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OLIVE (*Olea europaea* L.) LEAF EXTRACT BOOSTS ANTIOXIDANT STATUS AND ATTENUATES HEPATIC AND PANCREATIC CHANGES IN DIABETIC RATS

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ABSTRACT

The purpose of this study was to evaluate the antioxidant, oxidative DNA damage and hepatoprotective effects of olive (*Olea europaea* L.) leaf extract (OLE) on experimental diabetic rats. These effects of OLE were studied using streptozocin-induced diabetic rats. Antioxidant enzyme activities, oxidative DNA damage analysis and protein oxidation were evaluated spectrophotometrically methods. In addition, histological examination of the liver and pancreas was performed routinely with paraffin embedding and staining method. Protein carbonyl level and 8-hydroxy-2' –deoxyguanosine (8-OHdG) concentration decreased in OLE administrated group compared to diabetic group. Further, OLE treatment significantly increased superoxide dismutase and glutathione peroxidase activities. It was observed that OLE remarkably attenuated degenerative and necrotic changes in liver and pancreatic tissues. Consequently, it appears that OLE strengthens the antioxidant defense system, protects against oxidative DNA damage, and has a hepatoprotective effect on diabetic rats.

Key words: Antioxidant, diabetics, 8-OHdG, hepatoprotective, olive

INTRODUCTION

Diabetes mellitus is a heterogeneous metabolic syndrome characterized by hyperglycemia caused by relative or absolute deficiency of insulin (Harvey *et al*., 2011). In diabetics, insulin deficiency commonly results in characteristic abnormalities in the metabolism of carbohydrates, lipids, proteins and similar substances (Kuzuya *et al*., 2002). At the same time, insulin resistance in tissues and cells has an effect on hyperglycemia and the pathogenesis of diabetes (Tomas *et al*., 2002). Hyperglycemia triggers diabetic complications via certain signal mechanisms. These mechanisms are the activation of polyol pathway, increase in the formation of advanced glycation end-products (AGE), activation of protein kinase C and activation of hexosamine pathway. These mechanisms and nonenzymatic glycosylation, glucose auto-oxidation and monocyte dysfunction caused by hyperglycemia lead to the continuous production of free radicals (Hegde *et al*., 2013). In addition, these self-recurring causes and consequences affect the increase of reactive oxygen species and the development of complications (Ceriello, 2005). Augmenting the antioxidant status helps both to reduce free radicals and to prevent the activation of these pathways (Ayepola *et al*., 2014).

In recent years phytochemicals have been reported to effectively treat and prevent diseases. As the benefits of phytochemicals for health have become more widely appreciated, their

prophylactic and therapeutic uses have expanded. In daily life, olives (*Olea europaea* L.) and olive oil are the most widely used products in traditional Mediterranean diet. Olive leaf has rich phenolic content, mainly oleuropein, which is most abundant and active phenolic compound (Bock *et al*., 2013). Olives and olive leaf have a wide spectrum of physiological and pharmacological activities. Esmailidehaj *et al*. (2016) showed that oleuropein would lead to improved myocardial dysfunction following ischemic-reperfusion injury due to antioxidant potential of oleuropein. Previous studies have indicated that olive oil and olive leaf extract were effective in ameliorating hyperglycemia and antioxidants (Jemai *et al*., 2009; Wainstein *et al*., 2012; Santangelo *et al*., 2016). Gilani *et al*. (2006) reported that olive fruits provide spasmolytic (Ca^{+2} antagonist) and spasmogenic (cholinergic) activities, so act on gastrointestinal disorders. Recently, Şahin *et al*. (2017) notified that olive leaf extract showed antimicrobial activity as an antioxidant. The present study was aimed to investigate whether olive leaf extract is antioxidant and hepatoprotective and assess its effectiveness against oxidative DNA damage in experimental diabetes.

MATERIALS AND METHODS

The chemicals and reagents used in the present study were of analytical grade and procured from Sigma, Inc. (St. Louis, MO, USA) and Merck.

