The Effect of Alcoholic Extract of Thymus Vulgaris on Hepatic Enzymes Activity and Apoptosis-Related Gene Expression in Streptozotocin-Induced Diabetic Rats

Mohammadreza Azimi
Islamic Azad University Saveh Branch
Elnaz Ahmadi
Islamic Azad University
Fatemeh Aghaie
Shahid Beheshti University of Medical Sciences School of Nursing and Midwifery
Mohammad Orafei
Ferdowsi University of Mashhad
Armita Ahmadi
Ferdowsi University of Mashhad
Ali Ghorbani Ranjbary (alighorbani.r@ut.ac.ir)
Tehran University: University of Tehran

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Abstract

Many diseases, including diabetes, are involve in the development of liver disorders through changes in the expression of genes such as apoptosis-related gene. In the present study, the effect of alcoholic extract of Thymus Vulgaris on Hepatic Enzymes Activity and apoptosis-related gene expression in streptozotocin-induced diabetic rats. In this study, 50 adult male Wistar rats weighing approximately 200–220 g were divided into five groups. Diabetes was induced by intraperitoneal injection of STZ (60 mg/kg). Following 18 days, all the animals in different groups were weighed and blood samples were taken from their cardiac veins. GC analysis revealed 45 different compounds in the Thymus Vulgaris, including; thymol (39.1%), p-cymene (20.63%), γ-Terpinene (14.85%). The results showed a significant increase in liver enzymes (AST, ALT and ALP) in diabetic or streptozoic mice compared to the control group (healthy mice) ($p < 0.0001$). The level of liver enzymes (AST, ALT and ALP) in rats treated with doses 200 mg/kg and 400 mg/kg of thymus vulgaris extract showed a significant decrease in these enzymes in comparison with diabetic rats ($p < 0.0001$). The expression of caspase 3 and 9 genes in the groups treated with thyme significantly decreased compared to diabetic mice ($P < 0.0001$) and the expression of Bcl in the group receiving 400 mg / kg of thyme significantly increased compared to diabetic mice ($P = 0.0001$). Due to its antioxidant compounds, thyme improves the liver tissue cells in streptozotocin-induced diabetic mice by reducing caspases 3 and 9 as well as increasing Bcl-2.

Introduction

Different genetic factors leading to types I and II diabetes mellitus. Both of them are prone to complications such as nephropathy, retinopathy, peripheral nerves disorders, and blood pressure [1, 2]. Diabetes is as the leading causes of hepatic disorders in the United States of America. In many studies have shown that liver disease is an important cause of morbidity and mortality in type II diabetes [3]. Besides, the high prevalence of liver disease in diabetic patients, the incidence of diabetes is higher in patients with liver disease. It seems that the incidence of liver disease in diabetes type II occurs due to complications such as abnormal liver enzymes, non-alcoholic fatty liver, cirrhosis, hepatocellular carcinoma and acute liver failure is more prevalent [4].

Streptozotocin is a drug used in chemotherapy, and it also specifically kills pancreatic beta cells, thereby lowering insulin levels [5]. The streptozotocin model is a common method for inducing experimental diabetes in rodents and has been used repeatedly in various studies to induce type 1 diabetes [5, 6]. Recent studies have shown that streptozotocin causes inflammation by disrupting the relationship between ROS production and radical scavenging effect [7, 8]. The rise in the production of free radicals or ROS formation may result in oxidized LDL (Ox-LDL), a crucial step in the chain of events leading to atherosclosis sustained hyperglycemia and increased oxidative stress, which are the key players in the development of secondary diabetes problems. These abnormalities result in pathologies such as vasculopathies, neuropathies, ophthalmopathies and nephropathies, among various other medical problems [9]. Through in vitro cell culture as well as in vivo diabetic rodent models for STZ-induced toxicity, it has been shown that STZ induces cellular oxidative stress and mitochondrial respiratory

dysfunction [10, 11, 12]. A number of experiments have been conducted to assess the changes in cell mitochondrial functions from brain, heart, liver, and kidney of diabetic rats [13]. Nevertheless, the results have been at times controversial for the reason that experimental conditions such as age and strain of used animals have been different.

The synthetic drugs for liver diseases, including corticosteroids, antiviral and immunosuppressant agents, might cause serious adverse effects up to hepatic impairment, such as cholestatic jaundice with azathioprine and elevation of serum transaminases by interferon and virazole [14]. As such, it is of paramount importance to explore other sources to treat the liver disease more effectively and safely.

