their main organs, including the heart, kidney, spleen, brain, and liver, were removed and, after weighing, frozen with liquid nitrogen and stored at -80°C for use in subsequent studies.

Examining the expression of the phosphofructokinase-1 gene level

The total cell RNA extraction process from the whole liver tissue was carried out according to the instructions of the RTM-Hybrid Geneall kit (Pisgam Company). Then, cDNA was synthesized, and finally, the expression level of the target gene was evaluated by the Real-Time PCR method (The results represent three replicates/group). After extracting RNA, its quantity and quality were evaluated by spectrophotometry and agarose gel electrophoresis. First Aid Revert Strand cDNA Synthesis Kit was used for cDNA synthesis. The sequence of specific primers for the expression of PFK1 and also the TATA3-box

binding protein gene (TBP) as an internal standard is shown in Table 1. In this study, the websites <http://www.NCBI.com> and <http://www.Primer.com> and Beacon Designer software were used to prepare the desired primers. Then, through the NCBI website, the selected primer was blasted, and the degree of overlap and its similarity with other genes and species were obtained. Finally, only the primers that uniquely and specifically bind to the desired gene were selected, made, and then sent by the pioneer company. The complete sequence of the PFK-1 gene and mRNA sequence was obtained from the database with the retrieval number EC 2.7.1.11. Investigations demonstrated that this gene has three transcripts. Primers for the PFK-L transcript and the TBP reference gene in rats were designed with the retrieval numbers NM_008826 and NM_001004198, respectively. Primer sequences and melting curves of the TBP and PFK-1 genes are given in Table 1.

Table 1. Sequence of primers of the genes studied

Tagged ORF Clone code	Primer name		Primer sequence	The length of the piece (bp)
NM-008826	PFK1	F	AGGCTCTCGGCTGAACA	191
NM-008826		R	CCATCTTGCTACTCAGGATTCG	
NM-001004198.1	TBP	F	GAGCCAAGAGTGAAGAACA	126
NM-001004198.1		R	TCACATCACAGCTCCCCA	

Data analysis

Statistical analysis of data was carried out using SPSS software, and a comparison of averages was conducted using the ANOVA test and Dunnett T3 multi-range test for blood glucose levels and weights of rats. A P value of $P < 0.05$ was considered significant.

Results

Evaluation of the weight results among the studied rats

At the beginning of the study, no difference was observed between the diabetic group

treated with the extract and the other two groups at the beginning of the study. There was no difference among the healthy group in the three determined stages (0, 15, and 30th days). On the 15th day, a significant weight loss was observed in both diabetic groups (treated and untreated) compared to the healthy group. On the 30th day, although the weight of the rats in both diabetic groups (treated and untreated) had increased, their average weight was lower than the average weight of healthy rats, and the difference was significant. However, there was no significant difference between the weight of treated and untreated diabetic rats.

