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Original Article

Anti-diabetic Effects of Caucasian whortleberry and Ferula Extract on Blood Biochemical Factors, Liver and Kidney Histology in Streptozotocin-induced Diabetic Mice

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ABSTRACT

One of the biggest issues facing modern societies is diabetes, which is becoming more prevalent as a result of changes in daily routines. Numerous studies are currently being conducted to determine the impact of herbal medications on diabetes, given the negative side effects of chemical treatments used to treat the condition. The present study aimed to compare the antidiabetic effects of Caucasian whortleberry (*Vaccinium arctostaphylos* L.) methanolic extract and Ferula brewed extract (Ferula tea). To this end, 40 mice were given 200 mg/kg of streptozotocin intraperitoneally to induce diabetes in them. The mice were then divided into six groups, each consisting of ten mice: Group 1 included healthy mice that did not receive any treatment and was mentioned as the control; Group 2 was considered the diabetic control group and received a daily treatment of water; Group 3 and 4 received a daily treatment of the chemical drugs Acarbose or Metformin, which are regularly and separately used at a dose of 100 mg/kg of each drug. Group 5 received a daily treatment of 250 mg/kg of Caucasian whortleberry methanolic extract, and group 6 received a daily treatment of 300 mg/kg of Ferula tea. After the 40-day trial, the results showed that Caucasian whortleberry methanolic extract, Ferula tea, and Acarbose significantly reduced serum glucose levels compared to the control. In addition, Caucasian whortleberry methanolic extract caused a significant reduction in triglycerides and alkaline phosphatase.

Abbreviations: STZ: Streptozotocin, HDL: High-Density Lipoproteins, LDL: Low-Density Lipoproteins, SGOT: Serum Glutamic Oxaloacetic Transaminase, ALP: Alkaline Phosphatase, HbA1c: Hemoglobin A1c, SGPT: Serum Glutamic Pyruvic Transaminase, CGA: Chlorogenic Acid. FBS: Fast Blood Sugar.

INTRODUCTION

Diabetes mellitus is a metabolic disorder in which patients lose their ability to secrete insulin or the body becomes resistant to insulin. As a result, the secreted insulin fails to function normally. This disorder increases blood glucose levels and disrupts carbohydrate, fat, and protein metabolism [1]. Based on predictions and considering changes in lifestyle habits in human communities, the prevalence of diabetes is following an ascending trend [2]. In the long run, diabetes causes vascular and nonvascular complications. Vascular complications can be classified as microvascular (small blood vessels) complications such as retinopathy (eyes), neuropathy (nerves), nephropathy (kidneys), or macrovascular (large blood vessels) complications like peripheral vascular disease (PVD) and cerebrovascular disease. Nonvascular complications include gastroparesis, impotence, and skin changes [3]. There are different classifications for diabetes. In the most important classification, this disease is divided into type 1 diabetes and type 2 diabetes [4]. Type 1 diabetes is caused by viral infections or autoimmune disorders damaging the pancreatic beta cells [5], whereas obesity and insulin resistance are the main causes of type 2 diabetes [6]. Although insulin injection and administration of blood glucose-lowering drugs such as Metformin, Acarbose, Sitagliptin, and Liraglutide are the main treatments for diabetes. Metformin is another drug

that reduces blood glucose through the inhibition of gluconeogenesis, reduction of gluconeogenesis by the liver, and reduction of hepatic glucose excretion. Moreover, it reduces the absorption of glucose from the digestive system, increases subcutaneous adipose tissue, and decreases insulin resistance. By inhibiting the intestinal α-glucosidase, Acarbose prevents the breaking down of oligosaccharides into glucose and thereby hinders the absorption of intestinal carbohydrates. Therapeutic strategies for type 2 diabetes are usually based on reducing insulin demand, stimulating endogenous insulin secretion, improving insulin performance in the target tissue, and preventing the degradation of oligosaccharides and disaccharides.

The long-term complications of these drugs have created a need for drugs with fewer side effects. Furthermore, the use of herbal medicines and their derivatives has long been considered for treating diabetes and controlling its complications [7].

Caucasian whortleberry (*Vaccinium arctostaphylos* L.), with the scientific name *V. arctostaphylos L.*, a member of the family *Ericaceae*, grows in northern Iran in provinces like Gilan, Mazandaran, and Ardebil [8]. Caucasian whortleberry is rich in phenolic acid and various phenolic compounds [9] and exhibits useful effects like anticancer, antihypertensive, and antidiabetic ones [10]. It also contains phytochemical compounds such as flavonoids, anthocyanins, coumarin, and benzoic acid [11]. Its aerial organs reduce glucose and triglyceride levels in patients with type 2 diabetes [12].