Thymus vulgaris (called “wild thyme” in Persian) is a plant from *Labiatae* and *Plantae* family [15]. It grows in many parts of Europe, particularly in southern Europe, north of Africa, as well as large parts of Asia. Wild thyme is used in traditional medicine as an antiseptic, antispasmodic, anti-worm, and carminative as well as for relieving liver and bile problems [16, 17, 18, 19]. Wild thyme has 1% oil and a large part of it consists of phenols, monoterpene hydrocarbons, and alcohol. Wild thyme essence generally contains 25 compounds such as thymol (26.9%), carvacrol (40.7%), and γ-terpenen (7.3%) [20, 21]. This research aimed to achieve the protection role of T. Vulgaris on Hepatic Enzymes Activity and apoptosis-related gene expression in streptozotocin-induced diabetic rats.

Materials & Methods

Collecting and Identifying the Plant

Leaves and twigs of the wild thyme plant were collected from the areas near Shiraz, Iran, in March-April 2019, dried at 25˚C in the shade, and then powdered by a mechanical mill. The dried powder was kept in plastic bags in a freezer until testing.

Extraction Method

Twenty grams of the obtained powder was poured into an Erlenmeyer flask and 200 ml of ethyl alcohol at 70˚C was added. Next, the flask was closed by its cap and the solution was held for 48 hours, while the content of the flask was shaken once every 12 hours. Following 48 hours, the content of the flask was filtered into a beaker using a filter paper and a funnel glass. Then, the filtered solution was poured into a flask and placed in a rotary device at 75˚C with average rotation speed. After solvent evaporation, the resultant concentrated liquid was spread on the glass surface and left to dry. The obtained powder containing approximately 2.59 percent of the concentrated extract was collected after drying. Finally, the yielded powder was used to prepare doses of 100, 200, and 400 mg/kg. All the solutions were prepared by distilled water.

Plant Compounds

First, thymus vulgaris essential oil was prepared and the compounds were then isolated by GC/MS device (HP-6840/5973) in the central laboratory of Ferdowsi University of Mashhad. The constituting

elements were identified by comparing their mass spectra with the existing standard spectra.

Animals

Small rats, each with an approximate weight of 200±5 g, were kept in clean cages at 25±2˚C and in a diurnal cycle of 12 hours of light and 12 hours of darkness, with relative humidity of 40-60 percent. The animals had access to water and food.

Preparation of Diabetic Animals

Streptozotocin (Pharmacia & Upjophn, USA) (60 mg/kg) solved in sterile saline just shortly prior to the test was intraperitoneally injected into rats. Animals with up to 180 milligrams/deciliter of glucose level of serum were used in the test five days after the injection.

Treatment Method

The wild thyme hydroalcoholic extract was inraperitoneally used as treatment in different doses for 18 days. The number of animals in each group was 10. The first control group (Group 1) received merely regular food and water. The second control group (Group 2) received saline daily and the three experimental groups (Groups 3-5) daily and intraperitoneally received a low dose (100 mg), a medium dose (200 mg), and a high dose (400 mg) of wild thyme hydroalcoholic extract in two groups of healthy and diabetic for 18 days.

Assay of Hepatic Marker Enzymes

The hepatic marker enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in serum were measured using diagnostic kits (Parsazmon, Iran). As such, glucose, cholesterol, HDL cholesterol in serum, as well as LDL and VLDL cholesterol were measured [22].

Histopathology

The liver tissue was fixed in 10% formalin for 48 h. It was then followed by dehydration by passing through a series of graded alcohol, beginning with 50% alcohol and progressing in graded steps to 100% (absolute) alcohol, and was finally embedded in paraffin. Liver slices (5–6 μm thick) were prepared using a semi-automated rotator microtome, stained with Hematoxylin and Eosin dyes, and observed microscopically.

RT-qPCR Assays

Total RNA from liver tissues was extracted using the Column RNA Isolation Kit (DENAzist Asia Co., Iran) and reverse-transcribed with cDNA synthesis kit (Thermo Fisher Scientific., USA).

The designed primers (Beacon Designer v8) were as follows: for BCL2, F: 5'- GAGCGTCAACAGGGAGA-3' and R: 5'- GCCAGGAGAAATCAAACA-3'; for BAX, F: 5'- ACTAAAGTGCCCGAGCTGA-3' and R: 5'- ACTCCAGCCACAAAGATGGT-3'; for C3, F: 5'- GGAGCTTGGAACGGTACGCT-3' and R: 5'- AGTCCACTGACTTGCTCCCA-3'; for C9, F: 5'- AGCCAGATGCTGTCCCATAC-3' and R: 5'- CAGGAGACAAAACCTGGGAA-3'; for β-actin: F: 5'-ATCAGCAAGCAGGAGTACGAT-3' and R: 5'- AAAGGGTGTAAAACGCAGCTC-3'.

