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RESEARCH ARTICLE

Comparative effects of metformin and *Cistus laurifolius* L. extract in streptozotocin-induced diabetic rat model: oxidative, inflammatory, apoptotic, and histopathological analyzes

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Abstract

Interest in phytochemical therapy methods in the treatment of diabetes is increasing day by day. Although the antidiabetic and antioxidant effects of *Cistus laurifolius* L. (CL) have been mentioned, the systemic effects remain unknown. The present study aims at evaluating the antidiabetic effects of the CL aqueous extract via metformin on streptozotocin (STZ)-induced diabetic rats. Forty male *Wistar albino* rats were divided into five groups of eight animals each: control, diabetic group (55mg/kg STZ), STZ+125mg/kg CL, STZ+250mg/kg CL, and STZ+100mg/kg metformin. The effects of CL and metformin on oxidative, apoptotic, and inflammatory pathways were comparatively investigated. In addition, nuclear factor-κB (NFκB), tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-1β expressions analysis were carried out. CL treatment resulted in a significant improvement in blood glucose levels, lipid profile, pancreatic markers, and liver and kidney function tests. A 250mg/kg CL treatment decreased by 67.9%, 31.6%, 66.8%, 28.3%, and 31.4% in the total oxidant capacity, NFκB, TNF-α, IL-1β, caspase3, and cytochrome c levels, respectively, compared to the diabetic group. Additionally, CL treatments showed a dose-dependent reduction in NFκB, TNF-α, and IL-1β expression levels. A 250mg/kg CL treatment exhibited a greater increase (by 9.6%) in total antioxidant capacity than metformin. CL treatment provided histologically more improvement in the brain, heart, pancreas, spleen, liver, kidney, and testicular tissues compared to the metformin group. Our results suggest that the single treatment of CL aqueous extract at the low doses may have stronger short-term anti-diabetic effects than metformin. Therefore, further studies are needed regarding the long-term hypoglycemic effect or treatment of CL aqueous extract.

Keywords Diabetes · *Cistus laurifolius* L. · Metformin · Oxidative stress · Apoptosis · Inflammation · Histopathology

Introduction

Diabetes is a multifactor metabolic disease that causes significant health abnormalities such as neuropathy, nephropathy, and cardiovascular diseases (Lee et al. 2012). There are two main types of diabetes; type I is determined by reduced insulin production, which causes the destruction of T cell-mediated pancreatic beta cells, and the other is type II defined by insulin

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resistance and increased glucagon production (Leslie et al. 2016). Diabetes is a serious global health problem that affects over 425 million people aged 20 to 79 years. Besides, the diabetes prevalence is expected to be 377 million in 2030, and if there is no attempt to reduce the global burden of diabetes, the number of patients may increase to 649 million by 2045 (International Diabetes Federation 2017).

Excessive formation of reactive oxygen species (ROS) is the primary cause of chronic hyperglycemia. Extremely high levels of ROS and the simultaneous deterioration of the antioxidant defense mechanism might lead to the development of insulin resistance, increased lipid peroxidation, and cellular damage (Rehman and Akash 2017). The release of hyperglycemia-induced ROS increases the pro-inflammatory protein levels that could cause local and systemic inflammation by altering the redox balance. Redox-sensitive nuclear factor-κB (NFκB), strategically located at the junction between oxidative stress and inflammation, is involved in the expression of many inflammatory markers [tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), etc.] and cell activation responsible for immune responses (Muriach et al. 2014). Additionally, TNF-α, one of the major cytokines secreted during inflammation, initiates the activation of the caspase cascade, which contributes to the induction of apoptosis by modulating NFκB (Van Opdenbosch and Lamkanfi 2019).

Metformin (*N*, *N*-dimethyl biguanide) as an antihyperglycemic agent has been used for the treatment of diabetes for many years. Recently, in vitro and in vivo studies have revealed the versatile properties of metformin, which reduces hyperinsulinemia and weight gain, regulates lipid profile, overcomes cancer, and inhibits inflammatory processes (Kothari et al. 2016). Metformin is recommended as an adjunct therapy in diabetes, as it reduces glucose levels more effectively than insulin therapy and gradually reduces the need for insulin (Livingstone et al. 2017). Therefore, metformin is a diabetes drug that acts mostly as an insulin sensitizer, so it may be a suitable control in a diabetes model. Additionally, metformin exhibits anti-inflammatory and anti-apoptotic effects that reduce the synthesis of pro-inflammatory cytokines and inhibit NFκB activity. NFκB is stimulated apoptotic protein levels (e.g., cytochrome c and caspases) and oxidative stress. Interestingly, some researchers were reported that metformin caused the overproduction of ROS, and also increased apoptosis by downregulating the expression of anti-apoptotic proteins and NFκB activation (Sena et al. 2018). However, there are contradictory data that metformin has inductive and suppressive effects on apoptotic processes.

Recently, there has been a growing interest in herbal therapies to treat diabetes. Many traditional herbal extracts are used to treat various diseases. One of these plants, *Cistus laurifolius* L., is used to reduce hyperglycemia and inflammatory diseases (Polat and Satıl 2012). Research by Sadhu et al. (2006) demonstrated that the bioactive content of extracts from the leaves and branches of *Cistus laurifolius* L. was analyzed by nuclear magnetic resonance and mass spectrometry techniques. They emphasized that *Cistus laurifolius* L. could scavenge free radicals thanks to its antioxidant properties. Likewise, Loizzo et al. (2013) reported that essential oils isolated from different *Cistus* species exhibited antioxidant activity as well as acetylcholinesterase and butyrylcholinesterase inhibitory effects. In a previous study, researchers suggested that there was a positive correlation between the phenolic content of the *Cistus laurifolius* L. extract and its inhibitory activity on α-glucosidase and α-amylase (Orhan et al. 2013). Moreover, this study revealed that the plant contained various phenolic components that could regulate many metabolic pathways, particularly ROS-related oxidative stress. Additionally, extracts isolated from various species of the Cistaceae family have promising evidence in the treatment of inflammatory diseases. Kupeli and Yesilada (2007) reported that extracts from leaves and branches of *Cistus laurifolius* L exhibited potent inhibitory effects on carrageenan-induced hind paw edema and acetic acid-induced increased vascular permeability in mice. Similarly, *Cistus salviifolius* L. and *Cistus monspeliensis* L. aqueous extracts from Morocco caused significant inhibition in the treatment of inflammatory diseases such as paw edema and showed analgesic activities in nociceptive models (Sayah et al. 2017). However, although the antioxidant and inflammatory effects of *Cistus laurifolius* L. have been investigated more extensively in the literature, the evidence for its antidiabetic effects remains limited. On the other hand, there is still uncertainty about the systemic effects of *Cistus laurifolius* L. on tissues.

In the present study, we aimed at comparatively investigating the potential roles of metformin and the *Cistus laurifolius* L. aqueous extract in the oxidative, apoptotic, and inflammatory pathways in streptozotocin (STZ)-induced diabetic rats. Therefore, in order to examine the systemic effects of metformin and aqueous *Cistus laurifolius* L. extract, the brain, heart, spleen, liver, pancreas, kidney, and testicular tissues of diabetic rats were analyzed. To determine the diabetes-related complications, total oxidant status (TOS), total antioxidant status (TAS), caspase 3 (CASP3) activities, cytochrome c (CYC) levels, NFκB levels, TNF-α levels, and interleukin-1 beta (IL-1β) levels were measured biochemically in diabetic rats; histopathological examinations were performed with hematoxylin-eosin staining.