Ferula is a genus of the herbaceous perennial family *Umbelliferae*. This genus has over 130 species around the world. 30 of which grow in Iran, so that half of these 30 species grow only in Iran and the other half also grow in Anatolia, Central Asia, and Afghanistan [13]. These plants have anti-seizure, emmenagogue, anti-dyspeptic, and laxative properties. They are also used to treat uterine diseases. Plants in the *Ferula* genus also show strong antibiotic, anticancer, and antiviral (anti-HIV virus) effects [14, 15]. Recent studies indicate that these plants can reduce the side effects of morphine [16] and that they also have antitumor [17] and antihypoglycemic [18] effects. Photochemical studies conducted on extracts prepared from the roots of these plants demonstrate that they contain coumarin and terpenoid compounds that exhibit anticancer and antioxidant properties [19]. Considering the beneficial effects of the mentioned plants, the present study aimed at evaluating and comparing their positive effects on biochemical parameters of blood serum and on liver and kidney histology in streptozotocin-induced diabetic mice.

MATERIALS AND METHODS

Extract Preparation

To prepare a methanolic extract of Caucasian whortleberry (*V. arctostaphylos* L.), 5 g of the plant powder (bought from a perfumery in Sabzevar County) was dissolved in 100 ml of the nonpolar solvent N-hexane at a ratio of 1:20 (w/v). The solution was incubated in a shaking incubator (VS-8980) at 180 rpm at 37 °C for 48 hours. The resulting extract was filtered using filter paper and condensed at 37 °C by a rotary evaporator (Heidolph2) at 100 rpm. It was then vacuum-dried under a chemical fume hood and stored as non-polar compounds. The remaining part of the plant, whose non-polar compounds were previously removed, was soaked in 100 ml of methanol in a shaking incubator for 48 hours under the same conditions. The supernatant was filtered, and the rotary evaporator removed the solvent. The resulting extract was dried and used. To prepare Ferula tea, 5 g of the powdered plant was added to 100 ml of distilled water at 80˚C. Then put it in a water bath at 50 °C for 6 hours. At the end, the supernatant was filtered and dried.

The used Mice and its Maintenance Conditions

In this study, 40 male mice weighting approximately 6-8 weeks old and 25 ± 2 g were obtained from Sabzevar University of Medical Sciences and kept in standard cages under standard lighting conditions at 22 ± 2 °C with enough food and water. All experiments were conducted according to the guidelines recommended by the American National Institutes of Health (NIH) for taking care of and using laboratory animals and in compliance with practical strategies in Iran [20].

Induction of Diabetes and Treatment

The selected male mice were used to create an animal model of diabetes. Diabetes was induced in the mice with a single-dose intraperitoneal injection of 200 mg/kg streptozotocin dissolved in sodium citrate (pH 4-4.5). Most researchers utilized acidic

(pH 4.0-4.5) citrate buffer solutions to make STZ for injection [21-23]. Symptoms of diabetes, including blood glucose levels above 250 mg/dl, polydipsia, and polyuria, were observed in the mice from 24 hours to one week after the injection. The groups were kept in the laboratory for 15-30 days in order to stabilize their diabetic conditions. Then they were randomly assigned to 5 groups of 10 and treated for 40 days. At the end of the treatment period, the mice were anesthetized with chloroform after 6 hours of fasting in order to take blood samples from the heart and to remove their liver and kidneys for histological studies.

The mice were randomly assigned to 5 groups of 10 as follows:

First group (control): This group included healthy mice that did not receive any treatment, and diabetes was not induced in them (10 mice).

Second group (diabetic control group): This group included streptozotocin-induced diabetic mice that only received water by gavage during the treatment period (10 mice).

Third and fourth group (experimental Acarbose or Metformin group): This group consisted of streptozotocin-induced diabetic mice treated with drugs at 100 mg/kg by oral gavage administration (10 mice).

Fifth group (experimental group of Caucasian whortleberry): This group included streptozotocin-induced diabetic mice intraperitoneally treated with Caucasian whortleberry methanolic extract at 250 mg/kg (10 mice).

Sixth group (experimental *Ferula* group): This group consisted of streptozotocin-induced diabetic mice treated with Ferula tea at 300 mg/kg by oral gavage (10 mice).

All mice were weighed before the experiment. Their blood glucose after 6 hours of fasting was normal (80-130). After the induction of diabetes, all mice had glucose levels above 250. The mice in different groups were treated over a 40-day period. The first group, the control, contained healthy mice who received no treatment. Moreover, diabetes was not induced in them. The second group, the diabetic control group, received water in the form of oral treatment by gavage needle. The third group received Acarbose and Metformin at 100 mg/kg by oral gavage. The fourth group was injected intraperitoneally with the Caucasian whortleberry extract at 250 mg/kg. The fifth group received Ferula tea at 300 mg/kg.

At the end of the treatment period, blood samples taken from the mice were poured into glass tubes and kept at room temperature for an hour. They were then centrifuged at 2000 rpm for 10 minutes, and the obtained serum was separated by a sampler and kept in a vial at -20 °C until the experiments were carried out. Glucose, SGOT, ALP, and A1C values were measured using kits manufactured by Pars Azmoon, and cholesterol, triglycerides, HDL, and LDL levels were measured using an enzymatic procedure (Bionik Co. Kit; Tehran, Iran). The immunoturbidimetry method was employed for the A1C test. A BT3500 instrument that was made in Italy performed all experiments.

