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Original article

Vinca herbacea Extract Suppresses NF-kB Signaling and Modulates SIRT1/AMPK/PGC1α Axis to Exert Antidiabetic Effects in Streptozotocin-Induced Diabetic Rats

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Abstract

Background and objectives: AMPK/SIRT1/PGC1α signaling pathway has an important role in diabetic condition. Some natural products exert anti-diabetic effects by modulating this pathway and also by inhibition of NF-κB. *Vinca herbacea* has potent antioxidant and anti-inflammatory activities. In the present study, we investigated the effects of this plant on the AMPK/SIRT1/PGC1α axis and NF-κB genes expression as well as glucose, insulin levels and total antioxidant capacity in streptozotocin- induced diabetic rats. **Methods:** Streptozotocin induced diabetic male Sprague-Dawley rats were assigned to six groups: control, diabetic, diabetic + different doses of *Vinca herbacea* extract (100, 200 and 400 mg/kg.b.w) and glibenclamide. Fasting blood glucose, serum insulin and total antioxidant capacity were measured. The histopathology of liver and pancreas were evaluated. Real-time PCR was performed to assess gene expression levels. **Results:** *Vinca herbacea* extract (100 and 200 mg/kg.b.w) significantly reduced fasting blood glucose and 2-h blood glucose and increased serum insulin levels and total antioxidant capacity compared to the control diabetic rats. Also, an improvement in lipid profile and liver enzymes levels was observed. According to the histopathological assay, different damages induced by streptozotocin to liver and pancreas tissues were largely eliminated by treatment with the extract. *Vinca herbacea* extract significantly upregulated the AMPK, SIRT1 and PGC-1α and downregulated the NF-κB mRNA expression compared to the diabetic control rats. **Conclusion:** Anti-diabetic effects of *V. herbacea* extract were

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indicated in streptozotocin-induced diabetic rats. The AMPK/SIRT1/PGC1α/NF-κB signaling pathway was suggested as the mechanism involved in the protective effects of this extract in diabetes.

Keywords: NF-κB; PGC1α; SIRT1; total antioxidant capacity; *Vinca Herbacea*

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Introduction

Diabetes mellitus involves a number of metabolic and complex disorders which is one of the main challenges in the whole world [1,2]. The main feature of this disease is high blood glucose which plays the significant role in the creation and progression of diabetic complications through the induction of inflammatory cytokines, chemokines, and reactive oxygen species [3,4].

Accumulating evidence indicates that hyperglycemia and oxidative stress activate nuclear factor kappa-light-chain-enhancer of activated β cells (NF-κB) [5,6] which mediates the expression of target genes involved in the inflammatory responses [7,8].

A highly conserved serine/threonine protein kinase, AMP-activated protein kinase (AMPK), as a key regulator of metabolism and Sirtuin 1 (SIRT1), a NAD-dependent class III protein deacetylase, interfere with the NF-κB signaling pathway. AMPK and SIRT1 activation lead to inhibition of NF-κB signaling and inflammatory responses [9-12]. The hyperglycemia changes the balance between acetylation and deacetylation of NF-κB via lowering the level of intracellular NAD and in turn reduces the expression of SIRT1 [13,14]. AMPK and SIRT1 represent a close interaction via increasing each other's activity to the regulation of energy, metabolism, and inflammation [15]. AMPK enhances the synthesis of cellular NAD and consequently upregulates SIRT1 activity. SIRT1 also deacetylates liver kinase B1 (LKB1), the upstream kinase of AMPK, which can in turn phosphorylate activate AMPK [16,17].

AMPK and SIRT1 as the two main energy-sensing molecules have many common target molecules including PGC1α [10,18]. Peroxisome proliferator-activated receptor (PARP)-ɣ coactivator 1α (PGC1α) is known as a principal transcriptional co-activator and modulates metabolic balance, and the function of mitochondria [19]. In stressful environments such as hyperglycemia, mitochondrial biogenesis is reduced. So phosphorylation and deacetylation of PGC1α by AMPK and SIRT1 respectively, reduce reactive oxygen species (ROS) production and inhibit NF-κB signaling and transcription of inflammatory cytokine genes [10,20,21]. Under diabetic conditions, the AMPK/SIRT1/PGC1α signaling is inactivated [22].

Recently, the use of herbal products as complementary and adjunctive therapies has received much attention [23]. *Vinca herbacea* which originates from *Vinca* genus (Apocyancea family) is an herbaceous perennial plant and is distributed in various geographical areas [24]. *Vinca* species contain large amounts of indole alkaloids [25]. Total alkaloids isolated from various parts of *Vinca herbacea* are known to possess different pharmacological activities, including antiproliferative, apoptotic, antioxidant, anti-inflammatory, antihypertensive, and anti-arrhythmic activities [26,27]. Also the alkaloidal extract, like the standards (a-tocopherol and butylated hydroxytoluene) has shown considerable lipid peroxidation inhibiting activity [28]. Considering the therapeutic effects of *V. herbacea* extract, this study has investigated the molecular mechanism of this plant in the control of type 1 diabetes. By reviewing previous studies, it has been determined that no investigation has been done in this field till now. In the present study, the effects of hydroalcoholic extract of *Vinca herbacea* on the SIRT1/AMPK/PGC1α axis and NF-κB genes expression as well as glucose homeostasis in animal model of diabetes have been investigated.

Material and Methods

Ethical considerations

Ethical approval from the Zanjan University of Medical Science was obtained (IR.ZUMS.REC.1398.014) and all procedures were done according to the Ethical rules of this Committee.

Chemicals

Streptozotocin was obtained from Sigma Aldrich (Germany). To measure serum insulin, we used rat insulin ELISA kit (Bioassay Technology Laboratory, China). The kits of measuring triglyceride, total cholesterol, high-density lipoprotein cholesterol, and liver enzymes were provided from Pars Azmoon, Iran. Ethanol, methanol, paraffin, eosin, and hematoxylin were obtained from MERCK (Germany). The total RNA isolation kit was purchased from FAVORGEN (Taiwan). Furthermore, the cDNA synthesis kit and SYBR Green Master Mix were provided from BioFACT (Korea) and TaKaRa (Japan), respectively.

