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THE EFFECT OF F. VULGARİS EXTRACT ON STREPTOZOTOCİN-İNDUCED DİABETİC RATS

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ABSTRACT

Aim: Medical plants are more importance in the treatment of diabetic complications. Diabetes mellitus is a metabolic disorder and its incidence is considered to be high all over the world. F. vulgaris (FV) might have a various pharmacological effects.
Objective: This study was designed to evaluate effect of hydro-alcoholic extract of FV against Streptozotocin (STZ)-induced diabetic rats on blood concentrations of glucose, insulin, nitric oxide and sirtuin1 gene expression in heart tissue.
Material and Method: In this experimental study, diabetes animals were induced by STZ (60 mg/kg). 68 male rats were divided in to 8 groups. Including saline group, non-diabetic by various doses of FV (50, 100, 150 mg/kg) and diabetic plus FV(50, 100, 150 mg/kg) treatment groups were injected interaperitoneally once a day for 28 consecutive days. Body weight, blood concentrations of glucose, insulin, nitric oxide and sirtuin1 gene expression in heart tissue were measured and compared.
Results: The results indicated that STZ administration significantly decreased sirtuin1 gene expression, Body weight, insulin serum level and increase blood concentrations of glucose and nitric oxide compared to saline group ($p<0.05$). However, in the FV and diabetic plus FV groups significantly decrease nitric oxide and glucose level ($p<0.05$), while significantly boosted sirtuin1 gene expression, Body weight and insulin serum level in all doses compared to diabetic group ($p<0.05$).
Discussion: It seems that administration of F. vulgaris exerts appropriate, useful changes and improves diabetic -induced adverse effects in diabetic rats.

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Introduction

Various plants have traditionally been used to reduce and promote the secondary effects of diabetes, and there is a lot of information in this regard in Iranian traditional medicine as well as other countries around the world [1]. Falcaria vulgaris (FV) is a member of Umbelliferae family, with branching stems and average height of 30 cm. It grows naturally and rapidly in the west of Iran and is used as a vegetable [2]. In traditional medicine, this plant is used for the treatment of cutaneous and gastric

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ulcers, liver diseases, kidney stone and gall bladder stone [3]. This plant has been reported to have anti-inflammatory effects on gastric ulcer, and contains tannins, saponins (with blood glucose reducing properties) [4], vitamin C, protein and phytosterol [5]. Evidence shows that FV extract has antibacterial and antioxidant properties [6]. Diabetes mellitus (DM) is a complex heterogeneous syndrome induced by genetic defects, insulin secretion defects or resistance to insulin against environmental tissues, leading to extensive changes in the metabolism of proteins, carbohydrates, fats and minerals [7]. DM, with an increasing global trend, is predicted to be the leading cause of death in the future [8]. Based on the estimate of International Diabetes Federation, the number of diabetic people will escalate to 552 million patients in 2030 [9]. DM is a metabolic disorder characterized by increased blood glucose due to insulin secretion deficiency (type I diabetes) and resistance to insulin (type II diabetes) [10]. Shortage or relative decline of insulin in this disease is followed by acute and chronic metabolic dysfunctions [11]. Diabetes has chronic microvascular and macrovascular complications. Microvascular complications include nephropathy, retinopathy and neuropathy and macrovascular ones include hypertension, peripheral artery complication and cardiovascular complications [12, 13]. Oxidative stress is defined as imbalance between the production of oxygen free radicals and antioxidant defense capacity of body, which is highly associated with diabetes and its complications. Oxidative stress increases resistance to insulin by enhancing lipid peroxidation [14]. Enhanced blood glucose increases oxidative stress and alters the expression of inflammatory genes, both factors having a significant role in the incidence of diabetes-induced cardiac illnesses [15]. In a study by Chen et al. on diabetes-induced myocardial structural changes, it was reported that hyperglycemia caused structural changes in heart and cardiomyopathy in STZ-induced diabetic mice [16]. Sirtuin$_1$ gene is a member of Sirtuins family that plays a pivotal role in different cellular processes such as inflammation, resistance to oxidative stress, genetic stability and longevity [17]. The results of Yar et al. indicated reduced expression of Sirtuin$_1$ in the heart of rats following hyperglycemia; Sirtuin$_1$ being considered one of the important factors involved in the incidence of cardiac diseases, especially diabetes-induced cardiomyopathy [18]. Sirtuins belong to the family of Histone Deacetylases (HDAC) that have recently been identified, and since they are dependent on NAD as cofactor, they are differentiated from other HDACs [19]. The function and structure of human Sirtuins [1-3] are unknown and there is little information about the performance of other Sirtuins [20]. Accelerated atherosclerosis process in diabetic patients can be associated with dysfunction in NO production. NO is normally secreted from vascular endothelium and causes vasodilation. On the other hand, NO protects the reaction of platelets and leucocytes to blood vessels walls as well as intravascular injuries [21]. Diabetes affects the expression of some cardiac genes and induction of inflammation, followed by diabetes-induced cardiac diseases. Different properties of FV, especially antioxidant properties and its positive effects on improving inflammation have also been reported. Furthermore, there are very few studies on the effects of this plant on blood glucose, NO, serum blood insulin and expression of Sirtuin$_1$ as a gene involved in cardiac inflammation. Hence, the current study was aimed to evaluate the impact of FV hydro alcoholic extract on insulin, NO, blood glucose and Sirtuin$_1$ expression in the heart of diabetic rats.