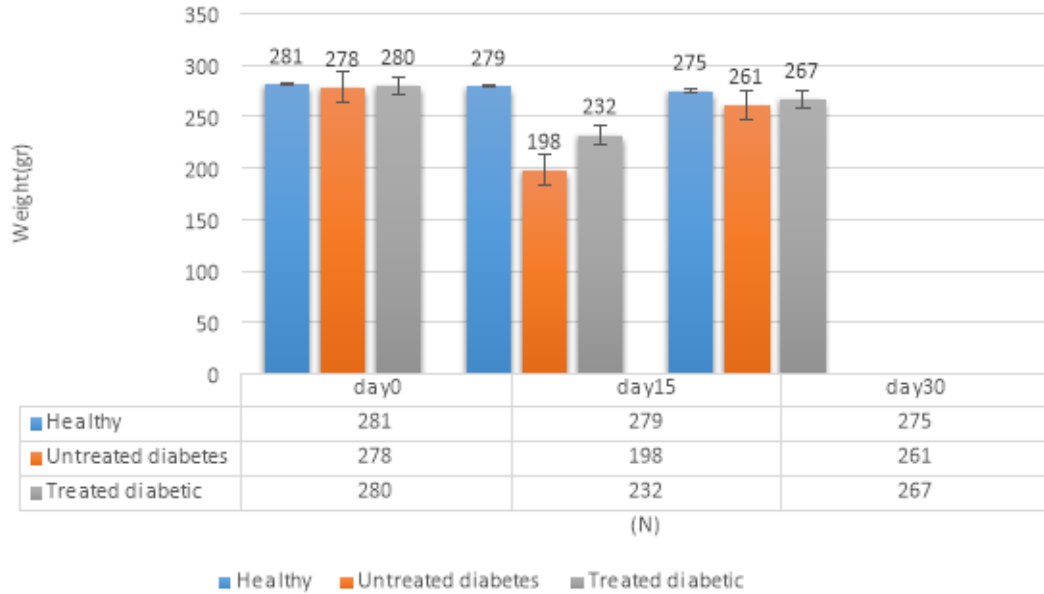


Figure 3. The effect of the hydroalcoholic extract of the rattle plant fruit on the weight of the rats in three groups

Evaluation of the blood glucose level in rats

According to the results, the blood glucose level in the healthy group was measured on 3 occasions, and it was found that the blood glucose level was within a normal range and there was no change. In the Untreated diabetic group, the witness also shows that the amount

of fasting blood glucose of the rats has increased significantly compared to the healthy group rats ($p < 0.05$). The diabetic group treated with rattle fruit extract shows a significant decrease in fasting glucose records on the 15th & 30th day compared with the untreated diabetic rat ($p < 0.05$) (Figure 4).

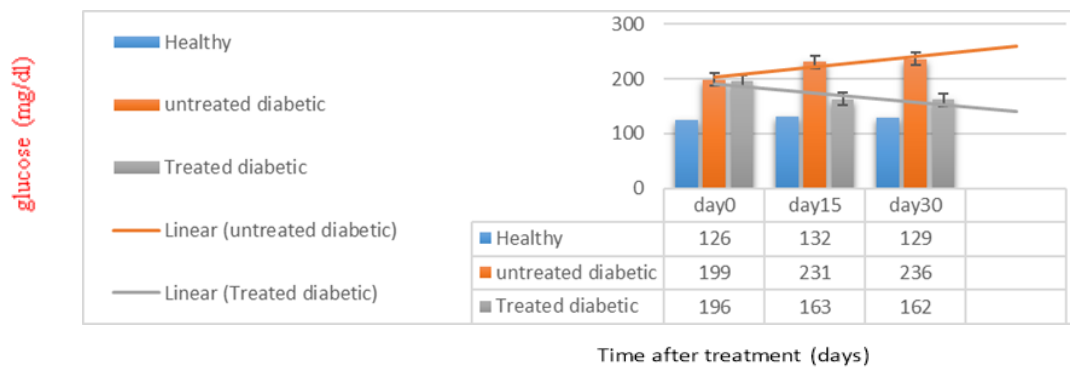


Figure 4. The effect of consuming the hydroalcoholic extract of the fruit of the rattle plant on the fasting glucose of rats (mg/dl) in three groups: a healthy control (blue), a diabetic treated with the fruit extract of the rattle plant (gray), and a diabetic control (orange). At three time points before extract administration (15th and 30th days). In the healthy control and diabetic control groups, only the rats received normal food. But in the treated group, in addition to consuming regular food, they also received the extract of the fruit of the rattle plant in the form of gavage.

Comparison of the weight of internal organs

According to Table 2, a comparison of the weight of different organs among the non-diabetic, untreated diabetic, and treated diabetic rats was carried out on the 30th day. The results showed that in untreated diabetic rats, the weight of organs such as the heart, spleen, and liver increased, and the weight of the kidney and brain decreased compared with healthy

rats, but the observed difference was not significant. Interestingly, in treated diabetic rats, the weight loss of organs such as the brain and kidney, and the increase in the weight of organs such as the heart, spleen, and liver were corrected. However, the difference observed between these rats and rats from both healthy and untreated diabetic groups was not statistically significant.

