Investigation of cytogenetic, electrophoretic, histopathological and biochemical effects of walnut (*Juglans regia* L.) leaf extract in rats experimentally induced diabetes by streptozotocin

ABSTRACT

In this study, the effects of *Juglans regia* L. (JR) leaf extract on histological damage and cytogenetic, electrophoretic, and biochemical parameters in the liver and kidney tissues of diabetic rats were investigated. In the study, 60 male rats (*Sprague-Dawley*) were separated into six groups. According to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) data, it was determined that there were increases and decreases in different serum protein expressions because of the treatment of JR leaf extract in the diabetes group. In the kidney tissue of the diabetes group, an increase in volume in the glomeruli and narrowing of the Bowman's space as well as thickening of the walls of tubules were detected. Vacuolization and shedding were observed in the epithelial cells of tubules in the cortical regions in kidney. In diabetic + JR extract groups administered JR leaf extract at doses of 250 mg/kg and 500 mg/kg, serum AST and ALT levels were reduced compared to the diabetic group. Diabetic rats' livers showed spotted necrosis and fibrosis in the portal area, biliary tract proliferation, mild inflammation, increased vascularization, unicellular necrosis in hepatocytes, and sinusoidal dilatation. The JR leaf extract group did not exhibit these problems. JR leaf extract influences reducing hepatotoxic and oxidative damage due to diabetes and increasing the level of antioxidant enzymes. As a result, it was concluded that the application of JR leaf extract may have a protective effect against damage caused by diabetes.

Keywords: Diabetes, *Juglans regia* L., kidney, liver, walnut

INTRODUCTION

Diabetes is a fatal disease that affects 285 million people worldwide and is a chronic metabolic condition that disrupts carbohydrate, protein, and lipid metabolism (Giacco and Brownlee, 2010). Therefore, early diagnosis is important and patients need to be treated appropriately and effectively. There are two types of diabetes: Type 1 (insulin dependent) and Type 2 (non-insulin dependent). Type 1 diabetes is an autonomic disease that appears suddenly in childhood. It is characterized by the autoimmune destruction of B cells. Type 2 diabetes occurs with insulin resistance and B cell dysfunction. It occurs in children and adults and is thought to be associated with obesity (Hoogwerf, 2020).

Walnut, which is widely available in the world, is traditionally a plant of great importance. Green walnuts, shells, kernels, bark, seeds, and leaves are widely used in the cosmetic and pharmaceutical industries (Stampar et al., 2006). While walnut leaf is used in traditional medicine in the treatment of venous insufficiency and hemorrhoid symptoms, the plant's antidiarrheal, anthelmintic, and antidiabetic properties are also used.

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Research Article

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A high amount of ellagic acid was found in the analyses made on the extract obtained from the leaf of the walnut (Ozer et al., 2007). The dried parts of walnut leaves are widely consumed in some European and Asian countries as tea. Green bark and leaf parts are rich in phenolic substances and flavonoids. These substances provide a protective effect against degenerative diseases by preventing oxidative stress and macromolecular oxidation. It also shows anti-carcinogenic properties with its free radical scavenging effects (Pereira et al., 2007). The most well-known active ingredient in walnuts is juglone (5-hydroxy-1,4-naphthoquinone), which is found in excess in young green leaves. This substance has very strong antioxidant and antimicrobial properties (Yiğit et al., 2009). Studies have shown that the antioxidant effects of the extracts obtained from plants are directly related to the phenolic content of the plant, and this feature does not change even after the plant has been stored for years (Halvorsen et al., 2002). Diabetes occurs with the insufficiency of insulin secretion of the pancreatic gland, which is characterized by hyperglycemia and the impairment of the response of the relevant tissues to insulin (Lipinski, 2001).

Mohammadi et al., (2011) found that due to the phenolic compounds in walnut leaf extract, such alkaloids, flavonoids, and saponins, it has potential in the control of type I diabetes.

In this study, it was aimed to define the cytogenetic, electrophoretic, histopathological, and biochemical effects of walnut leaf extract in rats with diabetes experimentally induced by streptozotocin.