Materials and methods

Animals

In this experimental study, 64 male Wistar rats, with mean weight of 220-250 gr, were purchased from Tehran Pasteur Institute and kept in standard cages in the animal house of Kermanshah University of Medical Sciences. Prior to the start of experiment, they were fed normal diet and water to get used to environment and adapt physiologically. In this time period, the rats were in similar conditions, 22±2 ºC with 12 hours light and 12 hours darkness and free access to water and food. All experimentation was conducted under approval of Ethics Committee of Kermanshah University of Medical Sciences [22].

Experimental design and treatments

The rats were randomly divided into eight groups (n=8). Group 1, saline group, received normal saline, other groups equivalent; Group 2, diabetes animals group were induced by STZ (60 mg/kg); groups 3 to 5, non-diabetic (FV groups) were given 50, 100 and 150 mg/kg FV respectively (5) and groups 6 to 8, diabetic plus FV groups, first were diabetic by STZ and then treatment by (50, 100 and 150 mg/kg) FV. In group 2, STZ was injected interaperitoneally at 60 mg/kg. To ensure the rats were diabetic, intravenous blood samples were taken from the animals' tail 72 hours after STZ administration. The rats with serum glucose >300 mg/dl were considered to be diabetic [23]. In groups 3 to 5 FV was injected interaperitoneally once a day for 28 consecutive days [5]. Rats in groups 6-8, Four weeks after induction of diabetes, received FV daily for 28 consecutive days [15]. The same volume of saline was injected in group 1.

Extract preparation

FV plant was purchased from a local store from which impurities were removed. After approval by a botanist, the plant was cleaned. The leaves and stems were dried in shadow for five days and powdered by a grinder. One hundred gr of the power was added to 70% ethanol (ratio of ¼). The obtained solution was kept in hot water bath (35 ºC) in dark condition. Then, the solution was gradually poured on Buchner funnel filter paper and cleared by vacuum pump. It was then transferred to rotary machine to get the extra solvent. The isolation process was continued until the concentrated extract was obtained. The extract was dissolved in distilled water and administered intraperitoneally per a kilogram of animal's weight [24].