Materials and methods

Plant material and extract preparation

Cistus laurifolius L. leaves were used as plant material in the study. The fresh leaves of *Cistus laurifolius* L. were collected from Tandır province of Eskişehir area (Eskişehir, Tandır

Village, 1150 m. North), Turkey, in July 2013 and brought to the laboratory in sterile containers. The identity of the sample was authenticated from the Herbarium of Anadolu University (ANES No. 15518). *Cistus laurifolius* L. extract was prepared according to the method of Sadhu et al. (2006). Following, 40g plant material was boiled in 800 mL distilled water for 1 h. After cooling, the extract was filtered through filter paper, centrifuged at 1032×*g* (Thermo Scientific SL 40) at room temperature, and then concentrated in a rotary evaporator (Thermomac RE1000L) and lyophilized. The extract was stored at +4°C until it was used.

Determination of total flavonoids and phenolic compounds

Total polyphenol and total flavonoid contents of *Cistusa laurifolius L.* were performed using 2,2-diphenyl-1-picrylhydrazil (DPPH, Sigma-Aldrich) with the help of ultraviolet (UV) analysis and Fourier transform infrared spectrophotometer (PerkinElmer FT-IR Spectrometer Frontier). Total phenolic content analysis was performed by Folin-Ciocalteu's phenol reagent method (Kumazawa et al. 2004). Gallic acid (GAE, Merck Millipore) was used as standard and the result of the plant extract was given as mg GAE/g extract. Total flavonoid content analysis was performed by the method based on the formation of an aluminum complex (Ramos et al. 2017). Quercetin (QE, Sigma-Aldrich) was used as the flavonoid standard and the result was given as a sample of mg QE/g extract. The scavenging activity of the plant extract against DPPH radical was performed spectrophotometrically (Pyrzynska and Pekala 2013). In antioxidant activity analysis, the results were given as scavenging 50% (concentration of sample required to SC50 of DPPH radicals) and Trolox (Merck Millipore) was used as the standard for comparison of results. The phenolic component of the extract was analyzed with the high-performance liquid chromatography (HPLC)-UV system (Prominence LC-20A) according to the method specified by Akyuz et al. (2014). This analysis was performed on a reverse-phase Zorbax Eclipse XDB-C18 column (4.6×150 mm, 5 um) using a two mobile phase gradient program. UV-visible absorption was performed with Thermo Scientific Evolution 201 UV-visible Spectrophotometer.

Animals and experimental procedure

Forty male *Wistar albino* rats aged 3–4 months and weighing 280–350 g were used in the experiments. The rats were housed in polypropylene cages and maintained under controlled conditions (24±2°C temperature and 50±5% humidity and 12 hour light/dark cycle). The animals were fed a normal pellet diet and water ad libitum. The rats were allowed to acclimate to the laboratory conditions for 7 days before the experiment. All experimental procedure on animals was confirmed by the Local Ethics Committee of Eskişehir Osmangazi University Animal Experiments (Approval number: 678-1).

Induction of diabetes in rats

Fifty-five milligrams per kilogram STZ (Sigma-Aldrich) injection was administered intraperitoneally to perform the experimental type 1 diabetes model in the rats (Wang-Fischer and Garyantes 2018). STZ was prepared in 0.1 M citrate buffer adjusted to pH 4.5. The animals fasted overnight on the seventh day after STZ injection, and diabetic rats were determined by measuring fasting blood glucose with a commercial glucose meter. Rats with blood glucose levels >200mg/dL were considered diabetic and used for subsequent experiments.

Experimental groups

The rats were divided into five groups, each group consisting of eight animals. According to previous studies, the effective doses of metformin and *Cistus laurifolius* L. were prepared freshly in 0.09% sodium chloride just before the experiments (Kurup and Mini 2017). *Cistus laurifolius* L. and metformin doses were administered as a single dose at the end of the seventh day. Daily body weight changes and feed-water consumption of the rats in all groups were recorded during the experimental period.

- Group I (normal control): The rats in this group were received 1 mL saline orally at the end of the seventh day.
- Group II (diabetic control): Seven days after STZ injection, rats with blood glucose levels above >200 mg/mL were included in this group. Then, 1 mL saline was given orally at the end of the seventh day.
- Group III (STZ+125 mg/kg *C. laurifolius* L.): After the formation of STZ-induced diabetes in rats, the aqueous extract was administered orally 1 mL at a dose of 125 mg/kg once at the end of the seventh day to determine the antidiabetic and metabolic effects of *Cistus laurifolius* L. Following, we measured the blood glucose levels of the rats at 30th, 60th, 120th, and 240th min in the blood (0.1 mL) taken from the tail vein.
- Group IV (STZ+250 mg/kg *C. laurifolius* L.): After the formation of STZ-induced diabetes in rats, the aqueous extract was administered orally 1 mL at a dose of 250 mg/kg once at the end of the seventh day to determine the antidiabetic and metabolic effects of *Cistus laurifolius* L. Subsequently, we measured the blood glucose levels of the rats at 30th, 60th, 120th and 240th min in the blood (0.1 mL) taken from the tail vein.
- Group V (STZ+100 mg/kg metformin): After the formation of STZ-induced diabetes in rats, the metformin (Merck

Millipore) was administered orally 1mL at a dose of 100 mg/kg at the end of the seventh day to determine its antidiabetic and metabolic effects. Following, we measured the blood glucose levels of the rats at 30th, 60th, 120th and 240th min in the blood (0.1 mL) taken from the tail vein.

After the completion of blood glucose measurements, the rats were sacrificed by cervical dislocation under light anesthesia [ketamine (15 mg/kg)-xylazine (2 mg/kg) intramuscularly] and then drawing all blood from the heart. The blood samples were taken into biochemistry tubes and centrifuged at 3000×*g* for 10 min (NUVE NF 400). The brain, heart, pancreas, spleen, liver, kidney, and testicular tissues were reserved for histological examination.

Biochemical measurements

Serum insulin, amylase, lipase, cholesterol, triglyceride, low-density lipoprotein (LDL), high-density lipoprotein (HDL), blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were measured on an autoanalyzer system (Roche Cobas C501). The remaining serum samples were stored at −80°C for other biochemical measurements.

We measured TOS and TAS levels in serum samples by a commercially available kit (Rel Assay Diagnostics, Turkey) using the enzyme-linked immune sorbent assay (ELISA, Epoch, BioTek) method. Briefly, oxidants that increase with oxidative stress oxidize the ferrous ion to ferric ion. Ferric ion forms a colored complex in the acid medium and the resulting color intensity was measured at 530nm. The results were expressed as μmol H_2O_2Equiv./L. Depending on the antioxidant levels in the serum samples, the colored 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) compound is reduced to a colorless form. The change in color intensity was determined at 660 nm. The results were indicated as Trolox Equiv./L.

NFκB, TNF-α, and IL-1β levels in the serum samples were measured using commercially available kits (MyBioSource, USA, Cat no. MBS453975, MBS2507393, and MBS264984, respectively). Shortly, NFκB, TNF-α, and IL-1 levels in the serum samples were determined using a microplate reader (Epoch, BioTek) at 450 nm according to the manufacturer's instructions. The NFκB, TNF-α, and IL-1β results were showed as ng/mL, pg/mL and μg/mL, respectively.

