Research Paper: Effect of the Aqueous and Hydro-Alcoholic Extracts of Viola odorata L. on Biochemical and Histologic Liver Parameters in Diabetic Wistar Rats

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ABSTRACT

Introduction: The liver, as an insulin target organ, undergoes numerous pathological changes in diabetes patients. This study investigated the effect of the aqueous and hydro-alcoholic extracts of violets on histologic changes and biochemical parameters of the liver in diabetic adult Wistar rats.

Methods: In total, 64 rats were examined in 8 groups of 8 rats (1 control group and 7 diabetic groups treated by streptozotocin). The rats were treated in 6 diabetic groups by different concentrations of the aqueous and hydro-alcoholic extracts of violets (100, 200, and 400 mg/kg). Biochemical tests were performed to evaluate the liver enzymes, glucose, and serum albumin using the photometric method on the blood of rats. Furthermore, Hematoxylin and Eosin (H & E) and Periodic acid-Schiff (PAS) stains were performed to investigate the number of Kupffer cells, hyper eosinophilia, inflammation, congestion, changes in the perimeter and the central vein area, and glycogenic deposits from the liver tissue of rats.

Results: The obtained results suggested a decrease of Kupffer cells in the concentration of 100 in extracts. Moreover, inflammatory accumulations decreased in the concentrations of 100 and 400 in the aqueous extract. In addition, a decrease of congestion in the concentrations of 400 in the aqueous extract and the concentrations of 100 and 200 in the hydro-alcoholic extract; a decrease of AST and ALT of serum in the concentrations of 100 and 400 in the aqueous extract; and a decrease of glucose in the concentrations of 100, 200, and 400 in the hydro-alcoholic extract and the concentration of 400 in the aqueous extracts were observed.

Conclusion: The prescription of the extracts of violets can improve the liver tissue in terms of Kupffer cell count, inflammation, and congestion. Furthermore, they reduced AST and ALT enzyme levels and serum glucose levels in diabetic rats.

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1. Introduction

The prevalence of diabetes, as the most important endocrine disease, is increasing [1]. According to the World Health Organization (WHO), diabetes will be the seventh cause of death in 2030 [2]. In Iran, the prevalence of diabetes is on the rise, and according to forecasts, in 2025, 5.2 million Iranian people will be suffering from diabetes [3].

The initial stage of type 2 diabetes is characterized by insulin resistance in target tissues, especially the liver, skeletal muscle, and adipokines. Insulin resistance in these tissues results in the production of excess glucose by the liver [4]. This disease is associated with a wide range of liver diseases, such as an abnormal increase in liver enzymes, non-alcoholic fatty hepatic disease, liver cirrhosis, carcinoma of liver cells, and chronic liver disorder [5]. In diabetes, the balance between antioxidants and free radicals is distorted; thus, the process of emergence of diabetic side-effects accelerates, due to increased antioxidants in the body [6].

Oxidative stress results from an imbalance between the production of oxygen-free radicals and the defense of antioxidants in the body [7]. Chronic hyperglycemia can cause abundant and irreparable lesions in the eyes, nerves, kidneys, liver, as well as heart and vessels [8]. Treatments based on medicinal plants is typically cost-effective, more feasible, and more accessible, compared to chemical drugs-based treatments. Furthermore, in some cases, it has fewer adverse effects. Therefore, in diabetes treatment, it is necessary to find effective compounds with fewer side-effects. More than 1200 medicinal plants have been identified as effective in diabetes treatment [9].

Violets (Violaceae), which have a wide variety and wide application in Iran, have been previously studied. Liver protecting flavonoids in Viola odorata species and anti-diabetic and antioxidant properties of Viola Mandshurica species are documented [10, 11]. The prevalence of diabetes is surprisingly increasing around the world and its associated complications are serious public health issues. Therefore, the present study aimed to evaluate the effect of violets on the liver enzymes and liver histologic parameters in diabetic adult Wistar rats.

2. Materials and Methods

Violets (Viola odorata L.), with the age of two weeks, were collected from the northern regions of Iran (Javaherdeh, Mazandaran Province). Then, they were vacuum dried at 55°C (with final moisture of about 8%) and powdered. Hydro-alcoholic extract (ethanol 70%) and aqueous extract of it were prepared by the distillation method with the balloons and water vapor based on the protocol proposed by M. Rabbani et al. (with some changes) [12].

This research was conducted according to the ethical considerations by the National Institutes of Health (NIH) as well as the ethical and caring guidelines for laboratory animals (Code: IR. GUMS. REC. 1930349905).

In total, 64 adult Wistar rats (age: 6-8 weeks; weight: 220-250 gr) (obtained from Razi Institute, Iran) were maintained in plexiglas with mesh doors at 23±2°C, the relative humidity of 40%-60%, and 12:12-h light–dark cycle. In the first week, the rats had free access to water and food (using ad libitum protocol) to get accustomed to the lab environment.

To induce diabetes, streptozotocin (made in the USA, ZanosarTM) was injected Intraperitoneally (IP) at a dose of 45 mg/kg. Moreover, 72 hours after the injection, the rats' blood samples were prepared through their tail tip. Then, their glucose levels were measured by glucometer, and rats with plasmatic glucose levels >250 mg/dL were considered as diabetic [13]. The injection was repeated only once again if they were not diabetic. Additionally, the day when injecting aqueous and hydro-alcoholic extracts was conducted, the body weight and blood glucose levels of rats were recorded; accordingly, those with <250 mg/dL were excluded from the study. The treatment of diabetic rats was performed IP by aqueous and hydro-alcoholic extracts of violets with the concentrations of 100, 200, and 400 mg/kg, for 4 weeks [10, 14, 15].