Plant material and extraction

Olive (*Olea europaea* L.) fresh leaves were collected from the province of Antalya, Turkey, in August 2013. The leaves were dried in outdoor under shadow (approx. 30^0C) for 4 days and ground. To prepare leaf extract 1 g of olive lead powder was first combined with 50 mL distilled water. Dynamic solvent extraction method (infusion) was used for dried olive leaf (Jiménez-Zamora *et al*., 2016). The dynamic infusion is a conventional extraction technique in which plant material is allowed to steep in the liquid for a period of time where the solvents are used according to their polarity to extract the interest compounds. The dynamic infusion process was performed with a magnetic hot plate (Wisd WiseStir MSH-20D). The extraction process was carried out for a period of 12 h at 80°C and stirring 750 rpm and then evaporated under reduced pressure. Further, 1.5 g leaf sample was infused in 100 mL water at 80°C for 5 min. to create a homemade extract.

Determination of extract content by HPLC

The amounts of oleuropein in the samples were measured quantitatively against external standard in extracted olive leaves. A Waters 1525 binary HPLC pump and Waters 2487 dual absorbance detector were used for content analysis. The measurement was made by adjusting chromatogram to 280 nm wavelengths for the determination and assignment of polyphenolic compounds. The dose administered to the rats was first standardized according to the amount of oleuropein because oleuropein is the main known bioactive compound in olive leaf extract (OLE). The amount of oleuropein was determined at 15335.55 µg g^{-1} in OLE by HPLC analysis.

Animals

Eighty healthy male rats *(Wistar albino)*, weighing 250 - 350 g, of 5-6 months age were procured from the Experimental Application and Research Center, Yuzuncu Yil University (Turkey). The rats were placed in standard plastic rat cages and housed at 22±2°C, 50% humidity, and 12-h day/night cycles. The present study was conducted with the permission of the Yuzuncu Yil University Animal Researches Local Ethic Committee (No. 2015/08).

LD_{50} study

In order to determine whether olive leaf extract has acute toxicity, an LD_{50} study was performed. The modified method of Amabeoku *et al*. (2010) was used to assess the acute toxicity of olive leaf water extract. Rats were used for the determining the acute toxicity. The rats were fasted for 12-h in

groups of plant extract and control (n = 8). The minimum dose chosen was equivalent to the highest dose confirmed in the literature conducted with antidiabetic study, 500 mg kg^{-1} body weight (bw) for rats (Eidi *et al*., 2009). OLE was administered orally with a bulbed steel needle to rats in graded doses (@ 500, 1000, 2000 and 4000 mg kg^{-1} bw). Eight rats in another group used as control, received 1 mL physiological saline orally. All the rats were allowed free access standard laboratory chow for rodents and tap water and observed for 7 days for any deaths or acute toxicity symptoms.

Experimental protocol

The rats were randomly divided into 5 experimental groups (n = 8). Streptozocin (STZ) was administered at 45 mg kg^{-1} bw intraperitonally (i.p.). The blood glucose levels were measured in the tails of rats 3 days after STZ injection. Rats with glucose levels ≥ 200 mg dL^{-1} were considered diabetic. The experimental groups were categorized as 1) control group (CG) administered 1 mL citrate buffer i.p. only, 2) diabetic group (DG) injected with a single dose of 45 mg kg^{-1} bw i.p STZ, 3) diabetic + OLE wherein diabetic rats were treated with 25 mg kg^{-1} bw OLE daily using an intragastric tube; 4) diabetic + infusion (Inf) wherein diabetic rats were treated with 1 mL infusion solution daily using an intragastric tube, and 5) diabetic + acarbose (Ac) wherein diabetic rats were treated with 150 mg kg^{-1} bw Glucobay (Bayer, Turkey) daily using an intragastric tube. The infusion and acarbose groups were designed for biochemical comparison and to monitor blood glucose level. Rats were fed standard chow and tap water *ad libitum* for 21 days (Girija *et al*., 2011). At the end of study, blood and tissue samples, taken from animals, were anesthetized with ketamine + xylazine.