Plant material

Vinca herbacea plant was collected in May 2018 from its natural habitat in Tarom, Zanjan Province, Iran, in its phase of flowering. The plant was identified and confirmed at the Department of Pharmacognosy, Faculty of Pharmacy, Zanjan University of Medical Sciences, and a specimen, with number 7100, was stored at the Herbarium of this faculty. The collected samples were cleaned and dried at room temperature away from sunlight. The dried specimens were powdered. The preparation of hydroalcoholic extract was done by drenching the powder of the dried plant in ethanol 70% at room temperature for 72h; plant: solvent ratio was 1:5. Finally, the solvent was removed by rotary evaporator and the solvent-free extract was stored in the dark airtight containers at 4 °C.

Animals

Healthy male Sprague-Dawley rats, weighing 180–240 g were used in the current experiment. Throughout the study period, the rats were under controlled conditions (temperature of 25 °C, and 12-h light/dark cycles), with free access to the standard diet and water.

Acute toxicity test

Acute oral toxicity test was performed on healthy Sprague-Dawley rats to rule out any toxicity of the different doses of the extracts, before conducting the study according to the principles of OECD guidelines 423 [29]. Ten rats were randomly divided to two groups including test and control group. Animals in control group received only vehicle (distilled water) and in test group received the *Vinca Herbacea* extract in doses of 50, 100, 200, 300 and maximum 4000 mg/kg. Any changes in behavior, acute signs of toxicity and mortality were followed up to 72 h. The animals were then examined for any signs of acute toxicity or mortality for up to two weeks [30,31].

Experimental diabetes induction

Streptozotocin was dissolved in freshly prepared cold citrate buffer (0.1M, pH 4.5). The type 1 diabetes model was induced by a single dose intraperitoneally injection of 60 mg/kg streptozotocin [32,33]. Seventy-two hours after streptozotocin injection, the blood glucose levels were measured by a glucometer (GLUCOCARD, ARKRAY, Japan). The rats with Fasting blood glucose (FBG)>250 mg/dL, frequent urination, and excessive thirst were chosen as diabetic rats for the current study. For normal groups, rats received intraperitoneal normal saline instead of streptozotocin.

Experimental design

The total number of 36 rats, normal and diabetic were divided into six groups randomly (n=6):

Group1 (the control group): normal rats + normal saline (0.5 mL/day, orally); group 2 (the diabetic control group): diabetic rats + normal saline (0.5 mL/day, orally); group 3: diabetic rats + low dose of *Vinca herbacea* (100 mg/kg/day, orally); group 4: diabetic rats + medium dose of *V. herbacea* (200 mg/kg/day, orally); group 5: diabetic rats + high dose of *V. herbacea* (400 mg/kg/day, orally); group 6: diabetic rats + glibenclamide as a positive control (10 mg/kg/day, orally).

Accordingly, in the entire 28 days of the study period, glibenclamide and different doses of *V. herbacea* extract were administered daily via the gastric gavage needle, and the rats of normal control and diabetic control groups received 0.5 mL/day normal saline orally. Fasting blood glucose was monitored weekly during the study, and body weight was evaluated on the first and last day of the study. The animals were euthanized by a carbon dioxide (CO2) chamber. On the last day, the serum and whole blood samples, as well as the liver and pancreas tissues, were collected from all rats for evaluation of biochemical parameters, gene expression, and histopathological study.

Fasting blood glucose

Fasting blood glucose level was monitored on the 7^{th}, 14^{th}, 21^{st}, and 28^{th} days of the experiment.

The blood samples were collected from the tail vein of 12-h fasted rats, and FBG was determined by digital glucometer.

Oral glucose tolerance test

On the 28th day of the study, the oral glucose tolerance test (OGTT) was performed after the evaluation of the FBG. A single dose of 2 g/kg oral glucose solution was administered to the rats and blood glucose levels measurement was done at 30, 60, 90 and 120 min of glucose administration, by the same digital glucometer [23].

Serum insulin determination

On the last day of the experiment, the levels of serum insulin were determined through the rat insulin enzyme-linked immunosorbent assays kit, according to the manufacturer's instructions.

Lipid profile, liver enzymes levels and TAC

Measurement of lipid profile (serum triglyceride (TG), total cholesterol (TC), and high-density lipoprotein (HDL-c) and liver enzymes levels (alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferease (ALT) was done by a commercial kit (Pars Azmoon, Iran). In addition, the Friedewald equation [34] was used for the calculation of LDL-C.

$$\text{LDL-cholesterol (mg/dL)} = [\text{TC-HDL-(TG/5)}]$$

Total antioxidant capacity (TAC) was evaluated using a colorimetric assay by a commercial kit (Kiazist, Iran) in the serum samples.

Histopathological study

The animals were euthanized by a CO2 chamber and the pancreas and liver were dissected on the last day of study. The tissues were fixed in 10% formalin, dehydrated in ethanol gradient (50–100 %), placed in xylol, and then embedded in paraffin. The sections with 5 μm thickness were then prepared with a microtome and stained with hematoxylin and eosin (H&E). Finally, the stained sections were evaluated by light microscopy.

Real-time PCR

The FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit (FAVORGEN) was used to extract the total RNA from the whole blood and liver tissues samples of rats using the manufacturer's instruction. RNA quantity and purity were detected by nanodrop, and the quality was assessed by agarose gel electrophoresis. The cDNA was synthesized from 8 μg of total RNA using a cDNA synthesis kit (BioFACT) according to the manufacturer's protocol. The Real-time PCR was performed for the determination of gene expressions of AMPK, SIRT1, PGC1a, and NF-κB. One μL of the synthesized cDNA was mixed with 0.5 μL of each forward/reverse primer, 5 μL SYBR Premix Ex TaqII (2x) (TaKaRa), as well as 0.2 μL Rox reference dye (50x), and deionized water was added for a total volume of 10 μL. Then, quantitative PCR was performed using the ABI system (Step One Plus model) and during these steps: 95 °C for 30 s, followed by 40 cycles of 95 °C for 20 s, 60 °C for 40 s, and 72 °C for 10 s, respectively. Gene Runner software and Oligo Analyser 3.1 software were used for the design and assessment of the primers of the target genes, listed in Table 1. In addition, β-actin was considered as the housekeeping control gene. Finally, the relative mRNA levels of the target genes were calculated by the comparative $2^{-\Delta\Delta Ct}$ method normalized to β-actin and untreated control group levels. The quality of the RT-PCR products was evaluated by 2% agarose gel electrophoresis.

