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PROTECTIVE EFFECTS OF *OLEA EUROPAEA* L. (OLIVE) LEAF EXTRACT AGAINST OXIDATIVE STRESS INJURY GENERATED WITH RENAL ISCHEMIA REPERFUSION

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ABSTRACT

In the present study, effect of olive (*Olea europaea* L.) leaf extract, known for its antioxidant quality, on ischemia/reperfusion (I/R)-induced injury in the kidney was investigated. A total of 3-4-month-old, weighing 200-250 grams each Spraque Dawley rats were divided into four groups (n=7): Group I (Sham), Group II (I/R+physiological saline solution), Group III (I/R+100 mg/kg of olive leaf extract), Group IV (I/R + 200 mg/kg olive leaf extract). A right kidney nephrectomy surgery was conducted to all groups under anesthesia. After 15 days of healing process physiological saline solution was orally given to the rats in Group I and II; olive leaf extract was given to Group III and IV. Forty five minutes ischemia and 24 hours reperfusion was conducted on all rats except those in Group I. At the end of the experiment, Blood Urea Nitrogen (BUN), Creatinine (CRE), and Uric Acid (UAC) values; and Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Cathepsin B and Cathepsin L enzyme activities and Malondialdehyde (MDA) levels were investigated. The results revealed that 200 $mg.kg^{-1}$ of olive leaf extract have a protective effect against oxidative stress damage caused by renal I/R.

Keywords: Kidney, Ischemia/Reperfusion, *Olea europaea* L. (Olive) Leaf Extract, Free Radicals, Oxidative Stress, Rats.

INTRODUCTION

Ischemia is the interruption or lack of blood flow to the organs for various reasons (Uyanoglu *et al*. 2011). Reperfusion is the restoration of blood flow to the tissue (Montalvo-Java *et al.* 2008). The mediators, released by polymorph nuclear leukocytes (PMNL) cells that settle on the tissue during ischemia and reactive oxygen species (ROS) are formed with reperfusion of the organs subjected to ischemia; and cause the pathology known as I/R damage. In addition, this pathology is also held responsible for the calcium that enters the cell to activate various proteases and phospholipases (Kurian *et al.* 2016; Malek and Nematbakhsh 2015). Cyclooxygenase and the lipoxygenase enzyme products, activated after arachidonic acid metabolism during I/R, also contribute to damage in cells (Kus, 2013). The pathology known as renal I/R damage has been the focus of attention after the spread of interventions such as kidney transplant, renal injury and renal artery surgery that reduce the blood flow in kidney (Akkoc, 2010).

As long as the speed of free radical formation is in balance with the defense system consisting of endogenous antioxidant enzyme, the organism is not affected by free radicals. However, if this free radical formation speed exceeds the speed of defense system, the free radicals begin to be harmful and emerge as oxidative stress in the organism (Kus, 2015; Senturk, 2008).

The radical damage is tried to prevent the potential harms of ROS with the help of protective antioxidant systems in the body (Kurian *et al.* 2016). Nowadays, the use of aromatic herbal oils and extracts as an alternative to antioxidants has become a current issue. For this purpose, some extracts have been widely used as natural antioxidants (Toptas, 2010). One of them is the olive leaf extract. It is reported that olive leaf extract exhibits a very strong antioxidant activity due to presence of some phenolic compounds in its structure such as hydroxytyrosol, oleuropein, verbascose, luteolin-7-glucoside, and diosmet (Benevante- Garcia *et al.* 2000; Visioli *et al.*2002).

Phenolic compounds in olive leaf such as oleuropein (Figure 1) and hydroxytyrosol have many biological effects in the organism and in the cells. Connecting free radicals (superoxide anion and hydrogen peroxide) (El and Karakaya 2009); preventing oxidation of Low Density Lipoprotein (LDL) (Tucker and Hayball 2002); and strengthening the endogenous antioxidant defense mechanisms are some of these effects (Visioli *et al.* 2002).

MATERIALS AND METHODS

The whole experimental study was carried out under the guide lines issued by Eskisehir Osmangazi University, Animal Experiments Local Ethics Committee vide No. 312-1/2013.