Furthermore, tissue processing stages (dehydration, clearing, and infiltrating), embedding, sectioning, making slides, and, finally, staining using hematoxylin and eosin stain) were carried out on tissue samples fixed in 10% formalin.

Statistical Analysis

The data were statistically analyzed by SPSS. An analysis of variance (ANOVA) was used to determine significant differences between the mean values for the groups. In addition, Tukey's test (if the variances of two groups were equal) and Dunnett's test (if the variances of two groups were unequal) were used for pairwise comparisons. All data were reported as Mean ±SEM and the selected level of significance was $p \leq 0.05$.

RESULTS AND DISCUSSION

The present study aimed to investigate the effects of plant extracts on diabetes and their side effects and compare them with commonly used chemical drugs like Acarbose and Metformin. In traditional treatment, the pharmaceutical properties of plants depend on the environment in which they are grown, the type of solvent used, and the extraction method employed. Depending on its concentration and polarity, the solvent can affect the quantities of the chemicals found in herbal extracts.

Previous studies on compounds existing in Ferula have shown that the roots of these plants are rich in tannin, anthocyanins, and phenol [24]. In addition, investigations have indicated that quinic acid, which exhibited the best controlling results in this study, was the dominant compound in Caucasian whortleberry. Quinic acid is known as a precursor of

chlorogenic acid (CGA), which is an antioxidant that reduces glucose release in the bloodstream after food intake [25].

Since the main objective of this study was to determine the effects of plant extracts on type 2 diabetes, and taking previous research into consideration, different methods and doses of streptozotocin were used. Finally, it was found that a single dose of streptozotocin (200 mg/kg) was effective for the induction of diabetes in the mice. From 24 hours to one week after this injection, the fasting glucose levels in the mice exceeded 250. Induction of diabetes by streptozotocin takes place by generating reactive oxygen species. This drug enters pancreatic beta cells through the glucose transporter (GLUT2) and causes DNA alkylation. DNA damage caused by streptozotocin-induced methylation activates the regeneration process of poly-(ADP-ribose), which plays a principal role in the development of diabetes by streptozotocin. This enzyme discharges NAD^+ and ATP from the cells, which is followed by increased activity of xanthine oxidase. The increased activity of this enzyme leads to the production of free radicals that cause pancreatic tissue destruction and the development of diabetes.

After the preparation of diabetic mice and in the first part of the study, a fasting glucose test was performed using an on-call plus glucometer for 6 weeks based on taking micro samples from each mouse's tail. Fig .1A and B show the diagram of the linear means of blood glucose in different groups during the treatment period.

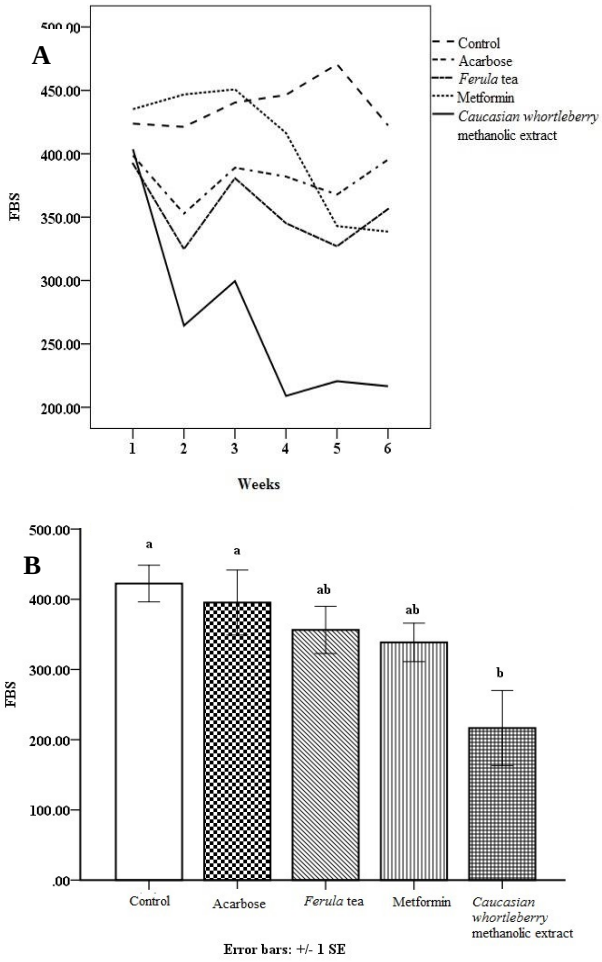

Fig. 1 A) Weekly control chart of FBS levels in studied groups. B) The comparison of the mean for FBS changes in mice groups on the last day of treatment.

Table 1 Mean and standard deviation of FBS on day 1 (A) and last day (B) of diabetic mice treatment.