Table 1. Primer sequences

Gene	Sequence
AMPK	F. 5′AGATAGCTGACTTCGGACTCTCT3′ R. 5′AACCTCAGGACCCGCATACA3′
NF-κB	F. 5′AGAGCAACCGAAACAGAGAGG3′ R. 5′TTTGCAGGCCCCACATAGTT3′
SIRT1	F. 5′TGGACGAGCTGACCCTTGA3′ R. 5′TCCTGCGGATGTGGAGATT3′
PGC-1α	F- 5′ CGATGACCCTCCTCACACCA3′ R- 5′TTGGCTTGAGCATGTTGCG3′
β-Actin	F. 5′TCACCCACACTGTGCCCATCTATGA3′ R. 5′CATCGGAACCGCTCATTGCCGATAG3′

Statistical analysis

The SPSS software version 18 was used for the statistical analysis of data. The data were expressed as the mean ± standard deviation (SD). The significant differences between the mean values were determined by one-way analysis of variance (ANOVA). For evaluation of the significant interrelation between the various groups, Tukey's multiple comparison test was used. P-value<0.05 was considered statistically significant.

Results and Discussion

Numerous natural products are efficient in

decreasing blood glucose, improving lipid profile, promoting insulin secretion, and improving insulin sensitivity [35]. These products are known as herbal medicines and due to their special anti-diabetic ingredients such as flavonoids, tannins, phenolic compounds, and alkaloids and also because of fewer side effects, lower costs, and low toxicity, they are considered notable options for diabetes treatment [35,36]. According to the findings of acute toxicity test in the present study, within 72 hours, no signs of acute toxicity and death were observed in rats at the maximum dose. So, in this study, the highest dose of the extract was selected as one tenth of the 4 g/kg b.w. (400 mg/kg b.w.) and two other doses were determined less than this dose (100 and 200 mg/kg b.w.) [30,31].

The effects of various doses of *V. herbacea* hydroalcoholic extract on body weight, serum glycemic control, lipid profile, hepatic function tests, TAC, as well as the liver and pancreas tissues histopathology in streptozotocin-induced diabetic rats were evaluated and compared with glibenclamide. In addition, the *V. herbacea* extract's effect on AMPK, SIRT1, PGC-1α, and NF-κB gene expression was investigated in the whole blood of the diabetic rats.

The findings of this study showed that the initial body weight of experimental groups was not different significantly ($p=0.791$). In comparison to control group, diabetic control rats showed a significant decrease ($p=0.003$) in body weight at the end of the study. The body weight of experimental groups at the end of study is shown in Figure 1A.

Hyperglycemia was recorded in diabetic control rats during the experimental period as compared to the control group. Treatment of diabetic rats with *V. herbacea* (100 and 200 mg/kg) or glibenclamide significantly reduced FBG levels compared to the control diabetic rats ($p<0.001$), while treatment with 400 mg/kg of *V. herbacea* could not improve FBG levels significantly ($p=0.860$) (Figure 1B).

The results of 2-hour postprandial blood glucose test at the end of study revealed a significant difference between normal and diabetic control rats ($p<0.001$). Treatment of diabetic rats with different doses of *V. herbacea* and glibenclamide showed similar results to fasting blood glucose test (Figure 1C).

Serum insulin level was measured at day 28. There was a significant decrease in the insulin levels in diabetic control rats compared to the control group ($p<0.001$). Treatment with 200 mg/kg of *V. herbacea* produced more significant effect on serum insulin level compared to glibenclamide ($p=0.005$), while treatment with 400 mg/kg of *V. herbacea* in diabetic rats showed no significant effect on serum insulin level compared to the control diabetic rats ($p=0.240$). As depicted in Figure 1D, the serum insulin levels in diabetic rats treated with 200 mg/kg of extract was almost the same as normal control ($p=0.071$).

The effect of *V. herbacea* on glucose tolerance test (GTT) pattern in control and experimental rats is shown in Figure 2. Blood glucose level was elevated to a peak at 60 min and declined to near basal level at 120 min in diabetic rats treated with 200 mg/kg of the extract (mean percentage change=3.65), 100 mg/kg of extract (mean percentage change=5.49) and control group (mean percentage change=5.93). According to the current study streptozotocin injection led to significantly enhanced blood glucose and reduced insulin levels in diabetic rats in comparison with the control group. Streptozotocin induces ROS production leads to pancreatic beta-cells cytotoxicity, followed by decreased production of insulin and development of diabetic symptoms such as hyperlipidemia and hyperglycemia [37,38]. Treatment with *V. herbacea* (100 and 200 mg/kg) similar to glibenclamide, decreased FBG levels as well as 2-hours postprandial glycemia and improved insulin levels compared with the diabetic control group. For evaluating timely abnormalities in glucose regulation, GTT is a more sensitive measure than FBG. During GTT, in the control group, glibenclamide group, and *V. herbacea* treated rats (100 and 200 mg/kg) the blood glucose reached to a peak then decreased to near fasting levels after 2 hours. While in diabetic control rats and *V. herbacea* high dose-treated rats, the level of blood glucose was elevated even after 2 h.

Lipid profile (TC, LDL-C, HDL-C, and TG) as well as liver enzymes levels (ALT and AST) and serum TAC were compared among control and experimental rats (Table 2). There were significant differences in TC, LDL-C, HDL-C and TG values between diabetic and control groups ($p<0.001$). Similar to glibenclamide, *V. herbacea* extract significantly decreased TC, LDL-c and TG levels at the dose of 100 and 200 mg/kg compared to the diabetic control rats ($p<0.001$). in comparison to the diabetic control rats ($p>0.05$).

