

Effect of Crataegus Azarolus on blood glucose, lipid profile and antioxidant status in streptozotocin diabetic rats fed zinc deficient diet

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Research Article

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Abstract

Objective

zinc plays an important role in insulin's biosynthesis and storage. Consequently, its deficiency may have a deleterious impact on the progression of diabetes and associated consequences. Thus, this study was conducted to investigate the effect of Hawthorn "*Crataegus Azarolus*" on blood biochemical parameters, tissue zinc status, and oxidative stress biomarkers in streptozotocin diabetic rats fed zinc insufficient diet.

Methods

Thirty-two males albino Wistar rats were divided into 4 groups: 2 groups were fed zinc-sufficient diet (One non-diabetic and the other diabetic), while the others 2 groups of diabetic rats were fed zinc insufficient diet. One non-treated group and the other treated with the extract of *Crataegus Azarolus* (150mg/kg Body weight). Body weight and food intake were recorded regularly. After 4 weeks of dietary manipulation, fasting animals were scarified

Results

zinc deficiency feed decreased body-weight, insulin, zinc tissues (femur, liver, kidney, and pancreas), glutathione concentrations, lactic dehydrogenase, catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase activities. It was also noticed that inadequate dietary zinc intake increased concentrations of glucose, cholesterol, triglycerides, urea, uric acid, creatinine, lipid peroxidation levels, and transaminases activities. However, oral administration of hawthorn extract ameliorated all the previous parameters approximately to their normal levels.

Conclusion

the present study showed that *Crataegus Azarolus* supplementation presumably acting as an antioxidant, and it can be a natural source for the reduction of diabetes development caused by zinc deficiency.

Introduction

Diabetes mellitus (DM) is a complex group of chronic diseases with different etiology, in which hyperglycemia is their common criteria, triggering defects in insulin secretion and/or its mechanism of action causing disorders in distinct organs and systems [1]. Around the world, 537 million grown-ups (20–79 years) are living with diabetes in 2021. The assessment is predicted to ascend to in excess of 643 million by 2030 and 784 million by 2045 [2]. Type 2 diabetes mellitus (T2DM) is the most pervasive type of diabetes mellitus, representing over 90% of all the morbidity and mortality [3]. Diabetes disease

is associated with deficiencies in insulin signaling transduction pathway components [4]. In diabetes, the presence of constantly raised glucose level contributes mostly to the process of overproduction of reactive oxygen species (ROS) through various mechanisms, especially glucose auto-oxidation, the activation of polyol, hexamine pathways, protein kinase C and height speed of advanced glycation end products (AGEs), which consequently adds to the increment of oxidative stress [5]. Assuming that ROS production isn't adjusted by the cellular antioxidant defenses, it may lead to oxidative harms (lipid peroxidation, protein degradation, disruption of DNA) and possibly cell death [6]. Oxidative stress might take place when antioxidant mechanisms are not functioning correctly as in dietary deficiencies of vitamins such as vitamin C and vitamin E or the essential trace elements like zinc, selenium and manganese [7]. Zinc performs its numerous biological functions in various aspects. First, it is indispensable by more than 300 enzymes for their catalytic activation, thus participating in several enzymatic and metabolic cellular processes in human body. Secondly, zinc ties to more than 2500 proteins, equivalent to 10% of total human proteome and keeps up with the structural integrity for many of them [8]. The trace element assumes a crucial role in the stabilization of insulin hexamer and insulin stockpiling in the pancreas and expands insulin condensation. Zinc actually favors phosphorylation of insulin receptors by improving transport of glucose into cells. Nevertheless, zinc deficiency increases the destruction of the islet cell in type 1 diabetes mellitus (T1DM) through the cytokine autoimmune attack [9]. The element plays a significant role in antioxidant defense as a cofactor of the superoxide dismutase enzyme, by modulating the glutathione metabolism and metallothionein expression, competing with iron and copper in the cell membrane and inhibiting nicotinamide adenine dinucleotide phosphate-oxidase enzyme [10]. Therefore, the insufficient intake reduced absorption and expanded loss of zinc result in zinc deficiency. Recent studies show that most *Crataegus* species have beneficial effects on human health. Extracts from aerial parts presented several biological activities such as anti-inflammatory [11], anti-hyperglycemic [12], vasorelaxing [13] and hypolipidemic properties [14]. In addition, researchers have referenced that leaves of *Crataegus azarolus* are rich in phenolic compounds and presented substantial antioxidant and antimicrobial activities [15]. It has also been demonstrated that extracts from the fruits and leaves of *Crataegus* are safe for human consumption [16]. Thus, the present investigation was carried out to examine the modulator effects of *Crataegus azarolus* administration for the prevention of diabetes pathology development by evaluating body weight gain, zinc status, carbohydrate metabolism and the antioxidant system in rats fed zinc deficiency diet.

Materials And Methods

Animals

Male albinos Wistar rats (weighing around 200 to 250 g, 10 to 12 weeks of age) were obtained from Pasteur Institute (Algiers, Algeria). Prior to experiments, the animals were allowed to acclimate to their surroundings for 2 weeks. Rats were housed in individual plastic cages with bedding. Standard food and tap water were available *ad-libitum* for the duration of the experiments unless otherwise noted. The temperature was around 22°C ± 2°C. A 12/12-hour light/dark cycle was maintained, with lights on at 6

am. The Institutional Animal Ethical Committee of Badji Mokhtar Annaba University (PNR-ANDRS 8/u23/332), approved the study protocol.

Preparation of extract

Crataegus azarolus aerial parts were collected in September 2020 from the commune of Ain-Berda, Region of Annaba (East of Algeria) and were authenticated by Dr. Hamel Tarek, Department of Biology, Faculty of Sciences, Badji Mokhtar University, Annaba, Algeria. The samples were washed several times, dried in a ventilated place at room temperature. Then, dried leaves were ground using a domestic blender until a very fine powder was obtained. The powders obtained were kept at room temperature in airtight containers away from light until the start of the experiment.

Qualitative phytochemical screening

The crude extract water of the plant were subjected to qualitative chemical screening for the identification of the various classes of active chemical constituent such as : alkaloids, flavonoids, tannins and saponins using the method described by Trease and Evans [17].

Detection of alkaloids

5 grams of powder are mixed with 50 ml of 1% HCl in a container. After maceration and filtration, a few drops of Mayer's reagent were added: the appearance of a white precipitate indicates the alkaloids.

Detection of flavonoids

10 g of powder are macerated in 150ml of 1% HCl for 24 hours, after filtering the mixture, the filtrate is made basic by adding a few drops of NH_4OH , after 3 hours; the appearance of a pale-yellow color in the upper part of the tube indicates the presence of flavonoids.

Detection of tannins

1 ml of water extract was mixed with 10mL of distilled water and filtered. Ferric chloride (FeCl_3) reagent (3 drops) was added to the filtrate. A blue-black or green precipitate confirms the presence of gallic tannins or catechol tannins respectively.

Detection of saponins

5 milliliter of water extract was vigorously shaken with 10 mL of distilled water for 2min. The appearance of stable foam that persists for at least 15 min, was taken as an indication of the presence of saponins.

Detection of anthocyanins

The detection of anthocyanins is based on the change in color of 10% extract with change in pH. A few drops of HCl were added to the extract, after observing the color change, a few drops of NH_4OH were added. A positive test is revealed by a pink-red coloration, which turns to purplish blue.

Quantitative phytochemical screening

Total Polyphenols content determination

An aliquot of 100 µl of an extract was mixed with 2.5 ml of Folin–Ciocalteu phenol reagent (10x dilution) and allowed to react for 5 min. Then, 2.5 ml of saturated Na_2CO_3 solution was added and allowed to stand for 30 min before the absorbance of the reaction mixture was read at 725 nm. The Total polyphenols concentration was calculated via the equation: $Y = 0.0073x - 0.3165$ ($R^2 = 0.994$) and expressed as milligram of gallic acid equivalent (GAE) per gram of dry weight (mg GAE/g DW) [18].

Determination of total flavonoids content

The flavonoids content was determined by aluminium trichloride method using catechin as reference compound. A volume of 125 µL of extract was added to 75 µL of a 5% NaNO_2 solution. The mixture was allowed to stand for 6 min. Then, 150 µL of aluminium trichloride (10%) was added and incubated for 5 min, followed by the addition of 750 µL of NaOH (1M). The final volume of the solution was adjusted to 2500 µL with distilled water. After 15 min of incubation, the mixture turned to pink and the absorbance was measured at 510 nm. The Total flavonoid concentration was calculated via the equation $Y = 0.0029x + 0.131$ ($R^2 = 0.994$), and expressed as milligram of quercetin equivalent (QE) per gram of dry weight (mg QE/g DW) [19].