To determine apoptosis, CASP3 and CYC levels in the serum samples were measured using commercially available kits (Cloud-Clone Corp., USA, Cat no. SEA626Ra and SEA594Ra, respectively). CASP3 and CYC levels were measured in the microplate reader (Epoch, BioTek) at 450 nm according to the manufacturer's instructions. The results were presented as ng/mL.

Quantitative real-time reverse transcriptase polymerase chain reaction

Total RNA of the brain, heart, pancreas, spleen, liver, kidney, and testis was isolated using an RNA extension kit (BS88136). Total RNAs isolated from tissues were transcribed to complementary DNA (cDNA) using the SuperScriptTM IV Single Step RT-PCR System. SYBR® Green Master Mix was used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, and StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) was used to amplify cDNA.

The NFκB (p65 subunit), TNF-α, IL-1β, and β-actin primers were as follows: NFκB forward primer 5'-ACG ATC TGT TTC CCC TCA TCT-3' and the reverse primer 5'-TGC TTC TCT CCC CAG GAA TA-3'; TNF-α forward primer 5'-GGG CTT GTC ACT CGA GTT TT-3' and the reverse primer 5'-TGC CTC AGC CTC TTC TCA T-3'; IL-1β forward primer 5'-CAC CTC TCA AGC AGA GCA CAG-3' and the reverse primer 5'-GGG TTC CAT GGT GAA GTC AAC-3'; β-actin forward primer 5'-GTG GGG CGC CCC AGG CAC CA-3' and the reverse primer 5'-CTT CCT TAA TGT CAC GCA CGA TTT C-3'.

β-actin mRNA levels were used for an internal standard and normalization of expression data. mRNA expression was determined using the $2^{-\Delta\Delta CT}$.

Histopathological examinations

Immediately after decapitation, the brain, heart, pancreas, spleen, liver, kidney, and testicular tissues of all groups were fixed for 24 h in 10% neutral formaldehyde for histological analysis. Following chemical fixation, tissues were dehydrated by passing 70%, 80%, 90% (1), 90% (1), 96% (1), 96% (2), and absolute ethyl alcohol for 1 h, respectively, in the ethyl alcohol series. Next, the tissues were made transparent by the application of xylol 2 times for 30 min, and the tissues were parafinized by keeping in the oven at 60°C for 3 h. The paraffin-embedded tissue blocks were sectioned 4 μm thick with a microtome (Leica RM 2025). Sections were stained using hematoxylin-eosin (H&E) and the preparations were closed with Entellan. Histopathological examination of H&E stained tissue sections under light microscopy was performed. H&E imaging was done with the Olympus BH-2 microscope and the Olympus DP-70 digital camera. Histomorphological changes in the tissues were evaluated in terms of the following features: cellular loss, cortical area injury, dilated vascular structures, necrotic cell structures, cellular inflammation, edema, vascular congestion, pericentral area, and karyolysis. In this study, tissues were evaluated by light microscopy.

Statistical analysis

The results were presented as means±standard deviation (SD). Statistical analyses between experimental groups were performed by GraphPad Prism 7.0 statistical software (San Diego, CA). One-way analysis of variance (ANOVA) was used for statistical analysis of the results. Differences between the groups were tested by Tukey's multiple comparison post hoc tests. $P < 0.05$ was considered to be statistically significant.

Results

Phytochemical analysis of the *Cistus laurifolius* L. aqueous extract

Total polyphenol, total flavonoid content, and SC50 level of *Cistus laurifolius* L. aqueous extract were 86.1168±1.46 mg GAE/g, 15.0476±0.68 mg QE/g, and SC50 was 0.0399±0.005 mg/mL, respectively.

The phenolic content analysis of the sample was performed using the following standards; gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, catechin, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, rutin, myricetin, resveratrol, daidzein, luteolin, *trans*-cinnamic acid, hesperetin, chrysin, pinocembrin, and caffeic acid phenyl ester (CAPE). Catechin and myricetin were not detected in the *Cistus laurifolius* L. extract. However, rutin and gallic acid were the main compounds that were defined in Table 1.

The phenolic chromatogram of the *Cistus laurifolius* L. extract was seen in Fig. 1. The extract exhibited maximum absorbance at 205 nm in the UV-visible area and showed some absorbances at 266 and 361 nm. This showed that the extract differed from the standard phenolic components.

According to the FT-IR analysis, the hydroxyl peak at 3244 cm^{-1} suggested that the extract had phenolic components. Furthermore, the phenolic components in the extract were shown with the carboxyl and carbon-oxygen stretching peaks at 1599 cm^{-1} and 1043 cm^{-1}, respectively (Fig. 1).

Effects of *Cistus laurifolius* L. and metformin on body weights, daily feed, and water consumption in STZ-induced rats

The effects of *Cistus laurifolius* L. and metformin on the body weight (BW) in the STZ-induced rats were shown in Table 2. After STZ injection, we observed that BW decreased by 14% in the STZ-induced diabetic rats ($P < 0.05$) whereas BW increased in the control group (3.6% increase). The 125 mg/kg *Cistus laurifolius* L., 250 mg/kg *Cistus laurifolius* L., and 100 mg/kg metformin treatment showed an increase by 4.7%, 14%, and 18.5%, respectively, in the diabetic rats BW ($P < 0.01$).

Table 1 Phenolic content analysis results of *Cistus laurifolius* L. aqueous extract (mg phenolic/g extract). *n.d.* not detected

Standards	*Cistus laurifolius* L. extract
Gallic acid	4.7538
Protocatechuic acid	0.7413
p-OH Benzoic acid	2.6165
Catechin	n.d.
Caffeic acid	0.0974
Syringic acid	0.0482
Epicatechin	0.1249
p-Coumaric acid	0.0263
Ferulic acid	0.1297
Rutin	7.2038
Myrecetin	n.d.
Resveratrol	0.0863
Daidzein	0.0259
Luteolin	0.0830
trans-Cinnamic acid	0.0042
Hesperetin	0.0156
Chrysin	0.456
Pinocembrin	0.217
CAPE	0.0429

The STZ-induced rats demonstrated typical features of diabetes such as polyuria, polydipsia, increased water and food intake, dehydration, and weight loss. Daily dietary and water consumption in diabetic rats increased through polyuria and weight loss (Table 3). *Cistus laurifolius* L. treatments showed a dose-dependent decrease in feed and water consumption in the STZ-induced rats ($P < 0.01$). Besides, metformin treatment caused the lowest feed consumption ratio among the experimental groups.