Animals: 3-4-month-old Spraque Dawley male albino rats weighing 200±50 grams were used in the experimental study. All animals were procured from Eskisehir Osmangazi University, Experimental Animal Production Laboratories (TICAM). The rats were allowed a week before the experiment to acclimatize to laboratory conditions. During the experiment, the animals were maintained in a room automatically set for 12 hour light/12 hour dark; in a temperature 22±2 °C with 45-50% humidity. They were fed with laboratory pellet chows and water was provided *ad libitum.*

The Application of *O. europaea* L. (Olive) Leaf Extract: A total of 3-4-month-old, weighing 200-250 grams each Spraque Dawley rats were divided into four groups (n=7): Group I (Sham), Group II (I/R+physiological saline solution), Group III (I/R+100 mg/kg of olive leaf extract), Group IV (I/R + 200 mg/kg olive leaf extract). In the experiment olive leaf extract with a high amount of oleuropein was used. Two dosages of *O.europaea* L. leaf extract (100 and 200 mg/kg) were orally administered once a day for 15 days by dissolving with 2 ml/kg 0.9 % sterile saline solution.

Experimental Protocol: Right kidney nephrotomy was performed to all groups under xylazine and ketamine anesthesia (Waynforth and Flecknell 1994). However, Group I had only laparotomy procedure. After 15 days of recovery period, while normal saline was given intragastrally to the animals in Group I and Group II once a day for 15 days, Group III and Group IV had olive leaf extract. The blood flow from left renal artery was stopped for 45 minutes with the help of atraumatic vascular clamp by isolating left renal artery and vein in the kidneys of the animals in Group II, III and IV. After 45minutes ischemia, the clamp was removed and reperfusion was performed for 24 hours (Sener *et al.* 2005; Waynforth and Flecknell 1994).

At the end of reperfusion process, the rats were sacrificed by removing the whole blood from their hearts under anesthesia. The blood samples were collected to estimate BUN, CRE and UAC values to assess the renal function; kidney tissue samples were examined to find out SOD, CAT, GPx, Cathepsin B, Cathepsin L enzyme activities and MDA values.

Biochemical Analyses: BUN, CRE and UAC levels were examined by using Crony Airone 200 RA autoanalyzer and BIOLABO, FRANCE commercial kits.

Determining enzyme activity in kidney tissue: The total protein amounts of SOD, CAT and GPx isoenzymes, activities of which will be determined with homogenates obtained from samples, were measured with Qubit device according to Bradford method. In kidney tissue sample homogenates, SOD, CAT and GPx isoenzymes were made run in the gel with Native Gel Electrophoresis technique and their enzyme activities were determined according to the reactions they performed with their substrates (Jayakumar *et al.* 2007). CAT activity was identified according to the method adopted by Woodbury *et al* (1971). SOD activity was identified according to Beauchamp and Fridovich's (1971) method. GPx isoenzyme activity was identified according to the method adopted by Lin *et al* (2002). Areas formed after enzyme activity were measured using a gel imaging system (Kodak Molecular Imaging Software in Kodak Gel Logic 1500 Imaging System).

The amount of MDA in kidney tissues was spectrophotometrically measured by using the method adopted by Ohkawa *et al.* (1979). The results obtained are expressed in nmol/ ml value.

Measuring Cathepsin B and Cathepsin L Enzyme Activity: Detection of cathepsins leaking from lysosomes into cytoplasm and remaining in intact lysosomes can give clues about cell damage. The ratio of cathepsin activities measured separately in cytosolic (C) and lysosomal (L) fractions (unit/mg) shows the amount of lysosomal damage. Protein levels of kidney tissue were determined with the biuret method (Layne 1957). Cathepsin B and L enzymatic activity was obtained according to the method modified by Kirschke *et al* (1983), by correlating cytosolic fraction results with lysosomal fraction results (C/L).

Histopathological Analyses: Kidney tissue samples taken for histological examination were placed into 10% neutral formaldehyde solution. After routine tissue processing stage, they were painted by using Hematoxylin and eosin (H&E) method. All tissue sections were histologically examined with CH40 model Olympus light microscope; and their images were taken with 3.2.0 model Spot Insight digital camera and Spot Advanced 4.0.6 software.

Statistical Analyses: The results were expressed as the mean ± standard error of seven animals per group. One way analysis of variance (ANOVA) and Tukey test were used for the analysis and comparison of data within and between groups (SPSS 20.0 for windows). Differences were considered significant at $P<0.05$.