The normalization and analyses of the qPCR data were performed using Genex Version 6 software (MultiD, Göteborg, Sweden) and Relative Expression Software Tool (REST; QIAGEN, Hilden, Germany).

Statistical Data Analysis

The collected data were analyzed using SPSS statistical software, and one-way analysis of variance (one-way ANOVA) and Tukey test at the significant level of $p<0.05$ were run to investigate between-group differences. All results were presented as Standard Error of Mean (SEM).

Results

The main compounds of thyme essential oil measured by GC/MS are shown in Table 1. Identification of compounds has been done by comparing their mass spectra with their mass indices with the reference spectrum and also comparing their inhibition indices with the inhibition indices of these compounds. As can be seen, the four compounds of thymol (39.1%) (Figure1), p-cymene (20.63%), γ-Terpinene (14.85%), and carvacol (4.65%) constituted an aggregate of 86.54% of the composition of Thymus Vulgaris essential oil.

The results show that the level of serum glucose has significantly increased in diabetic rats compared to healthy rats [Figure2A]. However, serum glucose levels in the groups receiving thyme extract showed a significant decrease compared to the diabetic group (C2) ($P<0.0001$). Regarding total serum cholesterol, a significant increase in the cholesterol level was observed in the diabetic control rats at the end of day 18 after the test compared to control group 1 [Table 2]. Thyme hydroalcoholic extract in the studied concentrations causes a significant reduction in total serum cholesterol levels compared to the diabetic control group ($P<0.05$).

Serum levels of ALT, ALP and AST in the diabetic group increased significantly compared with control group 1 [Figure2B-D]. Also, serum levels of liver enzymes in diabetic groups treated with thyme extract significantly decreased compared to the diabetic group ($P<0.0001$). Serum levels of liver enzymes in the diabetic group treated with thyme extract at a concentration of 400 mg/kg body weight significantly decreased, compared with the diabetic group treated with thyme extract at a concentration of 100 and 200 mg/kg body weight ($P<0.0001$). Serum HDL-C levels decreased significantly in the diabetic group compared to the control group and serum LDL-C levels increased significantly [Table 2]. However, in the groups treated with thyme, the levels of HDL-C and LDL-C increased and decreased significantly compared to the diabetic group, respectively ($P<0.05$). Also, serum levels of VLDL in the diabetic group

increased significantly compared to control group 1 (P<0.05). Moreover, serum VLDL levels in the diabetic group treated with thyme extract significantly decreased in comparison to the diabetic group (P<0.05).

Pathology Results

The results of the present study showed that the number of Kupffer inflammatory cells in the diabetic group increased significantly compared to control group 1. Nonetheless, the number of blood vessels and hepatocytes in the diabetic group decreased significantly compared to control group 1 [Figure 3A and B]. Treatment of diabetic rats with thyme extract, in a dose-dependent manner, led to a significant increase in the number of blood vessels and hepatocytes and also a significant decrease in the number of Kupffer cells compared to the diabetic group [Figure 3C-F]. Comparison of the number of Kupffer cells, blood vessels, and hepatocytes between diabetic groups treated with thyme extract at concentrations of 100, 200, and 400 mg/kg body weight showed a statistically significant difference (P<0.05). Histological examination indicated that the structure of the portal ducts and liver sinusoids is normal in control group 1 and no pathological changes were observed. In the diabetic group, inflammatory cells entered the lobule from the port space. Moreover, severe cell necrosis exists around the port space and there are scattered foci of necrosis in different parts of the liver lobules; also, a noticeable decrease in the number of liver cells, changes in the structure, and irregularity of the liver sinusoids were observed. In the diabetic group treated with concentrations of 100 and 200 mg/kg of thyme extract, the rate of cell necrosis decreased, compared with the samples in the diabetic group; However, severe inflammation is still seen in the port area. In the diabetic group treated with a concentration of 400 mg/kg of thyme extract, a decrease in cell necrosis around the port space, a decrease in local inflammation of hepatocytes, and a decrease in hepatic sinusoids irregularity are observed, compared with diabetic samples in which hepatocytes have an almost normal structure.