Effects of *Cistus laurifolius* L. and metformin treatments on blood glucose levels in the diabetic rats

The effects of *Cistus laurifolius* L. and metformin treatments on blood glucose levels of the experimental groups were indicated in Table 4. The blood glucose levels were measured in all groups at 0th, 30th, 60th, 120th, and 240th min following the treatments. The blood glucose levels were significantly enhanced in the diabetic rats compared to the normal rats ($P < 0.01$). The *Cistus laurifolius* L. treatments reduced the blood glucose levels in a time-dependent manner. Furthermore, metformin treatment increased the blood glucose in the first 30 min and decreased following periods. The 125 mg/kg *Cistus laurifolius* L., 250 mg/kg *Cistus laurifolius* L., and 100 mg/kg metformin treatments reduced the blood glucose levels between 0th and 240th min by 15.1%, 30.3%, and 22.4%, respectively

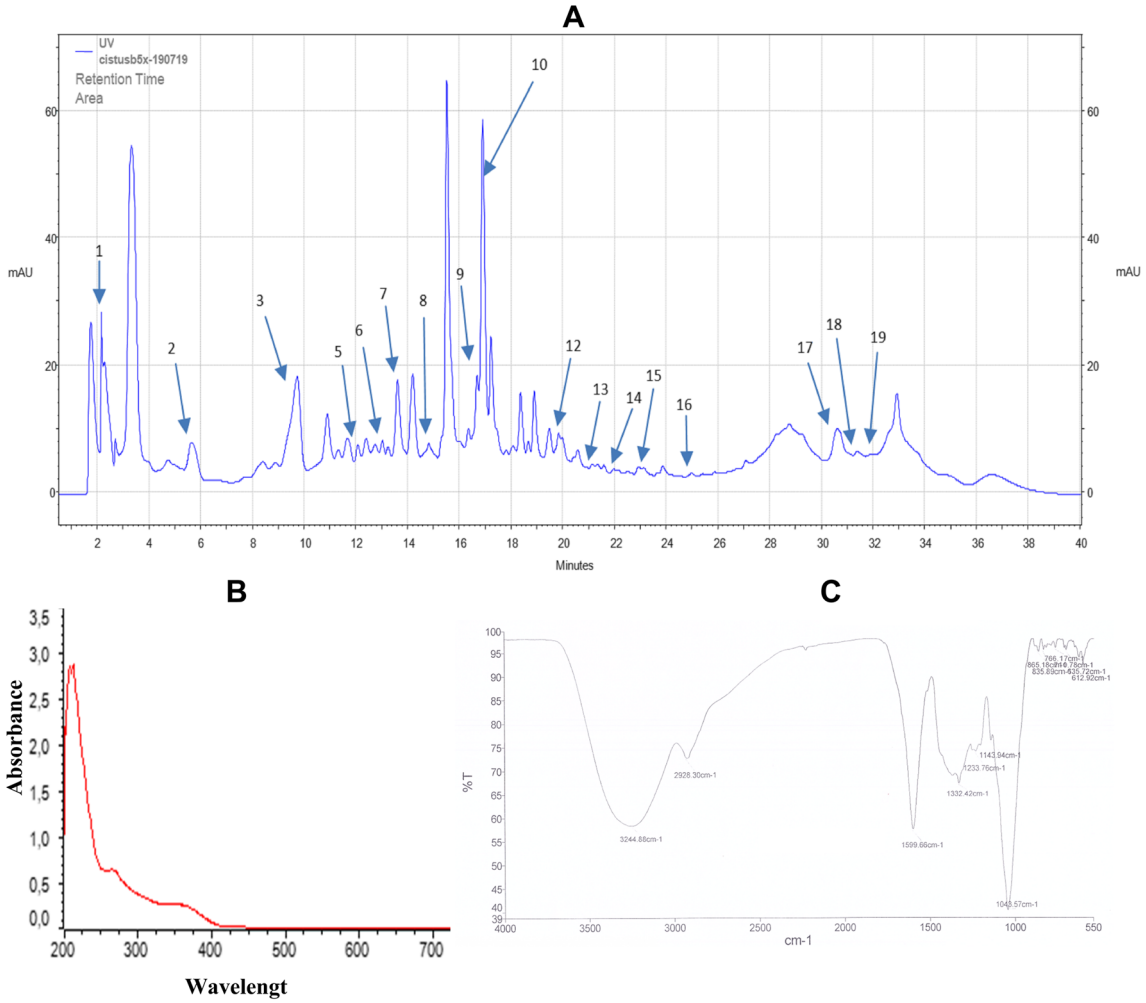

Fig. 1 Phytochemical analysis of the *Cistus laurifolius* L. aqueous extract. **A** Chromatogram of phenolic content analysis. **B** UV-visible spectra. **C** FT-IR spectra. 1: Gallic acid; 2: protocatechuic acid; 3: *p*-OH benzoic acid; 4: catechin; 5: caffeic acid; 6: syringic acid; 7: epicatechin; 8: *p*-coumaric acid; 9: ferulic acid; 10: rutin; 11: myrecetin; 12: resveratrol; 13: daidzein; 14: luteolin; 15: *trans*-cinnamic acid; 16: hesperetin; 17: chrysin; 18: pinocembrin; 19: CAPE

(P <0.01). The blood glucose levels in the STZ+250 mg/kg *Cistus laurifolius* L. group at 240th min reduced by 7.1% compared to the STZ+125 mg/kg *Cistus laurifolius* L. group (P <0.05). Additionally, metformin treatment decreased the blood glucose levels by 21.5% and 16.1%, respectively, compared to the STZ+250 mg/kg *Cistus laurifolius* L. and STZ+125 mg/kg *Cistus laurifolius* L. groups at 240th min (P <0.01).

Effects of *Cistus laurifolius* L. and metformin treatments on biochemical parameters in the diabetic rats

Table 5 showed the pancreatic (insulin, amylase, and lipase levels), hepatic (AST and ALT levels), and renal (BUN and creatinine levels) markers and lipid profile in all groups. The insulin, amylase, and lipase levels were significantly reduced

Table 2 Body-weight of the rats at the beginning and end of the experiment belonging to all experimental groups. *BW* body-weight; *STZ* streptozotocin; * compared with control group P <0.05; # compared with STZ group P <0.01

Groups	Initial (BW) (g) (1st day)	Final (BW) (g) (7th day)
Normal control	286.75±3.37	288.65±5.82
Diabetic control	289.15±3.48 *	265.87±4.12 *
STZ+125 mg/kg *C. laurifolius* L.	291.24±2.69 *, #	250.37±6.31 *, #
STZ+250 mg/kg *C. laurifolius* L.	293.83±4.59 *, #	247.36±3.72 *, #
STZ+100 mg/kg Metformin	290.71±2.36 *, #	233.42±5.25 *, #

Table 3 Daily food and water intake mean values in all experimental groups for 7 days. *BW* body weight; *STZ* streptozotocin; * compared with control group $P < 0.01$; # compared with STZ group $P < 0.01$

Groups	Daily food intake (g/BW)	Daily water intake (mL/BW)
Normal control	48.37±1.06	51.84±2.61
Diabetic control	52.25±1.28 *	219.84±10.17 *
STZ+125 mg/kg *C. laurifolius* L.	51.77±1.83 *, #	165.35±8.19 *, #
STZ+250 mg/kg *C. laurifolius* L.	53.73±1.36 *, #	152.42±11.04 *, #
STZ+100 mg/kg metformin	50.12±1.58	196.66±7.21 *, #

in STZ-induced diabetic rats ($P < 0.01$ vs control group). *Cistus laurifolius* L. and metformin treatments indicated a statistically significant increase in insulin, lipase, and amylase levels ($P < 0.01$ vs control and diabetic groups). However, AST, ALT, BUN, and creatinine levels were increased in diabetic rats compared to the control group ($P < 0.01$). *Cistus laurifolius* L. and metformin treatments resulted in a significant improvement in the liver and renal function tests ($P < 0.01$ vs diabetic group). *Cistus laurifolius* L. treatments showed a dose-dependent decrease in AST, BUN, and creatinine levels. The diabetic rats showed an increase in triglyceride, cholesterol, and LDL levels and a decrease in HDL levels ($P < 0.01$ vs control group). Conversely, diabetic rats treated with *Cistus laurifolius* L. and metformin were significantly reduced in triglyceride, cholesterol, and LDL levels and enhanced in HDL levels ($P < 0.01$ vs diabetic group). Interestingly, 25 mg/kg *Cistus laurifolius* L. treatment increased the insulin levels by 11.4% and 6.2%, respectively, compared to the STZ+125 mg/kg *Cistus laurifolius* L. and metformin groups ($P < 0.05$).