Biochemical analyses

The liver tissue was homogenized with phosphate salt buffer (PSB; pH 7.4). Protein oxidation (carbonyl content) was measured spectrophotometrically at 360 nm as specified by the supplier (Cayman Chemical, Michigan, USA). The analysis is based on the principle that carbonyl groups form 2,4-dinitrophenyl hydrazone by reacting with 2,4-dinitrophenyl hydrazine (Levine *et al*., 1990). Carbonyl content was standardized on protein concentration basis (Lowry *et al*., 1951). Reduced glutathione (GSH) content was measured at 412 nm by the amount of yellow colour formed as a result of the reaction of sulfhydryl groups in tissue samples with DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)] as per the method of Beutler *et al.* (1963). Superoxide dismutase (SOD) activity was measured at 505 nm by percentage inhibition of the formation of formazan dye (McCord *et al*., 1969). Glutathione peroxidase (GPx) activity was analyzed at 340 nm based on the catalytic oxidation of cumene hydroperoxide to reduced glutathione (Paglia and Valentine, 1967). Catalase (CAT) activity was determined as per Aebi (1984) which is based on the principle of water and oxygen breakdown of H_2O_2. Analyses of serum enzyme (ALT, AST and LDH) levels were conducted using a commercial kit on the Roche Modular PP auto-analyzer.

Oxidative DNA damage was determined using a commercial kit (ADI-EKS-350, Enzo Life Sciences, NY, USA) through enzyme-linked immunosorbent assay (ELISA) method with the detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in serum. This method is based on the principle of measuring tetramethylbenzidine substrate at 450 nm with a microreader.

Histopathology

At the end of experiment, the liver and pancreas tissues were taken, routinely embedded in paraffin and then stained with hematoxylin-eosin. Tissue samples were evaluated under a light microscope (Leica ICC50 W). In order to determine the differences between groups, the Z-ratio test was performed using Minitab 14 statistical software package.

Statistical analyses

Data were expressed as mean and standard deviation ($\overline{X} \pm$ SD). One-way analysis of variance was used to determine the significant differences between groups followed by Tukey's HSD test. A value of $p<0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

Diabetes mellitus under hyperglycemic conditions increase oxidant levels in oxidant/antioxidant balance (Tabak *et al.*, 2011). It is known that liver damage results from impaired liver function in diabetic rats, and thus serum AST and ALT enzymes are elevated (Eidi *et al.*, 2009). In present study there was a significant increase in diabetic group in the levels of AST and ALT enzymes, which exacerbate acute liver damage in hepatocytes and induce infiltrative disorders, as well as in LDH which causes parenchymal liver damage. However, AST and ALT levels showed a significant decrease in OLE-treated group. Domitrovic *et al*. (2012) reported that the administration of 100 and 200 mg kg^{-1} purified oleuropein exhibited both inhibitory and protective effects with regard to liver dysfunction in CCl_4-induced liver damage. Besides, no death occurred in any group as a result of LD_{50} administration. It is, therefore, assumed that high doses of OLE may be safe and non-toxic.

Table 1: Serum parameters of control and diabetic rats

Serum (U L^{-1})	CG	DG	OLE	Inf	Ac
AST	95.0±11.2	263.0±24.0[a]	171.7±15.5[a,b]	175.8±16.1[a,b]	193.7±13.4[a,b]
ALT	44.8 ± 4.0	123.3±17.3[a]	87.0±9.3[a,b]	76.7±12.2[a,b]	81.7±7.7[a,b]
LDH	448.3±54.5	656.8±56.0[a]	602.5±57.2[a]	488.8±38.5[b,c]	620.8±81.2[a,d]

CG: Control group; DG: Diabetic group; OLE: Olive leaf extract group; Inf: Infusion group; Ac: Acarbose group; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase.
[a]: It was significantly different from control (p<0.05); [b]: It was significantly different from DG (p<0.05);
[c]: It was significantly different from OLE (p<0.05); [d]: It was significantly different from Inf (p<0.05).