In total, 64 adult Wistar rats (56 diabetic and 8 healthy rats) were studied in 8 groups of 8 rats, as follows: group A: The healthy control group; group B: Diabetic rats (prescribed Streptozotocin: 45 mg/kg); groups C-E: Diabetic rats treated with the concentrations of 100, 200, and 400 mg/kg of aqueous extract, and group F-H: Treated with the concentrations of 100, 200, and 400 mg/kg of hydro-alcoholic extract.

Blood sampling was performed for biochemical studies after 4 weeks of intervention initiation. In addition, after the anesthesia induction with ketamine-xylene, the liver tissue was removed, dissected for histological investigation, and stained by Hematoxylin and Eosin (H & E) and Periodic acid–Schiff (PAS) methods. In the H&E staining, the cells' nucleus is purple, and the cytoplasm is pink. This staining was performed for histological

purposes, including congestion, inflammatory cells, cytoplasm hyper eosinophilia, the accumulation of active Kupffer cells, and central venous changes. Moreover, the PAS staining was used to investigate glycogen reserves in hepatocytes. From each tissue, 5 slides, and in each slide, 10 fields with a 400× magnification were investigated.

Serum glucose level was measured by the serum glucose kit (obtained from MerckTM, Germany), according to the glucose oxidase method using the photometric method and on 546 nm wavelength.

Using the Bromocresol Green kit method (obtained from MerckTM, Germany), by a special measurement kit to the measurement of serum albumin, rats' serum albumin was measured. Then, wavelength was investigated using the photometric method on 546 nm.

Serum of liver enzymes was measured by a special measurement kit (obtained from MerckTM, Germany) by the photometric method. The sensitivity of the measurement method for ALT, AST, and ALP was 4, 2, and 3 international units per liter, respectively.

The collected data were analyzed using the Kolmogorov-Smirnov (K-S) method to ensure the normality of data distribution. The obtained results were reported as mean and standard deviation. Comparing the results of 8 groups was performed using one-way Analysis of Variance (ANOVA). The inter-group comparison was conducted using the Tukey method. All statistical analyses were conducted by SPSS. $P<0.05$ were considered as significant.

3. Results

During injecting aqueous and hydro-alcoholic extracts, rats' glucose and weight were periodically measured. All rats' weight significantly decreased during the experiment, compared to the study onset ($P<0.05$). The rats' weight and Fasting Blood Sugar (FBS) level, which were measured by blood samples from their tail tip and examined by the glucometer are reported in Table 1.

The obtained results indicated that AST significantly decreased in the concentrations of 100 and 400 mg/kg in the aqueous extract. In addition, ALT revealed a significant decrease in the concentration of 100 mg/kg in the aqueous and hydro-alcoholic extracts as well as the concentration of 400 mg/kg in the aqueous extract. Meanwhile, IP injection of different aqueous and hydro-alcoholic concentrations suggested no change in serum ALP. The AST/ALT ratio also significantly reduced by the IP injection of different concentrations of aqueous extract, compared to the control group (Table 2).

The present study revealed a significant decrease in the glucose level of the streptozotocin-diabetic group, compared with the controls. This finding confirmed the rats becoming diabetic ($P<0.05$). In addition, IP injection of 100, 200, and 400 mg/kg of hydro-alcoholic extract of violets, and the concentration of 400 mg/kg of the aqueous extract significantly decreased the serum glucose level in diabetic rats ($P<0.001$). Moreover, the albumin level of the streptozotocin-diabetic group remained unchanged, compared to the controls (Table 2).

We observed the slides produced by the optical microscope in the control group, diabetic group, and diabetic group receiving different concentrations of the aqueous

Table 1. FBS and weight changes of the studied rats in different groups during the experiment

Groups	Primary Glucose	Glucose After 72 Hours	Final Glucose (Before Sacrificing Rats)	Initial Weight	Wk1	Wk2	Wk3	Wk4
	(mg/dL)			(kg)				
Control	74.9	83.4	107.8	311	397.8	405.2	412.8	415.4
Diabetic	103.3	367.6	539	286.2	260.3	269.4	269.3	277.3
Aqueous 100	68.3	348	414	311	314.8	318	319.5	320.6
Aqueous 200	74.3	357.1	513.2	301.6	282.6	262.8	280.6	282.8
Aqueous 400	80.2	336.3	475.3	251.5	227.3	222.3	213	206.8
Hydro-alcoholic 100	79.8	378.2	407.3	290.7	292	296.6	290	302.5
Hydro-alcoholic 200	105.1	450	485.5	288.8	267.4	277	254.4	277.7
Hydro-alcoholic 400	109.6	449.2	313.7	281.8	238.8	241.8	252	250.7

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Table 2. Results of biochemical and histological investigations