MATERIALS AND METHODS

Walnut leaf extract

In June 2020, walnut leaf samples were collected near Kars-Kağızman and identified at Kafkas University's Department of Botany. The samples were dried in the dark, sunlight-free laboratory. The samples were dried in a sunlight-free laboratory, ground with an IKA A11 (Staufen, Germany) grinder, and 50 g of the ground material was placed in a 500 mL Soxhlet extractor. Ethanol was used as a solvent, and extraction was performed for about 10 hours (10-15 siphons) until it became clear. The liquid extracts obtained were filtered through a <2 µm pore size blue band filter paper (Grade 589/3, Whatman, UK), and the solvents were evaporated at 50ºC with a rotary evaporator. The resulting walnut leaf extract was weighed with 0.1 mg sensitivity, stored at +4ºC, and prepared for further study (Gundogdu et al., 2016; Uluman and Aksu-Kılıçle, 2020).

Animals and experimental design

In this study, we used 60 male rats (*Sprague Dawley*), aged 2-3 months, which had never been mated or used in previous research. The rats were kept in standard cages at 22 ± 2ºC, under a 12-hour light-dark cycle, and were fed ad libitum with pellet feed and drinking water for a one-week acclimatization.

The rats were divided into six groups: (G-I) Control group (n = 10), (G-II) Diabetic group (n = 10), (G-III) 250 mg/kg JR leaf extract group (n = 10), (G-IV) 500 mg/kg JR leaf extract group (n = 10), (G-V) D+250 mg/kg JR leaf extract group (n = 10), and (G-VI) D+500 mg/kg JR leaf extract group (n = 10). Diabetes was induced in the Diabetic group using 50 mg/kg streptozotocin (STZ) administered intraperitoneally, and fasting blood glucose levels were measured 3 days after STZ application from tail veins using glucometers. Rats with a fasting blood glucose level of 250 mg/dL were accepted to have diabetes (Yapislar et al., 2022). The JR leaf extract was administered orally for 21 days in the treatment groups, with dosages based on previous research (Çelik and Koç, 2019).

Determination of mitotic index

The femoral bones of rats were removed, with one used for the mitotic index and the other for the micronucleus test Bone marrow from the femur was collected in a centrifuge tube with fetal calf serum. Mitotic activity was assessed following Preston's method, with metaphase preparations stained using 10% Giemsa for 10 minutes. Under an Olympus CX21 microscope, 1000 cells were randomly counted from each animal sample at 1000x magnification to determine mitotic activity. Cell numbers in the metaphase stage and their percentages were recorded (Preston et al., 1987).

Detection of micronucleus frequency

The femur bone was cut at both ends. Bone marrow was transferred to a centrifuge tube containing 3 mL of calf serum with the help of an injector. Then, it was prepared and stained using a method developed by Schmid and adapted to our laboratory's working conditions. Under an Olympus CX21 brand light microscope at 1000 magnification, 2000 pieces of PCE were randomly selected from each preparation, and the number of MNPCEs within them was determined, and their percentages were calculated (Schmid, 1975).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Sds-Page) method

Blood samples were centrifuged for 10 minutes at + 4ºC and 3000 rpm and their sera were removed and kept at - 20ºC until the study period. Protein concentrations of the samples were measured by the biuret method (Eisenthal and Danson, 1993). The SDS-PAGE procedure was performed according to Laemmli, (1970) and O'Farrell, (1975) methods. Bovine albumin (66 kD), egg albumin (45 kD), trypsinogen (24 kD) and lysozyme (14 kD) were used as protein standards in electrophoresis applications. The molecular weights of the proteins were determined according to the method of Weber et al., (1972).

Histopathological procedures

At the end of the experiment, liver and kidney tissue samples were taken from the rats by cervical dislocation under anesthesia. Tissue samples were fixed in 10% formalin and blocked-in paraffin after routine histological procedures. Crossman's triple staining technique and Hematoxylin Eosin staining were applied to the 5-7 µm sections taken from the blocks to show the general structure of the tissue (Luna, 1968). To observe the plasma cells and pyroninophilic cells, tissue samples were also fixed in alcohol-formol solution for 48 hours and methyl green pyronin staining was performed on the sections.