Weight (gr) Group	Kidney	Heart	Spleen	Brain	Liver
Healthy group (238gr)	0.99	0.9	0.74	17.1	7.56
Untreated diabetes (223gr)	0.9	1.23	0.76	15.9	11.66
Diabetics under treatment on 30th day (235gr)	1.3	1	0.6	16.5	10.83

Table 2. Comparison of the weight of different organs in rats

The results of cDNA production on an agarose gel

The results of the cDNA production demonstrated the correctness of the synthesis because it was designed using TBP reference

gene primers to perform PCR on the cDNA. Also, the presence of a specific band of the desired size and the absence of other non-specific bands confirmed the success of cDNA production (Figures 5 and 6).

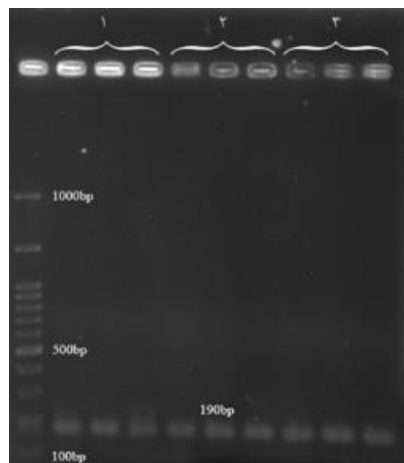


Figure 5. Amplification of the fragment (190 bp) related to the PFK gene. The wells of the nondiabetic group (1), the wells of the diabetic group (2), and the wells of the diabetic group treated with the extract (3).

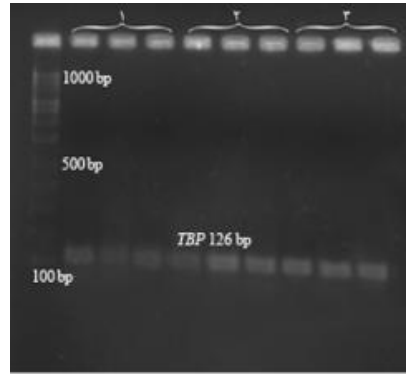


Figure 6. Amplification of the fragment (126 bp) related to the TBP gene. The wells of the non-diabetic group (1), the wells of the diabetic group (2), and the wells of the diabetic group treated with the extract (3)

The results of the PFK-1 gene expression level

After obtaining the CT of both TBP and PFK-1 genes for all samples, analysis was done using SPSS software. Statistical analyses were considered significant if $P < 5\%$. The PFK-1 gene was expressed in the three groups of healthy control, diabetic control, and diabetic under treatment at three time points on days 0, 15, and 30 of the study as follows (Figure 7), (Table 3).

According to the results of Figure 7, the expression of PFK has increased in the healthy

group, considering that $P > 0.05$, but it is not expressed at a significant level. In the untreated diabetic group, there was a decrease at three time points, but it was not a statistically significant difference. In the treated diabetic rat, it increased compared to the untreated diabetic rat; there was no significant difference. So we conclude that the extract of the rattle plant fruit does not affect the expression of the phosphofructokinase gene. (p-value was equal to 0.815, more than 0.05).