Variabels	Mean±SD							
	Control	Diabetic	Aqueous Extract (mg/kg)			Hydro-Alcoholic Extract (mg/kg)		
			100	200	400	100	200	400
AST (IU/L)	113.62±8.23	191.83±61. 38[a]	89.27±12.57b	173.71±26.57	126.89±22.25 [b]	172.95±41.69	172.43±37.46	226.13±50.34
ALT (IU/L)	45.38±6.21	161.44±71.23[a]	79.50±24.22[b]	149.86±57.84[a]	81.59±17.85 [b]	101.78±31.60	118.21±22.86	96.94±19.00
ALP (IU/L)	246.31±45.05	556.75±128.04[a]	543.01±101.48[a]	680.95±41.89 [a]	644.42±42.85 [a]	589.14±170.31[a]	634.38±99.17 [a]	580.77±212.16 [a]
AST/ALT	2.53±0. 35	1.43±0.42 [a]	1.02 ±0.35 a	1.02 ±0.33 [a]	1.62 ±0.56 [a]	1.80±0.53	1.48±0.36	2.47±1.05
Glucose (mg/dL)	165.71±24.26	713.26±55.81 [a]	720.85±60.80 [a]	664.75±69.18 [a]	584.78±76.94 [ab]	575.78±87.25 [ab]	553.81±81.56[ab]	525.99±43.26[ab]
Serum albumin (mg/dL)	2.08±0.54	2.13±0.42	2.54±0.44	2.55±0.39	2.26±0.35	2.41±0.34	1.76±0.35	2.18±0.08

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[a] shows significant values, compared to the controls

[b] shows significant values, compared to the diabetic group

and hydro-alcoholic extracts of violet. The relevant data suggested a significant increase in the number of Kupffer cells in the diabetic group, compared to the controls. In addition, the IP injection of 100 mg/kg of the aqueous and hydro-alcoholic extracts of violet significantly decreased the number of Kupffer cells. However, the concentrations of 200 and 400 mg/kg of the aqueous and hydro-alcoholic extracts failed to change the number of Kupffer cells (Figure 1).

Reduction of rats' hypereosinophilia hepatocyte cells at the concentration of 100 mg/kg

Observing the slides produced by the optical microscope demonstrated that the number of hypereosinophilia cells increased in the diabetic group, compared to the controls. In addition, the IP injection of 100 mg/kg of the aqueous extract of violet decreased the number of hypereosinophilia cells, compared to the diabetic group.

Reduction of inflammatory accumulations at the concentrations of 100 and 400 mg/kg of the aqueous extract

Observing the slides produced by the optical microscope suggested that the inflammation rate increased in the diabetic group, compared to the controls. The IP injection of 100 and 400 mg/kg of the aqueous extract of violet significantly reduced inflammatory accumulations, in comparison with the diabetic groups.

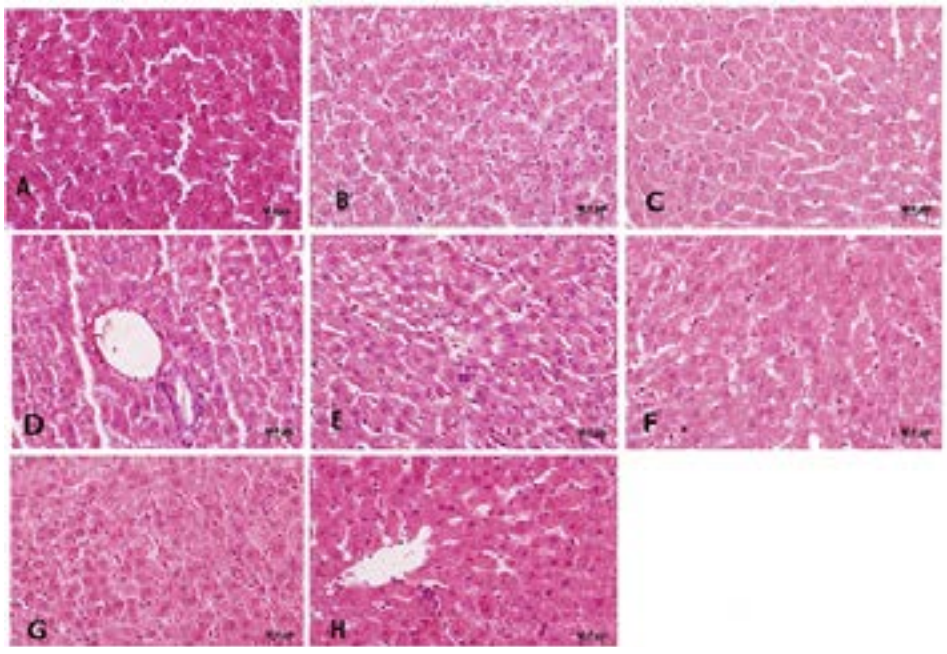

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Figure 1. The number of Kupffer cells in the controls

A. the diabetic group; B. the groups receiving the concentration of 100; C. 200 ; G. 400; H. the hydro-alcoholic extract of violet in diabetic rats (H & E staining with a 400× magnification)