Table 6 showed the antioxidant and oxidant status and inflammatory and apoptotic biomarkers in all groups. We found that oxidative stress, inflammation, and apoptosis increased in STZ-induced diabetic rats whereas antioxidant levels decreased ($P < 0.01$ vs control group). After *Cistus laurifolius* L. treatments, TOS, NFκB, IL-1β, CASP3, and CYC levels showed a statistically significant dose-dependent decrease in the diabetic rats. In addition, TOS, NFκB, TNF-α, IL-1β, CASP3, and CYC levels were decreased by 39.1%, 18.5%, 40.3%, 12.8%, 34.8%, and 45.9%, respectively, in the 125 mg/kg *Cistus laurifolius* L. group ($P < 0.01$ vs diabetic group). Similarly, TOS, NFκB, TNF-α, IL-1β, CASP3, CYC, and TAS levels showed 45.2%, 24.1%, 33.6%, 22.1%, 53.3%, and 56.8% decreases, respectively, in the 250 mg/kg *Cistus laurifolius* L. group ($P < 0.01$ vs diabetic group). Metformin treatment also decreased TOS, NFκB, TNF-α, IL-1β, CASP3, and CYC levels by 25.2%, 15.8%, 41.8%, 16.3%, 31.2%, and 40.1%, respectively ($P < 0.01$ vs diabetic group). The 125 mg/kg *Cistus laurifolius* L., 250 mg/kg *Cistus laurifolius* L., and 100 mg/kg metformin treatments showed an increase by 37.9%, 69.7%, and 55.1% in the TAS levels, respectively ($P < 0.01$ vs diabetic group). Besides, 250 mg/kg *Cistus laurifolius* L. treatment showed a further decrease in TOS, NFκB, IL-1β, CASP3, and CYC levels compared to 125 mg/kg *Cistus laurifolius* L. and 100 mg/kg metformin groups ($P < 0.01$).

Effects of *Cistus laurifolius* L. and metformin treatments on NFκB, TNF-α, and IL-1β expressions in the diabetic rats

The effects of *Cistus laurifolius* L. and metformin treatments on the expression levels of NFκB (p65 subunit), TNF-α, and IL-1β were analyzed in the brain, heart, pancreas, spleen, liver, kidney, and testis by qRT-PCR. As shown in Fig. 2, NFκB, TNF-α, and IL-1β expressions

Table 4 Effects of *Cistus laurifolius* L. aqueous extract and metformin treatments on blood glucose values in all experimental groups. *SD* standard deviation; *STZ* streptozotocin; * compared with control group $P < 0.01$; # compared with STZ group $P < 0.01$; & before *Cistus laurifolius* L. and metformin treatment

Groups	Mean blood glucose concentrations (mg/dL)±SD				
	Initial& (7th day)	30th min	60th min	120th min	240th min
Normal control	52.75±8.13	56.28±4.77	66.14±10.29	61.24±3.78	58.62±5.94
Diabetic control	389.28±15.38 *	363.58±21.75 *	365.37±14.35 *	367.52±15.82 *	355.17±15.82 *
STZ+125 mg/kg *C. laurifolius* L.	331.14±30.11 *	316.82±17.04 *, #	278.53±35.69 *, #	273.21±41.74 *, #	270.21±17.39 *, #
STZ+250 mg/kg *C. laurifolius* L.	413.28±64.37 *, #	371.34±21.55 *, #	330.73±46.88 *	300.5±19.14 *, #	288.12±33.45 *, #
STZ+100 mg/kg metformin	395.83±28.71 *, #	320.25±12.29*, #	307.59±13.71 *, #	271.46±16.52 *, #	229.63±25.48 *, #

Table 5 Effects of *Cistus laurifolius* L. aqueous extract and metformin on serum parameters. *STZ* streptozotocin; *AST* aspartate aminotransferase; *ALT* alanine aminotransferase; *LDL* low-density lipoprotein; *HDL* high-density lipoprotein; *BUN* blood urea nitrogen; * compared with control group $P < 0.01$; # compared with STZ group $P < 0.01$

Groups ↓	Insulin (μU/mL)	Lipase (U/L)	Amylase (U/L)	AST (U/L)	ALT (U/L)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)
Normal control	21.4±1.8	40.8±3.5	1347.2±61.5	131.1±15.9	39.5±5.7	32.9±6,.2	33.5±1.8	8.3±1.4	19.5±2.6	19.4±3.6	0.33±0.08
Diabetic control	9.5±2.2 *	21.5±3.2 *	811.5±43.8 *	164.7±9.5 *	73.8±9.6 *	48.1±3.8 *	47.5±2.2 *	11.6±1.8 *	14.3±2.1 *	48.5±4.2 *	0.64±0.1 *
STZ+125 mg/kg *C. laurifolius* L.	13.1±1.6 *, #	26.9±1.4 *, #	1098.6±52.4 *, #	110.3±16.9 *, #	62.4±4.1 *, #	35.63±1.4 #	35.1±2.9 *, #	7.2±1.6 #	23.8±1.8 *, #	35.1±1.3 *, #	0.4±0.02 *, #
STZ+250 mg/kg *C. laurifolius* L.	14.8±2.3 *, #	29.1±2.5 *, #	1023.8±33.1 *, #	122.7±6.8 *, #	57.5±6.3 *, #	40.6±2.8 *, #	27.6±3.1 *, #	4.8±1.1 *, #	30.4±3.7 *, #	28.4±1.5 *, #	0.23±0.04 *, #
STZ+100 mg/kg metformin	14.1±3.7 *, #	31.2±4.6 *, #	1134.6±48.3 *, #	127.5±11.3 *, #	98.6±10.2 *, #	57.4±1.2 *, #	40.5±1.7 *, #	6.4±0.7 *, #	31.1±4.6 *, #	32.7±2.1 *, #	0.37±0,.05 *, #

Table 6 Effects of *Cistus laurifolius* L. aqueous extract and metformin on oxidative, inflammatory, and apoptotic pathways. *STZ* streptozotocin; *TOS* total oxidant status; *TAS* total antioxidant status; *NFκB* nuclear factor-κB; *TNF-α* tumor necrosis factor-alpha; *IL-1β* interleukin-1 beta; *CASP3* caspase 3; *CYC* cytochrome c; * compared with control group $P < 0.01$; # compared with STZ group $P < 0.01$

Groups ↓	TOS (μmol H_2O_2Equiv./L)	TAS (Trolox Equiv./L)	NFκB (ng/mL)	TNF-α (pg/mL)	IL-1β (μg/mL)	CASP3 (ng/mL)	CYC (ng/mL)
Normal control	8.4±1.8	50.8±3.5	811.5±43.8	131.1±15.9	39.5±5.7	32.9±6.2	33.5±1.8
Diabetic control	21.5±2.2 *	19.5±3.2 *	1347.2±61.5 *	184.7±14.5 *	73.8±1.4 *	76.1±8.8 *	68.5±2.2 *
STZ+125 mg/kg *C. laurifolius* L.	14.1±1.6 *, #	26.9±1.4 *, #	1098.6±52.4 *, #	118.3±16.9 *, #	62.4±1.1 *, #	49.6±7.4 #	37.1±2.9 *, #
STZ+250 mg/kg *C. laurifolius* L.	12.8±1.3 *, #	33.1±2.5 *, #	1023.8±33.1 *, #	110.7±4.8 *, #	57.5±3.6 *, #	35.6±5.8 *, #	29.6±3.1 *, #
STZ+100 mg/kg metformin	16.1±2.7 *, #	30.2±4.6 *, #	1134.6±48.3 *, #	105.1±10.3 *, #	61.8±2.5 *, #	52.4±6.2 *, #	40.5±2.7 *, #