Biochemical analysis method

Serum total antioxidant status (TAS), total oxidant status (TOS) (Rel Assay Diagnostics, Clinical Chemistry Solutions, Türkiye) (Erel, 2004). aspartate aminotransferase (AST) (EASTR-100; EnzyChrom™ Aspartate Transaminase Assay Kit, USA), alanine aminotransferase (ALT) (EALT-100; EnzyChrom™ Alanine Transaminase Assay Kit, USA), quantities were determined spectrophotometrically using the commercial kit. AST and ALT values were calculated by measuring absorbances at 340 nm at 5^{th} and 10^{th} minutes as specified in the kit procedure.

Statistical analyses

The Kolmogorov-Smirnov test was utilized to assess the normality of the group data. Data determined to be normally distributed were tested with ANOVA followed by post-hoc Tukey HSD test. P values below 0.05 ($P < 0.05$) were considered statistically significant. Results are presented as mean ± standard deviation (SD). SPSS 22 (IBM Corp., New York, USA) was used for all calculations.

RESULTS

Cytogenetic results

Statistical analysis revealed a significant increase in MN numbers for the diabetic control

group and other diabetic groups (P < 0.001). However, MN numbers did not differ significantly in groups treated with walnut extract alone (P > 0.05). However, it was determined that the MN numbers of the groups in which diabetes was created and walnut extract was applied decreased compared to the diabetes group (P < 0.001) and this decrease was proportional to the dose of walnut extract applied. Mitotic index values decreased in diabetic groups (P < 0.001) but started to increase when different doses of walnut extract were applied (P < 0.001) (Table 1 and 2; Figure 1 and 2).

Table 1. Control and experimental groups mitotic index data.

Group	Number of subjects	Total cell count	Number of interphase cells	Number of metaphase cells	Group Mean ±SD	Metaphase cell ratio average (%)
Negative control	10	10000	9685	315	31.5 ±1.65[a]	3.15
D	10	10000	9833	167	16.7 ±2.06[d]	1.67
250 mg/kg JR	10	10000	9684	316	31.6 ±1.26[a]	3.16
500 mg/kg JR	10	10000	9678	322	32.2 ±1.99[a]	3.22
D+250 mg/kg JR	10	10000	9726	274	27.4 ±1.07[b]	2.74
D+500 mg/kg JR	10	10000	9766	234	23.4 ±0.97[c]	2.34

[a-d]: The difference between groups in columns with different character is significant (P<0.001). D: Diabetic, JR: *Juglans regia* L.

Table 2. Control and experimental groups micronucleus data.

Groups	Total PCE	MNPCE	MNPCE (%)	Mean ± SD
NK	20000	58	0.29	5.80 ± 1.14[d]
Diabetes	20000	257	1.28	25.70 ± 1.49[a]
250 mg/kg JR	20000	56	0.28	5.60 ± 0.84[d]
500 mg/kg JR	20000	59	0.29	5.90 ± 1.45[d]
D+250 mg/kg JR	20000	227	1.13	22.70 ± 1.49[b]
D+500 mg/kg JR	20000	159	0.79	15.90 ± 1.20[c]

The MN numbers of the diabetic control group and other diabetic groups increased statistically significantly (P < 0.001), but there was no difference in the MN numbers of the groups that only applied walnut extract (P > 0.05). [a-d]: The difference between groups in columns with different character is significant. D: Diabetic, JR: *Juglans regia* L.