Real-time PCR Results

BCL-2 gene expression significantly increased in the diabetic group compared to control group 1, whereas BAX in the diabetic group significantly decreased compared to control group 1 (P<0.0001). On the other hand, in the groups receiving thyme extract, the expression of BCL-2 decreased and Bax showed a significant increase, compared to the diabetic group without treatment (P=0.0001). Nevertheless, the group receiving 100 mg/kg body weight of thyme did not show a statistically significant difference when compared to control group 1. Furthermore, the expression of caspase 3 and 9 genes in the diabetic group without treatment showed a significant increase compared to the control group (P<0.0001). However, the expression level of caspase 3 and 9 genes in the diabetic groups treated with thyme extract showed a significant decrease compared to the diabetic control group (P<0.0001) [Figure 4].

Discussion

Currently, therapies available for treatment of non-insulin dependent diabetes mellitus including improvement of diet, hypoglycemic factors, and insulin, have their own constraints [23]. Investigation on herbal medicine will present a natural key to unlock problems of diabetes in the future. Streptozotocine is

an antibiotic and anticancer agent that is used in different types of animals to induce diabetes by degeneration and necrosis of pancreatic β-cells. The liver is one of the organs that is damaged by diabetes. The results of the present study revealed that thyme has a protective effect in STZ diabetic mice against liver tissue damage and reduces liver enzymes and preserves the morphology of liver tissue cells in STZ-receiving mice. The histopathological results of diabetic rats indicated inflammation in the structure of hepatic lobules, so that the infiltration of mononuclear cells and the proliferation of Kupffer cells as well as the departure of Kupffer cells from the sinusoidal wall were observed in the liver tissue sections. Moreover, Kupffer cell accumulation and mononuclear cells were seen around the central vein. Tissue samples in this study showed irregularities in the structure of liver cell plates and central venous dilation in the hepatic lobule of the diabetic group. all of which were decreased by T. Vulgaris. ALT, ALP and AST enzymes are abundant in the liver and any damage to liver cells increases their levels in blood. These enzymes are used to evaluate hepatic disorders. An increase in the activity of the above enzymes reflects damage to the liver. Inflammatory disorders in hepatic cells lead to a sharp rise in transaminase levels [24, 25]. The results are in accordance with those of other studies in terms of the effects of wild thyme hydroalcoholic extract on hepatic cells and reducing of AST, ALT, and ALP enzymes [26, 27]. According to Yam (2007) and Janbaz (2004), it is proven that caffeic acid prevents the increase of serum enzymes and thus protects against methane tetrachloride-induced hepatic damage. These enzymes prevent liver protection activity through different mechanisms[28, 29]. Bampidis (2005), ALT and AST enzymes decrease by the rise in the antioxidant activity of the liver [30]. According to Matsuura (2003) and Bozin (2006), it appears that due to their antioxidant properties, flavonoid compounds in wild thyme such as Rosemarinic acid are able to neutralize free radicals of 1,1-diphenyl-2-picrylhyrazyl (DPPH) and prevent their destructive effects [31, 32].

T. Vulgaris does not normalize hyperglycemia, suggesting the action of T.Vulgaris on liver dysfunction does not pertain to systemic variables associated with the glucose metabolism. T. Vulgaris, nonetheless, tends to balance out the dyslipidemia in rats with STZ-induced diabetes. T. vulgaris extract proved to have a hypolipidemic effect represented by the decrease on TC as well as LDLC and increase HDL-C levels. When compared to control group 1, BCL-2 gene expression had a significant increase in the diabetic group, while BAX decreased significantly in the diabetic group. Alternatively, when compared to the diabetic group that did not receive treatment, the expression of BCL-2 decreased and the expression of Bax increased significantly in the groups that received thyme extract. Moreover, as compared to the control group, the expression of caspase 3 and 9 genes was significantly higher in the diabetic group without treatment. Nevertheless, as compared to the diabetic control group, the expression levels of caspase 3 and 9 genes in the diabetic groups that received thyme extract were significantly decreased. T. Vulgaris' antiapoptotic potential is sufficient to restore the cells’ normal conditions.

Furthermore, stimulation of DNA polymerase by flavonoid compounds causes an increase in rRNA synthesis and results in reconstruction of hepatic cells [33, 34]. Based upon Oktem (2006), lithospermic B, 12-hydroxy jasmonic acid, ursolic acid, and other phenolic compounds reduce hepatic inflammations by inhibiting the lipo-oxygenize cycle and preventing leukotrienes and free radicals productions in hepatic kupffer cells in mice [35]. According to the results of Subten Ocak (2007), caffeic acid, one of the

antioxidant compounds of thyme, prevents high production of nitric oxide and reduces nephrotoxic-induced damage [36]. All the above-mentioned studies confirm the results obtained in this study. Thyme extract contains important antioxidant compounds such as thymol, which can prevent streptococcal damage in liver cells by reducing the expression of apoptosis-related factors. It follows that it can be beneficial for diabetic hepatitis by reducing liver enzymes and improving the morphology of liver tissue cells.