RESULTS

Biochemical findings in serum samples: At the end of the experiments, BUN, CRE and UAC values obtained from serum samples of all groups were comparatively shown in Table 1. It was found out that Group II had meaningful differences ($P<0.05$) compared to Group I in terms of its BUN, CRE and UAC levels. When Group I and Group II were compared to Group III and Group IV, Group III had meaningful difference compared to Group I ($P<0.05$) while there is no significant difference between Group IV and Group I.

Table 1. Mean ± SE value of BUN, CRE and UAC found in blood samples of experimental group rats (n=7).

Group	BUN (mg/dl)	CRE (mg/dl)	UAC (mg/dl)
I	63,69 ± 1,048	0,57 ± 0,02	0,74 ± 0,03
II	226,02±21,57^{a}	2,61 ± 0,16^{a}	1,40 ± 0,42^{a}
III	160,18±3,23^{ab}	1,86 ± 0,22^{ab}	0,50 ± 0,03^{ab}
IV	81,76±5,25^{bc}	0,74 ± 0,07^{bc}	0,73 ± 0,05^{bc}

Different **a:** from Group I; **b:** Group II and **c**: Group III; $P<0.05$.

Renal Isoenzyme Activity: In tissue samples SOD isoenzyme was observed as two strips while CAT isoenzyme is one strip and GPx isoenzyme is four strips (Figure 2). The numeric field values obtained after SOD, CAT and GPx enzyme activities and observed on the gel were measured (Table 2).

When SOD, CAT and GPx strip fields were examined, the strip field has low-density in Group I kidney tissue while it has the highest density in Group II. On the other hand, it was observed that the strip fields of Group III and Group IV have lower density than Group II; especially the strip field of Group IV was seen to be very close to Group I (Figure 2).

Cathepsin B and Cathepsin L: As the Figure 3 shows, Group II had significantly higher Cathepsin B and Cathepsin L values ($P<0.05$). However, they were significantly low in Group I ($P<0.05$). As for Group III and Group IV, Cathepsin B and Cathepsin L values were found to be significantly lower than Group II ($P<0.05$).

Table 2. The average values of strip fields after SOD, CAT and GPx enzyme activities from kidney tissue samples of experiment group rats.

Group	CAT mm^2	SOD mm^2		GPxmm^2			
		1	2	1	2	3	4
I	18,27	29,99	27,71	9,54	7,98	6,30	3,68
II	34,89	43,80	25,51	14,36	15,81	9,02	5,47
III	27,65	38,21	23,57	11,21	11,45	8,06	6,53
IV	24,37	27,27	16,26	7,98	8,07	8,36	5,64

MDA Levels: While MDA levels in Group II were significantly higher than Group I, they were significantly low in Group I (Figure 4) ($P<0.05$). They were significantly lower in Group III and Group IV compared to Group II ($P<0.05$).

Histopathological analysis: Histological results obtained from the results of the study are shown in Figure 5. After the examinations with the light microscope, it was observed that kidney sections of Sham group were in normal appearance (Figure 5.A); but kidney sections belonging to I/R group of animals had glomerular deformation and tubular degeneration, vacuolization, inflammation and fluid accumulation inside intense tubules due to I/R damage (Figure 5.B). When we examined the 100 mg.kg^{-1} histologic preparations, we found out that the heavy damage in tubule cells in I/R group reduced leastwise (Figure 5.C). However, the renal sections of 200 mg.kg^{-1} treatment group rats showed that the damage observed in I/R group significantly reduced. The tubular cells and glomerular structure were similar to control group (Figure 5.D).

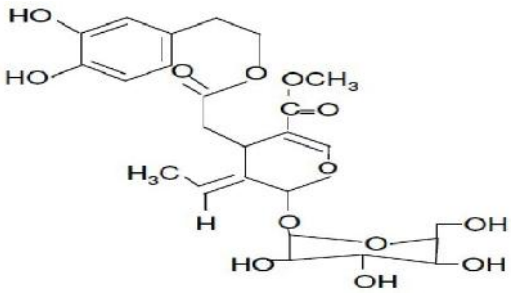

Figure 1. The Chemical structure formula of Oleuropein (Winkelhausen *et al*., 2005).