Descriptives			
A	N	Mean	Std. Deviation
Control	5	423.8000	88.08348
Acarbose	5	398.6000	67.77020
Ferula tea	5	392.2000	21.47557
Metformin	5	435.2000	60.20548
Caucasian whortleberry methanolic extract	5	403.6000	74.19434

B	N	Mean	Std. Deviation
Control- Diabetic	5	422.4000	58.24345
Acarbose	5	395.4000	103.53405
Ferula tea	5	356.4000	75.09194
Metformin	5	338.6000	61.35389
Caucasian whortleberry methanolic extract	5	216.6000	119.59431
Control-Non diabetic	25	345.8800	107.46950

According to the results of this study, Table 2 shows the mean and standard deviation of the treatment groups.

Table 2 Mean and standard deviation of test results in experimental groups.

Group	Control		Acarbose		Ferula tea		*V. arctostaphylos L.*	
	Mean	std. Deviation	Mean	std. Deviation	Mean	std. Deviation	Mean	std. Deviation
FBS	871.8	37.56	437.75	145.42	494.9	69.23	412.13	101.25
TG	157.6	35.49	111.75	57.26	137.5	8.06	78.44	22.33
CHO	141.8	.24.93	137.25	16.05	136	18.06	95	12.15
HDL	71.4	5.63	90	13.52	97.3	19.38	66.5	5.63
LDL	16.2	3.49	22.75	2.87	19	5.41	16.57	2.7
SDOT	253.4	37.13	429.4	264.1	345.65	193.74	219.5	38.31
SGPT	135.6	26.1	203.83	38.8	206	76.43	89.83	42.07
ALP	356.8	60.84	381.75	53.75	265.71	32.7	149	28.04
A1C	9.38	1.18	9.28	0.99	8.32	0.74	7.46	1.13

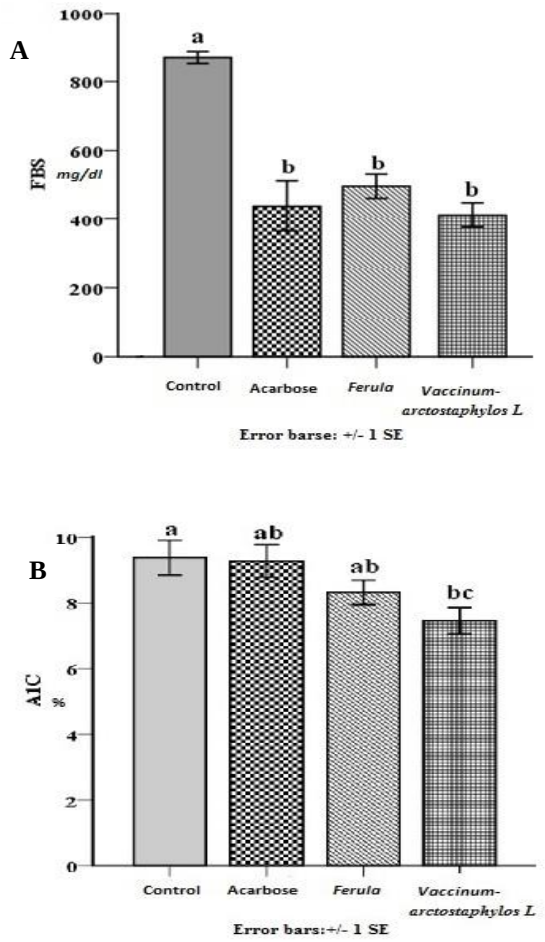

Fig. 2 A: FBS level in the diabetic control group and in the groups treated with Acarbose, Ferula tea, or Caucasian whortleberry methanolic extract; B: Diagram indicating A1C levels in the diabetic control group and the groups treated with Acarbose, Ferula tea, or Caucasian whortleberry methanolic extract. (a, b, c- there were significant differences between groups with different letters.

The best control was observed in mice treated with Caucasian whortleberry: FBS levels decreased from 403±74 on the first day of treatment (Table 1A) to 216±119 in the last week (Table 1B). Analysis of variance (ANOVA) and Tukey's test also demonstrated that there was a significant difference between Caucasian whortleberry treatment with Acarbose and control in the mean FBS on the last day of the trial. In this study, several biochemical factors were investigated.

After preparing blood sera, a study of the diagrams related to various biochemical factors showed that FBS, or fasting blood glucose, levels significantly decreased in all three groups treated with Acarbose and Metformin, Ferula tea, and Caucasian whortleberry methanolic extract compared to those of the control group (Fig. 2A). As the results showed, all three experimental groups showed a significant reduction in FBS ($P< 0.05$) compared to the control group.

However, only the group treated with Caucasian whortleberry methanolic extract exhibited a significant reduction in A1C ($P< 0.05$) compared to the control (Fig. 2B).

Furthermore, the reduction of triglyceride (TG) (Fig. 3A) and alkaline phosphatase (Fig. 4A) was also observed in this treated group. The Shapiro-Wilk test confirmed the normal distribution of the data ($P> 0.05$) and MANOVA showed that there were significant differences between the groups. Based on Levene's test, the equality of variance of the groups was confirmed with respect to the FBS, TG, LDL, ALP, and A1C variables but not for the other variables. Therefore, to determine the differences between which groups were significant, Tukey's test was used for variables for which variances in the groups were equal and Dunnett's test for the other variables.