Declarations

Ethical approval

Animals use and care were approved with national ethics committee of Tehran University (ethical code: IR.TU.VCR.REC.13992117) and were performed in accordance with the university's guidelines. Furthermore, all animal experiments comply with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised1978).

Compliance with ethical standards

Conflict of interest all authors declare that they have no conflict of interest.

Authors' contributions

AG and MZ wrote the primary draft; EA, FA, MO and AA critically revised the manuscript. All authors read and approved the fnal manuscript.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable

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Tables

Table 1: Main Compounds of Thymus Vulgaris Essential Oil

Compound	Percent	*RI	CHEMICAL FORMULA	**RT	*** LRI	IDENTIFICATION
α-Pinene	1.7	930	$C_{10}H_{16}$	6.76	939	RI, MS
α - Thujene	1.41	928	$C_{10}H_{16}$	7.11	936	RI, MS
β-Pinene	0.64	979	$C_{10}H_{16}$	14.16	984	RI, MS
α-terpinene	0.4	971	$C_{10}H_{16}$	11.61	985	RI, MS
Sabinene	0.51	964	$C_{10}H_{16}$	29.96	972	RI, MS
Borneol	0.32	1172	$C_{10}H_{18}O$	13.68	1180	RI, MS
Myrcene	1.64	871	$C_{10}H_{16}$	14.62	902	RI, MS
Thymol	39.1	2010	$C_{10}H_{14}O$	21.50	2015	RI, MS
Linalool	0.39	1080	$C_{10}H_{18}O$	12.53	1085	RI, MS
Carvacrol	4.65	1296	$C_{10}H_{14}O$	30.16	1330	RI, MS
trans-Sabinene hydrate	0.2	1050	$C_{10}H_{18}O$	15.36	1068	RI, MS
Pulegone	36.3	1214	$C_{10}H_{16}O$	18.11	1221	RI, MS
Bornyl acetate	0.9	1284	$C_{12}H_{20}O_2$		1291	RI, MS
p-cymene	20.63	1223	$C_{10}H_{14}$	35.40	1227	RI, MS
γ-Cadinene	0.1	1531	$C_{15}H_{24}$	38.60	1535	RI, MS
γ-Terpinene	14.85	1050	$C_{10}H_{16}$	11.16	1060	RI, MS

*RI: retention indices calculated on apolar; **RT: retention time (min.); *** LRI: retention indices of literature

Table 2: Lipid profile of streptozotocin-induced rat's liver injury treated with T. vulgaris leaves alcoholic extract.

Groups	TC	HDL-C	LDL-C	VLDL
Control 1	98.36±2.96	62.5± 3.45	26.35±2.1	20.72±1.96
Control 2	120.45±3.35	49.68±3.39	31.12±2.86	24.66±2.14
100	115.25±2.58	55.85±2.98	29.36±2.42	22.45±1.86
200	109.18±2.76	60.19±3.56	27.56±2.21	21.59±1.79
400	102.12±2.47	61.95±2.15	26.78±1.84	21.36±1.44

Data are presented as the means ± S.D of Five replicates. Data analyzed by T-test, P < 0.001, Value with the same letter has no significant but value with different letter has significant at 0.05. TC; total cholesterol, HDL-C; high density lipoprotein cholesterol, VLDL-C; very low density lipoprotein cholesterol, LDL-C; low density lipoprotein cholesterol.