Determination of total tannins content

0.1–0.5 mL of crude extract were taken and put into tubes covered with aluminium foil. 3 mL of 4% vanillin (w/v) in methanol was added, and the tubes were shaken vigorously with a mixer. Immediately 1.5 mL of concentrated HCl was pipetted and the tubes were shaken again. The absorbance was estimated at 500 nm against blank after being allowed to stand for 20 min at room temperature. The total tannins content was calculated via the equation

$Y = 0.0018x + 0.0737$ ($R^2 = 0.994$) and expressed as milligram of catechin equivalent (CE) per gram of dry weight (mg CE/g DW) [20].

Determination of DPPH radical scavenging activity

The DPPH radical-scavenging activity was determined using the method described by Brand-Williams *et al* [21]. A DPPH solution ($0.070 \text{ mg} \cdot \text{mL}^{-1}$) was mixed with sample solutions at different concentrations (25 to $100 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$). A control (Abs Control) containing methanol and DPPH solution was also realized. All solutions obtained were then incubated for 30 min at room temperature and absorbance was measured at 517 nm.

The standard used is: Quercetin (10 to $100 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$) and the radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 517 nm. When the reading was complete, the percentage of inhibition of samples was calculated from obtained absorbance by the equation:

% inhibition = $[(\text{Abs Control} - \text{Abs test}) / \text{Abs Control}] \times 100$

Then, curves were constructed by plotting percentage of inhibition against concentration in $\mu\text{g/mL}$. The equation of this curve allowed to calculate the half maximal inhibitory concentration (IC_{50}) corresponding to the sample concentration that reduced the initial DPPH[•] absorbance of 50%. A smaller IC_{50} value corresponds to a higher antioxidant activity. All test analyses were realized in triplicate.

Induction of experimental diabetes

Diabetes was induced by a fresh streptozotocine solution, which was intraperitoneally administered at a dose of 50 mg/kg body weight after being dissolved in citrate buffer (0,1 M, pH 4.5). A 10% glucose solution was given overnight to the Streptozotocin-treated animals to prevent STZ-induced hypoglycemia. One week later, the diabetic state was confirmed by estimating blood glucose levels from the tail vein using a glucose meter (*VitalCheck@MM1200*); only rats with glucose level over 14 mmol/L were considered diabetics animals.

Diet preparation

Basal diet of animals was prepared as described by Southon et al/[22] and it consisted (in g/kg diet) of: cornstarch 326, sucrose 326, protein 168 (Soja), lipids 80 (corn oil), fiber 40 (cellulose), vitamin mix (sigma) and mineral mix 40. The latter was formulated to contain either adequate (54 mg/kg) or inadequate (1.2 mg/kg) quantities of zinc, as determined by atomic absorption spectroscopy. The mineral mix was supplied (in g/Kg of diet) by calcium hydrogen orthophosphate, 13; disodium hydrogen orthophosphate, 7.4; calcium carbonate, 8.2; potassium chloride, 7.03; magnesium sulphate, 4; ferrous sulphate, 0.144; copper sulphate, 0.023; potassium iodide, 0.001; manganese sulphate, 0.180; and zinc carbonate, 0.1. The zinc-deficient diet contained no additional zinc carbonate.

Experimental design

After stabilization of diabetes, rats were divided into 4 groups (8 each). The first and second groups were non-diabetic (ND) and diabetic (DAZ) fed sufficient zinc diet containing 54 mg Zn/kg diet. The third and fourth groups were diabetics and received deficient zinc diet containing 1.2 mg Zn/kg diet. One was an untreated group (DZD), and the other was treated orally with aqueous extract of *C. Azarolus* at doses of 150 mg/kg (DZD + Az). The treatment of animals was carried out for 4 weeks.

Blood and tissue samples collection

Animals were killed by cervical decapitation. The collected blood samples were centrifuged at 3000 rpm and serum stored at -20°C until biochemical analysis. Pancreas, liver and kidney were excised and washed with ice-cold isotonic NaCl saline, and blotted to dry. The right femur was taken and the connective tissues and muscle were removed. Liver fragment, pancreas fragment, right kidney and femur were weighed and dried at 80°C for 16 h and then zinc concentrations were determined. The other fragment of liver and the left kidney was immediately processed for assaying malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and

glutathione peroxidase (GSH-Px). The second fragment of pancreas was utilized for the histological study.

Measurement of biochemical parameters

The biochemical parameters were assayed using a biochemistry analyzer system (Erba Mannheim XL600) and the principle of each test was determined according to the ERBA XL-600 data sheet.

Tissues zinc analyses

The dried kidneys, pancreas, livers and femurs were heated in silica crucibles at 480°C for 48 hours, and the ash was dissolved in hot 12M (HCl) acid for zinc analysis utilizing a flame atomic absorption spectrophotometer (Perkin-Elmer Atomic Absorption Spectrometer AAnalyst 400). Standard reference materials: bovine liver and wheat flour were used to check the accuracy of zinc recovery, which exceeded 96% in the reference materials. Zinc standards were prepared from 1 mg/mL zinc nitrate standard solution. All tubes were soaked in HCl (10% v/v) for 16 hours and rinsed with doubly distilled water to avoid zinc contamination from exogenous sources.

Measurement of stress oxidative parameters

Tissue preparation

About 1 g of liver and kidney was homogenized in 2 mL ice-cold TBS (50 mM Tris, 150 mM NaCl; pH 7.4). Then the homogenates were centrifuged at 9000×g for 15 minutes at 4°C, and the resultant supernatant was used for the determination of proteins, MDA, GSH, SOD, CAT, GSH-Px and GR.

Lipid peroxidation estimation

The lipid peroxidation level was measured as malondialdehyde (MDA), which is the end product of lipid peroxidation, and reacts with Thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) to produce a red colored complex with a peak absorbance at 532 nm according to Buege and Aust[23].

Estimation of reduced glutathione

The reduced glutathione was estimated utilizing the colorimetric technique of Jollow *et al* [24] based on the development of a yellow color when Ellman reagent (5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added to compounds containing sulfhydryl groups. The GSH concentration (nmol GSH/mg protein) was measured at 412 nm.

Assay of superoxide dismutase activity

The specific activity of superoxide dismutase (SOD) was determined according to the method described by Misra and Fridonich[25]. 10 µL of tissue homogenate were added to 970 µL of ethylene diamine tetra-acetic acid (EDTA) - Sodium carbonate buffer (0.05 M) at pH10.2. The reaction was started by adding 20 µL of epinephrine (30 mM) and the activity was estimated at 480 nm for 4 min. A unit of SOD is defined

as the amount of enzyme that inhibits by 50% the speed of oxidation of epinephrine and the results were expressed as UI/mg protein.

Assay of glutathione peroxidase activity

Determination of GSH-Px activity was carried out according to the method of Flohe and Gunzler [26]. This method was based on the reduction of hydrogen peroxide in the presence of reduced glutathione (GSH), the latter is transformed into (GSSG) under the influence of GSH-Px and the reading absorbance was measured at 412 nm. The enzyme activity was expressed as μ moles of reduced GSH/min/mg protein.

Assay of glutathione reductase activity

The estimation of glutathione reductase (GR) activity was based on the method of Goldberg and Spooner [27]. The enzymatic activity was assayed photometrically by measuring, NADPH consumption. In the presence of oxidized glutathione (GSSG) and NADPH, GR reduces GSSG and oxidizes NADPH, resulting in a decrease of absorbance at 340 nm.

Assay of catalase activity

The principally common method for measuring catalase activity is the UV spectrophotometric method, which depends on monitoring the change of 240 nm absorbance at high levels of hydrogen peroxide solution (≥ 30 mM). High levels of hydrogen peroxide (H_2O_2) immediately lead to inhibition of the catalase enzyme by altering its active site structure, although there is variation in the extent to which this occurs. The activity was measured at 240 nm every 15 s for 1 min according to the method of Aebi[28].