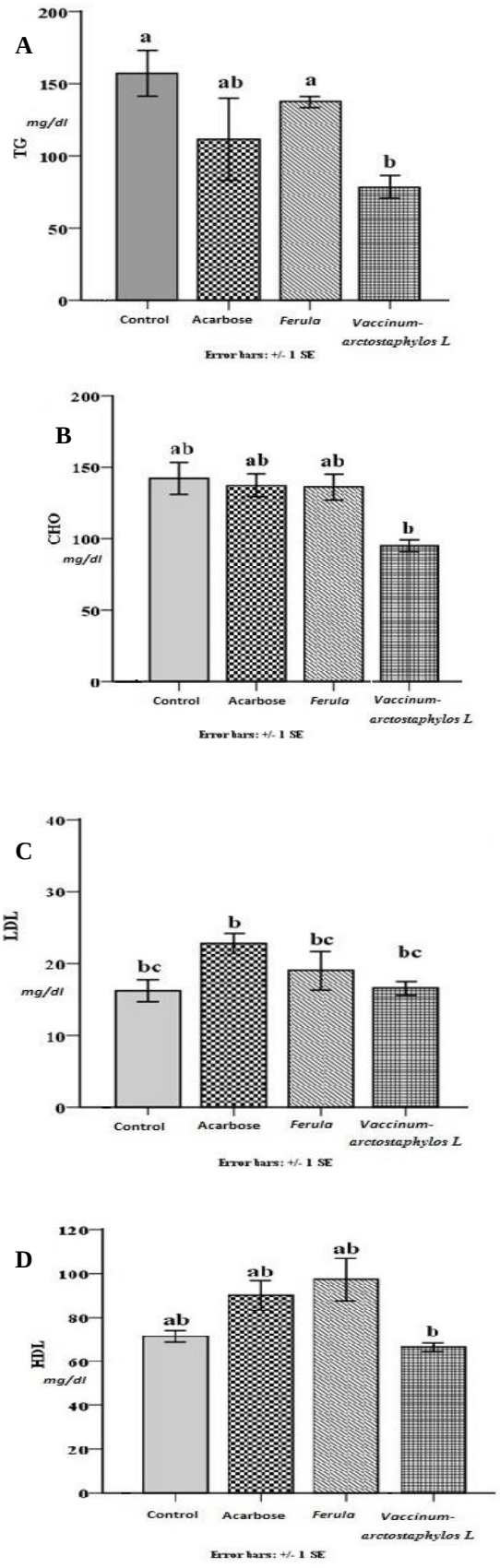

Fig. 3 Diagram showing TG levels (A), B: CHO levels (B), LDL levels (C), and HDL levels (D) in the diabetic control group and in the groups treated with Acarbose, Ferula tea and Caucasian whortleberry methanolic extract. (a, b, c- there were significant differences between groups with different letters).

As for the other measured biochemical parameters like cholesterol (Fig. 3B), LDL (Fig. 3C), aspartate aminotransferase (SGOT), and alanine aminotransferase (SGPT) (Fig. 4B and 4C, respectively), reductions were observed in the experimental groups, especially in the group treated with Caucasian whortleberry methanolic extract. These reductions were not significant ($P > 0.05$) compared to the control.

SGOT and SGPT levels are low in healthy mice, but any damage to liver cells or muscles causes the release of SGOT into the bloodstream. That is why the SGOT test is useful for diagnosing liver damage in uncontrolled diabetes. Uncontrolled diabetes and high blood glucose can lower HDL cholesterol levels. In this study, there was an increase in HDL (high-density lipoprotein) in all experimental groups, but the largest increase was observed in the group treated with Ferula tea. However, the increase in the group treated with Caucasian whortleberry extract was not significant (Fig. 3D). The measurement of ALP in the liver and bones is clinically important, so it is even used as a tumor marker in some cases. The data related to ALP in the present study indicated a reduction in groups treated with plant extracts compared to the control and the group treated with Acarbose. This reduction in the group treated with Caucasian whortleberry methanolic extract was significant compared to the control group and the group treated with Acarbose. CGA is one of the secondary phenolic metabolites produced by certain plant species and one of the most abundant polyphenol compounds in the human diet. Studies have shown that CGA has many biological properties, including antibacterial, antioxidant, and anti-cancer activities. The role and application of CGA in relation to glucose and lipid mechanisms have recently attracted the attention of researchers [26].

CGA also has potent antidiabetic effects [27]. Basilly *et al*. (2008) studied its effects on glucose content and glucose tolerance and reported that CGA significantly reduced blood glucose levels, which is very likely due to decreased absorption of glucose from the intestines [28].