Figures

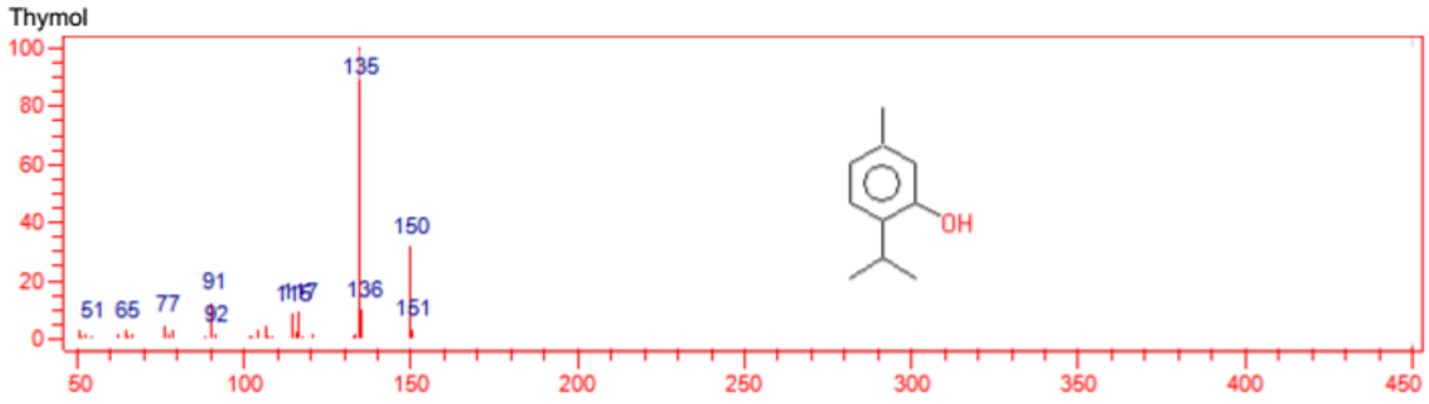

Figure 1

Thymus vulgaris. viridulum GC/MS chromatogram. Thymol.