Oral glucose tolerance test (OGTT)

Oral glucose tolerance tests were carried out according to Bonner-Weir method [29]. Briefly, animals divided into 4 groups (6 each), group 1 served as control. Groups 2, 3 and 4 received aqueous extracts of *C. Azarolus* at an oral dose of 100, 150 and 200 mg/kg respectively. Subsequently, OGTT was carried out after 14 days of treatment, during which the animals were fed with standard diet. On completion of 14 days of treatment, rats were fasted overnight and blood was withdrawn from tail-vein just prior to glucose administration (2g/kg) and at 30, 60, 90 and 120 min of glucose loading. Blood glucose level was measured immediately by using vital-check glucometer.

Histological study

Pancreas obtained by dissection was washed with isotonic saline (0.9%). Then, it was immediately fixed in Bouin solution for 24 hours, processed by using a graded ethanol series and embedded in paraffin. The paraffin sections were cut into 5 μ m slices and stained with hematoxylin-eosin. All pictures were taken using optic microscopy; the magnification was 100 $\times$.

Statistical analysis

The data were expressed as mean $\pm$ SEM. All data were analyzed by one-way analysis of variance (ANOVA) followed by "Tukey's multiple comparison test" using the Graph-Pad Prism software (Version

8.0.1). Differences were considered statically significant at $p < 0.05$.

Results

Phytochemical screening

The phytochemical analysis of *C. Azarolus* aqueous extract indicated are indicated in Table 1.

Table 1
Represent the phytochemical screening test result

Extract	Flavonoids	Tannins	Chlorophylls	Anthocyanins	Saponins	Alkaloids
<i>Crataegus Azarolus</i>	+++	+++	+++	-	+	++
+++ : High, ++ : Moderately, + : Trace, - = Absent or negligible						

Quantities determination of total phenolic, flavonoid and tannin contents

The quantitative measurement showed that *C. Azarolus* contains polyphenols (15.53 mg GAE/g DW), total flavonoids (23.14 mg QE/g DW), and total tannin contents (1.29 mg CE/g extract) (Table 2).

Table 2
Contents of total polyphenol (expressed as mg gallic acid equivalents/g dry weight), flavonoids (expressed as mg quercetin equivalents/g dry weight), and condensed tannin (expressed as mg catechin/g dry weight) and antioxidant activity determined by DPPH in *Crataegus Azarolus* aqueous extracts.

Extract	Total polyphenols	Total flavonoids	Total Tannins	DPPH assay	
	(mg GAE/g DW)	(mg QE/g DW)	(mg CE/g DW)	IC ₅₀ (µg/mL)	% Inhibition
<i>Crataegus Azarolus</i>	15,53 ± 1,37	23,14 ± 4,74	1,29 ± 0,60	14,79 ± 2,71	83,7
Values are means of triplicates ± SEM					
GAE: gallic acid equivalents, QE: quercetin equivalents, CE: catechin equivalents.					

Body weight gain and food Intake

Diabetes caused very significant reduction ($p < 0,001$) of body weights and high food intake ($p < 0.01$). Meanwhile, zinc deficiency slightly affected body weight of diabetic rats. However, treatment with *C. Azarolus* restored the growth rate and food consumption of zinc deficient diabetic rats (DZD) (Table 3).

Table 3

Initial body weight, final body weight, food intake and tissues zinc concentration of non-diabetic rats (ND), diabetic adequate zinc rats (DAZ), diabetic zinc deficient rats (DZD), and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD + Az).

Parameters	Experimental groups			
	ND	DAZ	DZD	DZD + Az
Initial body weight (g)	238,8 ± 2,54	249 ± 6,53	249,6 ± 5,27	239,4 ± 4,96
Final body weight (g)	244,4 ± 2,61	184,4 ± 4,94 ^{***}	174,8 ± 6,15	210,4 ± 12,35 ⁺
Food intake (g/day/rat)	25,8 ± 3,60	27,4 ± 3,89 ^{**}	23,15 ± 4,00 ^{###}	26,86 ± 5,65 ⁺⁺⁺
Femur (µg/g dry weight)	138,57 ± 3,17	98,98 ± 14,88 [*]	93,58 ± 9,61	128,31 ± 8,64 ⁺
Liver (µg/g dry weight)	42,26 ± 1,92	29,33 ± 1,70 ^{***}	25,77 ± 1,26 [#]	34,58 ± 3,19 ⁺
Pancreas (µg/g dry weight)	110,28 ± 34,07	78,23 ± 11,68	46,71 ± 9,44 [#]	85,55 ± 15,68 ⁺
Kidney (µg/g dry weight)	119,17 ± 4,36	101,92 ± 23,79	44,7 ± 24,64 [#]	123,76 ± 13,76 ⁺⁺
Values are mean ± SEM; number of samples = 8.				
[*] p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001: DAZ vs ND.				
[#] p < 0.05, ^{###} p < 0.001: DZD vs DAZ.				
⁺ p < 0.01, ⁺⁺⁺ p < 0.001: DZD + Az vs DZD.				

Tissues zinc concentrations

Zinc concentrations in femur, liver, kidney and pancreas are also illustrated in Table 3. The zinc levels in femur and liver of diabetic rats (DAZ) were significantly ($p < 0.05$, $p < 0.001$) lower than that of non-diabetic rats (ND). On the other hand, diabetic rats gave zinc deficiency feed, have significant ($p < 0.01$) low zinc concentration in liver, pancreas and kidney. However, the treatment with *C. Azarolus* improved zinc levels in the previous tissues.

Blood biochemical values

The results are illustrated in Table 4. As expected, diabetes state affected most the biochemical parameters. Therefore, the results showed the following blood parameters were significantly raised in the diabetic group compared to the non-diabetic group including glucose ($p < 0.001$), cholesterol ($p < 0.01$), triglyceride ($p < 0.001$), urea ($p < 0.01$), uric acid ($p < 0.001$) and creatinine ($p < 0.05$), aspartate aminotransferase ($p < 0.05$) and alanine aminotransferase ($p < 0.01$). The diabetes state provoked also a significant decrease of insulin ($p < 0.01$) and lactic dehydrogenase ($p < 0.01$). Simultaneously, it was observed that zinc deficiency resulted an increase of cholesterol ($p < 0.05$), GOT ($p < 0.05$), urea ($p < 0.01$) and creatinine ($p < 0.05$). Whereas, *C. Azarolus* administration restored the previous parameters.

Table 4

Mean blood glucose, serum insulin, serum triglycerides, serum cholesterol, serum uric acid, serum urea, serum creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and lactate dehydrogenase concentrations of non-diabetic rats (ND), diabetic adequate zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD + Az).

Parameters	Experimental groups			
	ND	DAZ	DZD	DZD + Az
Glucose (g/L)	1,132 ± 0,06	3,674 ± 0,34 ^{***}	3,316 ± 0,35	1,31 ± 0,13 ⁺⁺⁺
Insulin (µU/mL)	2,84 ± 0,71	0,5 ± 0,25 ^{**}	0,29 ± 0,10	2,20 ± 0,20 ⁺⁺⁺
Triglyceride (g/L)	0,96 ± 0,12	1,844 ± 0,15 ^{***}	1,828 ± 0,29	1,156 ± 0,07 ⁺
Cholesterol (g/L)	0,65 ± 0,15	1,86 ± 0,36 ^{**}	1,05 ± 0,13 [#]	0,79 ± 0,14
Urea (g/L)	0,27 ± 0,13	0,38 ± 0,06 ^{**}	0,48 ± 0,05 ^{##}	0,20 ± 0,02 ⁺
Uric acid (mg/dL)	1,20 ± 0,20	2,50 ± 0,22 ^{***}	3,00 ± 0,45	1,60 ± 0,24 ⁺⁺
Creatinine (mg/L)	4,80 ± 0,97	9,40 ± 0,24 [*]	11,12 ± 0,81 [#]	5,60 ± 0,51 ⁺⁺⁺
GOT (IU/L)	150,20 ± 4,75	174,20 ± 9,59 [*]	197,00 ± 2,17 [#]	149,60 ± 6,71 ⁺⁺⁺
GPT (IU/L)	72,80 ± 8,80	108,60 ± 6,34 ^{**}	104,60 ± 6,64	76,80 ± 4,53 ⁺⁺
LDH (IU/L)	1040,25 ± 126,82	549,58 ± 104,41 ^{**}	440,55 ± 73,13	888,77 ± 81,01 ⁺⁺
Values are mean ± SEM; number of samples = 8.				
*p < 0.05, **p < 0.01, ***p < 0.001: DAZ vs ND.				
#p < 0.05, ##p < 0.01: DZD vs DAZ.				
+p < 0.01, ++p < 0.01, +++p < 0.001: DZD + Az vs DZD.				