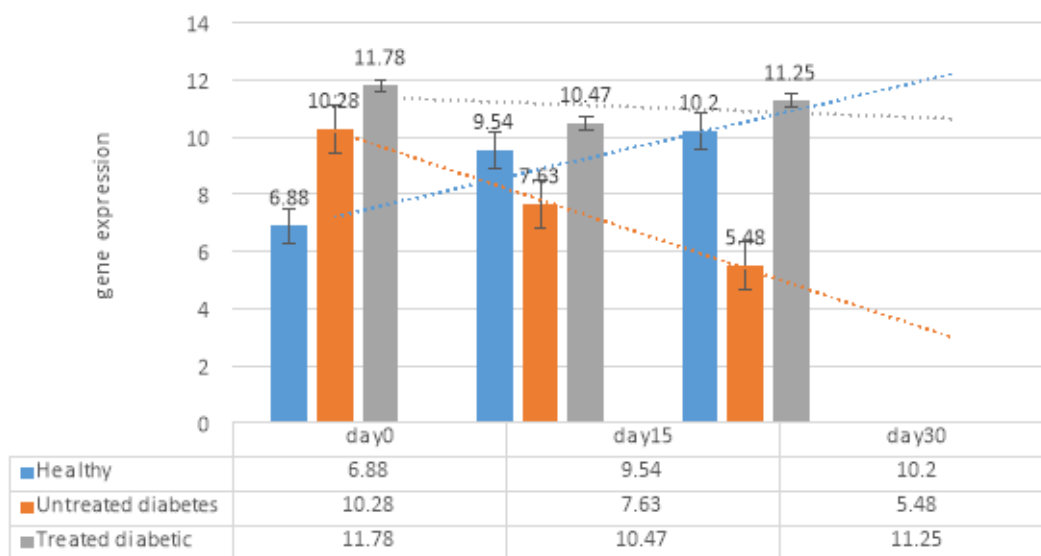


Figure 7. Changes in PFK-1 gene expression level among three groups of healthy, untreated diabetes, and diabetic treated with the extract at three determined time points; e.g., before extract administration, 15, and 30 days after extract administration.

Discussion and Conclusion

Antidiabetic drugs (e.g. sulfonylurea such as Glibenclamide) have some beneficial effects, but they also have undesirable side effects such as hypoglycemia, weight gain, skin reactions, acute porphyria, and hyponatremia. The use of plants induces relatively few side effects compared with synthetic drugs. Hence, investigation of novel natural product-based therapeutic agents is currently advocated (12).

Natural products can induce antidiabetic effects through diverse mechanisms, including suppression of glucose availability from the intestine or glucose production in the liver, enhancing the glucose uptake by tissues, increasing insulin secretion from β -cells, and increasing pancreatic tissue regeneration, inhibition of β -cell apoptosis, and enhancement of β -cell viability/ numbers (13, 14).

In the present study, we measured body weight and serum glucose levels in rats to investigate and determine the effect of rattle plant fruit's hydroalcoholic extract on diabetes, as the results of the our study showed, treatment with the extract did not affect total body weight, but it caused a relative correction of changes in the weight of internal organs such as the heart, spleen, liver, kidney, and brain in the treated diabetic rats. The relative increase in organ weight in treated rats is likely due to metabolic changes caused by extract consumption, but further studies are needed to understand and determine exactly which of the proposed metabolic changes (based on previous studies) caused them.

The results of measuring the body weight of rat show that on the 15th day of the study, the diabetic rat (treated and untreated) experienced severe weight loss and their weight difference with healthy rat was significant, while on the 30th day of the study, they experienced weight gain and their body weight did not differ significantly from the body weight of healthy rat. It seems that the severe decrease in body weight of diabetic rat on the 15th day of the study is due to the initial stress of diabetes, and since the weight of diabetic rat increased on the 30th day of the study in both treated and untreated groups, this weight gain cannot be

considered a result of treatment with the extract and is probably related to responses adapted to the stress of diabetes in rat.

Mehrafarin et al. reported that trigonelline is an alkaloid compound in the rattle plant, which has important medicinal benefits, including anti-cancer, anti-migraine, blood fat-reducing, and anti-diabetes effects (the reduction of blood glucose levels) that may occur due to the presence of this substance in the fruit extract of this plant [15].

In our study, rattle fruit extract shows a significant decrease in fasting blood glucose in treated diabetic in compared with untreated diabetic rats ($p < 0.05$). Kaneko et al. reported a significant decrease in fasting blood glucose levels in the study of aqueous extract consumption from the ginseng plant after 11 weeks, with the decrease only apparent after 6 weeks compared to the control group [16]. In another study, the level of blood glucose among diabetic rats treated with Polium Teucrium in the second and fourth weeks was significantly reduced compared to the control group [17]. Also, in another study, after 21 days, the oral treatment with an alcoholic extract of the Eucalyptus plant caused a significant decrease in the level of blood glucose and a significant increase in the level of insulin in the serum of diabetic animals. The antidiabetic effect of rattle fruit extract in the present study appeared at the 15th day. Therefore, the time during which diabetic rats are treated with the extract of rattle fruit can be effective in reducing the blood glucose level.