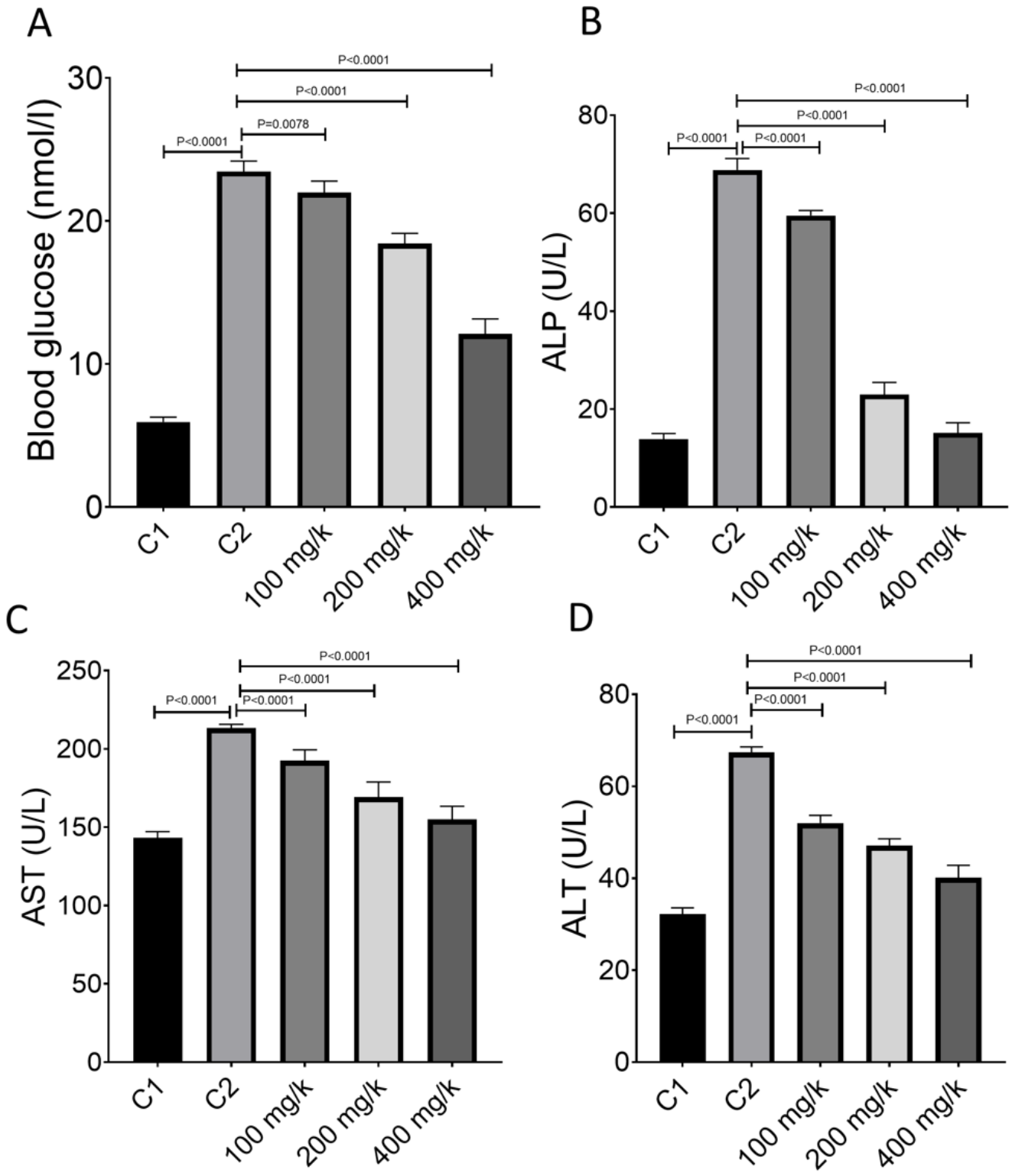

Figure 2

C1) control group, C2) Diabetic group, 100 mg/k, 200 mg/k and 400 mg/k) STZ+ Thymus Vulgaris.

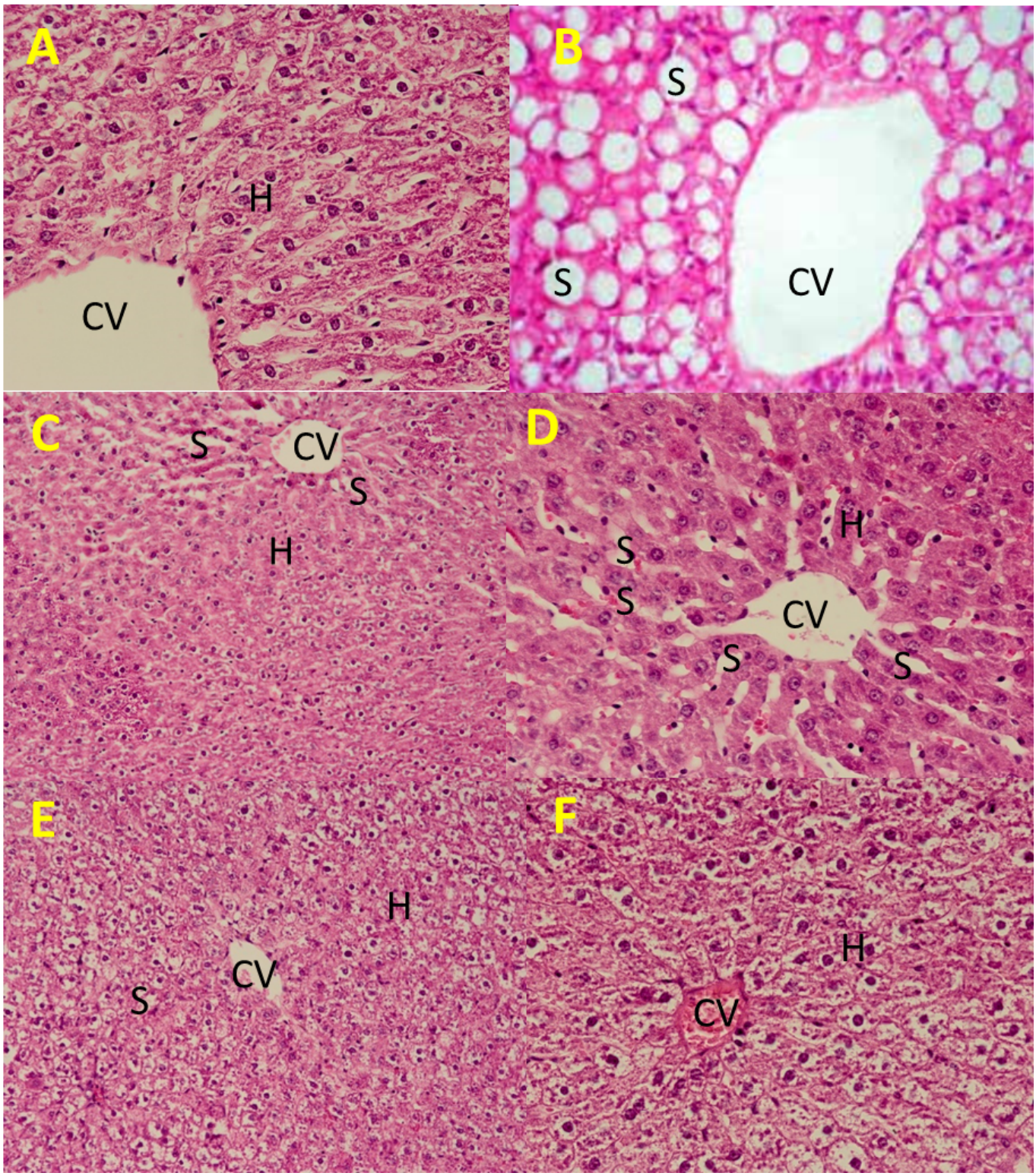

Figure 3

A photomicrograph of liver sections from: A) negative control rat showed normal structure of liver tissue; B) control 2(), C-F) Treated rats with extract and STZ showed normalization of liver tissue but with fine dilatation of main blood vessels and sinusoids. CV: Central Venuse Catheter, H: Hepatocyte, S: sinusoid (H& E × 200).