Blood glucose measurement

To assess blood glucose changes in the study groups, the blood glucose level of rats was measured in three stages, as follows: 1) At baseline, 2) 72 hours after diabetes induction and 3) at the end of treatment with extract. The study groups were kept hungry (fasting) for 24 hours before blood sampling. After taking venous blood samples from the tail, the samples were incubated for 15 minutes at 37 ºC to clot. The clotted blood was centrifuged for 15 minutes at 3000 rpm to isolate the serum. The isolated serum was stored at -20 ºC until the measurement of insulin, glucose and NO. Glucose oxidase (GOD-PAP) method was used to determine blood glucose concentration [23].

ELISA assay:

Serum insulin was measured by Monobind kit (USA, Sigma) using ELISA method. In brief, the formerly taken serum samples were defrozen (like blood glucose analysis, the samples were taken in three stages). One hour before the start of the test, the kit was exposed to room temperature. To prepare the irrigation solution, the contents of a vial (20 ml) were diluted with distilled water to obtain 1000 ml solution. The content of each vial was mixed with 2 ml distilled water. Fifty λ of sample was poured in the wells. One hundred λ of conjugated insulin solution was added to all wells and mixed afterwards. The surface of wells was covered with plate adhesive and was incubated for 60 min at room temperature. The content of the plate was removed and 350 λ diluted irrigation solution was added to all wells. Six λ of substrate solution was added to all wells and incubated for 15 min at dark ambient temperature. Fifty λ stop solution was added to all wells, and plates were shaken gently on the table surface for 20 seconds. After 30 minutes, plates were read by ELISA reader at 450 Nm. The tests in all stages were repeated independently three times [25].

Animals weight estimations

The body weight of each mature male rat was measured three times. Each rat was weighed by a digital scale (A&D / GF600) three times: at baseline (first day), 72 hours after diabetes induction and after treatment with FV hydroalcoholic extract (24 hours after the last injection of extract). The obtained weights were recorded, and mean changes were analyzed [23].

Reverse transcription and real-time PCR analysis

To evaluate the expression of Sirtuin1 in the heart of study groups, Real Time-PCR method was applied. The rats were sacrificed with a high dose of chloroform. The heart of rats was immediately removed, frozen in liquid nitrogen and stored in freezer at -80 ºC until analysis. In the first stage, RNA was extracted from the heart tissue using RNeasy mini kit (Qiagen co) according to the manufacturer's instructions. Using DNase set kit, the extracted DNA samples were treated to remove the genomic DNA. cDNA version was synthesized from the RNA extracted from the previous stage using RevertAid™ First Strand cDNA Synthesis Kit. The expression level of the given gene was measured by GAPDH primer (Glyceraldehyde 3-phosphate dehydrogenase) as endogenous control using Maxima SYBR Green/Rox qPCR master mix (Fermentas co) by Comparative Ct (ΔΔ Ct) technique. First denaturation at 59 ºC in 10 min, denaturation at 59 ºC in 15 sec, annealing & extension at 06 ºC in 1 min with 40 cycles and melt curve (increment 3.0 ºc, 06 ºc → 59 ºc) were drawn by Stepone plus (Applied biosystem). The sequence of primers used is presented in Table 1 [19].

Griess assay

Nitric oxide, measurement by Griess assay using microplate method. In sum, sulfonamide solutions, N-(1- naphthyl) ethylenediamine dihydrochoride (NEED) and nitrite standards were prepared. To measure nitrite concentration in serum, samples de-freezing, 100 µl of the sample serum was deproteinized by zinc sulfate (6 mg zinc sulphate powder was mixed with 400µL serum and vortexed for 1 minute.) and transferred to the wells. To recover nitrate to nitrite, 100 µl chloride vanadium, 50 µl sulfonamide, and 50 µl NEED solutions were added afterwards (vanadium chloride recovery (III) method). Samples' optical density (OD) was measured by ELISA reader at the wavelength of 540 nm [24].

Statistical Analysis

All data are presented as mean ± standard deviation. Statistical differences among groups was carried out 1-way analysis of Variance(ANOVA), followed by the LSD post hoc test, to determine the statistical significance between different groups using the SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL, USA). $P<0.05$ was considered significant.