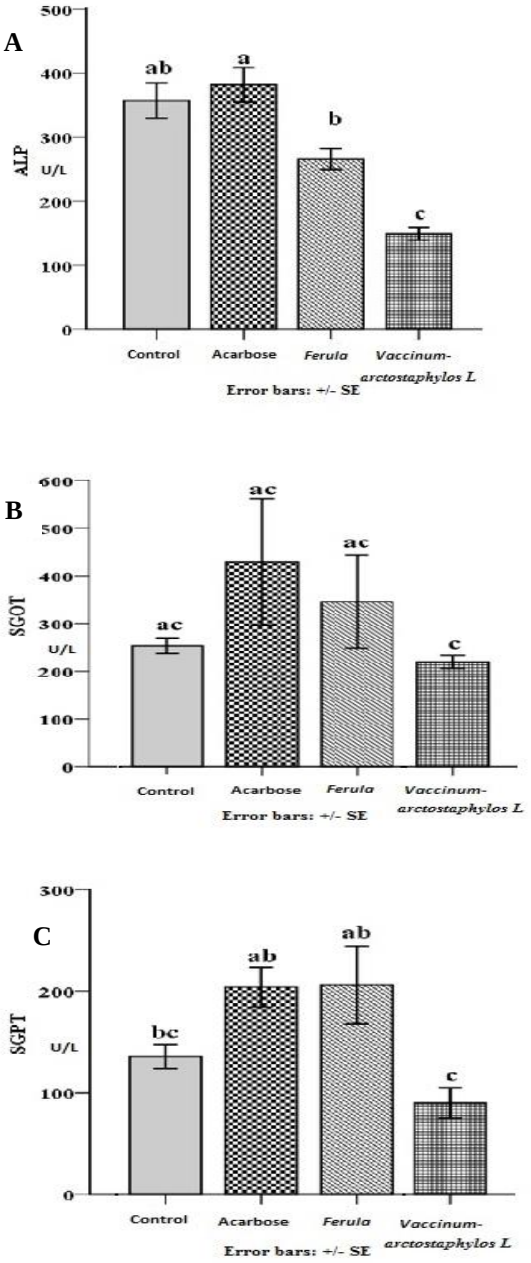

Fig. 4 A: Diagram showing ALP levels in the diabetic control group and in the groups treated with Acarbose, Ferula tea and Caucasian whortleberry methanolic extract, B: SGOT levels, and C: SGPT levels, (a, b, c- there were significant differences between groups with different letters).

This compound increases glucose absorption in muscle cells by stimulating insulin secretion. In addition, it has been shown that CGA is effective in the secretion of insulin from the islets of Langerhans in rats. Clinical observations indicated that CGA was able to intervene in glucose absorption and insulin secretion in humans [29]. As an effective factor in improving glucose tolerance and insulin resistance in mice, CGA is considered an antidiabetic compound. Moreover, it reduces blood lipid levels. Since CGA can reduce LDL oxidation in vitro, it is able to play a protective role against cardiovascular diseases. Inhibition of lipid absorption, activation of fat metabolism, and changes in lipids, lipoproteins, and enzymes associated with lipid metabolism are among the other functions of this compound. CGA reduces the concentrations of lipids (in plasma and in liver and kidney tissues), cholesterol, triglyceride, free fatty acids and phospholipids, and low-density lipoproteins (LDL and VLDL). It also increases the concentrations of high-density lipoproteins (HDL) in streptozotocin-induced diabetic mice. Lipid inhibition is triggered by inhibition of intestinal absorption and cholesterol biosynthesis in the liver [26]. Jian-hui *et al*. (2012) studied the effect that the improvement caused by CGA in antioxidant activity had on lipid metabolism in hyperlipidemic rats. Results demonstrated that serum levels of lipids significantly decreased, but the antioxidant level of the enzymes increased [30]. The presence of CGA in Caucasian whortleberry extract is the reason for its higher effectiveness in controlling diabetes compared to *Ferula* extract and Acarbose.

Histological studies showed that the complications of indirect hepatitis, including the reduced number of cells per unit area, cytoplasmic elongation and inflammation, interlobular inflammation, portal inflammation, necrosis, hyperemia, vacuolization, and picnozation of nuclei, were observed in the liver of diabetic mice. This is corroborated by the results of AST, ALT, and ALP tests. The liver is the main organ for detoxifying drugs in the body. Since the use of streptozotocin in the treatment of patients with pancreatic cancer only occasionally causes liver toxicity, changes in the liver tissue were caused by diabetes complications. Streptozotocin causes toxicity in the liver through the production of free radicals. This is followed by lipid peroxidation in the liver cell membrane. On the other hand, increased ROS by streptozotocin may lead to mitochondrial dysfunction in mice. Hypertrophy of hepatocytes is characterized by a dramatic increase in the number of mitochondria and a clear reduction in glycogen granules [31, 32].

As shown in Fig. 5, the greatest improvement in complications was observed in the groups treated with Caucasian whortleberry methanolic extract. The highest number of liver complications was found in the diabetic control group. They included reduced

number and swelling of hepatocytes, elongation and narrowing of sinuses, vacuolization, picnozation of nuclei, necrosis, hyperemia, portal inflammation, interlobular inflammation, increased cytoplasmic volume, etc. [33, 34].

In addition, reduced interlobular inflammation and decreased swelling of hepatocytes were clearly observed in the liver tissue of the group that was injected with Caucasian whortleberry at 250 mg/kg. However, there were no necrotic foci. The number of cells per unit area also increased compared to the diabetic tissue.