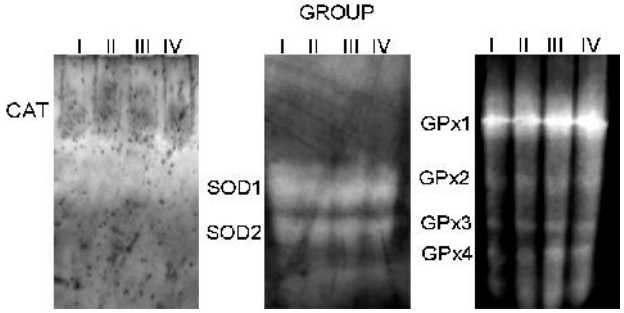

Figure 2. Electrophoretic strips after CAT, SOD and GPx enzyme activities.

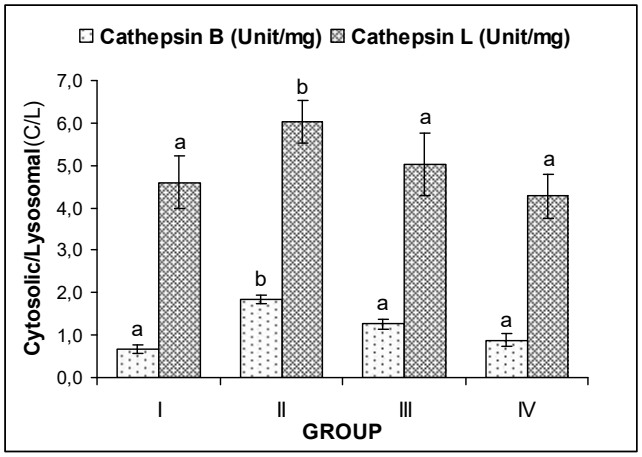

Figure 3. Cathepsin L and Cathepsin B activities of experiment groups. Mean ± standard error (SE) value (n=7). The use of the same letters on histograms is statistically similar (P< 0,05).

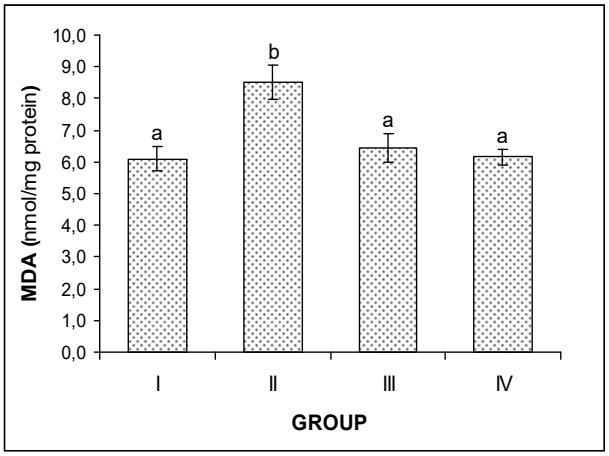

Figure 4.MDA levels of the groups. Mean ± standard error (SE) value (n=7). The use of the same letters on histograms is statistically similar (P< 0,05).