Results

Means of blood glucose and nitric oxide

In the present study, diabet caused a significant decrease in the means of blood glucose and nitric oxide compared to the saline group ($p<0.05$). In addition, FV and diabetic plus FV in all doses administration showed significantly increased means of blood glucose and nitric oxide compared to the diabetic group ($p<0.05$) (Figure.1 and figure. 2).

Means of blood insulin hormone

The means of blood insulin hormone significantly decreased in the diabetic group compared to saline group ($p<0.05$). However, means of blood insulin hormone were significantly improved in FV and diabetic plus FV in all treated groups compared with the diabetic group ($p<0.05$) (figure. 3).

Weight of animals

The effective dose of STZ (60 mg/kg) caused a significant decrease in mean weight of rats compared to Saline group ($p< 0.05$). FV improved mean weight in treated animals of all doses compared with the diabetic group ($p< 0.05$). Moreover, rats weight was significantly increased in treated animals with FV and diabetic plus FV in all doses in comparison with STZ group ($p< 0.05$) (figure. 4).

Real-time PCR

We examined the effect of STZ, FV and FV plus STZ on the mRNA expression of $Sirtuin_1$ in rat heart cells using real-time PCR. The $Sirtuin_1$ gene expression was dramatically down-regulated by STZ treatment compared to Saline group ($p< 0.05$). However, the effect of FV and diabetic plus FV treatment on $Sirtuin_1$ gene expression was significantly higher in all doses comparison with diabetic group ($p< 0.05$) (figure. 5).

Discussion

Diabetes is one of the most prevalent metabolic disorders in the current century. It is followed by reduced sensitivity to insulin, in addition to increasing blood glucose, and plays a key role in pathogenesis of cardiovascular diseases [12]. Diabetes can directly affect the structure and performance of heart and cause diabetic cardiomyopathy [26]. Considering the involvement of Sirtuin1 in cardiac inflammation and cardiomyopathy [18] and various effects of FV, especially anti-inflammatory effects [4], the present study evaluated the effects of FV on the expression of $Sirtuin_1$, glucose, insulin and NO as well as the weight of diabetic male rats. The results of rats' weight showed their weight was reduced significantly at the end of the study in diabetic group compared to saline group. Also, a significant increase in the rats' weight was observed in all diabetic plus FV extract groups in comparison with diabetic group. Streptozotocin induced hyperglycemia, reduced insulin through alkylation, necrosis and impairment of beta cells and induced diabetes consequently. It seems that body cannot use the blood glucose in diabetes type I due to deficient insulin production; therefore, it uses other sources like fats and even proteins, and the diabetic animal becomes very thin [27]. The presence of tannin and saponin (with blood glucose reducing properties) in FV extract [4] may prevent the use of other fatty resources in the body by stimulating the cells to use blood glucose, thereby increasing the weight of diabetic animals [28]. Further, saponin present in FV extract can regenerate pancreatic beta cells [29]. The findings of Neminia-audia et al. are in line with the results of the current study, indicating that administration of vernomia amygdalina extract (containing saponin) in diabetic rats recovered their lost weight [30]. The findings of this study indicated the reduction of insulin and increase of glucose in STZ-treated rats. Also, a significant increase in insulin level and significant decrease in serum glucose were found in diabetic plus FV extract group after treatment compared with diabetic group. It seems that STZ decreases insulin and increases blood glucose in rats through impairment of the membrane of pancreatic beta cells, DNA segmentation and reaction with enzymes such as glucokinase [31]. Chronic hyperglycemia increases the production of reactive oxygen species (ROS), and ROS in also increased in STZ-induced diabetes [32]. The hypoglycemic effect of FV extract in the study groups seem to be associated with the presence of tannin and saponin and to be dependent on alterations in the activity of hexokinase and glucokinase enzymes in liver [33]. Tannin and saponin can inhibit the absorption of glucose from intestines, increase the release or performance of insulin from unimpaired pancreatic beta cells due to STZ (via canals non-dependent to K-ATP and calcium canals) and reinforce the performance of insulin receptor in cells through interaction with Tyrosine Kinase [34, 35]. It seems that insulin absorption is due to the formation of mixed micelles between saponin molecules and insulin, which consequently helps to facilitate insulin crossing the cell membrane [36]. The results of Yu et al. showed that saponin in Astragaloside significantly reduces blood glucose and activity of glycogen phosphorylase and glucose-6 phosphatase in diabetic mice, confirming the findings of present study [37]. In the current study, serum NO level in diabetic groups was significantly increased compared with saline group. Moreover, a significant reduction was observed in diabetic plus FV extract groups than in diabetic group. IL-17 is a proinflammatory cytokine that is responsible for induction of NO synthase and release of NO, which can impair beta cells [38]. Diabetes can decrease the endothelium-dependent vasorelaxation capacity by interfering with its performance, which is largely mediated by NO. It seems that in oxidative stress conditions such as diabetes, iNOS-related mRNA expression and consequently NO level, as an oxidant, are increased [39]. As a free radical scavenger (5), FV extract seems to reduce blood NO in diabetic groups. The results of Akbarzadeh et al. show that STZ-induced hyperglycemia increases NO production, which is in line with the findings of present study [40]. The results of the current study revealed the reduced expression of Sirtuin1 in the heart tissue of diabetic rats and its reincrease after treatment with FV. Sirtuin1 expression through deacetylation of major substrates such as P53, FOXO transcription factors, receptors activating the proliferation of peroxisome and NF-Kβ has protective effects against cardiac diseases, especially myocardiopathy and oxidative stress-induced detoxification, intracellular calcium control, increased survival and angiogenesis [41]. The results of Vahtola et al. indicated that $Sirtuin_1$ in diabetic and non-diabetic groups was not significantly increased after MI induction, which is in contrast with the results of current research [42]. On the other hand, the findings of Brodsky et al. are in line with

