

Hepatoprotective effects of purified oleuropein from olive leaf extract against ethanol-induced damages in the rat

Alirezaei, M.^{1*}; Dezfoulian, O.²; Kheradmand, A.³; Neamati, Sh.⁴; Khonsari, A.⁵ and Pirzadeh, A.⁶

¹Department of Basic Sciences, School of Veterinary Medicine, Lorestan University, Khorramabad, Iran; ²Department of Pathobiology, School of Veterinary Medicine, Lorestan University, Khorramabad, Iran; ³Department of Clinical Sciences, School of Veterinary Medicine, Lorestan University, Khorramabad, Iran; ⁴BSc in Veterinary Medicine Lab. Technology, Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran; ⁵MSc in Herbal Physiology, Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran; ⁶BSc in Veterinary Medicine Lab. Technology, Laboratory of Pathobiology, School of Veterinary Medicine, Lorestan University, Khorramabad, Iran

*Correspondence: M. Alirezaei, Department of Basic Sciences, School of Veterinary Medicine, Lorestan University, Khorramabad, Iran. E-mail: alirezaei_m54@yahoo.com

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Summary

Olives and olive oil contain large amounts of oleuropein. This phenolic compound is responsible for their bitter taste and pungent aroma and has been recognized as a powerful hypotensive, hypoglycemic and antioxidant agent. Thus, the aim of the present study was to evaluate the antioxidant properties of oleuropein on ethanol-induced oxidative damage and to examine its beneficial effects on liver function. Thirty-two adult male Sprague-Dawley rats were divided into four equal groups: the first group served as untreated control. The second group of rats were given ethanol (4 g/kg) orally. Group 3 received oral oleuropein (15 mg/kg). The final group of rats were fed ethanol (4 g/kg), 120 min after oral administration of oleuropein (15 mg/kg). All of the treatments were applied for 4 weeks via gavage. Administration of ethanol to rats induced toxicity in their liver, as shown by the significant elevation in the serum levels of transaminases, total cholesterol as well as liver histopathological findings. Elevation of glutathione peroxidase activity, the hepatic main antioxidant enzyme, and total glutathione was observed to suppress oxidative stress in the ethanol group. TBARS (an indicator of lipid peroxidation) concentration is also increased in ethanol-treated rats. In contrast, oleuropein during ethanol treatment in rats resulted in a higher antiperoxidative enzyme activity, catalase, and inhibited toxicity to the liver, as monitored by the reduction in ALT and AST levels and TBARS concentration. It is suggested that oleuropein possesses beneficial antioxidant effects against ethanol-induced liver toxicity, and therefore use of olive leaf extract may have prophylactic value in reducing the common complications resulting from oxidative stress in alcoholism.

Key words: Olive leaf, Oleuropein, Ethanol, Rat, Liver

Introduction

It is well known that ethanol consumption induces oxidative stress in the liver, and is linked to the imbalance between the prooxidant and the antioxidant systems in favor of the former (Nordmann *et al.*, 1992). During the metabolism of alcohol, acetaldehyde is formed as the principal metabolite. When acetaldehyde is oxidized, it produces superoxide free radicals that are

able to react with hydrogen peroxide to form other types of free radicals, such as hydroxyl radicals (Smith *et al.*, 2005). The susceptibility of liver to ethanol toxicity can promote alcoholic liver disease (Lieber, 1994), and in recent years, a wide variety of antioxidants and diverse diets have been tested to offset the oxidative stress induced by ethanol abuse (Nanji *et al.*, 2003; Kasdallah-Grissa *et al.*, 2008; Ramírez-Farías *et al.*, 2008).

To preserve the integrity of biological membranes from detrimental oxidative stress caused by free radicals, both enzymatic and non-enzymatic mechanisms are present in the cell (McCord, 1993). The components of this defense system can be divided into two main groups: antioxidant enzymes (GPx, SOD, and CAT) and small endogenous antioxidant molecules such as glutathione (GSH), and coenzyme Q (Casalino *et al.*, 2002). Lipid peroxidation in biological membranes is a highly destructive phenomenon produced when reactive oxygen species and free radicals attack the double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid peroxides (Halliwell and Chirico, 1993). These compounds, when generated in biological systems, are cytotoxic and, if not scavenged, may initiate a chain reaction. In this regard, sensitivity of the LDL lipids to oxidative stress depends on an appropriate balance between their amount on polyunsaturated fatty acids and antioxidant concentrations (Gutierrez *et al.*, 2001). Previous studies have demonstrated that polyphenolic compounds are potent inhibitors of LDL oxidation *in vitro* and *in vivo* (Visioli and Galli, 1994; Visioli *et al.*, 1995; De La Cruz *et al.*, 2000). Inhibition of LDL oxidation is important because the *in vivo* oxidation of LDL is strongly linked to the formation of atherosclerotic plaques, which in turn contribute to the development of coronary heart disease (Edgecombe *et al.*, 2000). Indeed, it was reported that the decrease in LDL-cholesterol concentration and the increase of HDL-cholesterol level could accelerate the removal of cholesterol from peripheral tissues to liver for catabolism and excretion, subsequently inhibiting LDL-cholesterol oxidation (Young *et al.*, 2004).