Table 2 shows the oxidative stress and antioxidant parameters mean and standard deviation of control and diabetic rats. Also, Fig. 1 and 2 are presented as graphical interfaces that to facilitate comparison between groups. The increase of reactive oxygen species causes irreversible protein oxidation and formation of carbonyl groups (Tomlinson *et al.*, 2008). PC level was significantly elevated in the diabetic treated group (Fig. 1A). However, the administering of OLE maintained carbonyl levels at control group levels by inhibiting protein oxidation (p<0.05). On the other hand, the high carbonyl level observed in infusion group is similar to those reported by Andreadou *et al.* (2007). One of the oxidative DNA damage biomarkers that is another reliable indicator of oxidative stress, 8-OHdG, has shown increase in various degenerative diseases and diabetic patients (Chen *et al.*, 2011). The 8-OHdG box graph shows the top and bottom of quadrangle, including the 25th and 75th percentiles of data. The line in the box represents median (Fig. 1B). In present study, the concentration of 8-OHdG in diabetic group increased by exceeding the level of DNA glycosylase (p<0.001), thus causing oxidative damage. However, extract and infusion administration remarkably

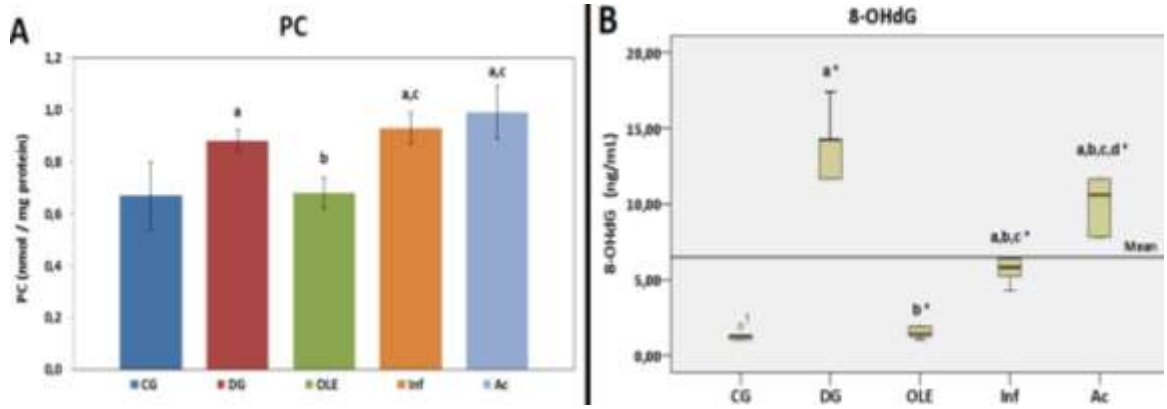

Fig. 1: Protein carbonyl levels (A) and 8-OHdG concentration boxplot (B) of control and diabetic rats; CG: Control group; DG: Diabetic group; OLE: Olive leaf extract group; Inf: Infusion group; Ac: Acarbose group; PC: Protein carbonyl content; 8-OHdG: 8-hydroxy-2'-deoxyguanosine

Table 2: Oxidative stress and antioxidant parameters of control and diabetic rats

Biochemical parameters	CG	DG	OLE	Inf	Ac
PC (nmol mg^{-1} protein)	0.67±0.13	0.88±0.04[a]	0.68±0.17[b]	0.93±0.06[a,c]	0.99±0.10[a,c]
8-OHdG (ng mL^{-1})	1.20±0.13	13.9±2.10[a*]	1.5±0.40[b*]	5.70±0.90[a,b,c*]	10.0±1.90[a,b,c,d*]
GSH (nmol mg^{-1} tissue)	4.92±0.18	3.55±0.29[a]	4.16±0.14[a,b]	4.53±0.30[b]	3.97±0.30[a,b,d]
SOD (EU mg^{-1} protein)	29.7±0.65	20.86±0.45[a]	24.13±0.50[a,b]	23.32±0.73[a,b]	21.90±0.65[a,c,d]
GPx (EU mg^{-1} protein)	11.02±0.86	3.61±0.31[a]	4.69±0.30[a,b]	6.16±0.76[a,b]	4.86±0.60[a,b]
CAT (EU mg^{-1} protein)	3.26±0.32	1.90±0.23[a]	1.98±0.11[a]	2.25±0.20[a]	1.88±0.19[a]