Diabetes increases the diameter of the glomeruli in the kidneys and disrupts blood filtration. Increased glomerular endothelial cells, a larger diameter of glomeruli and curved tubes, and vacuolized endothelial cells are other complications of diabetes in kidney tissues [35]. However, few complications were observed in the kidney tissue, including an increased number of glomerular and mesenchymal cells [36, 37]. Fig. 6 indicates that hepatic complications of diabetes decreased more significantly in the group receiving Caucasian whortleberry methanolic extract than in other groups.

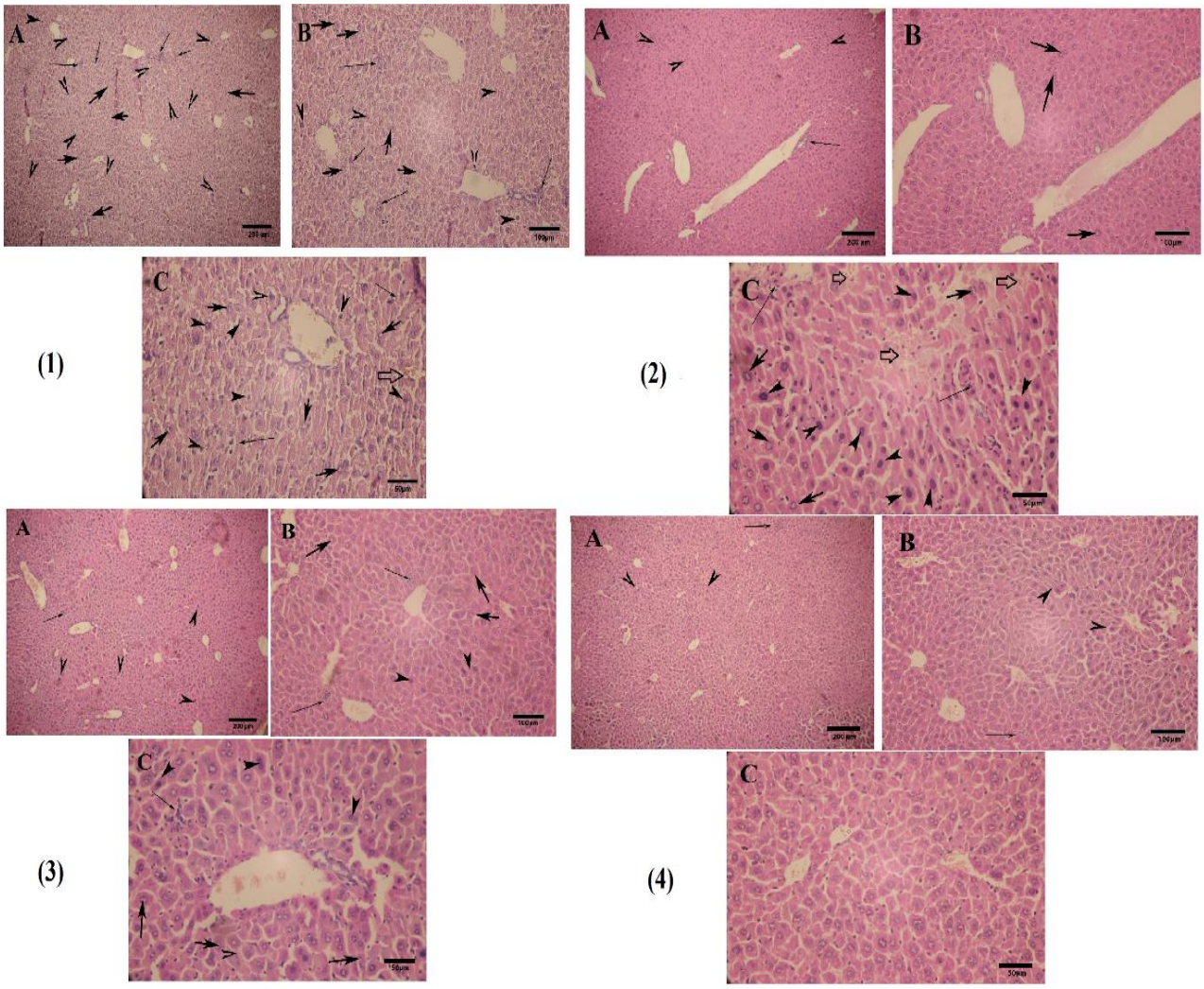

Fig. 5 Histological study of the liver in the different experimental groups: (1) A liver cross section of the diabetic sample at 10 X magnification (A), at 20 X magnification (B), at 40 X magnification (C). (2) A liver cross section of mice orally treated with Acarbose (100 mg/kg) at 10 X magnification (A), at 20 X magnification (B), at 40 X magnification (C). (3) A liver cross section of mice orally treated with Ferula tea (300 mg/kg) at 10 X magnification (A), at 20X magnification (B), at 40X magnification (C). (4) A liver cross section of mice treated with Caucasian whortleberry methanolic extract (250 mg/kg) at 10 magnifications (A), at 20 X magnification (B), at 40 X magnification (C). (Narrow arrow: necrosis, Wide arrow: nucleus vacuolization; hollow arrow: hyperemia; arrow point: picnozation of nuclei).