Oxidative stress parameters

As shown in Figs. 1, 2, 3, 4, 5 and 6, diabetes caused a rise in MDA level ($p < 0.01$; $p < 0.001$), with a decrease in GSH content ($p < 0.05$; $p < 0.01$), GSH-Px ($p < 0.001$), CAT ($p < 0.01$), SOD ($p < 0.01$; $p < 0.001$) and GR ($p < 0.05$) activities in liver and kidney respectively. Meanwhile, it was observed that zinc deficiency led a slight augmentation of liver and renal MDA with a decrease of GSH, GSH-Px, SOD, GR and liver catalase. However, *C. Azarolus* administration resulted an improvement the above-mentioned oxidative stress parameter.

Oral glucose tolerance test (OGTT)

OGTT was used to determine the anti-hyperglycemic effect of *C. Azarolus* extract. As presented in Fig. 7, glucose administration provoked a noticeable rise of blood glucose at 30 min, and 60 min after glucose loading in all studied experiment groups, but at 90 and 120 min, there was a decline in blood glucose concentration in the experimental treated groups as compared to the control group.

Pancreatic histopathologic results

The histology of the pancreas (Fig. 8) revealed that non-diabetic rats had an intact pancreatic islet. Whereas, both the diabetic adequate zinc rats (DAZ) and diabetic zinc-deficient rats (DZD) have depleted islet cells. Moreover, diabetic zinc deficient rats treated with aqueous extract of *C. Azarolus* (DZD + Az) showed preserved pancreatic islet cells.

Discussion

Oxidative stress plays an important role in the development of DM and it is not only responsible for the destroys islet β cells and insulin signaling pathways, but also may contribute to serious complications such as cardiovascular and nephropathy [30]. Recent studies showed that some medicinal plants are regarded as good sources for traditional medicines and from these plants many of the modern medicines are produced [31]. Nevertheless, the utilization of plants in medicine is basically based on its biologically active compounds, which have numerous therapeutic properties including antioxidant, antidiabetic, anti-inflammatory, and anti-hypercholesterolemic [32, 33]. Therefore, different studies have been devoted to assess the secrets of plants. Thus, the current study was focused on evaluating the potential antidiabetic and antioxidant effects of the aerial aqueous extract part of *C. Azarolus* on diabetes under nutritional zinc deficiency condition. The results of total phenolic, flavonoid and total tannin contents estimation indicated that *C. azarolus* was very rich of these chemicals compound, which reflects its strong biological antioxidant properties. Moreover, the antioxidant capacity of *C. azarolus* was evaluated through different tests. The findings revealed that the aqueous extract of *C. Azarolus* was more potent with an inhibition rate of 83.7%. This is evident for aqueous extract, because there is a close relationship between the phenolic and flavonoid contents and the antioxidant activity. Furthermore, IC_{50} value for the *C. Azarolus* extract was close to those used standards. The result is an agreement with previously studies about other species of *Crataegus* genus collected from many countries [15, 34]. As expected in the present investigation, the diabetic group showed remarkable decrease in body weight as compared to non-diabetic group; this is an accordance with some reports [35, 36]. The reduction of body weight was undoubtedly due to the disturbance of the metabolic state and suggests that weight-loss might be clarified by the powerlessness of body to use carbohydrates as source of energy and the high-rate catabolism of fat and protein mass. On the other hand, diabetic rats fed zinc-deficient diet had lower body weight gain and food intake as compared to rats fed zinc-adequate diet, which is consistent with published reports [37, 38]. It is known that zinc is required for the normal growth and development of animal species and human [39]. The treatment of zinc deficient diabetic animals with *C. Azarolus* ameliorated body weight. The noticeable increase of body weight of animals might be due to the rise food intake and protein synthesis. Additionally, it was documented that *Crataegus Azarolus* has the

ability to reverse gluconeogenesis and control protein loss [40]. Tissues zinc concentrations including femur, liver, pancreas and kidney of diabetic rats were lower than those of non-diabetic rats. It has been postulated that low level of zinc in patients with diabetes is usually related to excessive urinary output and gastrointestinal malabsorption [41]. The findings showed also that there was a significant decrease in zinc content in the above-mentioned organs of diabetic animals fed zinc-deficient diet compared to the diabetic animals fed zinc-sufficient diets; this coincides with some published investigations [37, 42]. The level of zinc in zinc deficient diabetic rats was restored after *C. Azarolus* extract administration. This was unlikely due to the antioxidant effect of this plant extract against the development of the diabetic state. It is possible explained to be that treatment with this aqueous extract positively influences oxidative stress harm through down regulation hyperglycemia and preventing kidney dysfunction resulting in reduction of zinc urinary losses. In the present study, when the time of feeding was strictly controlled, and the amount of food eaten by each animal before an overnight fasting was known to be similar, animals with diabetes fed an inadequate zinc diet and those under adequate zinc diet had no differences in blood glucose level. Notwithstanding the fact that zinc deficiency exacerbates often fasting hyperglycemia related with decreased circulating insulin. Blood glucose level was decreased in diabetic zinc-deficient rats, which were treated with *C. Azarolus*. This finding correlated with the histologic studies of the pancreas, where the plant extracts preserved islet cells. The hypoglycemic effect of *C. Azarolus* might be due to the presence of some α -glucosidase inhibitors including the polyphenols mainly containing quercetin, the epigallocatechin gallate (EGCG) and flavonoids [43, 44]. The phytochemical screening indicated the richness of this plant in these active constituents. These compounds can effectively reduce the insulin resistance, gluconeogenesis and increase the hepatic glycogen synthesis and storage [45]. The oral administration of *Crataegus Azarolus* at dose of 150 mg/Kg diet exhibited significant antihyperglycemic effect and marked improvement in glucose tolerance (OGTT), which confirm the effectiveness of *C. Azarolus* extract as a hypoglycemic agent through delaying carbohydrate digestion, thereby lowering blood glucose level [43]. The cholesterol, triglyceride, urea, uric acid and creatinine levels were higher in both zinc-deficient diabetic rats and zinc sufficient diabetic rats. Diabetes is generally associated with abnormal lipid metabolism, which is an important risk factor for diabetic vascular disease [46]. High concentration of cholesterol and triglyceride were definitely as a result of lipids metabolism variations under diabetic conditions, which the later led to suppress lipoprotein lipase activity in account of insulin deficiency and insulin resistance [47]. Moreover, variation of zinc status exhibited lipid and protein disturbance via highly significant elevation of cholesterol, triglycerides and creatinine. In other word, zinc deficiency provoked catabolism of lipids and proteins because of increased demand for energy [42, 48]. The treatment of zinc-deficient diabetic rats with *C. Azarolus* resulted an improvement of these altered parameters. An explanation, that the reduction in the serum total cholesterol and triglycerides levels is a complex process involving multiple steps in cholesterol metabolism. Among which, the total flavonoids contribute in the expression of two essential liver enzymes, hydroxy-methylglutaryl coenzyme A reductase (HMG-CoA) and cholesterol-7- α -hydroxylase (CYP7 α), which are key enzymes for lipid synthesis. Moreover, they play a role for inhibiting cholesterol absorption by downregulating the expression and activity of intestinal acyl-CoA cholesterol acyltransferase (ACAT) [49, 50]. Significant increases of serum GOT and GPT activities were observed in zinc deficient diabetic rats as compared to zinc sufficient