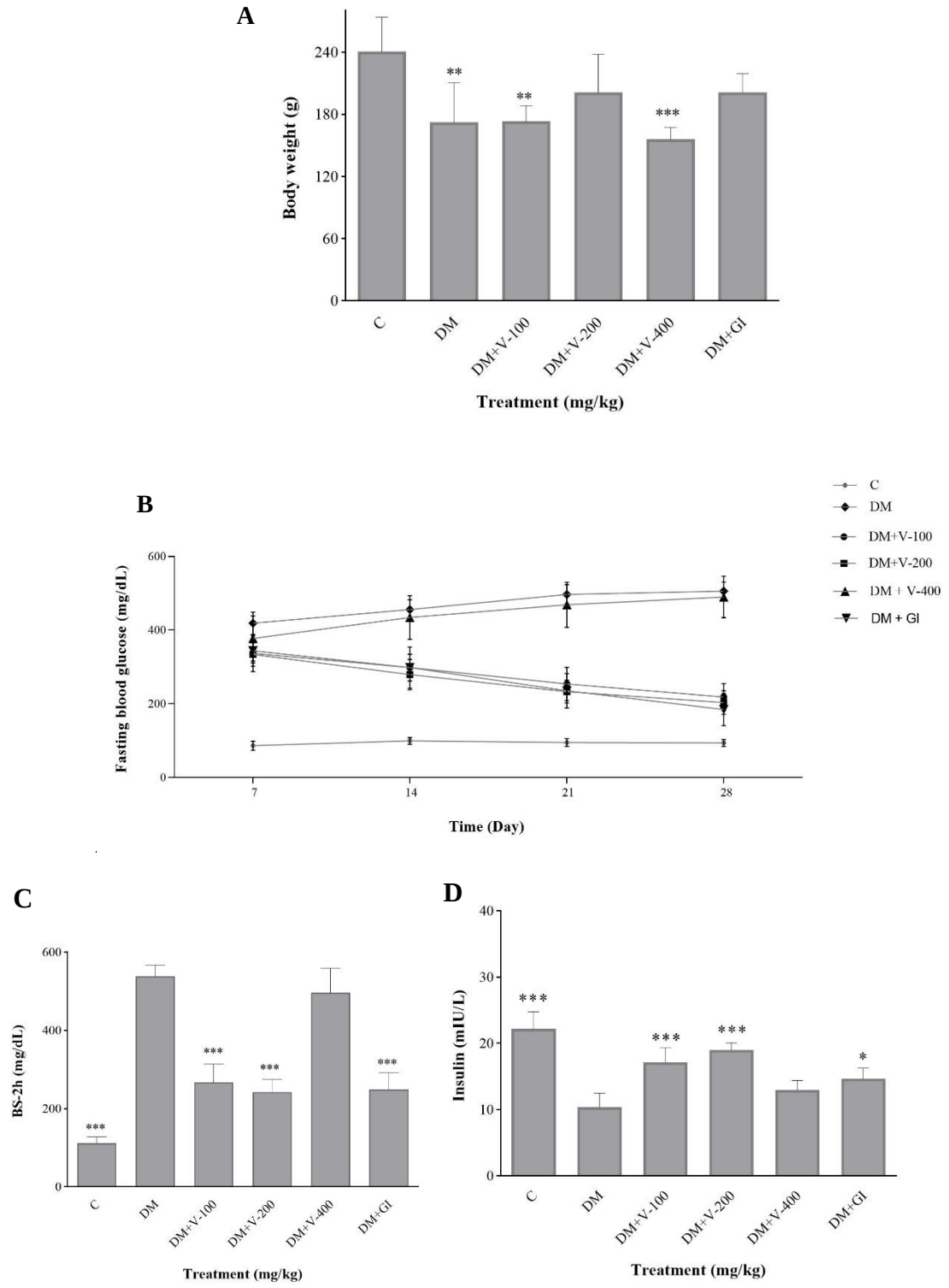

Figure 1. Effect of different doses of *Vinca herbacea* extract (100, 200 and 400 mg/kg body weight) and glibenclamide on (A) body weight, (B) fasting blood glucose, (C) blood sugar 2-hours, (D) serum insulin levels of normal and STZ induced- diabetic rats. Data were presented by mean ± SD. C: control group, DM: diabetic control, DM+V-100: diabetic rat + 100 mg/kg body weight extract, DM+V-200: diabetic rat + 200 mg/kg body weight extract, DM+V-400: diabetic rat + 400 mg/kg body weight extract, DM+GI: diabetic rat + glibenclamide; STZ: streptozotocin; (A): **, *** $p<0.01$, $p<0.001$ versus control group; (C & D); *, *** $p<0.05$, $p<0.001$ versus diabetic control group

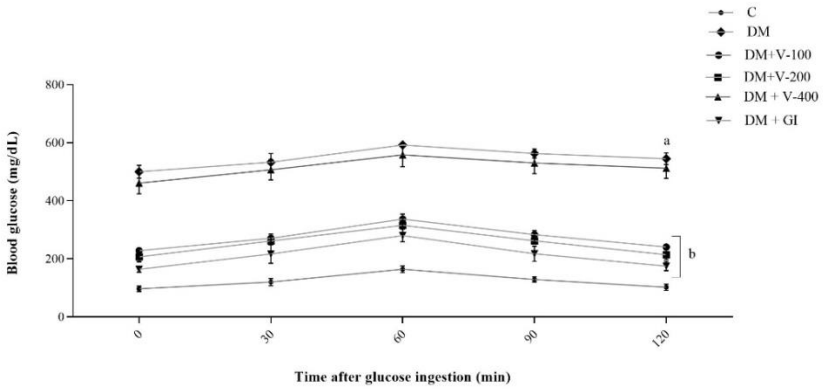

Figure 2. Effect of different doses of *Vinca herbacea* extract (100, 200 and 400 mg/kg body weight) and glibenclamide on oral glucose tolerance; data were presented by mean ± SD; C: control group; DM: diabetic control; DM+V-100: diabetic rat + 100 mg/kg body weight extract; DM+V-200: diabetic rat + 200 mg/kg body weight extract; DM+V-400: diabetic rat + 400 mg/kg body weight extract; DM+GI: diabetic rat + glibenclamide; a: $p<0.001$, diabetic control group versus control group; b: $p<0.001$, experimental diabetic groups versus diabetic control group