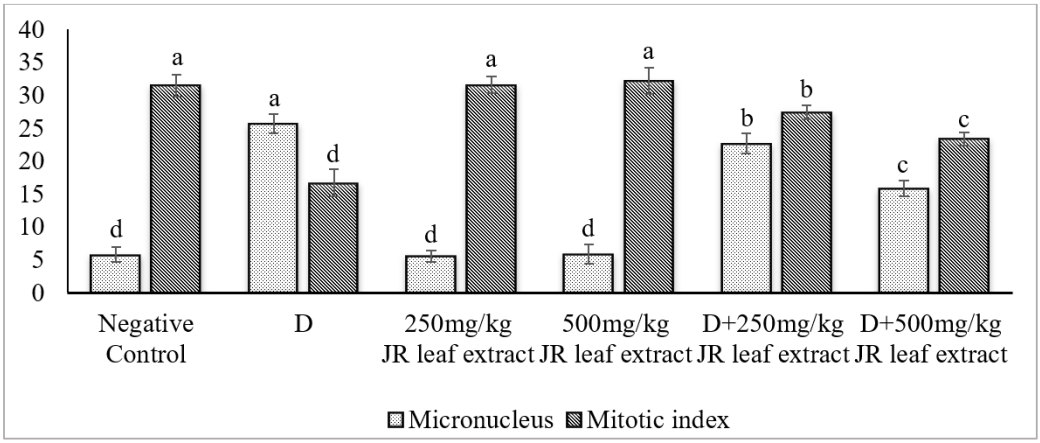

Figure 1. Micronucleus and mitotic activity values of control and treatment groups. D: Diabetic, JR: *Juglans regia* L.

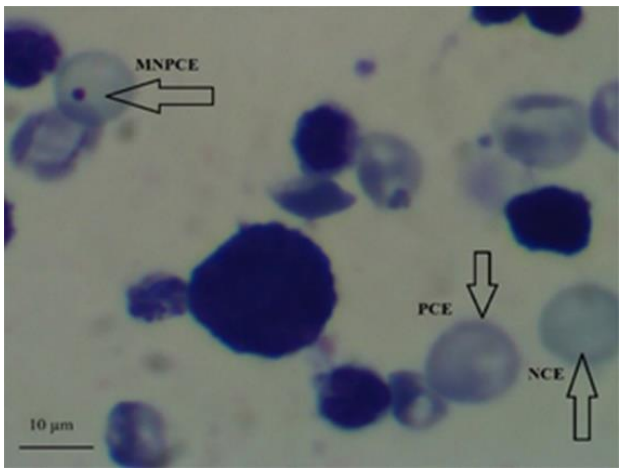

Figure 2. Polychromatic erythrocyte with micronucleus (MNPCE), Polychromatic erythrocyte (PCE) and Normochromatic erythrocyte (NCE) image.

Electrophoretic results

In the electropherogram obtained from sodium dodecyl sulphate polyacrylamide gel electrophoresis of serum samples, it was observed that the protein expressions of 13 kD, 40 kD and 45 kD increased, while the protein expression of 24 kD decreased in the samples belonging to the animals in the diabetic group. 13 kD, 16 kD, 40 kD, 45 kD, 56 kD, 82 kD and 103 kD protein expressions were increased in diabetic animals treated with JR leaf extract, and 24 kD protein expression decreased in diabetic animals treated with 500 mg/kg JR leaf extract (Figure 3).

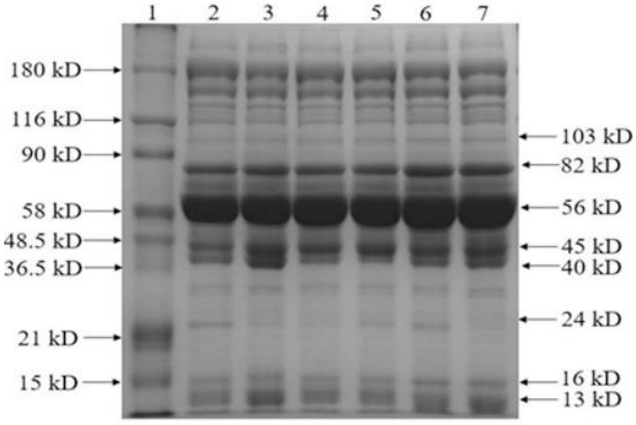

Figure 3. Electropherogram obtained from SDS-PAGE of animals in control and administration groups. 1: Standard proteins, 2: Negative control, 3: D, 4: 250 mg/kg JR, 5:500 mg/kg JR, 6: D+250 mg 7kg JR, 7: D+500 mg/kg JR. D: Diabetic, JR: *Juglans regia* L.