diabetic rats. These findings are coinciding with these published reports [37, 42]. Transaminases appear to be most sensitive to zinc restriction in that their activities are affected adversely by Zn-deficient diet [51]. Greeley and Sandstead [52] found evidence of decreased oxidation of the carbon chain of alanine when zinc was restricted and led to alanine accumulation in blood. A reduction in serum lactic dehydrogenase was observed in zinc deficient diabetic rats. The decrease of lactic dehydrogenase activity was certainly due to zinc depletion. Briefly, LDH is a metalloenzyme that requires zinc as a cofactor for its activity [35]. Considerable recent evidences suggest that cellular reduction-oxidation (redox) imbalance leads to oxidative stress and subsequent occurrence and development of diabetes complications by involving certain signaling pathways in β -cell dysfunction and insulin resistance [53]. Depending on the results obtained, there was an increment of MDA concentration, which affirms the harmful impact of zinc deficiency by expanding lipid peroxidation. Moreover, glutathione (GSH) level, catalase (CAT), glutathione-peroxidase (GSH-Px), superoxide dismutase (SOD) and glutathione reductase (GR) activities were decreased in zinc deficient diabetic rats. The reason for the depletion of glutathione could be explained to its scavenging free radicals role and the high consumption [54]. However, the observed decline of antioxidants enzymes activities could be due to the modification of sulfhydryl groups in these enzymes by the generation of reactive oxygen species or by auto-oxidation of glucose and non-enzymatic glycation of proteins [48]. The administration of *C. Azarolus* resulted an attenuation and decline of oxidative stress and cellular damage. In other words, the findings showed a significant reduction in the formation of malondialdehyde and an augmentation of GSH concentration plus an improvement in CAT, GSH-Px, SOD and GR activities in zinc deficient diabetic animals treated with *C. Azarolus*. Simply, it has been reported that several polyphenol compounds isolated from *C. Azarolus*, such as alkaloids, tannins and flavonoids, which have a strong antioxidant property and play an important role in inhibiting and scavenging free radicals [55]. In addition, it has been documented that *C. Azarolus* extract can elevate the expression of several antioxidant genes, including RP-n11, CAT, GSH-Px, SOD and GR [56].

Conclusion

In conclusion, this study indicated that the administration of aqueous extract of *C. Azarolus* has a potential effect for reducing the development and complications of diabetes due to zinc deficiency by improving growth rate, zinc status, carbohydrate metabolism and antioxidant system. Further clinical investigations are required to assess the effectiveness of this plant and its bioactive compounds in the management of diabetes complications linked to zinc deficiency.

Declarations

Authors Contributions

ZK formulated the present hypothesis. ZK and RT were responsible for writing the report. RT, KB, MC were responsible for analysis of the data.

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Data availability

The data presented in this study are available within the text and figures of the paper and in supplementary material. Original data is available from the corresponding authors.

Declaration of Competing Interest

The authors report no declarations of interest.

Ethics Approval, consent to participate and consent for publication: not applicable.

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Figures

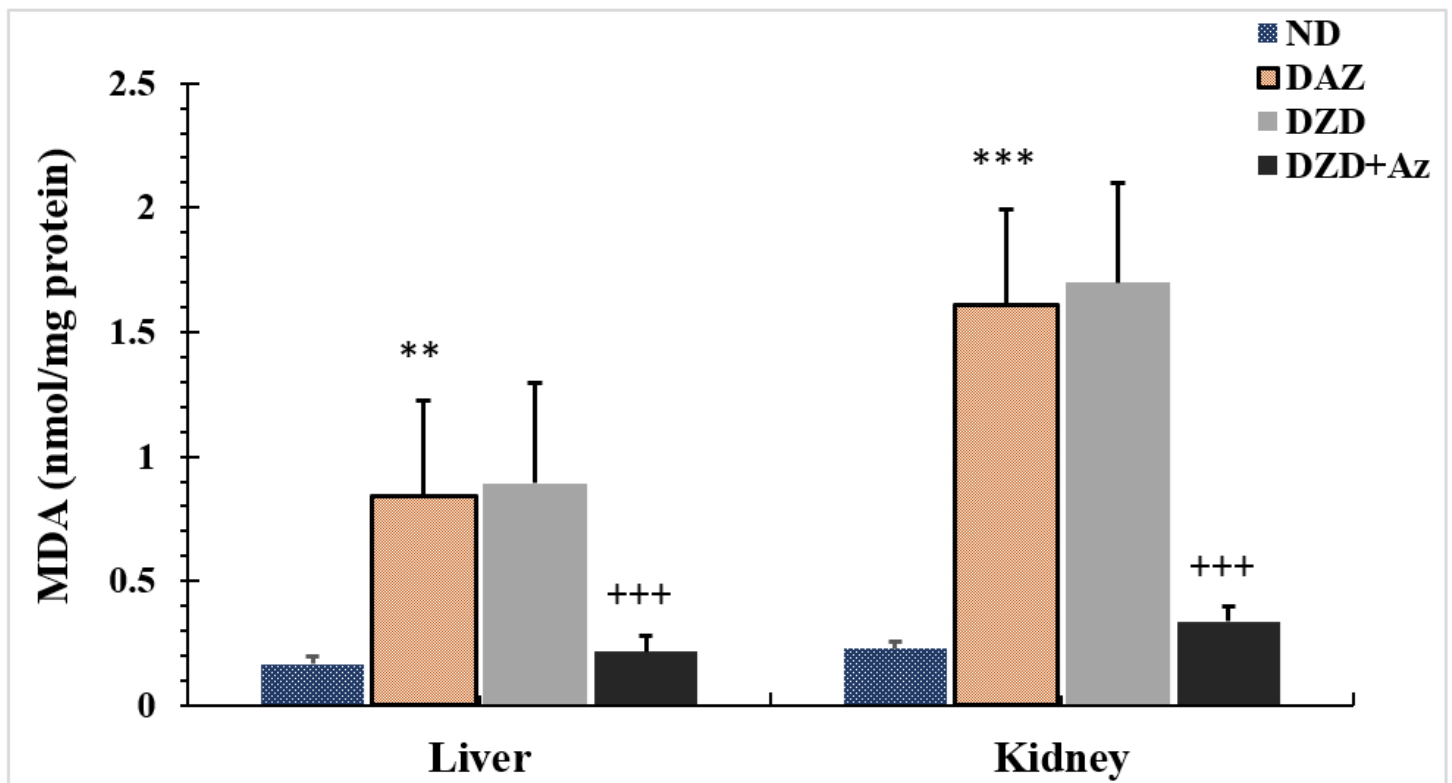


Figure 1

Malondialdehyde (MDA) level of non-diabetic rats (ND), diabetic adequate-zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az). Values are mean $\pm$ SEM; number of samples = 8. ** $p < 0.01$, *** $p < 0.001$: DAZ vs ND, +++ $p < 0.001$: DZD+Az vs DZD.