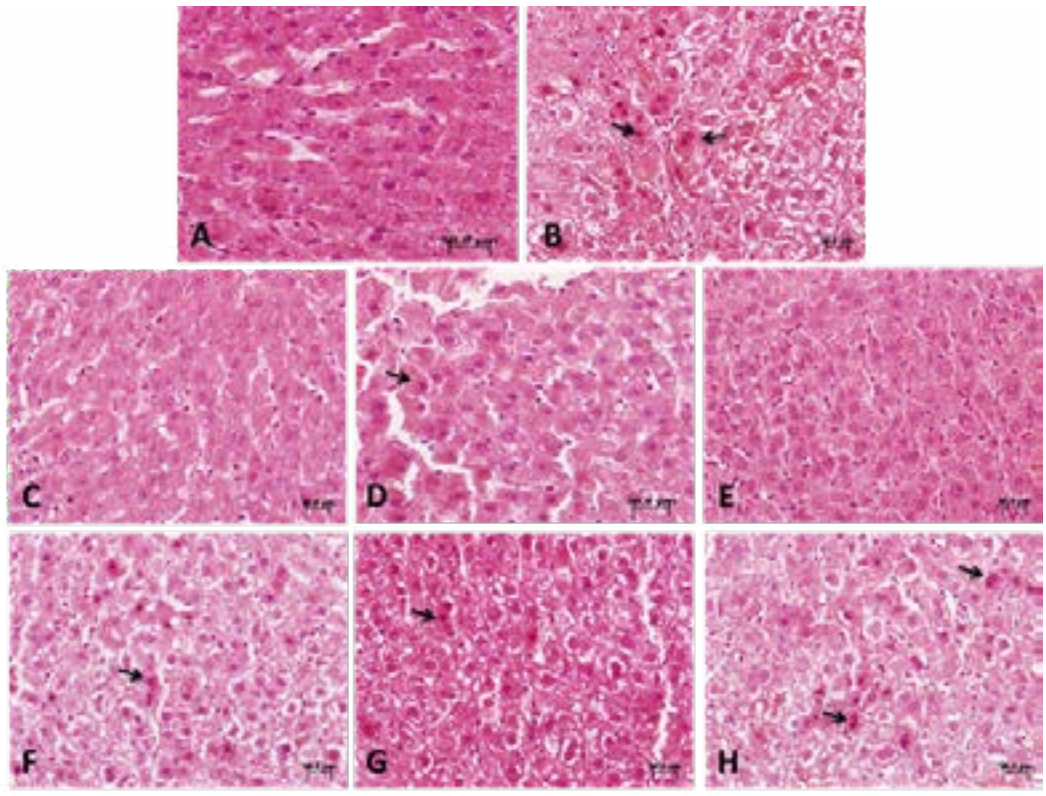

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Figure 2. The hypereosinophilia cells in the control group

A.the diabetic group; B. the groups receiving the concentrations of 100; C. 200; D. 400; E. of the aqueous extracts and the concentrations of 100; F. 200; G. 400; H. of the hydro-alcoholic extract of violets in the diabetic rats (H & E staining with a 400× magnification)

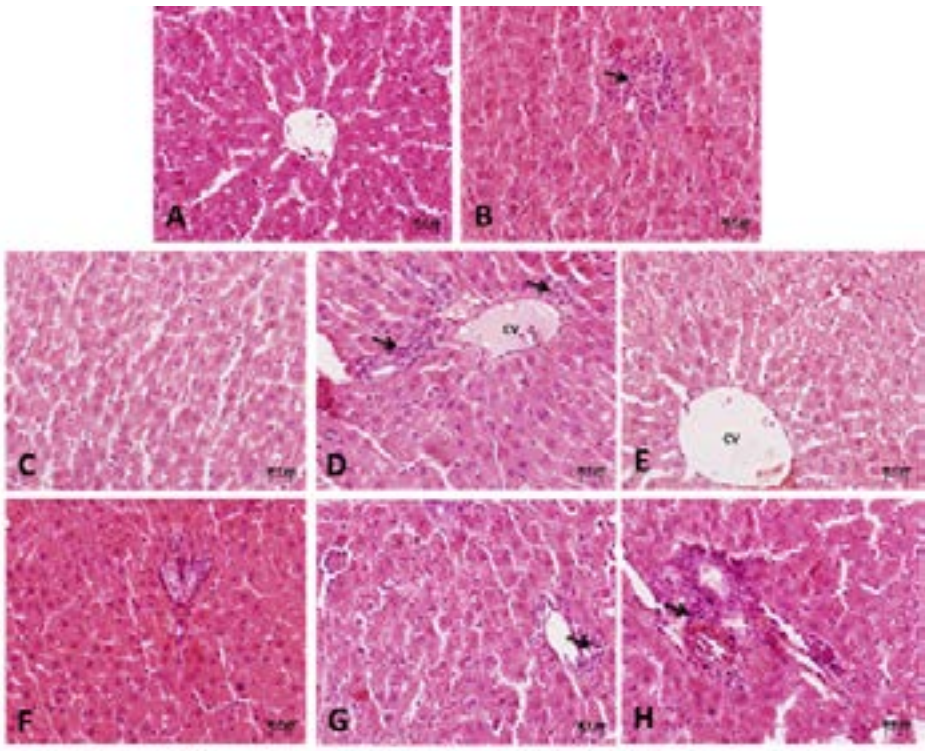

Figure 3. The inflammatory accumulations in the control group

A. the diabetic group; B. the groups receiving the concentrations of 100; C. 200; D. 400; E. of the aqueous extracts and the concentrations of 100; F. 200; G. 400; H. of the hydro-alcoholic extract of violets in the diabetic rats (H & E staining with a 400× magnification)