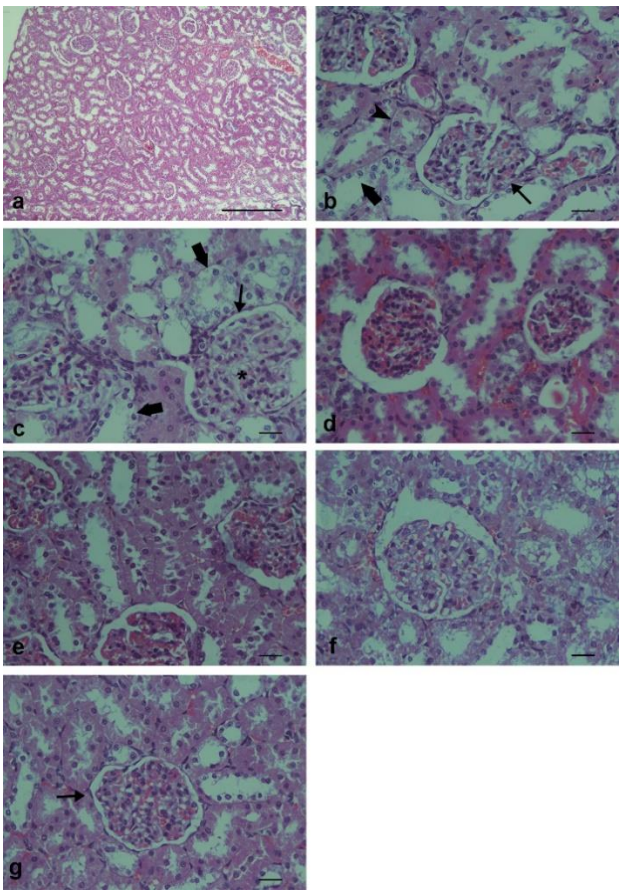

Figure 4. Rat kidney tissue. a: Control group. Crossman's triple staining, 100 µm, b: Diabetes group, increase in Glomerus volume (asterisk), narrowing in Bowman's space (arrow), thickening of the walls of the distal (thick arrow) and proximal tubules (arrowhead), c: Diabetic group, Increase in glomerus volume (asterisks) and Bowman area narrowing (arrow), Vacuolization in epithelial cells of proximal and distal tubules (thick arrow), d: D+250 mg/kg JR leaf extract group, e: D+500 mg/kg JR leaf extract group, f: 250 mg/kg JR leaf extract group, g: 500 mg/kg JR leaf extract group, constriction in Bowman capsules in glomeruli (arrow). H&E staining, 10µm. D: Diabetic, JR: *Juglans regia* L.

Histopathological results

Normal histological structure was detected in kidney tissues from the G-1, G-III, G-V, and G-VI groups. In the kidney tissue of diabetic rats, an increase in glomerulus volume, narrowing in Bowman's space, and thickening of the walls of the distal and proximal tubules were detected. Vacuolization and epithelial cell shedding were observed in the epithelial cells of the proximal and distal tubules. The narrowing of the Bowman capsules of the glomeruli was detected in the G-IV (Figure 4).