In the Mediterranean area, olive leaves are one of the by-products of farming of the olive grove; they can be found in high amounts in the olive oil industries and they accumulate during pruning of the olive trees (Tabera *et al.*, 2004). Olive leaves are considered a cheap raw material and a useful source of high-added value products (Briante *et al.*, 2002; Jemai *et al.*, 2008). The main phenolic compound in olive leaves is the glycosylated form of oleuropein (Amro *et al.*, 2002; Visioli *et al.*, 2002). It is a

natural phenolic antioxidant, which is present in high concentration in olives, olive oil and olive tree leaves (Andreadou *et al.*, 2007). Despite the wide body of evidence linking the antioxidant properties of olive oil with positive health outcomes (Kasdallah-Grissa *et al.*, 2008), the current study focused on the antioxidant effects of purified oleuropein from olive leaves (in our laboratory) against ethanol-induced oxidative damage to the liver. Therefore, the aim of this study was to investigate the beneficial effects of oleuropein on many biochemical indicators of liver function and antioxidant enzyme activities as well as lipid peroxidation in liver of rats.

Materials and Methods

Oleuropein purification

The oleuropein was isolated from *Olea europaea* leaves according to the method described previously (Andreadou *et al.*, 2006).

Experimental design

Thirty-two adult male Sprague-Dawley rats were housed in temperature-controlled conditions under a 12:12-h light/dark photocycle with food and tap water supplied *ad libitum*. After one week acclimatization period, the rats were divided into four equal groups (n=8 rats per cage), weight gain and food consumption were determined at weekly intervals and treated daily in the following order: control group received 1 ml normal saline, ethanol group received ethanol (4 g/kg), oleuropein group received oleuropein soluble in water (15 mg/kg) and oleuropein plus ethanol group received oleuropein (15 mg/kg) and after 120 min, feeding with ethanol solution (4 g/kg), and all treatments were applied orally by gavage for 4 weeks. Dose of ethanol was determined according to the previous studies (Wu and Cederbaum, 2003; Turner and Lysiak, 2008) and oleuropein based on the average consumption of olive drupes and olive oil in the Mediterranean diet in humans (Andreadou *et al.*, 2006, 2007). All rats were treated humanely and in compliance with the recommendations of the Animal Care Committee for the Lorestan University of

Medical Sciences (Khorramabad, Iran). One the day after the last gavage, the rats were sacrificed using diethyl ether anesthesia (Dagenham, UK) by decapitation. Immediately after rat killing, blood sample was collected via cardiac puncture and serum was obtained by centrifugation at 3,000 g for 5 min and stored at -70°C in aliquots until the analysis. Livers were excised immediately, washed with ice-cold physiologic saline solution; the same lobes of livers were stored at -70°C, other lobes were removed for histopathological analysis.

Histopathological analysis

Liver tissues were fixed in 10% formaldehyde solution. The washed tissues were dehydrated in increasing gradient of ethanol and finally cleared in toluene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 µm thickness and stained with haematoxylin and eosin. The sections were then viewed under light microscope to detect eventual histopathological changes.

Biochemical parameters of liver function

Concentrations of total cholesterol (TC), total triglycerides (TG), and activity of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in serum were measured by using commercially available diagnostic kits supplied by Ziest Chemistry Laboratories, Ltd. (Ziest Chemistry, Tehran, Iran).

Tissue preparation for protein measurement and enzyme assay

Rat livers were thawed and manually homogenized in cold phosphate buffer (0.1 M, pH = 7.4) containing 5 mM EDTA using liquid nitrogen, and protein content of tissue homogenates was determined using a colorimetric method of Lowry with bovine serum albumin as standard (Lowry *et al.*, 1951).

Total glutathione (GSH content)

Total glutathione was estimated by the model as described previously (Sedlak and Lindsay, 1968).

Measurement of GPx activity

Glutathione peroxidase activity (GPx) was evaluated with Randox GPx detection kit according to the manufacturer's instructions as described previously (Randox, UK). One unit (U) of GPx was defined as 1 µmol of oxidized NADPH per min per milligram of tissue protein. The GPx activity was expressed as unit per milligram of tissue protein (U/mg protein).