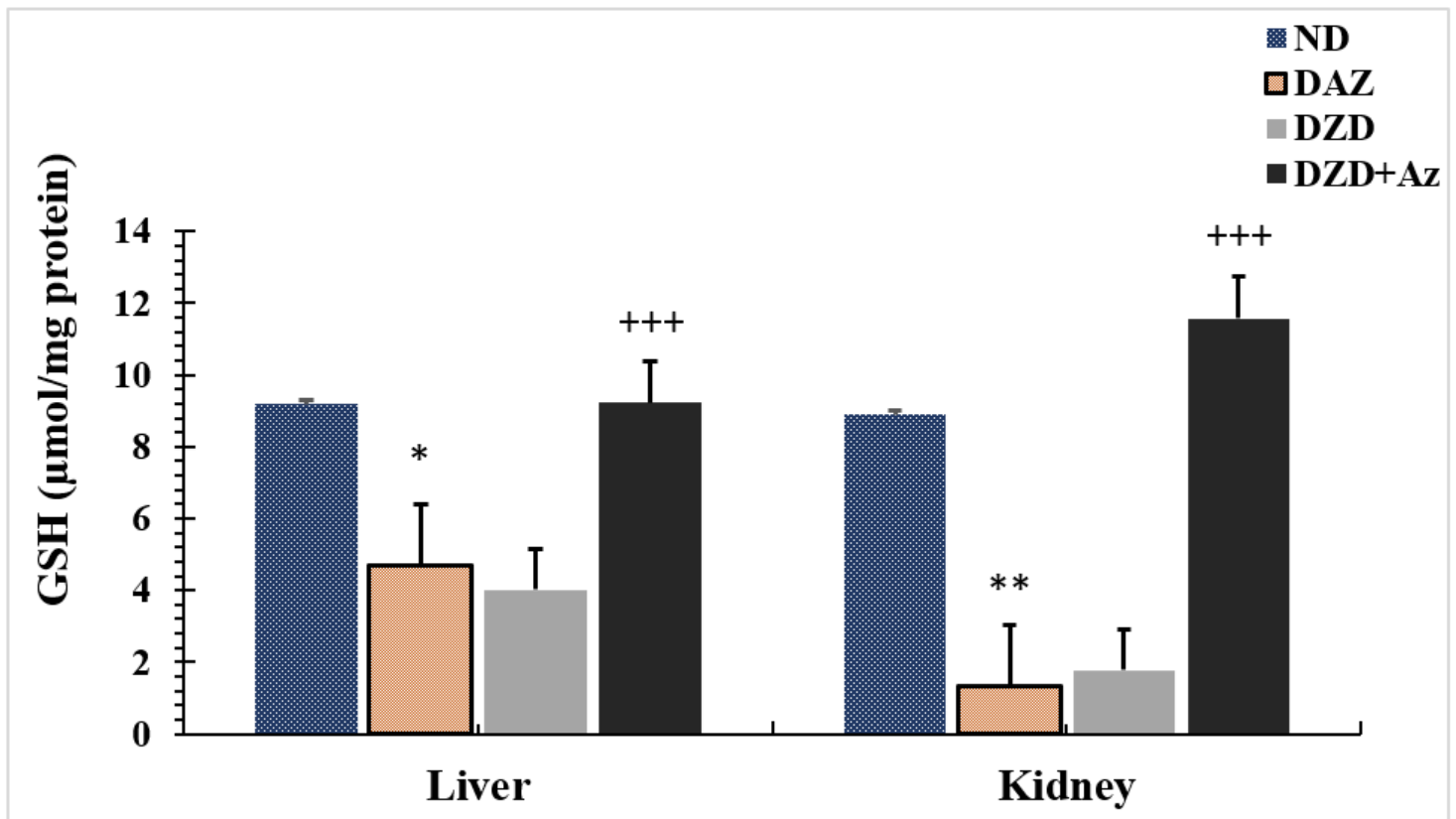


Figure 2

Reduced glutathione (GSH) concentration of non-diabetic rats (ND), diabetic adequate-zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az). Values are mean $\pm$ SEM; number of samples = 8. * $p < 0.05$, ** $p < 0.01$: DAZ vs ND, *** $p < 0.001$: DZD+Az vs DZD.

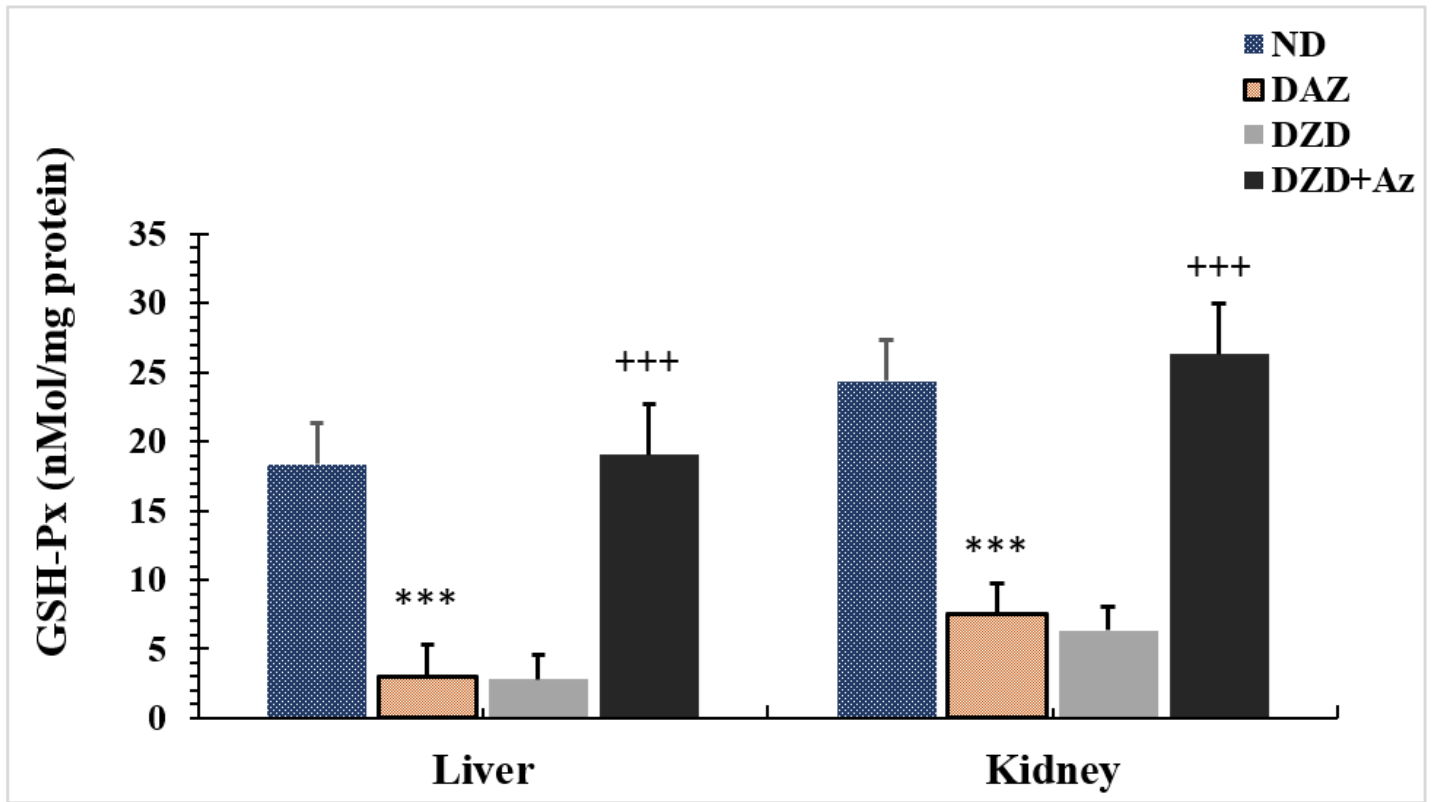


Figure 3

Glutathione peroxidase (GSH-Px) activity of non-diabetic rats (ND), diabetic adequate-zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az). Values are mean $\pm$ SEM; number of samples = 8. ***p<0.001: DAZ vs ND, ***p<0.001: DZD+Az vs DZD.