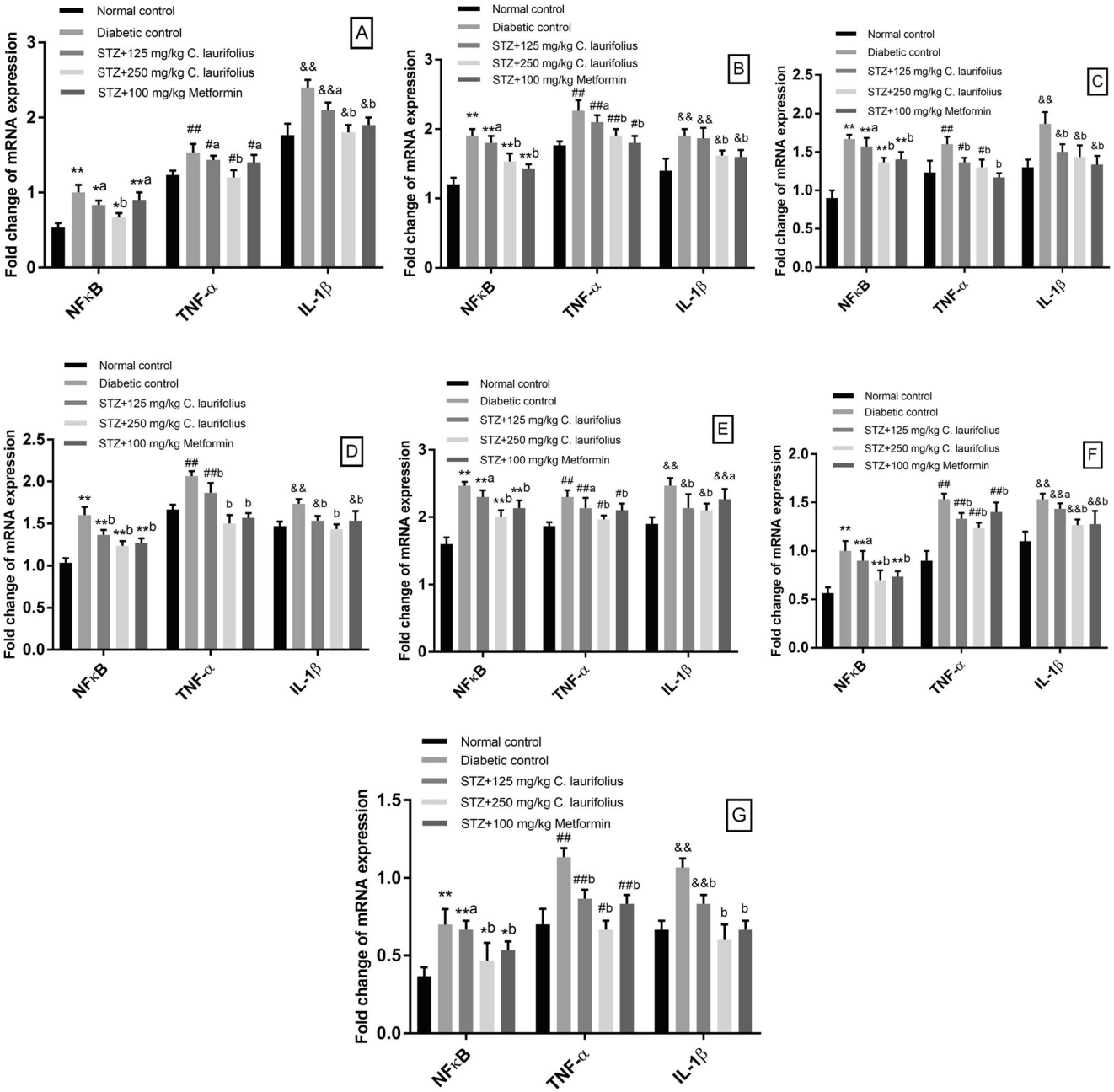

Fig. 2 Effect of *Cistus laurifolius* L. and metformin on NFκB, TNF-α, and IL-1β mRNA expressions in the diabetic rats. **A** Brain, **B** heart, **C** pancreas, **D** spleen, **E** liver, **F** kidney, and **G** testis. * (P <0.05) and ** (P <0.01) compared to the control for NFκB; # (P <0.05) and ## (P <0.01) compared to the control for TNF-α; & (P <0.05) and && (P <0.01) compared to the control for IL-1β. a (P <0.05) compared to the diabetic group; b (P <0.01) compared to the diabetic group

were significantly enhanced in the STZ-induced diabetic rats (P <0.01). Consistent with the ELISA results, *Cistus laurifolius* L. treatment showed remarkably a dose-dependent manner decrease in NFκB, TNF-α, and IL-1β expressions in the diabetic rats. In addition, diabetic rats treated with metformin strongly downregulated NFκB, TNF-α, and IL-1β mRNA levels compared to the STZ-induced diabetic group.

Effects of *Cistus laurifolius* L. and metformin treatments on histopathological changes in STZ-induced diabetic rats

Normal-looking neurons and glial cells were detected in the control group (Fig. 3). Cortical area injury, dilated vascular structures, and necrotic cell structures were observed in the diabetic rats' brain tissues. In the diabetic rats treated with 125