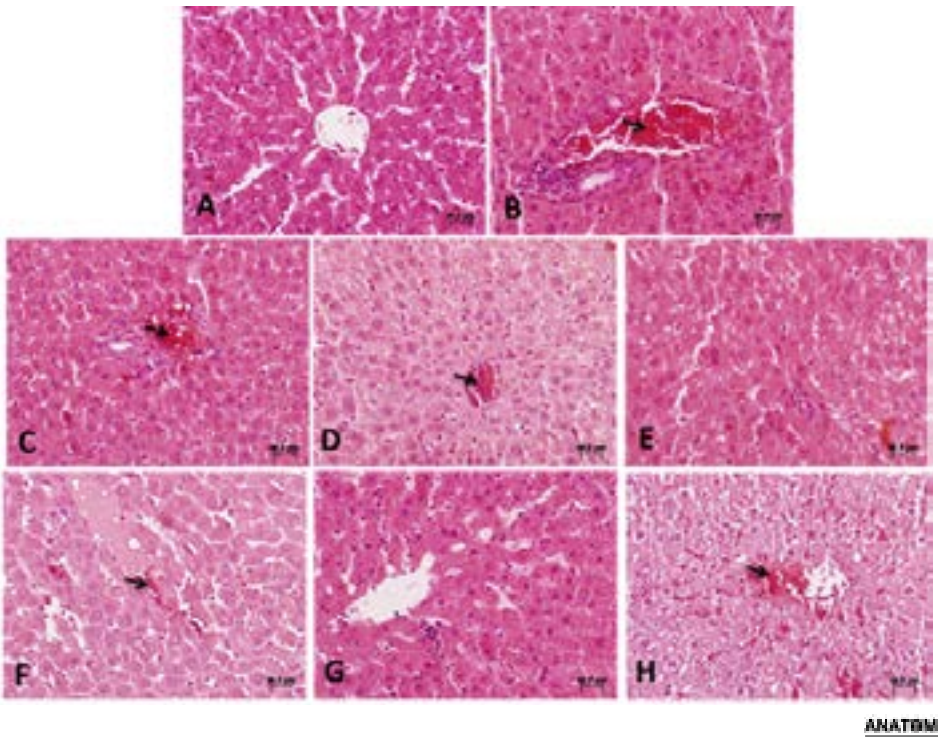

Figure 4. The congestion in the controls

A. the diabetic group; B. the groups receiving the concentrations of 100; C. 200; D. 400; E. of the aqueous extracts and the concentrations of 100; F. 200; G. 400; H. of the hydro-alcoholic extract of violets in the diabetic rats (H & E staining with a 400× magnification)

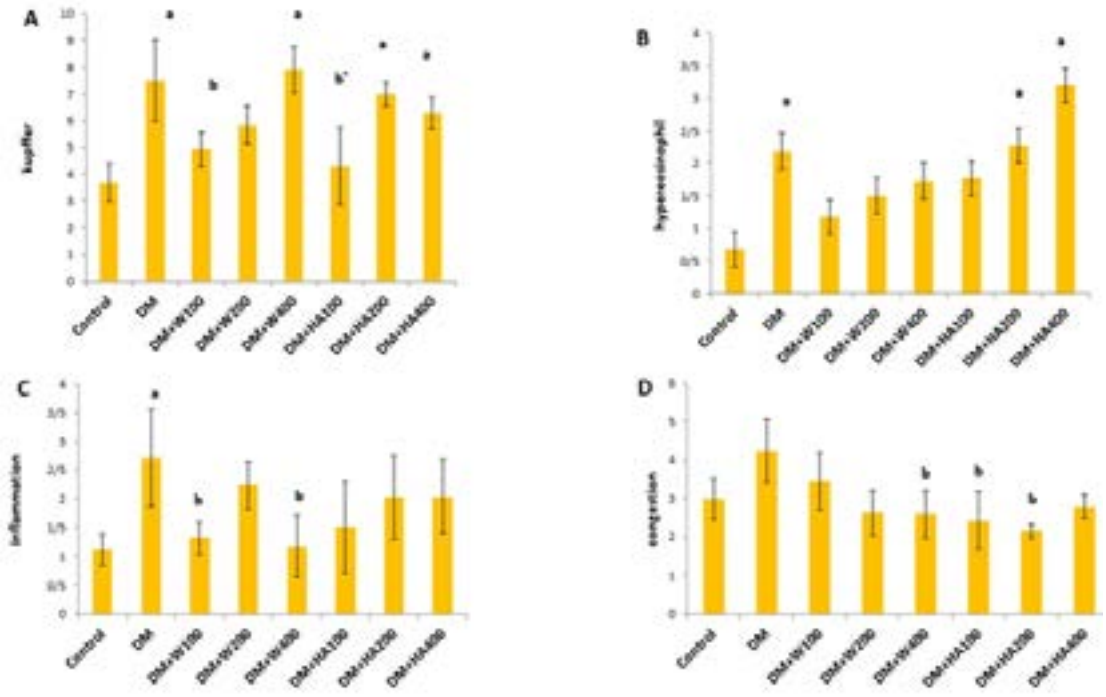

Figure 5. The effect of the aqueous and hydro-alcoholic extracts of violets (Viola odorata L.)
A. the number of kupffer cells; B. the number of hypereosinophilia cells, (C) inflammatory accumulations, (D) congestion changes in the groups under investigation (all data are reported as mean±SDM)
(a shows P<0.001, compared to the controls, and b shows P<0.05, compared to the diabetic group, DM stands for diabetized group, W stands for the aqueous extracts of violets, and HA stands for the hydro-alcoholic extract of violets.)