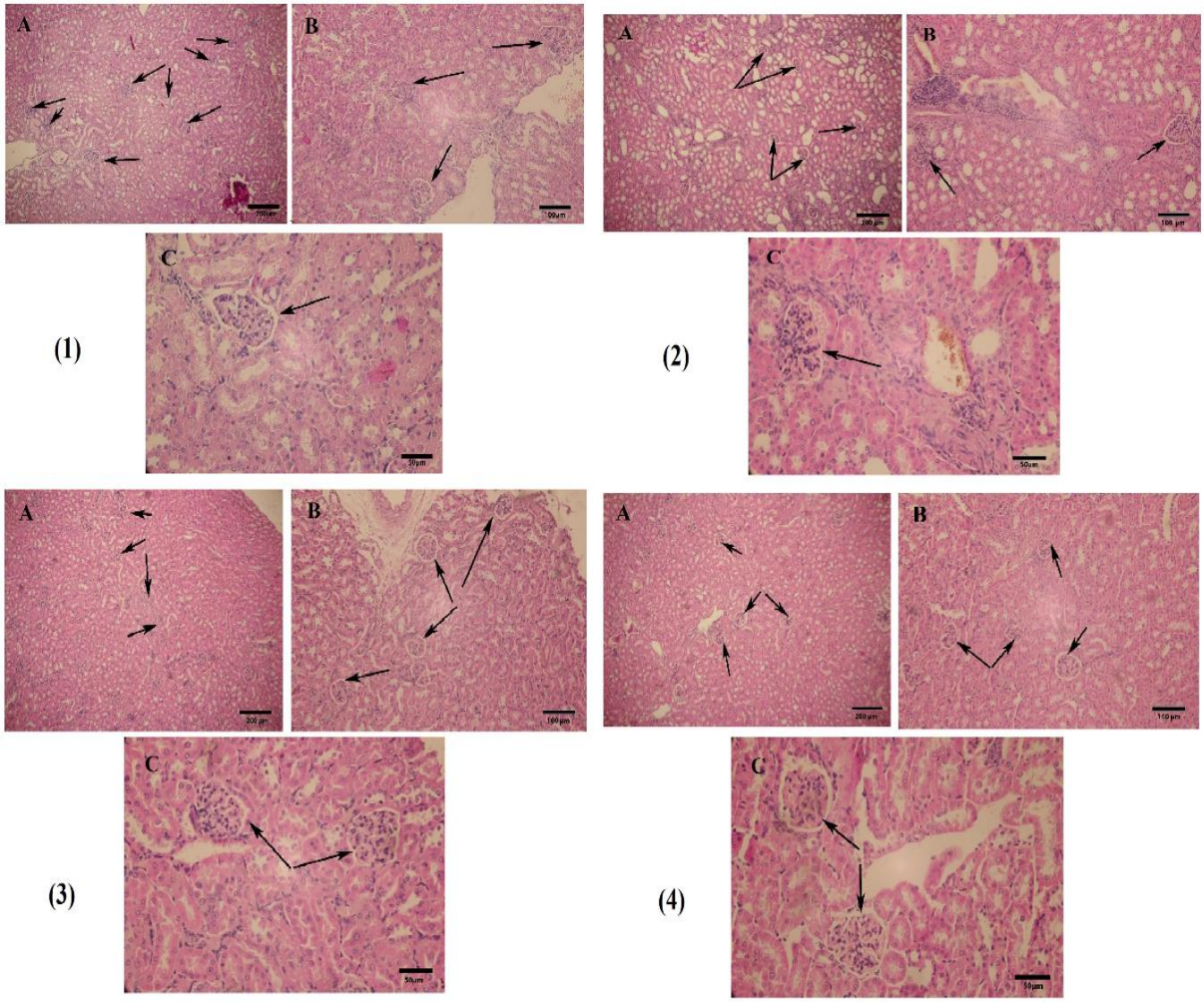

Fig. 6 Histological study of the kidney in different experimental groups (1) A kidney cross section of the diabetic sample at 10X magnification (A), at 20 X magnification (B), at 40 X magnification (C) (2) A kidney cross section of mice orally treated with Acarbose (100 mg/kg) at 10 X magnification (A), at 20X magnification (B), at 40X magnification (C) (3) A kidney cross section of mice orally treated with Ferula tea (300 mg/kg) at 10X magnification (A), at 20X magnification (B), at 40X magnification (C) (4) A kidney cross section of mice treated with Caucasian whortleberry methanolic extract (250 mg/kg) at 10X magnification (A), at 20 X magnification (B), at 40 X magnification (C). (Arrow: glomerulus).

The study findings generally suggest that Caucasian whortleberry methanolic extract has the best control over diabetes complications. Therefore, more studies are needed to find drugs that are more effective in combination with the composition of this plant for diabetic patients.

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