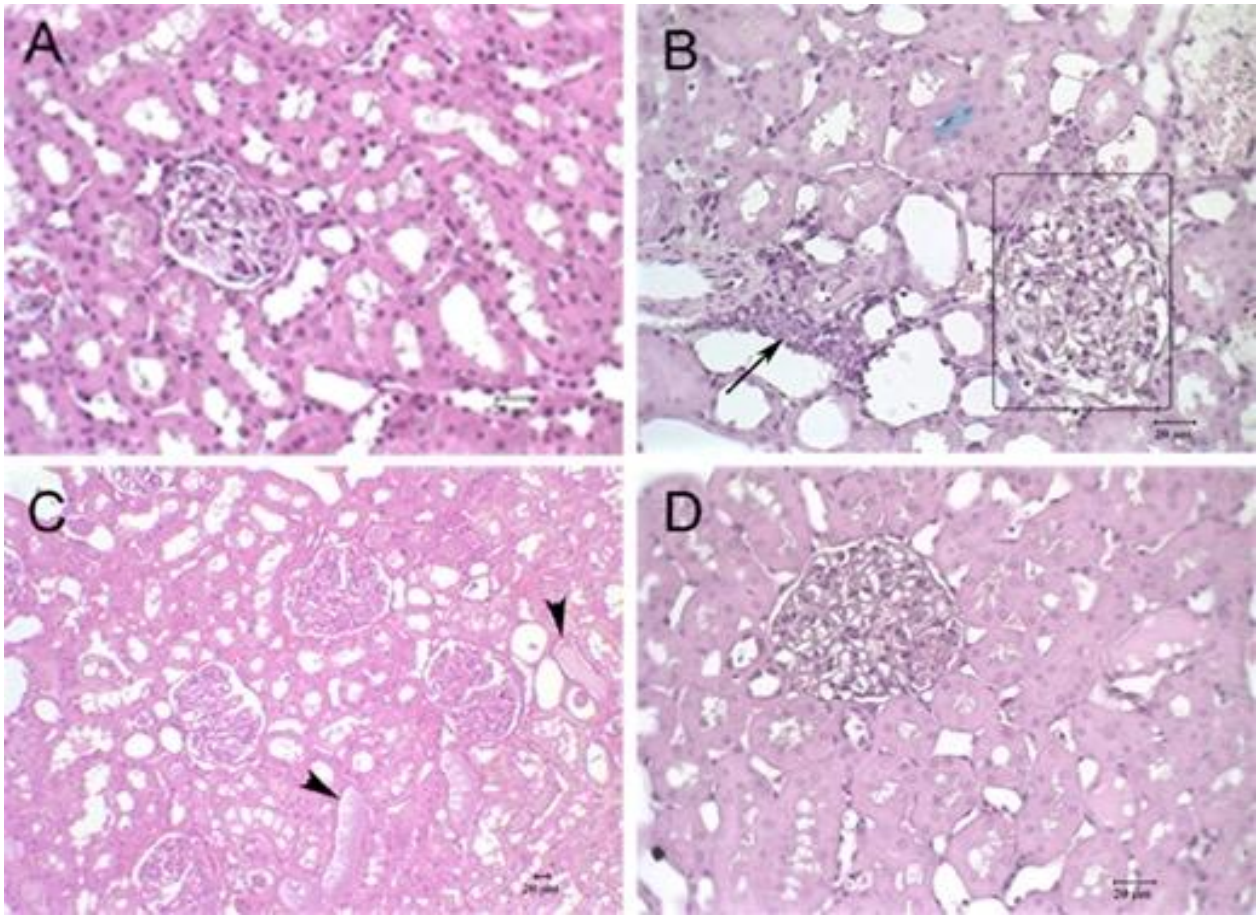

Figure 5. Histologic images of sham and experiment groups. A: Sham group. Normal glomerulus and tubular structure; B: I/R group. PMNL Infiltration (➔) and deformation in glomerular structure (□); C: 100 mg.kg^{-1} olive leaf extract administration group. Fluid decrease inside tubules (➔); D: 200 mg.kg^{-1} olive leaf extract administration group. Near normal glomerulus and tubule cells.

DISCUSSION

Renal I/R damage is one of the common causes of acute renal failure, and is a complex pathophysiological process leading to increased vascular permeability, tissue necrosis, cell injury and eventually to cell death (Bi *et al*. 2009; Salehipour *et al.* 2010). Organisms have developed defense systems against ROS after I/R, most important of which is antioxidant enzyme systems (Demirkıran, 2011).

In this study, it was observed that SOD, CAT and GPx enzyme activity reached to their highest level in Group II. The increase of ROS production in the renal samples of I/R group might be the cause of this situation. In addition, the low antioxidant enzyme activity indicates less ROS exposure. It is suggested that the significant decline of the treatment group is due to antioxidant properties of olive leaf extract.

In a study conducted by Bayramoglu *et al.* (2011), SOD and CAT values in I/R group were significantly high but relatively low in Echinacea administered treatment group rats in renal failure cases during 24 h reperfusion after 45 min ischemia. In another study on the antidiabetic effects of olive leaf extracts, a diabetes model was created using streptozotocin and they wanted to find out the possible effects of these extracts in reducing the oxidative stress caused by diabetes and its complications. Olive leaf ethanol extract was administered as a single oral dosage for 14 days and at the end, a decrease in SOD, GSH-Px activities in the liver of rats was observed (Aggul, 2012). Those findings are consistent with the results we have obtained in our study.

One of the most used methods in evaluating the renal functions after I/R damage is to determine the BUN and CRE levels known as the indicators of glomerular dysfunction. Many studies report that the increase BUN and CRE levels in kidney can be due to free radical induced tissue damage after I/R (Gunduz, 2010; Korkmaz and Kolonkaya 2010).

The CRE level of serum is an important parameter in the diagnosis of renal failure and in monitoring the progress (Gunduz, 2010). The increase observed in serum CRE level which directly shows the glomerular filtration after I/R damage is a condition that indicates the dysfunction of kidney proximal tubule cells (Thiemerman *et al.* 2003).