CG: Control group; DG: Diabetic group; OLE: Olive leaf extract group; Inf: Infusion group; Ac: Acarbose group; PC: Protein carbonyl content; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; GSH: Reduced glutathione; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase.
*: $p<0.001$; [a]: It was significantly different from control; [b]: It was significantly different from DG; [c]: It was significantly different from OLE; [d]: It was significantly different from Inf.

ameliorated the 8-OHdG concentration ($p<0.001$). Kendall *et al.* (2009) reported a reduction in the concentration of 8-OHdG as compared to the placebo group as a result of the olive leaf extract administration in capsule and liquid forms to healthy male and female subjects. Another study reported that olive leaf extract and pure oleuropein orally administrated inhibited the increase of 8-OHdG-positive cells in acute ultraviolet-B induced skin damage (Sumiyoshi and Kimura, 2010). OLE is thought to function due to its antioxidant effect against oxidative damage in diabetes by upregulating the expression of the protective and/or DNA glycosylase enzyme, and by activating DNA repair.

Although GSH content and SOD, GPx, and CAT activities decreased significantly in diabetic group as compared to the control group, the other antioxidant markers (excluding CAT activity) showed significant increase in OLE and infusion groups (Fig. 2A-D). It has been reported that extra

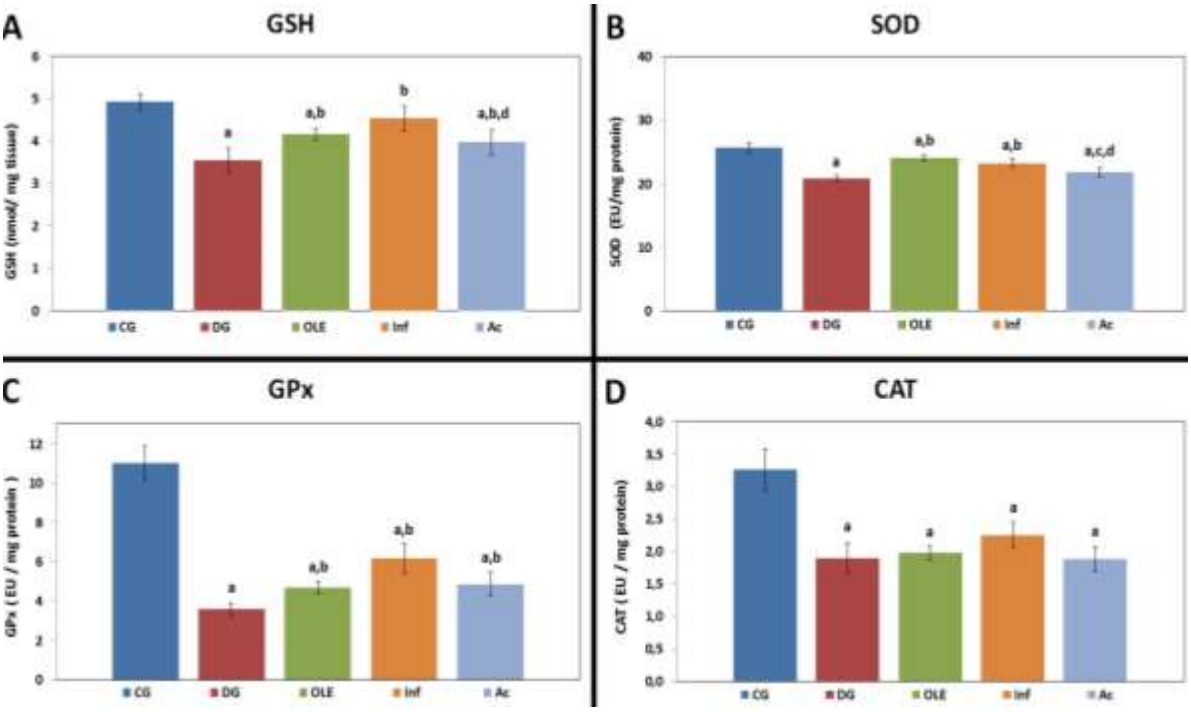

Fig. 2: GSH levels (A), SOD (B), GPx (C) and CAT (D) activities of control and diabetic rats; CG: Control group; DG: Diabetic group; OLE: Olive leaf extract group; Inf: Infusion group; Ac: Acarbose group; GSH: Reduced glutathione; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase.

Table 3: Histopathological changes in liver and pancreas of control and diabetic rats

Groups	Liver		Pancreas		
	Degeneration	Necrosis	Degeneration	Necrosis	LIA
CG	-/6	-/6	-/6	-/6	-/6
DG	6/6[a]	5/6[a]	6/6[a]	4/6[a]	4/6[a]
OLE	2/6[b]	2/6[b]	2/6[b]	2/6	2/6

CG: Control; DG: Diabetic group; OLE: Olive leaf extract group; LIA: Langerhans islets atrophy.

virgin olive oil biophenols inhibit the redox imbalance by scavenging released radicals, thus preserving the intracellular GSH content (Masella *et al.*, 2004). Similarly, Weinbrenner *et al.* (2004) showed that biophenolic compounds in extra pure olive oil regulate redox balance in humans. Parallel with previous findings, the present study can be explained by the different classes of biophenols which help to improve the activities of enzymes involved in GSH synthesis and metabolism in different cell types, and also increase the concentration of GSH (Moskaug *et al.