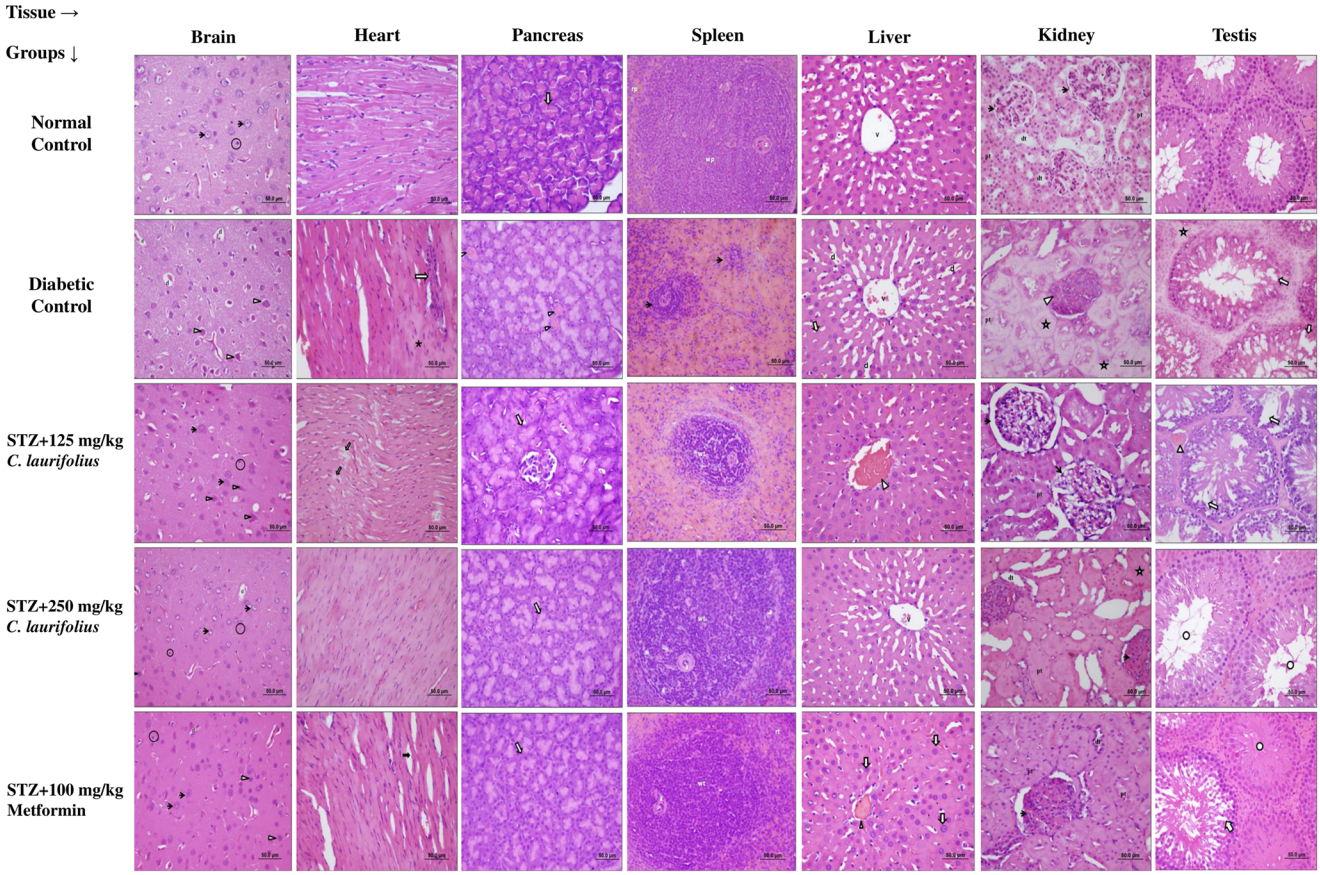

Fig. 3 Hematoxylin-eosin stained light microscopy images of the brain, heart, pancreas, spleen, liver, kidney, and testis tissues of rats in all experimental groups. →: Necrotic cell, ○: Dilated vessel structure, ➡: Congestions,⇨: Aciner cell, *: Necrotic muscle fibers, a: Arteriola centralis, wp: White pulp, rp: Red pulp, v: Vena centralis, k: Karyolysis, dt: Distal tubule, pt: Proximal tubule, #: Seminiferous tubule. Scale bar: 50μm

mg/kg *Cistus laurifolius* L., several dilated vascular structures and necrotic cells were seen in the cortical area, but there was improved cortical area damage compared to the STZ-induced group. Furthermore, a few numbers of necrotic cells in the cortical area and neuronal and glial cell structures were seen in the 250 mg/kg *Cistus laurifolius* L. treatment group. Additionally, a small number of necrotic cells and normal-looking neurons and glial cells were observed in the metformin treatment group.

Muscle cells with normal histological structure and no cellular inflammation, edema, and necrosis were monitored in the control group heart tissues (Fig. 3). Myofibrillar degeneration, necrotic muscle fibers, perivascular inflammation, and congestion were seen in the heart muscle cells of the diabetic group. The 125 mg/kg *Cistus laurifolius* L. treatment showed asymmetric muscle fibers in some places. Moreover, myofibrillar degeneration, cellular inflammation, and edema were not detected in the 250 mg/kg *Cistus laurifolius* L. treatment. However, degenerations of the heart muscle fibers were determined in the 100 mg/kg metformin treatment.

Figure 3 showed normal Langerhans islet histology and serous acinus structures in the control group pancreatic tissue. In the STZ-induced rat pancreas, there was vascular congestion and inflammation in the interlobular area. *Cistus laurifolius* L. treatments showed a significant decrease in pancreatic damage and normal-looking acinar cell structures compared to the diabetic group. On the other hand, histology of partial vascular congestion in the interlobular region was detected in the metformin treatment group.

Parenchymal tissue damage, white pulp degeneration, and red pulp congestion were detected in the diabetic rat spleens (Fig. 3). *Cistus laurifolius* L. treatments in the STZ-induced rats showed improvement in the white pulp, red pulp, and arteriola centralis structures. Conversely, metformin treatment showed less amelioration in spleen parenchyma tissue than *Cistus laurifolius* L. treatments.

The diabetic rats showed necrotic areas in the liver tissues, sinusoidal dilatation in the pericentral area, and karyolysis in several hepatocyte cells (Fig. 3). Normal hepatocyte cells, sinusoidal structures, and vena centralis structures were determined in the 250 mg/kg *Cistus laurifolius* L. treated group. However, the congestions of vena centralis were occasionally seen in the 125 mg/kg *Cistus laurifolius* L. and metformin treatments.

Malpighi bodies and tubular structures with normal histological appearance were observed in the control group kidney tissues, whereas the STZ-induced diabetic rats were seen necrotic tubules and Bowman's capsule contraction in the renal cortex (Fig. 3). The reduction of renal cortex damage appeared in *Cistus laurifolius* L. treatments. Besides, renal tubular structures in the metformin group had similar histology to the control group.

Normal-looking seminiferous tubules, spermatogenic cell series in the tubule wall, Sertoli cells, spermatogonium, primary spermatocytes, spermatogenesis, and spermatogenesis were seen in the control group rat testis (Fig. 3). Contrarily, cellular losses in the tubule wall, necrotic tissue, and edema in the interstitial area were noteworthy in the diabetic rat testis. *Cistus laurifolius* L. treatments decreased the histopathological findings related to diabetes in the testicular tissue (Fig. 3). In the metformin treatment group, renal tubular structures had similar histology to the control group. However, the mild tubular injury was still present in the metformin group.

Discussion

Diabetes is an important endocrine disease characterized by abnormalities in carbohydrate, lipid, and lipoprotein metabolisms. Diabetes-related complications are still among the most important causes of morbidity and mortality in the world. Treatment methods depending on the type of diabetes lead to major complications including neuropathy, nephropathy, retinopathy, multiple organ failure, and especially lipoatrophy. Therefore, phytochemical analyses of hundreds of plants for medicinal uses have been extensively investigated for many years as a source of new antidiabetic compounds to treat diabetes. *Cistus laurifolius* L., which has various biological activities, is a plant species used in the treatment of diabetes in Turkish traditional medicine. By taking into consideration the complications caused by diabetes, we wanted to show both the damage caused by diabetes in selected tissues and the effects of *Cistus laurifolius* L. and metformin treatments on all body tissues in a comprehensive way. This is the first study to investigate the histopathological and systemic effects of the *Cistus laurifolius* L. aqueous extract on the blood, brain, heart, spleen, pancreas, liver, kidney, and testicular tissues, and also examined its effects on oxidative, inflammatory, and apoptotic pathways. Moreover, systemic effects of *Cistus laurifolius* L. aqueous extract were compared with metformin. Our results revealed that the *Cistus laurifolius* L. extract not only increased the insulin, lipase, amylase, and HDL level but also decreased the AST, ALT, triglyceride, cholesterol, LDL, BUN, and creatinine levels. In addition, we showed that *Cistus laurifolius* L. aqueous extract could have more effective therapeutic agent than metformin in diabetes treatment. In the literature, a previous study reported that the *Cistus laurifolius* L. active fraction significantly improves the number of inflammatory cell infiltration and edema in the gastric and duodenal tissues against gastric and duodenal lesions (Yesilada et al. 1997). Recently, Sayah et al. (2020) showed that *Cistus salviifolius* L. treatment reduced the damage of Langerhans islets in pancreatic tissue of STZ-induced diabetic mice. Consistent with the aforementioned studies, we found that *Cistus laurifolius* L. treatment improved the damage caused by diabetes more than metformin in the brain, heart, spleen, pancreas, liver, kidney, and testicular tissues of rats in the STZ-induced diabetes model. Therefore, it is reasonable to say that *Cistus laurifolius* L. is more effective in diabetes-induced histopathological changes in diabetic rats.