Table 2. Effect of *Vinca herbacea* extract on biochemical parameters

	Control group	Diabetic control	Diabetic+extract (100 mg/kg)	Diabetic+extract (200 mg/kg)	Diabetic+extract (400 mg/kg)	Diabetic +glibenclamide
TC, (mg/dL)	75.50 ± 4.64	127.40 ± 2.30[a]	99.67 ± 4.13[b]	101.50 ± 2.88[b]	131.67 ± 3.93	89.83 ± 3.34[b]
LDL-C,(mg/dL)	15.17 ± 4.58	55.00 ± 8.37[a]	33.83 ± 4.40[b]	29.33 ± 2.94[b]	57.67 ± 6.83	27.33 ± 8.66[b]
HDL-C,(mg/dL)	49.50 ± 4.46	25.40 ± 5.81[a]	37.83 ± 4.21[b]	35.50 ± 3.51[b']	20.00 ± 3.16	39.17 ± 3.66[b]
TG, (mg/dL)	86.17±10.57	184.40 ± 8.88[a]	109.67 ± 11.83[b]	109.83 ± 9.28[b]	192.50 ± 7.06	90.33 ± 15.53[b]
ALT, (U/L)	81.00±10.45	161.40 ± 7.20[a]	142.33 ±11.20[b'']	129.33 ± 5.50[b]	161.67 ± 7.42	94.33 ± 13.22[b]
AST, (U/L)	126.00±8.69	224.20±15.12[a]	135.00 ± 5.97[b]	130.83 ± 10.22[b]	203.83 ± 18.46	134.67 ± 9.31[b]
ALP, (U/L)	190.83±6.97	414.60 ± 7.20[a]	372.33 ± 13.56[b]	367.83 ± 9.15[b]	413.67 ± 13.35	259.00 ± 20.39[b]
TAC,(nmol/mL)	0.71 ± 0.04	0.32 ± 0.02[a]	0.48 ± 0.05[b]	0.53 ± 0.04[b]	0.30 ± 0.03	0.34 ± 0.03

Data were expressed as mean ± SD; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TAC: Total antioxidant capacity; diabetic control group versus control group [a] ($p<0.001$); experimental diabetic groups versus diabetic control group [b] ($p<0.001$), [b'] ($p<0.01$), [b''] ($p<0.05$)

Significant increase in HDL-C serum levels were observed in both diabetic rats treated with *V. herbacea* extract at the doses of 100 ($p<0.001$) and 200 mg/kg ($p=0.005$) and the diabetic rats treated with glibenclamide ($p<0.001$) in comparison to the diabetic control. *Vinca herbacea* extract at the dose of 400 mg/kg had no significant effect on serum lipid profile variables Significant increase was identified in the levels of serum ALT and AST in the diabetic control rats compared to the control group ($p<0.001$). The doses of 100 ($p=0.030$) and 200 mg/kg body weight or glibenclamide ($p<0.001$) resulted in considerable reduction in the levels of ALT and AST when compared to the diabetic control rats (Table 2).

The levels of serum TAC were significantly decreased in diabetic control rats compared to control group ($p<0.001$). The extract at doses of 100 and 200 mg/kg efficiently improved serum TAC levels compared to diabetic control rats. Diabetes is associated with hyperlipidemia [39]. In diabetes, more free fatty acids are delivered into the blood circulation [40]. The reduced insulin function enhances the activity of lipase and hence liberating free fatty acids from the adipose tissue which causes hyperlipidemia [41]. The rise in the concentration of fatty acid enhances the fatty acids' β-oxidation and generating further acetyl-CoA and cholesterol [40]. Furthermore, as insulin activates lipoprotein lipase, the beta-cell destruction leads to hyperlipidemia by plasma insulin depletion [42]. Thereby, in this study, the lipid profile was evaluated in diabetic rats. Comparison with the diabetic control group, treated rats with 100 mg/kg and 200 mg/kg *V. herbacea* showed a meaningful decrease in TC, TG, and LDL-C levels, as well as significant enhancement in HDL-C levels. The significant regulation of serum lipid profile suggests that the extract may act by ameliorating insulin secretion. Hyperglycemia can elevate hepatic enzymes in the blood by inducing hepatocellular damages [43,44].