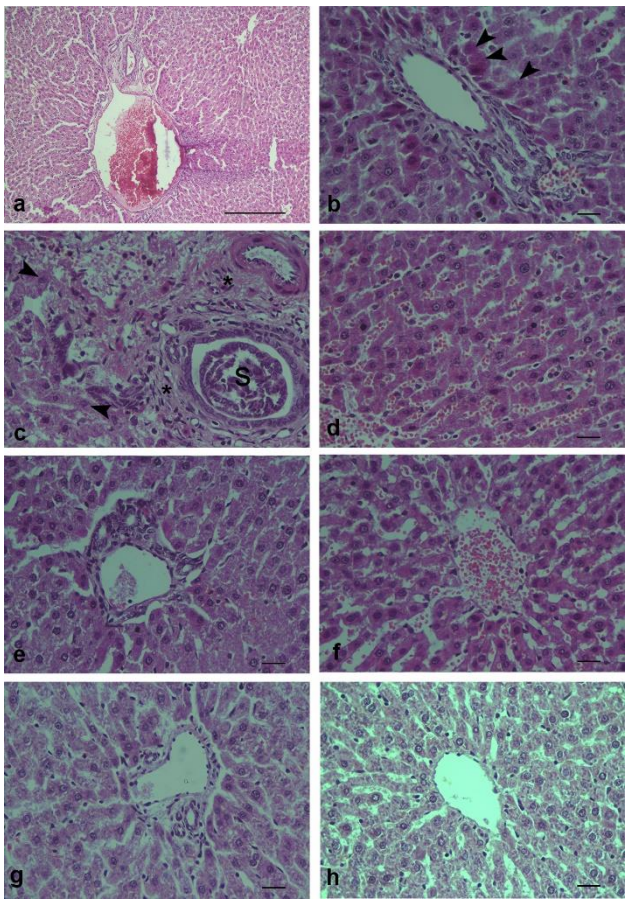

Figure 5. Rat liver tissue. a: Control group. Crossman's triple staining, 100µm, b: Diabetes group, Spotty necrosis areas (arrowheads), c: Diabetes group, Proliferation in bile ducts (S), portal area fibrosis (stars), mild inflammation, increased vascularity and single cell necrosis in hepatocytes (arrowheads), d: Diabetic group, Dilatation in sinusoids, e: D+250 mg/kg JR leaf extract group, f: D+500 mg/kg JR leaf extract group, Dilatation in sinusoids, g: 250 mg/kg JR leaf extract group, h: 500 mg/kg JR leaf extract group. H&E staining, 10µm. D: Diabetic, JR: Juglans regia L.

The liver tissue of rats in the G-I, G-III, G-IV, and G-V groups was shown to have normal histological structure. In the liver of rats with diabetes, spotty necrosis and fibrosis in the portal area, proliferation in the bile ducts, mild inflammation, increased vascularization, single cell necrosis in hepatocytes, and dilatation in sinusoids were observed. In the G-VI group, dilatation was detected in the sinusoids (Figure 5).

Biochemical results

Due to the application of JR leaf extract at doses of 250 mg/kg and 500 mg/kg, the liver AST ($P < 0.001$) and ALT ($P < 0.05$) enzyme levels were increased in rats with diabetes with streptozotocin compared to the G-I group. It has been determined that it has a decreasing effect on diabetes-related hepatotoxic damage, thus reducing the levels of this enzyme. However, the decrease in enzyme levels did not show a statistical difference compared to the G-I group ($P > 0.05$) (Table 3).

Antioxidant enzyme level increased against oxidative damage caused by diabetes ($P < 0.001$), but no statistical difference was found in oxidant enzyme level ($P > 0.05$). JR leaf extract application was found to increase the antioxidant enzyme level ($P < 0.001$), and the oxidant enzyme level was similar to the G-I group ($P > 0.05$) (Table 3).

Table 3. Liver and antioxidant / oxidant enzyme levels of animals in control and treatment groups.

Groups	AST (U/L)	ALT (U/L)	TAS (mmolTrolox Equiv./L)	TOS (µmol H_2O_2 Equv./L)
Control	136.57 ± 17.06 ^{c}	31.00 ± 5.35 ^{b}	0.46 ± 0.14 ^{c}	17.95 ± 3.63 ^{ab}
Diabetes	188.14 ± 33.14 ^{a}	38.14 ± 6.07 ^{a}	1.27 ± 0.37 ^{a}	19.58 ± 3.94 ^{a}
250 mg/kg JR	138.71 ± 19.21 ^{bc}	31.86 ± 3.98 ^{ab}	1.36 ± 0.28 ^{a}	17.99 ± 3.40 ^{ab}
500 mg/kg JR	135.71 ± 13.82 ^{c}	32.29 ± 2.63 ^{ab}	0.84 ± 0.20 ^{b}	13.18 ± 2.94 ^{b}
D + 250 mg/kg JR	168.14 ± 7.24 ^{ab}	37.29 ± 2.29 ^{ab}	1.07 ± 0.18 ^{ab}	17.46 ± 3.49 ^{ab}
D + 500 mg/kg JR	161.71 ± 12.75 ^{abc}	35.71 ± 3.15 ^{ab}	1.08 ± 0.20 ^{ab}	17.27 ± 5.66 ^{ab}

AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; TAS: Total antioxidant status; TOS: Total oxidant status. $^{a-d}$: The difference between groups in columns with different character is significant. D: Diabetic, JR: *Juglans regia* L.

DISCUSSION

Diabetes mellitus is one of the diseases with high rates of mortality and morbidity in the World (Memisogullari, 2005). In addition, diabetes hyperglycemia can both increase the formation of free radicals and cause the endogenous antioxidant defense system to deteriorate (Saxena et al., 1993). Superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide, and transition metals are blamed for oxidative stress in diabetes (Memisogullari, 2005). Although some other living things, especially humans, have antioxidant defense systems that protect and restore oxidative damage caused by free oxygen radicals, these systems may fail to prevent oxidative damage (Yoshikawa et al., 2000). Oxidative stress is an important tool in the development of chronic complications (neuropathy, nephropathy, and retinopathy) in diabetes (Lipinski, 2001; Memisogullari, 2005). Today, the therapeutic potential of many natural and nanotechnology-based compounds against diabetes is being investigated (Caylak and Nur, 2024a; Caylak and Nur, 2024b; Deprem et al., 2015; Yıldız et al., 2015).

Walnuts are cancer-preventing and also contain polyphenols that destroy free radicals and have metal chelate (binding) activity qualities. For example, ellagic acid, a polyphenol, is known for its anticancer properties as well as enhancing the immune system (Cerda et al., 2005). Walnut leaves have high anti-diabetic properties due to the highly hydrophilic components in the *Juglans regia* leaf extract (Forino et al., 2016).

In a study, the cholesterol profile of type II diabetes patients who ate 30 g of walnuts per day improved. It is stated that the reason for this is probably due to the high content of omega-3 acids in walnuts (Tapsell et al., 2004). Omega-3 fatty acids also play a key role in the fluidity of cell membranes, and membrane stiffness, which develops in the absence of these fatty acids, negatively affects transport functions, receptor interactions and numbers. For example, while the increase in membrane fluidity increases the number of insulin receptors, the solidification of the membranes causes a decrease in the number of these receptors and leads to diabetes and insulin resistance (Konukoglu, 2008).

Genotoxicity was investigated by micronucleus tests in rat bone marrow cells and peripheral blood in which experimental diabetes was induced by STZ. Arora et al., (2005) reported that extracts obtained from three medicinal plants (*Acacia nilotica*, *Juglans regia* and *Terminalia chebula*), using vitotox and comet assay tests, showed antimutagenic properties at certain concentrations. An anti-apoptotic effect of walnut mallow extract against UVB-induced apoptotic responses has been reported. This protective effect is due to the high amount of flavonoid and antioxidant content in the extract (Muzaffer et al., 2018). We think that the walnut leaf extract used in our study may have genotoxicity properties similar to the above studies and the decrease in micronucleus number may be due to the antimutagenic properties of the extract.

While it was determined that the total protein levels in the brain and kidney tissues of rats treated with carbon tetrachloride increased and decreased in the liver tissue, it has been reported that due to the application of *Juglans regia* L. extract with CCl_4, due to the phytochemical compounds it contains, the total protein levels in the brain and kidney tissues decreased and increased in the liver tissue (Aydin et al., 2015). In another study, it was reported that serum total protein levels decreased in rats treated with CCl_4, and total protein levels increased due to the application of *Juglans regia* L. extract (Hosseini et al., 2018). In this study, an increase and a decrease in different protein expressions were found in the diabetic groups in the serum protein expressions and in the diabetic groups treated with JR extract.