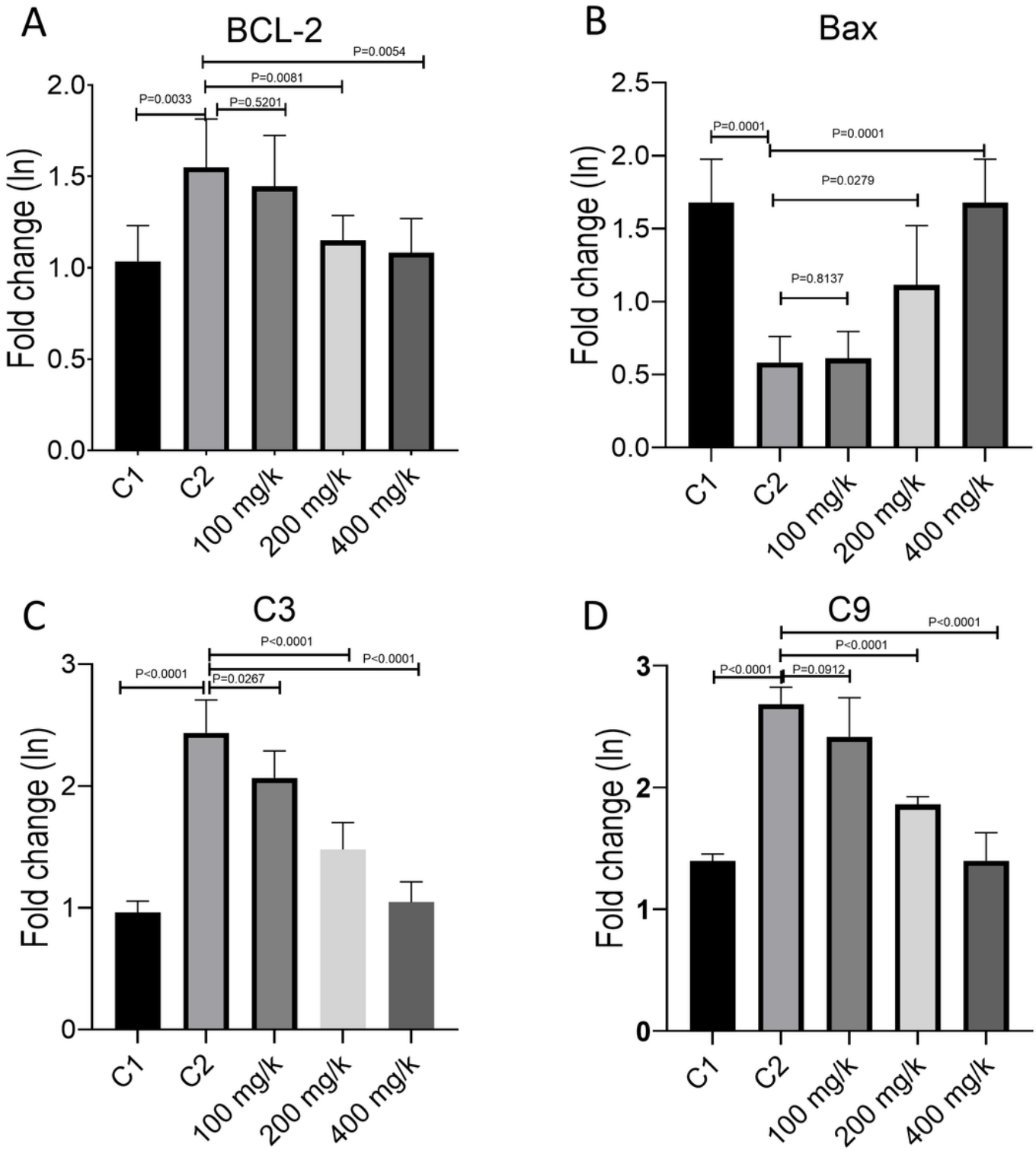

Figure 4

C1) control group, C2) Diabetic group, 100 mg/k, 200 mg/k and 400 mg/k) STZ+ Thymus Vulgaris.