Reduction of congestion at the concentrations of 400 mg/kg of the aqueous extract and 100 and 200 mg/kg of the hydro-alcoholic extract

Observing the slides prepared by the optical microscope revealed that the congestion rate remained unchanged in the diabetic group, in comparison with the controls. The IP injection of 400 mg/kg of the aqueous extract and 100 and 200 of the hydro-alcoholic extract significantly reduced congestion.

Observing the slides prepared by the optical microscope suggested that the diabetic group remained the same, in comparison with the controls in the perimeter and the area of the central vein. However, the IP injection of 200 mg/kg of the aqueous extract and 100 mg/kg of the hydro-alcoholic extract of violet significantly decreased (P<0.05).

Observing the slides produced by the optical microscope demonstrated that the diabetic group remained unchanged, compared to the controls in glycogen deposits. Although the level of glycogen was increased by injecting the hydro-alcoholic extract, compared to the diabetic group, the changes were not significant.

4. Discussion

Researchers keep investigating the potential effects of plants in the treatment of diseases. In this study, histological changes and functional tests of the liver were evaluated in the diabetic rats treated with different concentrations of the aqueous and hydro-alcoholic extracts of violet.

The prescription of streptozotocin with 45 mg/kg concentration in 72 hours induced diabetes in male rats. Streptozotocin, after being absorbed by the pancreas beta cells, by causing changes in the DNA and production of the oxygen-reactive ROS, leads to the degradation of these cells, dysfunction of insulin production, increased blood glucose (hyperglycemia), and the suffering of type 1 diabetes [16]. The changes obtained from streptozotocin on the liver include the dilation of the arteries, irregularity of hepatocyte plates, hepatic fibrosis, and the reduction of glycogen deposits [17].

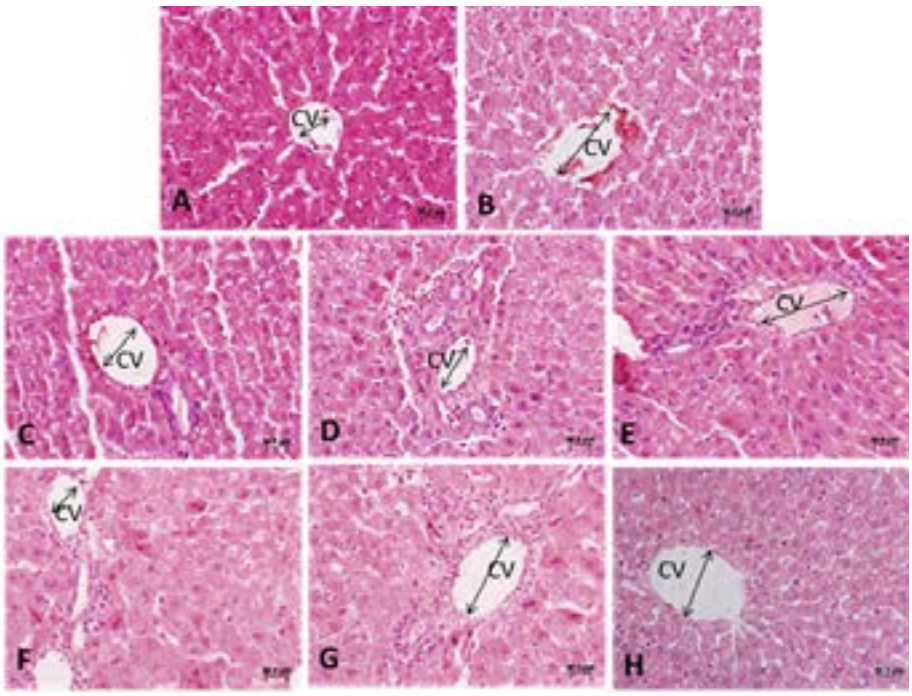

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Figure 6. The central venous changes in the control group

A. the diabetic group; B. the groups receiving the concentrations of 100; C. 200; D. 400; E. of the aqueous extract and the concentrations of 100; F. 200; G. 400; H. of the hydro-alcoholic extract of violet in the diabetic rats (H & E staining with a 400× magnification)