*, 2005). Antioxidant enzyme activities may be reduced by the dysfunction of proteins due to glycation. Furthermore, the activities may be diminished due to the consumption of antioxidant enzymes through excessively elevated ROS (Irudayaraj *et al.*, 2012). In present study, the administration of both extract and infusion to diabetic rats boosted SOD and GPx activities. It has been reported that purified oleuropein and hydroxytyrosol increase the activity of antioxidant enzymes such as SOD, GPx, and CAT in diabetic rats (Jemai *et al.*, 2009; Hamden *et al.*, 2009). Further, Vina *et al.* (2006) showed that polyphenolic compounds increase the expression of SOD and CAT enzymes at the level of transcription.

Histopathological findings in liver and pancreas tissues were evaluated according to the areas of degeneration and necrosis as shown in the summary of histopathological changes and *p* values of liver and pancreas in Table 3 and 4. Hyperglycemia especially causes damage to liver, kidney and components of hematopoietic system (Sabu *et al.*, 2002). The effects of diabetes on liver are usually in the form of ultrastructural changes such as hypertrophy in hepatocytes and the appearance of autophagic vacuoles. In addition, the nucleus of hepatocytes is usually enlarged and sometimes observed to have irregular contours and intranuclear inclusions (Kume *et al.*, 2004). In present study, microscopic examination of liver revealed necrosis, vacuolar and hydropic degeneration of hepatocytes and dilation in sinusoids of diabetic group (Fig. 3B). Nonetheless, the histological features in OLE-treated group attenuated in the current study. Further, perisinusoidal cells rarely increased and sinusoidal focal mononuclear cell infiltrations were seen (Fig. 3C). Domitrovic *et al.* (2012) have reported that inflammatory responses such as NF-κβ, TNF-α and COX-2 decreased in massive liver necrosis with oleuropein administration, and that histopathological findings improved by inhibiting caspase-3 activation. The present findings are in line with previous studies which showed that biophenolic compounds of OLE reduce degenerative and necrotic changes, possibly by helping to protect the cell integrity of hepatocytes, and thereby have a protective effect on liver (Domitrovic *et al*., 2012; Kumral *et al*., 2015; Al-Attar *et al*., 2017; Osman and Tantawy, 2017).