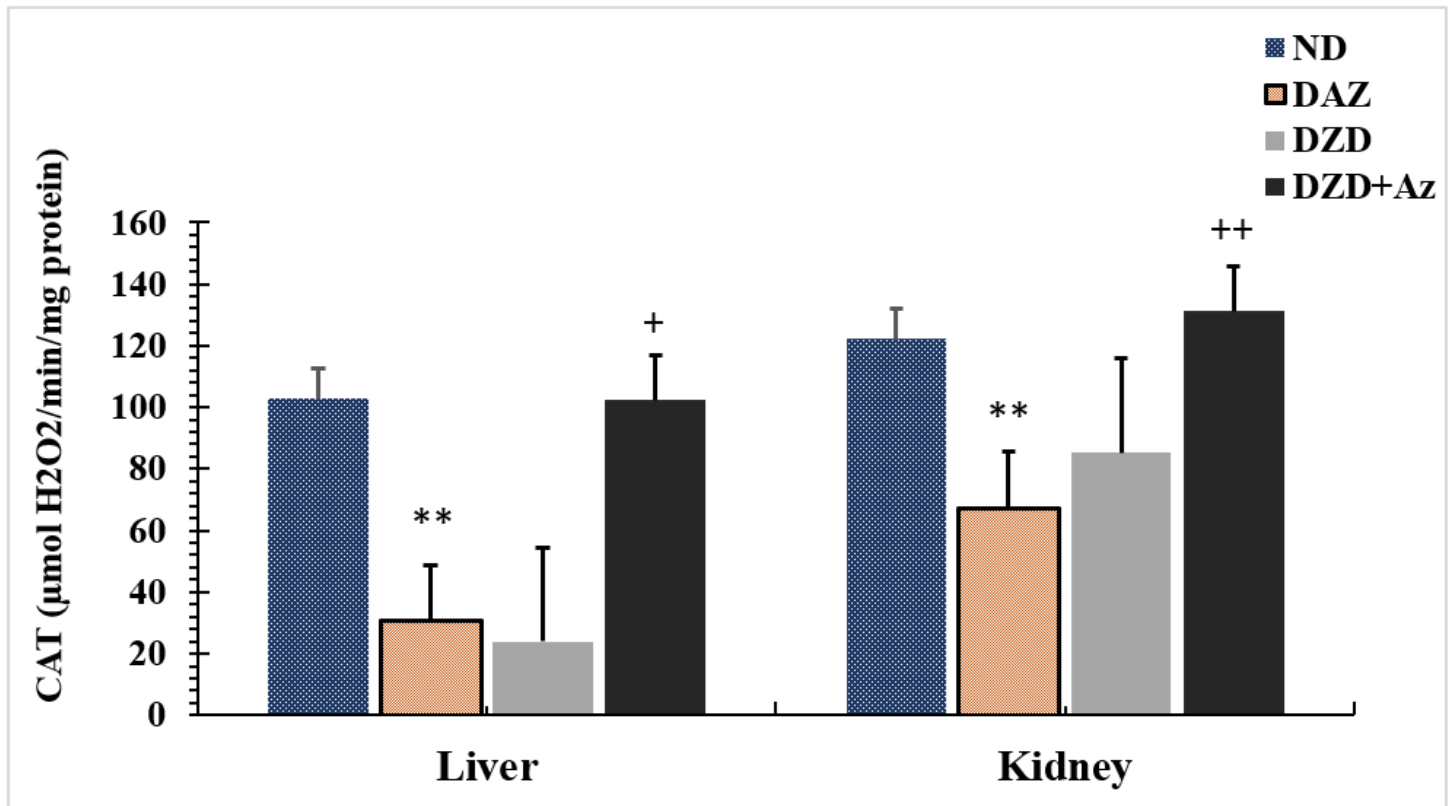


Figure 4

Catalase (CAT) activity of non-diabetic rats (ND), diabetic adequate-zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az). Values are mean $\pm$ SEM; number of samples = 8. ** $p < 0.01$: DAZ vs ND. + $p < 0.05$, ++ $p < 0.01$: DZD+Az vs DZD.

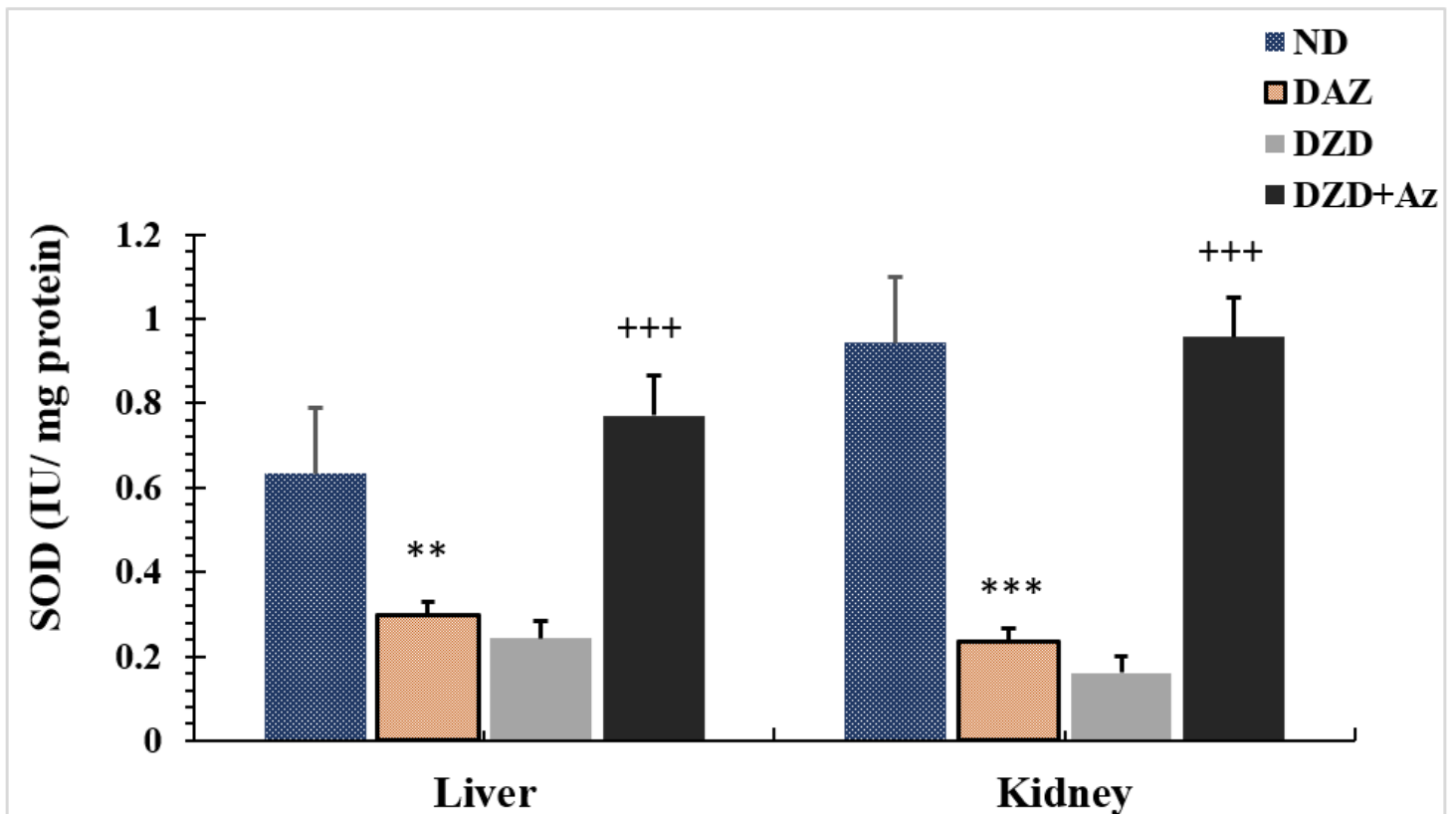


Figure 5

Superoxide dismutase (SOD) activity of non-diabetic rats (ND), diabetic adequate-zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az). Values are mean $\pm$ SEM; number of samples = 8. ** $p < 0.01$, *** $p < 0.001$: DAZ vs ND. *** $p < 0.001$: DZD+Az vs DZD.