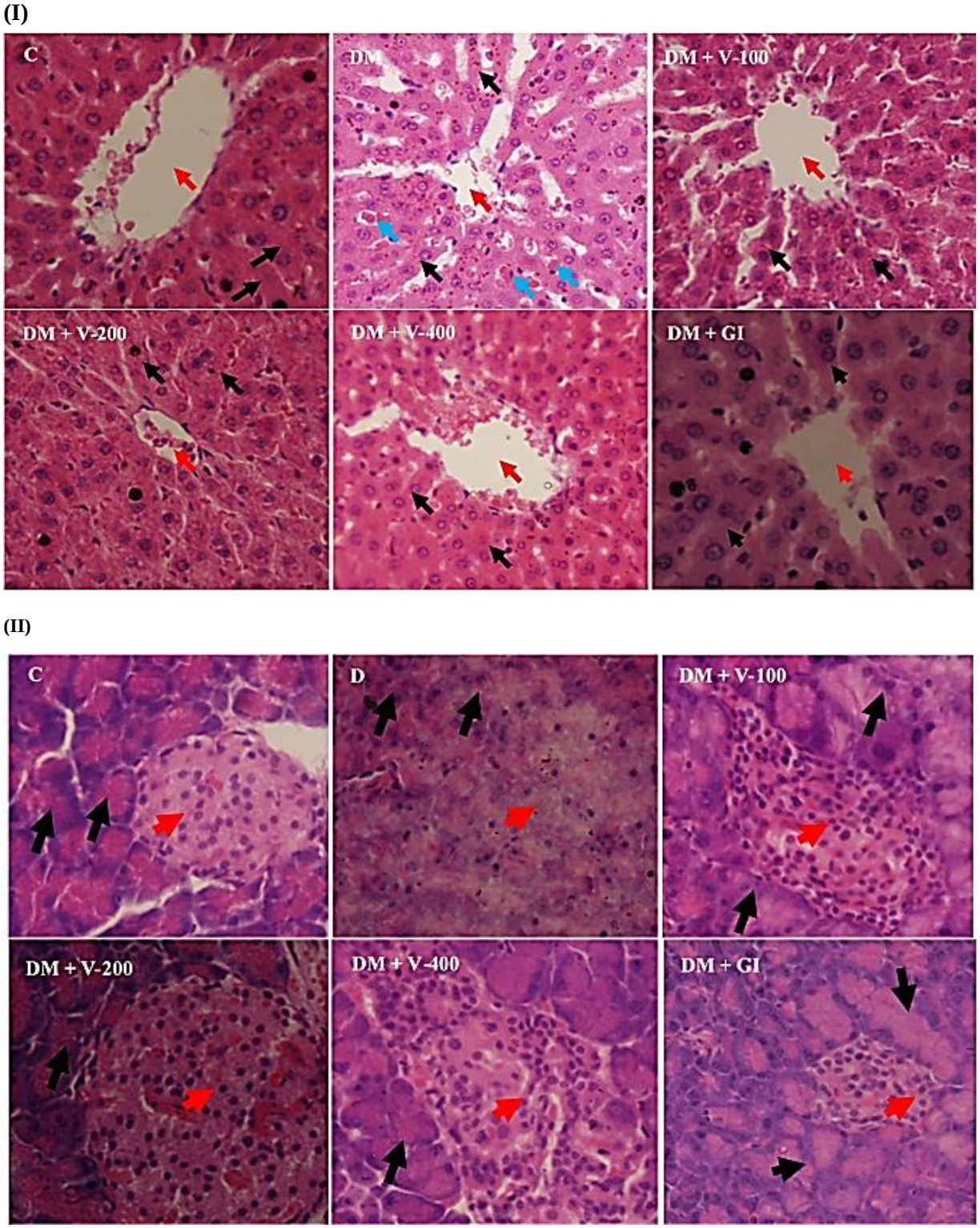

Figure 3. Histopathological findings of the liver and pancreas following treatments with different doses of *Vinca herbacea* extract (100, 200 and 400 mg/kg body weight) in SZT-induced diabetic and normal rats. (I): liver tissue; (II): pancreatic tissue. In the liver: red, black and blue arrows respectively show: central vein, plates of hepatic cells and sinsoids. In the pancreas: red and black arrows respectively show pancreatic islets and exocrine parts of pancreas

Our study showed that *V. herbacea* administration (100 and 200 mg/kg) significantly reduces ALP, ALT, and AST levels.

According to the histopathological examination, the liver of the diabetic control rats showed disarrangement of hepatic strands, accumulation

of fat droplets, inflammation, and dilation of sinusoids. While, treating diabetic rats with 100 and 200 mg/kg of *V. herbacea* extract attenuated the hepatic inflammation and lesions. Treatment of diabetic rats with high dose of extract did not show any improvement in liver tissue according to histopathological examination. Some tissue changes such as sinusoidal space dilation and decreased tissue integrity were obsereved (Figure 3). The pancreatic tissues of control group demonstrated usual construction with arranged islets of Langerhans, whereas the pancreas of diabetic control rats exhibited signs of degeneration and inflammation in addition to the decrease in the number of β-cells. Treatment of diabetic rats with 100 and 200 mg/kg of *V. herbacea* extract showed efficient regeneration and improvement in pancreatic tissue compared to the diabetic rats treated with high dose of the extract (Figure 3). The histopathological findings showed that the acinar cells were swollen, and the size of the islets was reduced. In addition, there was no clear boundary between the endocrine and exocrine segments of the pancreas of diabetic control rats. Efficient improvement and regeneration were observed in pancreatic tissue after diabetic rats' treatment with 100 and 200 mg/kg of *V. herbacea* and in contrast, pancreatic tissue degeneration was observed in diabetic rats which received high dose of the extract. Moreover, according to the histopathological findings, 100 and 200 mg/kg *V. herbacea* administration to diabetic rats caused improvement of the hepatic inflammation and lesions. These results suggest the hepatoprotective effect of *V. herbacea* hydroalcoholic extract that probably due to the presence of active compounds which have antioxidant properties. In contrast, the dose of 400 mg/kg of *V. herbacea* did not show favorable effects in hepatic enzymes evaluation and the histopathological study. These findings suggest that high doses of *V. herbacea* (400 mg/kg) did not have tissue-protective and hepatoprotective properties and even may have toxic effects.

These findings show the antidiabetic, antihyperlipidemic, and hepatoprotective effects of *V. herbacea* extract. Hence, to find out the probable molecular pathways responsible for these properties, the effects of the extract on the expression levels of AMPK, SIRT1, NF-κB and PGC-1α were evaluated in whole blood by Real-time PCR. It should be noted that only the difference between control and diabetic groups and also diabetic rats treated with different doses of extract with diabetic control rats were shown in the Figure 4. Results of this investigation revealed that treatment of diabetic rats with high dose of the extract (400 mg/kg) did not show significant effect on gene expression level of mentioned genes compared to the diabetic control rats ($p>0.05$) (Figure 4).

In comparison to the normal control rats, gene expression levels of AMPK in diabetic control rats were reduced, but the difference was not significant ($p=0.560$). The treated diabetic rats with low dose of the extract (100 mg/kg) exhibited increased expression of AMPK compared to the diabetic control rats ($p=0.026$). A similar upward trend was observed in diabetic rats treated with 200 mg/kg of extract ($p=0.008$). The difference in gene expression between diabetic rats treated with low dose and 200 mg/kg of the extract with high dose 400 mg/kg extract was significant ($p=0.009$ and $p=0.002$, respectively).

The gene expression of SIRT1 in diabetic control rats was reduced compared to the normal control rats ($p=0.026$). Treatment of diabetic rats with 100 and 200 mg/kg of *V. herbacea* extract significantly upregulated the mRNA expression of SIRT1 in comparison to the diabetic control rats ($p<0.001$). The increase in gene expression levels in diabetic rats treated with low dose and 200 mg/kg of extract compared to diabetic rats treated with high dose of extract was significant ($p<0.001$).

NF-κB mRNA expression was significantly upregulated in the diabetic control rats compared to the normal controls ($p<0.001$). *Vinca herbacea* extract at the dose of 200 mg/kg significantly reduced the gene expression of NF-κB compared to the diabetic control rats ($p=0.029$).