the results of the present study, indicating that Sirtuin$_1$ expression in hyperglycemia is reduced in heart and vessels [43]. It seems that Sirtuin$_1$ plays a pivotal role in glucose metabolism in the liver, pancreas muscle and adipose tissue, and is one of the most important regulators of this reaction is deacetylation of PGC-1α by activation of Sirtuin$_1$ [44]. The study of Duan showed that saponin can induce cardiac protection against induced hyperglycemia in mice through increased expression of Sirtuin$_1$, confirming the results of the resent research [45]. Therefore, the present study showed that treatment with FV extract can reduce the risk factors causing cardiovascular complications during diabetes by increasing SIRT 1 expression. Based on the results of the current study and those of other studies, it seems that FV hydroalcoholic extract can have a potential role in decreasing diabetes-induced impairments through different mechanisms. Nevertheless, further complementary and extensive studies are required to evaluate the exact molecular and cellular mechanisms involved in the pharmacological performance of this extract in the treatment of diabetes and other diseases in order to gain more understanding on its potential effects.

Conclusion

The present study showed that hydro-alcoholic extract of F. vulgaris can significantly improve some of symptoms of diabetes in rats treated with Streptozotocin. The results also suggest the potential effects of F. vulgaris especially antioxidant effects against toxic effects of STZ -treated male rats and may provide a novel therapeutic approach.. However, further research in animal models is warranted to obtain more conclusive evidence for the molecular interaction between extract of F. vulgaris and diabetes mechanism.