The review of the sources declares that few studies have been conducted to investigate the gene expression level of glycolysis and gluconeogenesis metabolic pathway enzymes under the influence of plant extracts. Some researches show that treatment with plants changes the activity of genes involved in glycolysis, such as hexokinase, glucokinase, glycogen synthase, glycogen phosphorylase, glucose dehydrogenase, and phosphofructokinase. This will reduce the blood sugar of diabetics [18, 19]. Based on these findings, in order to understand the mechanism of the effect of the extract, the expression level of the gene PFK-1, which is a

key enzyme of glycolysis in the liver and small intestine, was investigated. The PFK-1 gene is present in skeletal muscles, liver, and blood cells. This gene is located on chromosomes p12 and 20 in the rat liver and contains 780 amino acids. There are 3 known isoforms regarding the PFK-1 gene: PFK-L, PFK-M, and PFK-C (also known as PFK-P), which is a tetramer. Phosphofructokinase is an enzyme that is both allosteric and inducible, and its activity rate plays a major role in regulating glycolysis reactions [20]. In the present study, the antidiabetic effect of the hydroalcoholic extract of rattle fruit and expression of the PFK-1 gene were investigated among diabetic rats. In our study, the expression level of the PFK-1 gene among the treated diabetic rats did not exhibit any significant difference compared to the diabetic and nondiabetic rats. In another study, the expression level of the PK enzyme gene under the influence of the hydroalcoholic extract of the rattle fruit significantly increased among diabetic rats, and the researchers concluded that the administration of the hydroalcoholic extract of the rattle fruit plant probably increased the expression level of the PK gene, causing a decrease in blood glucose level among diabetic rats [21]. Also, in another study, the effect of an aqueous extract of fenugreek seeds on the expression level of the PFK-1 gene in STZ-treated diabetic rats and its effect on blood sugar and fat concentration was investigated. The findings of their study showed that the level of gene expression in the liver and mucosa of the extract-treated group is lower compared to the control group ($P < 0.05$). The two diabetic groups treated with 1 M of fenugreek seed extract showed a significant increase in the activity of the liver and intestinal mucosa by 54% and 75%, respectively ($P < 0.0001$). The blood glucose level among diabetic rats treated with fenugreek seeds with a concentration of 0.5 and 1 M was significantly reduced by 32% and 43%, respectively ($P < 0.0001$). It was reported that Persian shallot significantly reduced the blood glucose level, probably through the reduction of the gene expression level of an enzyme of gluconeogenesis named phosphoenolpyruvate carboxykinase in the liver [22]. They also

suggested Persian shallot as a hypoglycemic and antidiabetic agent, which acts by increasing insulin secretion levels and reducing hepatic glucose output by suppressing the expression level of phosphoenolpyruvate carboxykinase [23]. Another study demonstrated that the use of aloe vera extract increased the expression level of the glucokinase enzyme gene and decreased the expression level of the phosphoenolpyruvate carboxykinase enzyme gene, followed by a decrease in the blood glucose level among diabetic rats [24]. Also, in another study, the essential oil of *Satoria khustanica* Jamzad caused a moderate increase in the gene expression level of glucokinase and glycogen phosphorylase enzymes and a decrease in the gene expression level of the phosphoenolpyruvate carboxykinase enzyme, causing a decrease in the blood glucose level among diabetic rats [25]. In another study, a decrease in the level of blood glucose and an increase in the level of insulin secretion, along with an increase in the expression level of glucokinase, aldolase, and pyruvate kinase genes, were observed under the influence of *Costus speciosus* plant extract among diabetic rats [26]. In general, according to the results of the present study, the extract of the rattle plant fruit had an impact on blood glucose level reduction, but it did not affect the expression level of the PFK-1 gene, so the extract likely affected other genes of the glycolysis enzyme pathway or other aspects of glucose metabolism [27].

As far as the expression level of the PFK-1 gene also showed no significant difference, the extract likely affected other genes of the glycolysis pathway (The detailed mechanism of action remains to be elucidated). Further studies are, however, required to establish the exact antidiabetic mechanism of the rattle plant fruit and its active component(s). Therefore, to obtain more accurate findings and more complete results, we suggest that the following should be done in future studies:

- 1- Suggesting the evaluation of the effect of other concentrations of the hydroalcoholic extract of the rattle fruit.

2- In addition to evaluating the blood glucose level in rats, other liver enzymes should also be evaluated.

3- In addition to evaluating the fasting blood glucose level in rats, the HbA1c level should also be evaluated.

4- In addition to the liver tissue of the rats under study, other organs should also be examined.

5- Study of other glycolysis pathway genes.

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