Table 4: The *p* values of control and diabetic rats the binary group comparisons of the histopathological findings on the liver and pancreas

Binary Groups	Liver		Pancreas		
	Degeneration *P*	Necrosis *p*	Degeneration *P*	Necrosis *p*	LIA *P*
CG – DG	0.000	0.000	0.000	0.001	0.001
CG – OLE	0.083	0.083	0.083	0.083	0.273
DG – OLE	0.001	0.042	0.001	0.221	0.221

CG: Control group; DG: Diabetic group; OLE: Olive leaf extract group; LIA: Langerhans islets atrophy; *p*: significance value.

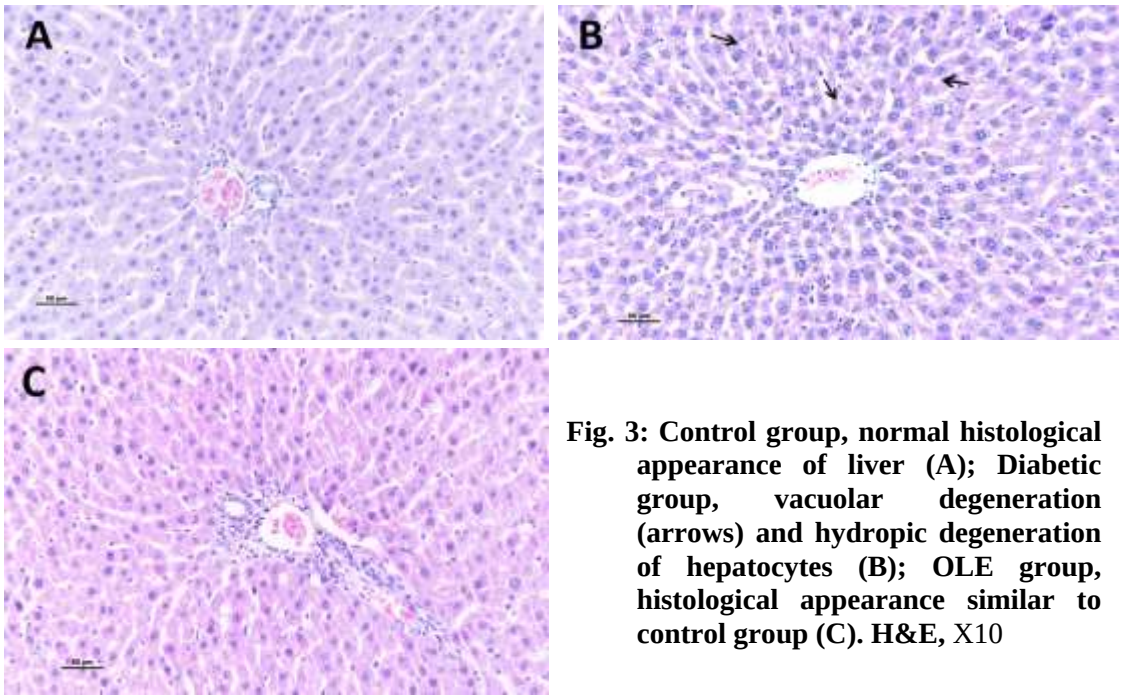

Fig. 3: Control group, normal histological appearance of liver (A); Diabetic group, vacuolar degeneration (arrows) and hydropic degeneration of hepatocytes (B); OLE group, histological appearance similar to control group (C). H&E, X10

Degenerative and necrotic (arrows) changes in the Langerhans islet cells of diabetic group were clearly seen. The cytoplasm of islet cells was swollen and the nuclei were pyknotic (Fig. 4B). The current investigation confirmed previous findings that common necrotic changes with reduced islet size damage β-cell populations. Fibrosis and atrophy were detected in STZ-induced diabetic rats (Irudayaraj *et al*., 2012). Despite severe degenerative and necrotic changes in diabetes, atrophy in the islets nevertheless improved with OLE therapy (Fig. 4C). The improvement in these histological findings was attributed to the potential therapeutic effect of the natural compounds in plants (Patel *et al.*, 2012; Lin *et al.*, 2016; Choudhury *et al*., 2018).