The gene expression of PGC-1α in diabetic control rats was reduced significantly compared to the normal control rats ($p<0.001$). Treatment of diabetic rats with 100 and 200 mg/kg of *V. herbacea* extract significantly increased the mRNA expression of PGC-1α in comparison to the diabetic control rats ($p<0.001$). The effect of 200 mg/kg of extract was significantly greater than low dose ($p=0.003$). The increase of gene expression levels in diabetic rats treated with low dose and 200 mg/kg of extract compared to diabetic rats treated with high dose of extract was significant ($p=0.008$ and $p<0.001$, respectively).

Compared with the diabetic control group, diabetic rats' treatment with doses of 100 and 200 mg/kg of *V. herbacea* caused significant upregulation of the AMPK, SIRT1, and PGC-1α expression. In addition, 200 mg/kg *V. herbacea* downregulated the NF-κB expression. However, the high dose of extract didn't lead to significant changes in any one of the investigated genes.

AMPK is a principal sensor of cellular energy and a major metabolic homeostasis regulator which dysregulations of its activity have a role in the metabolic disorders' pathogenesis and progress [45]. Activation of AMPK will stimulate oxidation of fatty acids in the skeletal muscle and liver, prevent lipogenesis and further augment insulin secretion [46]. AMPK activation enhances the NAD+/NADH ratio and thereby increases the SIRT1 expression and activation [47]. Upregulation of SIRT1 has been associated with amelioration of lipid profile, insulin sensitivity, and glucose homeostasis in animal models [48].

Evidence shows that the AMPK signaling activation can suppress the NF-κB system-induced inflammatory responses by its downstream mediators such as SIRT1 [49,50]. NF-kB is one of the transcription factors which lead to transcription of the apoptotic and inflammatory genes in response to oxidative stress. In diabetic rat models, NF-kB is activated, and pro-apoptotic and pro-inflammatory genes are increased in the liver [51-54]. Some plant ingredients such as flavonoids, resveratrol, and alkaloids are potential activators of SIRT1 and AMPK [55-57].

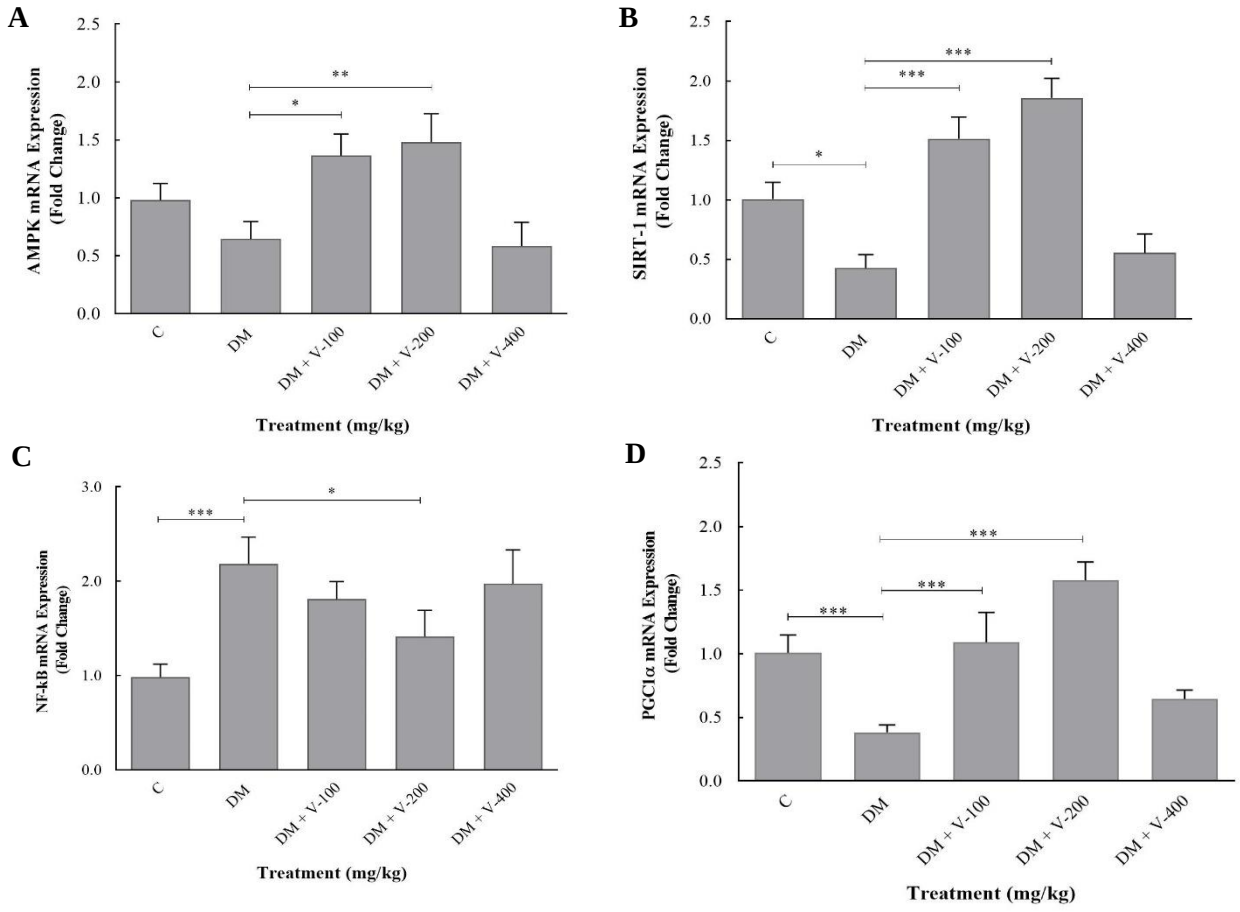

Figure 4. Effect of different doses of *Vinca herbacea* extract (100, 200 and 400 mg/kg body weight) on the gene expression of (A) AMPK, (B) SIRT1, (C) NF-κB and (D) PGC1α in the study groups (data are presented as mean±SD); C: Control group; DM: diabetic control; DM+V-100: diabetic rat + 100 mg/kg body weight extract; DM+V-200: diabetic rat + 200 mg/kg body weight extract; DM+V-400: diabetic rat + 400 mg/kg body weight extract; DM+GI: diabetic rat + glibenclamide; *, **, *** $p<0.05$, $p<0.01$ and $p<0.001$, respectively