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Table1. Primers used in real-time PCR

Primer ID	Primer sequences
GAPDH-F	AAGCTCATTTCCTGGTATG
GAPDH-R	CTGCCACAAGAACTAGAGGATAAGA
$Sirtuin_1$-F	TGGCAAAGGAGCAGATTAGTAGG
$Sirtuin_1$-R	CTGCCACAAGAACTAGAGGATAAGA

GAPDH: Glyceraldehyde3-phosphatedehydrogenase, as endogenous Control

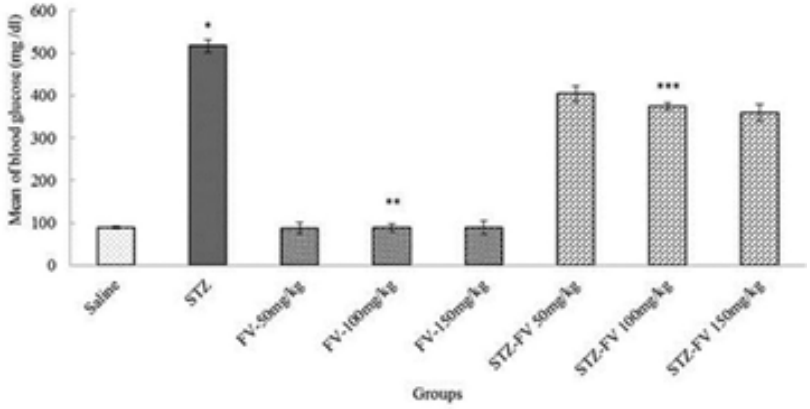

Fig 1. Effects of STZ (diabetic), FV (non- diabetic) and STZ + FV (diabetic plus FV) on means of blood glucose levels in rats (n=8 for each group). *Significant increase in diabetic group compared to Saline group ($p<0.05$). ** Significant decrease in all doses non- diabetic groups compared to diabetic group ($p<0.05$). *** Significant decrease in all doses diabetic plus FV groups compared to diabetic group ($p<0.05$).

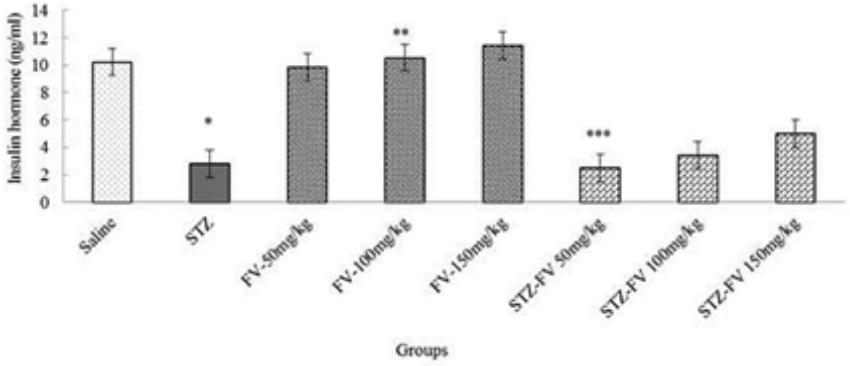

Figure 2. Comparison of the means of nitric oxide in blood serum in the STZ (diabetic), FV(non- diabetic) and FV + STZ (diabetic plus FV) treatment groups in rats. *Significant increase in diabetic group compared to Saline group ($p<0.05$). ** Significant decrease in all doses non- diabetic groups compared to diabetic group ($p<0.05$). *** Significant decrease in all doses diabetic plus FV groups compared to diabetic group ($p<0.05$).