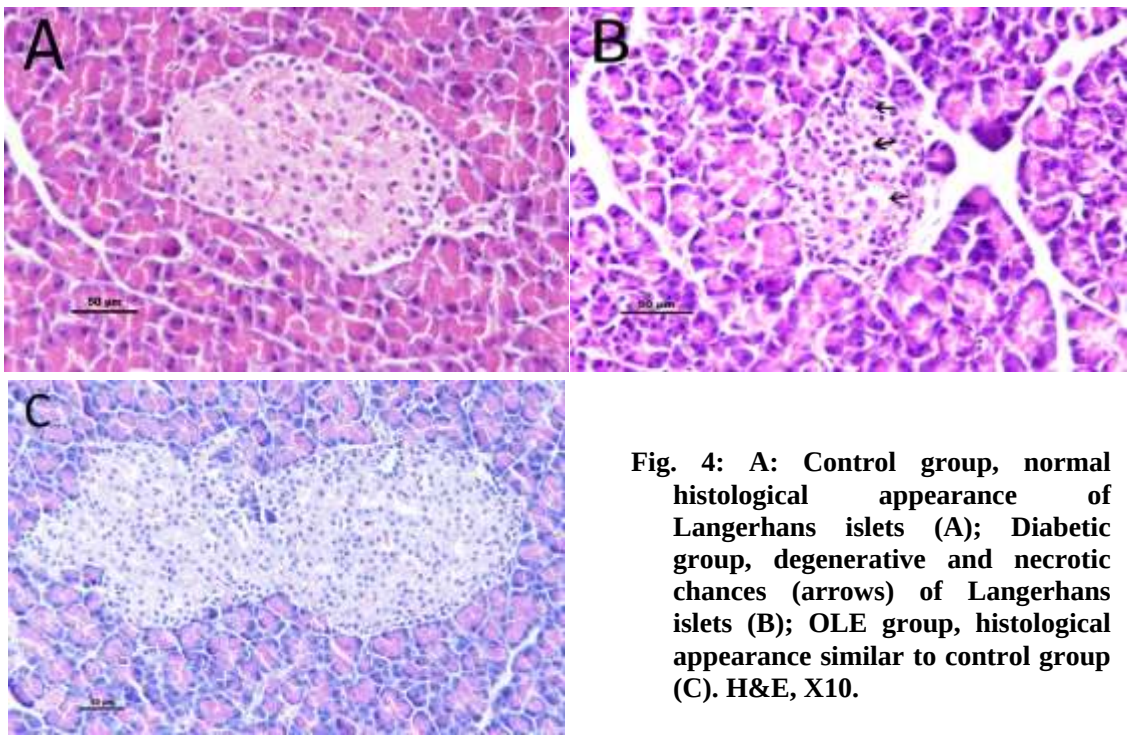

Fig. 4: A: Control group, normal histological appearance of Langerhans islets (A); Diabetic group, degenerative and necrotic chances (arrows) of Langerhans islets (B); OLE group, histological appearance similar to control group (C). H&E, X10.

Conclusion: Olive leaf extract has been demonstrated to be effective in increasing redox balances, augmenting antioxidant parameters, protecting against oxidative DNA damage and reversing histological findings in hepatocytes, and ameliorating atrophy in islet cells. Olive leaf extract may exhibit these effects by possessing an enhanced synergistic antioxidant action and by suppressing oxidative stress, thanks to the biophenolic compounds in OLE.

Ethical statement: The present study was conducted with prior permission from the Yuzuncu Yil University Animal Researches Local Ethic Committee vide their communication No. 2015/08.

Conflict of interest: The authors declare that there is no conflict of interest.

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