Their anti-inflammatory properties seem to be mediated by NF-κB signaling suppression via AMPK/SIRT1. PGC-1α is a major regulator for mitochondrial biogenesis and performance, which includes ROS elimination and oxidative phosphorylation [58]. Deregulation of its expression leads to metabolic disturbances that result in metabolic disease and inflammation. PGC-1α is clearly associated with metabolic syndrome pathogenesis and its main complications. And as it has the ability of metabolic pathways modulation, can be an interesting therapeutic target in metabolic disease [59]. Reduction of PCG-1α expression is associated with insulin resistance in nonalcoholic fatty liver disease and diabetes [60]. PCG-1α activation stimulates insulin sensitivity through activation of PPARα [61]. The enhanced hepatic PCG-1α ameliorates the homeostasis of glucose in vivo [60]. PGC-1α expression and mitochondrial biogenesis were mediated by AMPK [62]. AMPK activates PGC-1α via sirtuins [47]. Activation of AMPK increases gene expression of GLUT4 by phosphorylation of PGC1-α [62]. SIRT1 acts in the homeostasis of glucose as a PGC-1a modulator, which has effective implications for the energy homeostasis basic pathways, diabetes, and lifespan. SIRT1 prompts genes involved in gluconeogenesis and glucose production of the liver via PGC-1a [63]. *Coptis Chinensis* has a well-known anti-diabetic activity. It can increase insulin secretion by stimulating the regeneration of pancreatic Langerhans islets and also stimulates the phosphorylation of AMPK in skeletal muscle and liver [46]. It is reported that quercetin, a plant flavonol, stimulates GLUT4 translocation via AMPK activation and therefore increases glucose uptake [64]. Besides, a recent study showed that treatment with quercetin in streptozotocin - induced diabetic rats improved glucose and lipid metabolism and also attenuated histomorphological injury of hepatic tissue, which is probably associated with the SIRT1 activity upregulation by quercetin [65]. It has also been reported that resveratrol, a natural polyphenol, shows anti-hyperglycemia, and anti-hyperlipidemia effects, improves insulin sensitivity and beta-cell function and alleviates hepatic glucose production by the SIRT1 activation [66,67]. In another study, it is revealed that hesperetin, a citrus polyphenolic flavonoid, has a stimulatory effect on SIRT1and AMPK signaling pathway in HepG-2 cells which is even stronger than that of resveratrol. *Momordica charantia*, bitter melon, has been utilized greatly for its antidiabetic and antioxidant effects. Its extract downregulates MAPKs and NF-kB, in order to ameliorate the impaired signaling of insulin, moreover, protects pancreatic beta cells [35]. Results from the study of Muthukumaran Jayachandran et al. [38] showed the great capacity of guava leaf extract to reduce plasma glucose and oxidative stress as well as inflammation improvement in streptozotocin-induced diabetic rats. In their study, streptozotocin administration caused hyperglycemia, intensive oxidative stress, and inflammation, which was proved by the alterations in the inflammatory cytokines such as NF-kB. Similar to the glibenclamide treatment group, guava leaf extract meaningfully diminished inflammatory cytokines expression, including NF-κB. Therefore, the mechanism of relieving the diabetes symptoms by guava leaf extract is through hyperglycemia regulation followed by regulation of the NF-kB pathway and oxidative stress.

These findings are consistent with the present study. The current study indicated the advantageous effects of *V. herbacea* in glucose and lipid metabolism were related to increased gene expression of AMPK, SIRT1, and PGC1α and decreased NF-κB gene expression in the diabetic rats. Thereby, adjustment of the AMPK/SIRT1/PGC1α /NF-κB signaling pathway by *V. herbacea* was most likely responsible for its anti-diabetes, hepatoprotective, and anti-hyperlipidemia effects.

In the study of Morin et al., nobiletin showed activation of sterol regulatory element-binding proteins in its lower doses and their inhibition with its higher doses [68]. These findings are also consistent with our study. In the present research, although *V. herbacea* with doses of 100 and 200 mg/kg augmented AMPK, SIRT1, and PGC1α and decreased NF-κB gene expression, its effect was not significant in the dose of 400 mg/kg. Therefore, *V. herbacea* may show different responses in various doses and likely be more effective at lower doses.

Conclusion

Despite the numerous studies that have investigated the bioactive phytochemicals effects on cellular metabolic pathways, there is almost

no evidence about the possible effects *V. herbacea* extract on AMPK, SIRT1, NF-KB, and/or PGC1α levels in diabetes. To the best of our knowledge, our experiment was the first investigation for indicating anti-diabetic and anti-hyperlipidemic effects of *V. herbacea* in experimentally diabetic models and suggested the AMPK/SIRT1/PGC1α/NF-κB signaling pathway adjustment as a mechanism for the *V. herbacea* protective effects in diabetes. Nevertheless, protein levels and activations for investigation genes were not evaluated in the present study and there is a need for further research.

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Author contributions
Nasim Abedimanesh was involved in conceptualization, formal analysis, methodology, writing the original draft and revising the manuscript; Kosar Mohammadnejad contributed in writing the original draft and revision; Morteza Nouri, Maedeh Barati, Elham Dabardani Elahe Kakavand and Seyed Hojjat Hosseini contributed in investigations and validation; Maryam Noubarani and Sina Andalib were involved in data curation and formal analysis; Momeneh Mohammadi and Iraj Jafari Anarkooli contributed in histopathological evaluation; Mohammad Reza Eskandari, Alireza Yazdinezhad and Behrooz Motlagh contributed in conceptualization, methodology, revising; supervision and project administration.

Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMPK: 5' adenosine monophosphate activated protein kinase; ANOVA: analysis of variance; AST: aspartate transaminase; DM: diabetes mellitus; ELISA: enzyme-linked immunosorbent assay; FBG: fasting blood glucose; GLUT4: glucose transporter type 4; GTT: glucose tolerance test; HDL-c: high-density lipoprotein cholesterol; MAPK: mitogen-activated protein kinase; NADH: nicotinamide adenine dinucleotide; NF-κB: nuclear factor kappa B; PCR: polymerase chain reaction; PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARα; peroxisome proliferator-activated receptor alpha; ROS: reactive oxygen species; SIRT1: sirtuin 1; STZ: streptozotocin; TC: total cholesterol; TG: triglyceride.