Diabetes, one of the chronic metabolic diseases, induces oxidative stress by increasing ROS production. Oxidative stress is particularly known to be an important pathway in the development of chronic complications of diabetes mellitus (Asmat et al. 2016). In diabetes, increased ROS and decreased antioxidant levels make tissues susceptible to oxidative stress. A previous study was reported that *Cistus laurifolius* L. exhibited antioxidant activity and anti-hyperglycemic effects with polyphenolic flavonoids (Akkol et al. 2012). In this study, according to the phytochemical analyzes, *Cistus laurifolius* L. showed a high antioxidant capacity according to the DPPH radical scavenging analysis. Metformin, an antihyperglycemic drug that reduces plasma glucose levels by suppressing gluconeogenesis and increasing glucose uptake in peripheral tissues, was reported to support antioxidant capacity by lowering lipid peroxidation and increasing antioxidant levels (Diniz Vilela et al. 2016). On the other hand, it was noticed that metformin also ameliorated dyslipidemia in diabetic patients beyond the hyperglycemia-lowering effect (Saisho 2015). However, Diaz-Morales et al. (2017) declared that metformin treatment (daily 1700mg for 12 months) did not cause any change in ROS, superoxide dismutase, and catalase levels in patients with diabetes. On the contrary, in this study, we demonstrated that the *Cistus laurifolius* L. aqueous extract and metformin promoted effectively antioxidant capacity and could normalized blood glucose levels by regulating insulin secretion. Intriguingly, besides *Cistus laurifolius* L., metformin also supported its antioxidant defense capacity in this study.

Mitochondrial dysfunction may occur during oxidative stress. Mitochondrial dysfunction leads to disruption of mitochondrial membrane integrity, the release of cytochrome c into the cytoplasm and following caspase activation. The glucotoxic and lipotoxic effects of diabetes encourage oxidative stress and trigger apoptosis with cytochrome c release and caspase activation. In a previous study, it was shown that flavonoids regulated insulin secretion and might also have direct effects on the prevention of apoptosis (Soares et al. 2017). According to our results, *Cistus laurifolius* L. aqueous extract treatment decreased caspase 3 and cytochrome c levels

in a dose-dependent manner in STZ-induced diabetic rats. Although metformin is one of the recent drugs used for the treatment of diabetes, there is controversial information about its effects on apoptosis. Currently, He et al. (2018) have demonstrated that metformin might induce or inhibit apoptosis by affecting mitochondrial functions. In a recent study, metformin decreased therapeutic efficiency by increasing apoptosis in mesenchymal stromal cells in diabetic mice. Interestingly, a previous study stated that metformin contributes to preventing the development of insulin resistance in diabetes by regulating mitochondrial functions (Wang et al. 2019). Unlike the aforementioned studies, in this study, metformin inhibited apoptosis as well as hampered ROS production. Our results suggested that increased caspase 3 and cytochrome c levels showed a significant decrease with metformin treatment in diabetic rats.

IL-1β and TNF-α, produced by macrophages and T cells, were reported to bind to surface receptors found in many cell types and activate the transcription factor NFκB (Ortis et al. 2010). NFκB, a pleiotropic transcription factor, induces intracellular signaling pathways and enhances transcription of oxidative, apoptotic, and inflammatory mediators (Liu et al. 2017). Consistent with our results, Datusalia and Sharma showed that NFκB-mediated inflammation was promoted in STZ-induced diabetic rats (Datusalia and Sharma 2016). Furthermore, NFκB activation promotes the production of excess superoxide anions by acting as a pro-oxidant (Soskić et al. 2011). NFκB was also triggered by oxidative stress, which explained the relationship between inflammatory processes and metabolic abnormalities in diabetes (Zhong et al. 2017). In this study, NFκB levels were consistently increased with oxidative stress, inflammation, and apoptosis in STZ-induced diabetic rats. Noteworthy, IL-1β and TNF-α initiated immunological responses, which were effective on the inflammation and the progression of diabetes-related complications (Tsalamandris et al. 2019). Our data demonstrated that IL-1β and TNF-α levels significantly upregulated in the STZ-induced diabetic rats; however, diabetic rats treated with *Cistus laurifolius* L. aqueous extract as well as metformin showed a reduction in IL-1β and TNF-α expressions compared with the control. Synergic activation of the NFκB signaling pathway and pro-inflammatory cytokines TNF-α and IL-1β are the main reasons for the progression of diabetes-induced pathophysiological processes. Gu et al. (2014) reported that metformin had anti-inflammatory effects by inhibiting NFκB-induced cytokine activation. In a previous study, methanol, chloroform, and ethyl acetate extracts of *Cistus laurifolius* were shown to inhibit IL-1β and TNF-α inflammatory cytokines (Kupeli and Yesilada 2007). Additionally, we showed that *Cistus laurifolius* L. aqueous extract also had anti-inflammatory effects. According to histopathological and biochemical analysis, *Cistus laurifolius* L. could provide greater amelioration than metformin in diabetic rats. Therefore, it could be considered to say that *Cistus laurifolius* L. shows protective effects by preventing oxidative stress, apoptosis, and inflammation related to diabetes-induced complications, thanks to its rich bioactive content.

Conclusion

To conclude, our results suggested that *Cistus laurifolius* L. treatment showed a dose- and time-dependent reduction of hyperglycemia in diabetic rats. At the same time, *Cistus laurifolius* L. aqueous extract could maintain improvements in oxidative, apoptotic, and inflammatory pathways in diabetes. Although single administration at doses of 125 mg/kg and 250 mg/kg of *Cistus laurifolius* L. aqueous extract showed anti-diabetic effects, the effects of high doses and long-term treatments of *Cistus laurifolius* L. require further investigations in diabetes models.

Availability of data and materials Not applicable.

Ethical approval The experimental procedure on animals was confirmed by the Eskisehir Osmangazi University Animal Experiments Local Ethics Committee (Approval number: 678-1).

Consent to participate Not applicable.

Consent to publish Not applicable.

Author contribution CH, FK, and GK made experimental design and participated in data collection. CH, FK, and HS formed the experimental diabetes model in rats. CH, FK, and GK analyzed and interpreted biochemical measurements. EY provided the plant material and prepared its extract. YK analyzed the total flavonoid and phenolic compounds in the plant extract. DBD performed the histological examination of the brain, heart, pancreas, spleen, liver, kidney, and testis. CH was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Conflict of interest The authors declare no competing interests.

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