Diabetes have effects such as hypertrophy in the glomeruli of rat kidneys, enlargement of the mesangium, glomerular sclerosis, thickening of the glomerular basement membrane, enlargement of the kidneys, and increased permeability through pores (Li et al., 2019). In addition, with STZ injection, it was found that the rate of kidney structure deterioration and apoptosis was more intense, but renal damage was significantly reduced by treatment with paricalcitol (Liu et al., 2019). In another study, walnut leaf application removes the damage caused by diabetes in kidney and liver tissues (Mollica et al., 2017). In our study, an increase in glomerular volume, narrowing of Bowman's space and thickening of the walls of distal and proximal tubules were detected in the kidney tissue of diabetic rats. In addition, vacuolization and epithelial cell shedding were observed in epithelial cells of proximal and distal tubules. The liver tissue of diabetic rats has degeneration and necrosis in hepatocytes, especially the cytoplasm of hepatocytes in the periacinal region has single or multiple small vacuoles, enlargement in the portal spaces, fibrosis, bile duct proliferation and inflammatory cell infiltration, but it has been reported that these damages are significantly reduced with the application of acorn extract (Yaman and Doğan, 2016). Diabetic groups showed apoptotic nuclei, hydropic degeneration and central vein occlusion in liver tissue (Asokan et al., 2019). In our study, the liver of diabetic rats showed punctate necrosis and fibrosis in the portal region, proliferation of bile ducts, mild inflammation, increased vascularization, single cell necrosis in hepatocytes and dilatation of sinusoids.

There are many studies on the use of natural compounds as preservatives against hepato and nephrotoxic agents (Deveci et al., 2021; Nur et al., 2023). The application of *Juglans regia* extract in rats with liver damage with carbon tetrachloride reduces liver enzyme levels by showing hepatoprotective effect, but also increases antioxidant enzyme activities such as superoxide dismutase and catalase (Eidi et al., 2013). In a study in which *Juglans regia* L. leaf extract was applied to diabetic patients, the ALT level, which was high before the extract application, decreased significantly (Rabiei et al., 2018). AST and ALT enzyme levels decreased significantly due to the application of the ethanolic extract of the walnut fruit core to diabetic rats (Ghiravani et al., 2016). The application of *Juglans regia* extract against the oxidative damage caused by isoprenaline showed quite important antioxidant properties (Sharma et al., 2021). Similarly, Rusu et al., (2020) stated that the *Juglans regia* extract significantly reduced the level of reactive oxygen species. In the present study, applying different doses of JR leaf extract to diabetic rats decreased serum AST and ALT levels, as well as increased TAS levels and decreased TOS levels in rats by showing protective properties against liver damage due to diabetes.

CONCLUSION

As a result of this study, walnut leaf extract has an effect on reducing kidney damage due to diabetes, hepatotoxic and oxidative damage, increasing the antioxidant enzyme level, and also decreasing the increase in micronucleus frequency and increasing the mitotic index.

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Conflict of interest: There is no conflict of interest between the authors.

Ethical statement or informed consent: This study was carried out at Kafkas University Research Animals Application Center. This research was approved by The Ethics Committee of the Faculty of

Veterinary Medicine, Kafkas University (KAU-HADYEK, Ref No: 2020/045 Date: 24.03.2020).

Author Contributions: In this study, the distribution of tasks is as follows: Conceptualization was carried out by PAK., EK, EKS, AD, HA, and YYA. Formal Analysis was conducted by PAK, EK, YYA, and HA. Investigation was performed by PAK, EK, HA, and YYA. Methodology was developed by PAK, EK, EKS, AD, HA, and YYA. Project Administration was carried out by PAK. Resources were provided by PAK, EK, EKS, AD, HA, and YYA. Validation was done by PAK, EK, and HA. Visualization was performed by HA. Writing – Original Draft Preparation was carried out by PAK, EK, EKS, AD, HA, and YYA. Writing – Review & Editing was conducted by PAK, EK, YYA and HA.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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