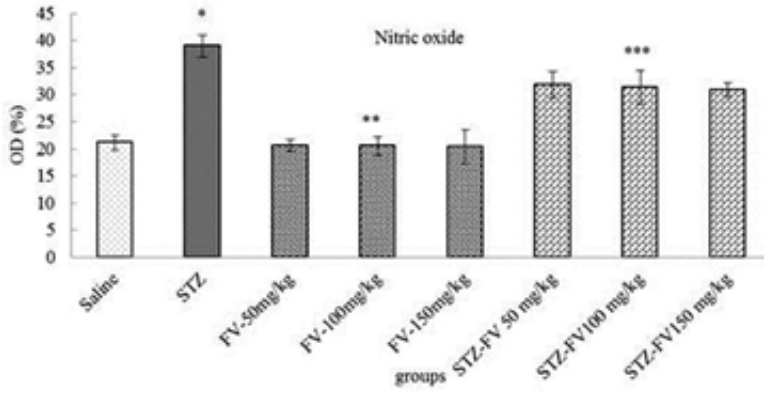

Fig 3. Correlation analysis between treatment groups STZ (diabetic), FV (non- diabetic) and FV + STZ (diabetic plus FV) in rats and insulin level in blood serum. *Significant decrease in STZ group compared to Saline group ($p<0.05$). ** Significant increase in all doses non- diabetic groups compared to STZ group ($p<0.05$). *** Significant increase in all doses diabetic plus FV groups compared to diabetic group ($p<0.05$).

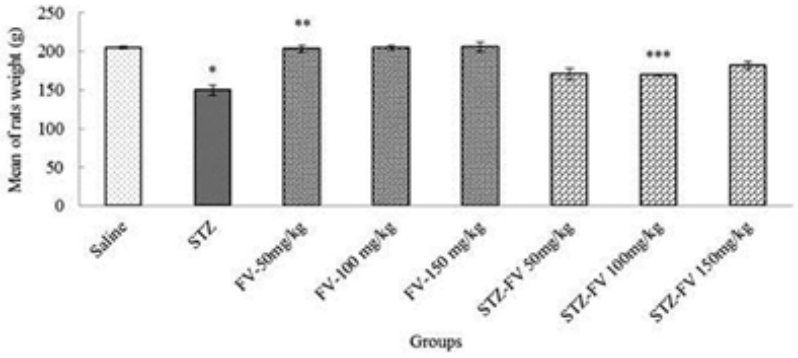

Fig 4. Comparison of the mean of weight between treatment groups ((STZ (diabetic), FV (non- diabetic) and FV + STZ (diabetic plus FV)). *Significant decrease in STZ group compared to Saline group ($p<0.05$). ** Significant increase in all doses FV groups compared to STZ group ($p<0.05$). *** Significant increase in all doses diabetic plus FV groups compared to STZ group ($p<0.05$).

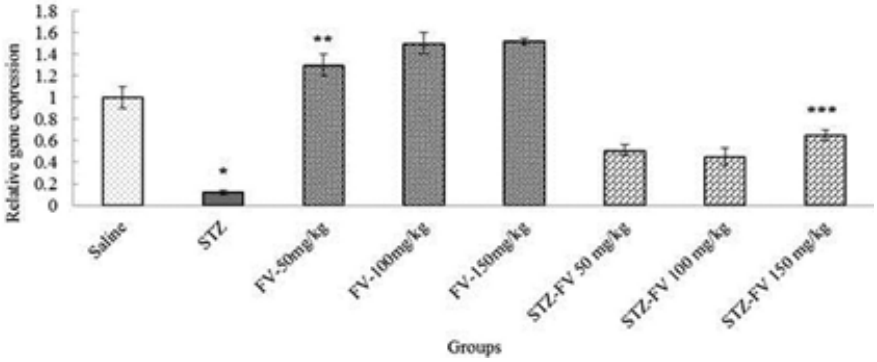

Fig 5. Results of Real-time quantitative PCR on the sirtuin$_1$ mRNA expression in rats heart tissue treatment with STZ (diabetic), FV (non- diabetic) and FV + STZ (diabetic plus FV). Relative expression levels of each gene were obtained by using the Comparative Ct ($\Delta\Delta$ct) method. *Significant decrease in STZ group compared to Saline group ($p<0.05$). ** Significant increase in all doses non- diabetic groups compared to STZ group ($p<0.05$). *** Significant increase in all doses diabetic plus FV compared to diabetic group ($p<0.05$).