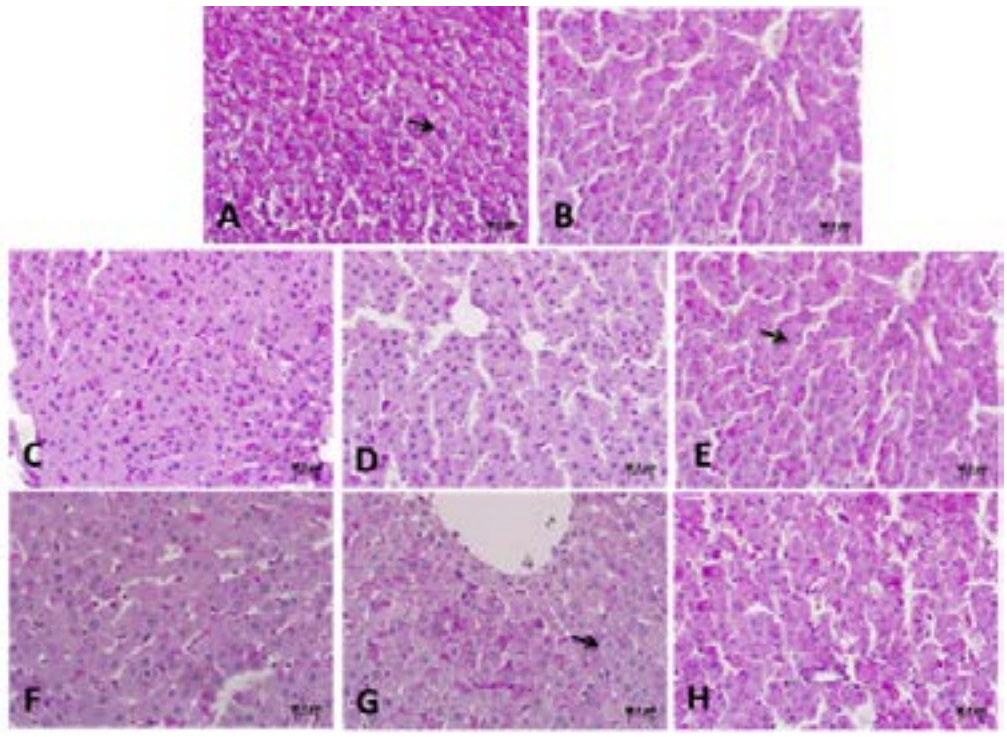

Figure 7. Glycogen deposits in the control group

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A. the diabetic group; B. the groups receiving the concentrations of 100; C. 200; D. 400; E. of the aqueous extracts and the concentrations of 100; F. 200; G. 400; H. of the hydro-alcoholic extract of violets in diabetic rats (PAS staining with a 400× magnification)

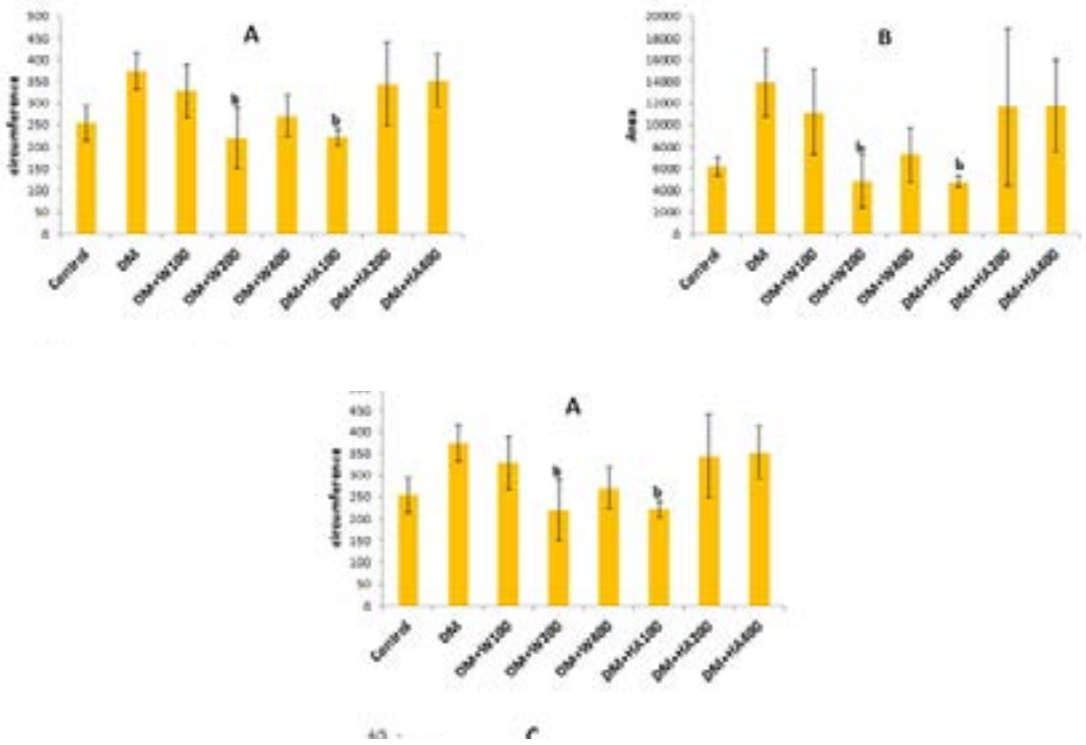

Figure 8. The effect of the different concentrations of the aqueous and hydro-alcoholic extracts of violets
A. perimeter changes of the central vein; B. area changes of the central vein; and C. changes of glycogen deposits in the groups under investigation (all data are reported as Mean±SDM) (DM stands for diabetic group, W stands for the aqueous extracts of violets, and HA stands for the hydro-alcoholic extract of violets).

The liver significantly affects maintaining healthy concentrations of glucose after eating; thus, it is involved in diabetes mellitus [18]. Diabetes leads to morphological and morphometric changes in hepatocytes, which complies with our findings in terms of histological assessments. Most studies on the liver have focused on biochemical and physiological changes, and the histological aspects and liver structure are less considered. In this respect, our results suggested an increase in the number of Kupffer cells, hypereosinophilia, and lymphomas inflammation in the diabetic group. Furthermore, different concentrations of the aqueous and hydro-alcoholic extracts of violet reduced the number of Kupffer cells, hypereosinophilia cells, inflammation, and congestion, and increased glycogen deposits.