In terms of their serum CRE levels, Group II and Group III had significantly higher serum CRE levels compared to Group I ($P<0.05$). This situation is consistent with existing literature. In the study conducted by Tavafi *et al*. (2012), the rats were given 100 $mg.kg^{-1}$ gentamicin intraperitoneal for 12 days. After this period, they investigated the nephrotoxicity damage caused by renal oxidative stress. 25, 50 and 100 mg/kg doses of olive leaf extract were administered with gavage 1 hour before the gentamicin injection for 12 days. The results showed that there was a positive decline in serum BUN and CRE levels in olive-leaf-extract-administered groups. In our study, the BUN and CRE levels increasing in I/R group rats showed a decline in olive leaf extract administered groups depending on the dosage. This finding is consistent with Tavafi *et al.* (2012) findings.

The similar experiments indicated that serum BUN and CRE levels increased in control groups and decreased in treatment groups in I/R related damages (Koga *et al.* 2012; Korkmaz and Kolonkaya 2010).

After oxidation, especially after peroxidation in cellular membrane lipids, one of the resulting product is MDA and the size of the damage can be determined with Thiobarbituric Acid (TBARS) test. After renal I/R, the amount of MDA increases due to the oxidation of unsaturated fatty acids. In a study conducted using gingko biloba, MDA values from renal homogenates after 45min ischemia and 6 h reperfusion were examined. MDA value in I/R group increased depending on lipid peroxidation compared to control group. After I/R damage simulated by giving 50 mg/kg Slymarin extract 60 min before ischemia alongside I/R, MDA level decreased (Sener *et al.* 2005).

According to findings obtained in our study, MDA values are higher in I/R group compared to the control group. Since the values from olive-leaf-extract administered group were similar to the control group, it can be suggested that olive leaf extract has positive effects in preventing lipid peroxidation due to its antioxidant properties.

Histologic evaluation of the microtome kidney tissue sections revealed that Group I tissue samples showed glomeruli and bowman capsule of renal tubule cells in normal appearance and that there is no fluid accumulation inside and between the tubules. As for the samples of Group II, we observed serious tubular damage, bloodstains in glomerulus, expansion in the range of bowman capsule, vacuolization in renal tubule cells and inflammation in some areas. Depending on these conditions, we also observed tubular degeneration, bloodstains within cells, cell loss in the tubules, fluid accumulation in the tubule cells. The olive leaf extract in 100mg/kg dose is observed to be reducing the damage compared to I/R group. We had similar results with the sham group in the 200 mg/kg dose of olive leaf extract. This is an indication that this extract could prevent ROS effect.

In a similar study conducted by Kurcer *et al.* (2007), when the kidney tissues were histopathologically examined after 24h reperfusion period, they observed intense necrosis in proximal tubules, apoptosis, mild congestion in glomeruli and intense congestion in medulla. In melatonin administered group, the melatonin protected the renal histology, which might be due to its antioxidant properties.

In another study, the results obtained from histological data on I/R damage showed that vacuolization in tubule cells, tubular dilation, intercellular bloodstains, expansion of bowman capsule range after I/R damage (Senturk, 2008).

Cathepsins are group C1 cysteine proteases called as papain family. They are synthesized as inactive proenzymes and glycated as posttranslations. They are directed towards lysosomal sections using cellular mannose-6-phosphate receptors. They can be released into the cytoplasm from lysosomes and can catalyze substrate division there. ROS causes dissolution of lysosomal permeability (Chwierals *et al.* 2006). As the result of degradation of lysosomes, Cathepsins leak into cytoplasm (Kaasik *et al.* 2005). The ratio of intact cathepsin activities remaining after lysosomes move into cytoplasm shows the amount of lysosomal damage. For this reason, while there is an increase of free oxygen radicals after the ischemia the cells are undergoing and the subsequent reperfusion, Cathepsin B and Cathepsin L values were high in I/R group in this study. The-olive-leaf-extract-administered Group III and Group IV had low level Cathepsin B Cathepsin L values. It is argued that this is due to the antioxidant properties of olive leaf extract.

Under the light of histological and biochemical results obtained from this study, it is assumed that oral administration of olive leaf extract has protective effects depending on the dosage. However, these findings need to be supported by further, extensive mechanism-based molecular experiments.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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