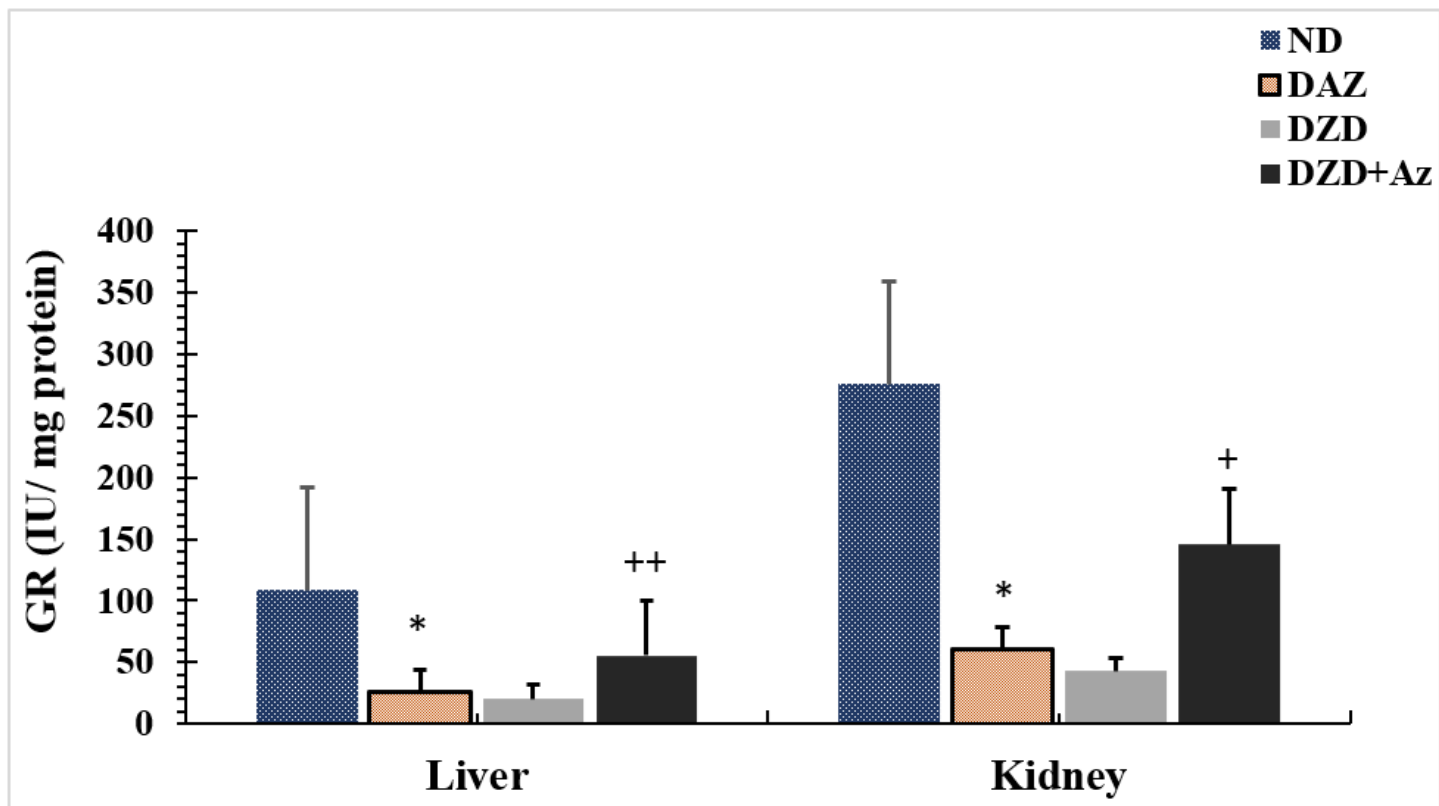


Figure 6

Glutathione reductase (GR) activity of non-diabetic rats (ND), diabetic adequate-zinc rats (DAZ), diabetic zinc-deficient rats (DZD) and diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az). Values are mean $\pm$ SEM; number of samples = 8 * p <0.05: DAZ vs ND. + p <0.05, ++ p <0.01: DZD+Az vs DZD.

OGTT in mice

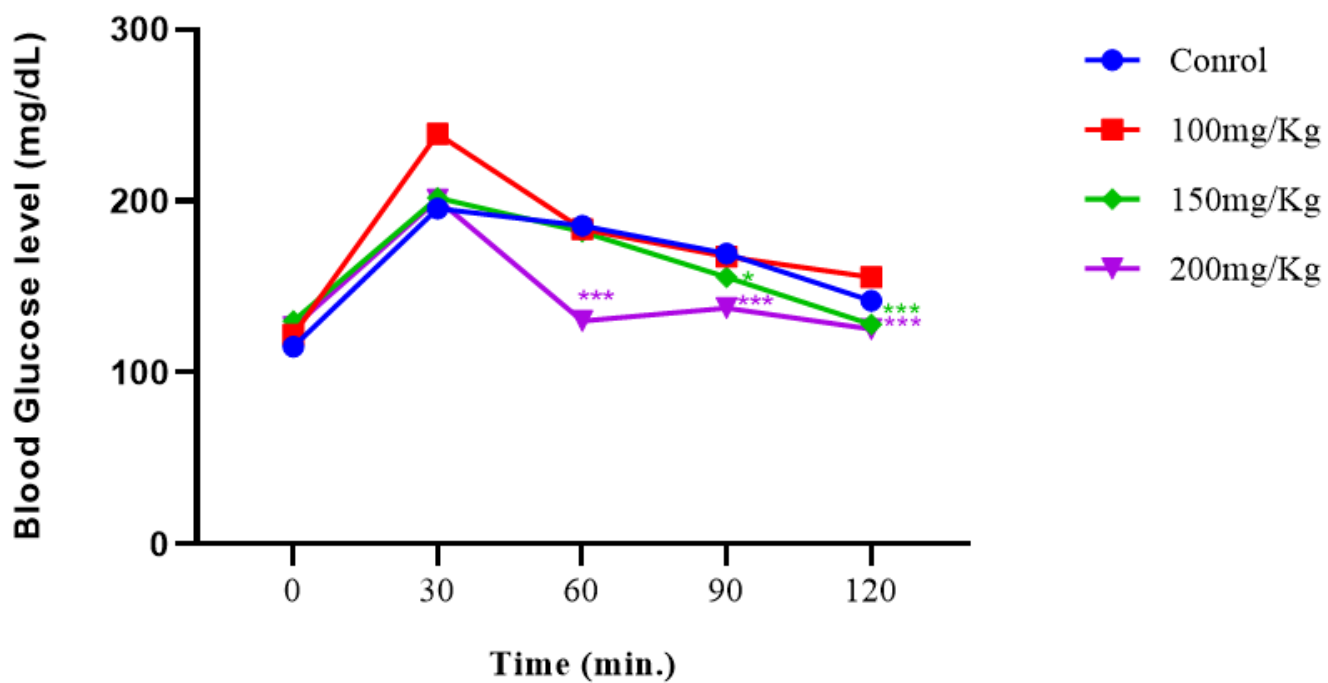


Figure 7

Effect of *Crataegus Azarolus* extract on blood glucose (hyperglycemia) in mice following 120 min of glucose administration. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$ as compared to control group.

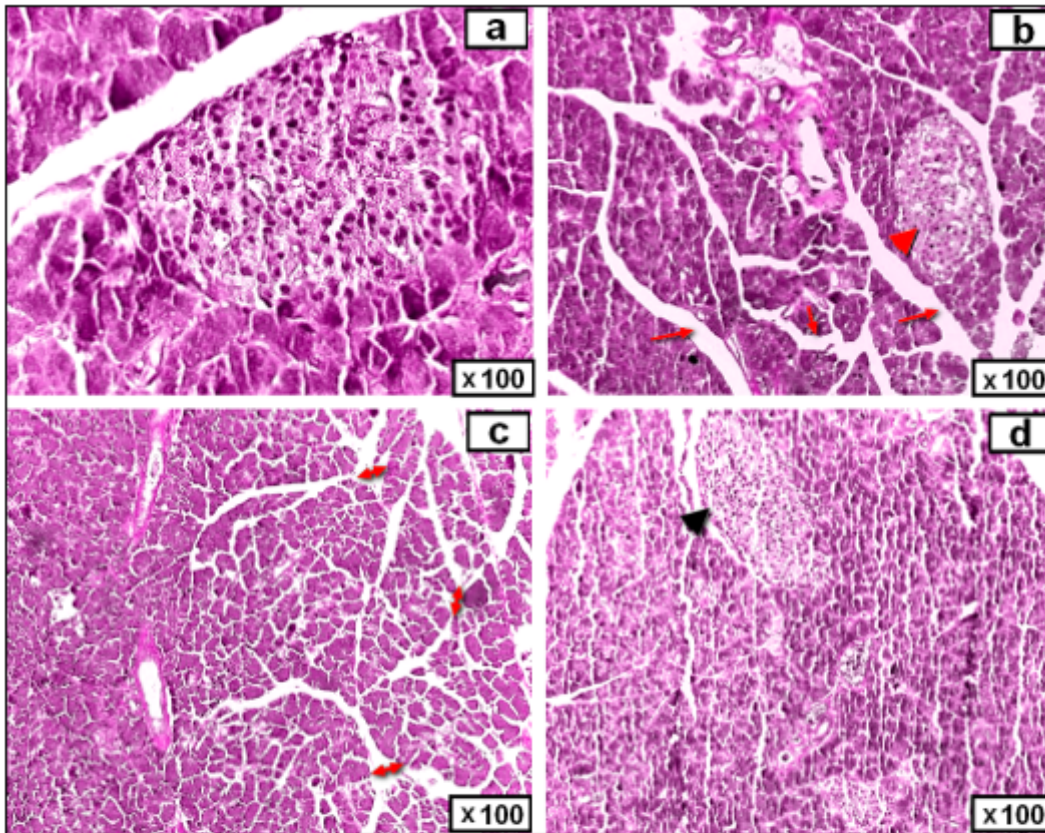


Figure 8. Effect of *Crataegus Azarolus* on histopathologic damage in the pancreas after 4 weeks of treatment: **a**, Section of pancreas tissue from non-diabetic rats (ND) showing normal architecture. **b**, Section of pancreas tissue from diabetic adequate-zinc rats (DAZ) showing reduced *B-cells* (►) size and degenerative vascular changes in pancreatic islets (→) . **c**, Section of pancreas tissue from diabetic zinc-deficient rats (DZD) indicating more degenerative vascular changes in the pancreatic islets (↔). **d**, Section of pancreas tissue from diabetic zinc-deficient rats given *Crataegus Azarolus* (DZD+Az) showing an apparently normal and preserved pancreatic islets population (►). Optic microscopy: sections were stained using the hematoxylin-eosin method (100×).

Figure 8

See image above for figure legend