According to our results, the concentrations of 100 mg/kg of the aqueous and hydro-alcoholic extracts of violet significantly decreased the number of Kupffer cells. Additionally, 100 and 400 mg/kg of the aqueous extract significantly decreased inflammatory accumulations. Therefore, the aqueous and hydro-alcoholic extracts reduced the complications of diabetes, such as increased number of Kupffer cells, inflammation, and congestion.

The deposits of glycogen granules inside hepatocytes of the cytoplasm remained unchanged in all the treated groups by PAS staining [19]. The increased activity of hepatic enzymes of serum, which reflects liver damage due to the increase of the activity of ALP, AST, and ALT enzymes, results from their leakage from hepatic cytosol into the bloodstream [20]. Increasing the protein catabolism along with gluconeogenesis and urea formation in diabetic patients may increase these transaminases in the blood [21]. In our study, AST, ALT, and ALP levels of serum significantly increased in diabetic rats. However, after the treatment of diabetic rats with aqueous and hydro-alcoholic extracts, ALT, AST, AST to ALT ratio, and serum glucose decreased in some concentrations.

The obtained results indicated that the IP injection of different concentrations of 400 and 100 mg/kg of the aqueous extract of violet, in comparison with the diabetic group, significantly decreased AST and ALT concentrations in the diabetic rats. The hydro-alcoholic extract also reduced the ALT level in the treated groups, compared to the diabetic group; however, the change was not significant. With the injection of the violet extract, no significant decrease was observed in the ALP level of the diabetic rats. Although the ALP level increased, the in-

creasing trend continued in liver degradation, and violets had no favorable impact on this enzyme. This might be due to the short treatment period. However, it could have favorable effects on this parameter in long-term use.

Research has suggested diabetes-induced oxidative stress among major factors causing tissue damage and biochemical disturbances in diabetic patients' body cells [22]. Hyperglycemia by glucose oxidation, protein glycation, and poly-alcohol metabolism activation can accelerate the production and the oxygen-reactive ROS, increase the oxidation of fats, DNA, and proteins in different tissues [23]. Producing free radicals is a cause of change in liver enzymes activity in patients with diabetes [24]. Serum albumin is exclusively produced by the liver cells and has a long half-life (18 to 20 days). Moreover, approximately 4% of it is decomposed daily and, the degradation rate of albumin is low; thus, serum albumin levels are not an appropriate indicator for mild or acute hepatic dysfunction [24].

In our study, there were no significant differences in the serum albumin level between the diabetic group and the controls. One possible mechanism related to the beneficial effects of violet extract on the histological and biochemical changes of the liver can be caused by the alkaloids, flavonoids, and natural polyphenolic compounds in this plant. Vessal et al. concluded that the IP prescription of some flavonoids in the diabetic rats with streptozotocin significantly decreased the serum glucose levels; i.e. dependent on concentration. However, the same flavonoids do not affect blood glucose concentration in healthy animals [25]. A part of the beneficial effects and hypoglycemic flavonoids can probably increase the hexozakinaz and glucokinase of liver activity and protect and even increase the density of Langerhans-adrenergic cells due to their antioxidant properties. Lee et al. investigated the physiological activities of Viola Mandshurica to measure its antioxidant properties by the total phenolic content.

Moreover, its antidiabetic activity was estimated with the activities of the inhibitors on α-amylase and α-glucosidase. They concluded that methanol extract has the highest amount of phenol content. Acetone extract represents a more powerful radical scavenging activity. The acetone extracts inhibitory activity against α-amylase and α-glucosidase is higher than 100% at a concentration of 1000 µg/mL. The obtained results suggested that V. Mandshurica may have potential anti-oxidant and antidiabetic activities [26].

The violet extract contains active morphologic factors, such as alkaloid, glycoside, tannin, steroid, terpenoid, saponins, flavonoid, methyl salicylate, mucilage, and vitamin C [27-29]. Some of these compounds seem to somehow reduce the severity of self-safe reactions and the inflammation process leading to beta-cell destruction. Thus, preventing residual cells' destruction may provide adequate opportunity for the proliferation of these cells and the reconstruction of Langerhans. In addition, about 30 cyclotides of the aerial and root sections of V. Odorata were identified [30]. Several reports have indicated that the anti-diabetic property of medicinal plants is due to the presence of saponins [4]. Compounds containing saponins are hayperglismic antioxidants and increase insulin secretion [31]. The blue compounds of the VO reveal the existence of anthocyanin [32]. Anthocyanins are antibacterial, antihistamine, anti-allergic, anti-malarial, fat- and cholesterol-reducing, and anti-diabetic agents [4].

Empirical evidence has revealed that some effects of ethanol lead to the induction of metabolic processes, resulting in an increased production of ROS [33]. A study reported that ethanol and atrazine have toxic effects on rats' liver and kidney and cause severe destructive effects on catalase activity and superoxide dismutase [34]. According to these studies, due to the destructive effects of alcohol on the liver tissue, the hydro-alcoholic extracts, unlike the aqueous extracts were unable to have more effect on liver enzymes.

The obtained results suggested that the prescription of the aqueous extract of violet in 100 and 400 mg/kg doses can improve the liver tissue in terms of cell count, inflammation, and congestion. Moreover, they could significantly decrease AST and ALT enzymes. The 400 mg/kg dose of the aqueous extract and all used doses of the hydro-alcoholic extract significantly decreased serum glucose levels in the diabetic rats.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article.

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Authors' contributions

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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