Measurement of CAT activity

Tissue catalase activity was assayed using the method described previously (Claiborne, 1986). The CAT activity was expressed as the unit that is defined as 1 µmol of H₂O₂ consumed per min per milligram of tissue protein (U/mg protein).

Measurement of lipid peroxidation

The amount of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the liver. Tissue TBARS was determined by following the production of thiobarbituric acid reactive substances as described previously (Subbarao *et al.*, 1990). TBARS results were expressed as nmol per milligram of tissue protein (nmol/mg protein).

Statistical analysis

All results are presented as mean ± SEM. Data were compared by one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. Previously, all variables were tested for normal and homogeneous variances by leven's statistic test. A calculated p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the statistical package SPSS version 11.5.

Results

The mean values (±SEM) of the biochemical indicators of liver function (ALT, AST, TG, TC) of rats are presented in Table 1. The activity of serum AST was significantly higher in the ethanol group compared to the other groups (P<0.05). Also, administration of ethanol significantly increased ALT activity in ethanol-treated

rats compared to the oleuropein group. Oleuropein supplementation prevented the adverse effects of ethanol on transaminase (ALT and AST) activities and returned them to the normal value. Total cholesterol concentration of treated rats by oleuropein showed a significant decrease compared to the ethanol group ($P < 0.05$). Although the concentration of total triglyceride in oleuropein-treated rats was lower than the ethanol group, this reduction was not statistically significant ($P = 0.707$). Taken together, when oleuropein was administered prior to ethanol, it could decrease the level of these parameters to near the control group.

Changes in antioxidant enzyme activities including CAT and GPx, as well as glutathione (GSH) content in the liver of rats are shown in Table 2. Administration of oleuropein significantly increased CAT activity in the oleuropein group compared to the other groups ($P < 0.05$). In contrast, GSH content (in order compensatory) increased significantly in the ethanol group compared to oleuropein-treated rats ($P < 0.05$). In this sense, GPx activity tended to approach significance in ethanol-treated rats compared to oleuropein-treated animals ($P = 0.076$).

Treatment of rats with ethanol significantly increased lipid peroxidation (as shown by the TBARS concentration), while in contrast, administration of oleuropein to

the oleuropein plus ethanol group could prevent an increase in TBARS concentration ($P < 0.05$). In addition, TBARS concentrations in oleuropein and the control group were remarkably lower compared to the ethanol-treated rats ($P < 0.05$; Fig. 1).

The histopathological analysis of liver in the ethanol, oleuropein, and oleuropein plus ethanol treatment is presented in Fig. 2. The scattered necrosis of several hepatocytes as well as infiltration of both polymorph and mono nuclear cells in the portal spaces and occasionally into the parenchyma were similar to the striking histopathological findings in the ethanol-treated rats (Figs. 2A-C), while the highest population of cells had no pathological changes in the oleuropein plus ethanol-treated rats (Fig. 2D). Indeed, oleuropein treatment allowed, to a certain extent, overcoming the hepatic architecture aberrations with the preservation of parenchymal structure.

Discussion

The major findings of this work in rats are (1) ethanol administration at a dosage of 4 g/kg BW for 4 weeks is associated with an oxidative stress and lipid peroxidation; (2) administration of purified oleuropein from olive leaves is able to reduce the oxidative damage caused by ethanol in the liver; and (3) ethanol enhances GSH content

Table 1: Biochemical indicators of liver function (alanine aminotransferase, aspartate aminotransferase, total triglyceride, and total cholesterol) in serum of experimental groups

Groups	ALT (U/L)	AST (U/L)	TG (mg/dl)	TC (mg/dl)
Control	75.0 ± 7 ^{a,b}	180.6 ± 29.2 ^a	103.6 ± 9.8 ^a	71.0 ± 5.7 ^{a,b}
Ethanol	90.2 ± 6.4 ^a	332.8 ± 16 ^b	118.4 ± 25.7 ^a	85.8 ± 7.4 ^a
Oleuropein	53.2 ± 6.2 ^b	152.2 ± 14.7 ^a	78.2 ± 7.9 ^a	57.4 ± 2.7 ^b
Oleuropein and Ethanol	71.0 ± 7.7 ^{a,b}	227.2 ± 17.2 ^a	107.6 ± 10.6 ^a	58.2 ± 4 ^{a,b}

^{a, b} Means in the same columns with different superscripts differ statistically (One-way ANOVA followed by Tukey's post hoc test). Data are expressed as mean ± SEM (n = 8 rats per group)

Table 2: Antioxidant enzyme activities (catalase, glutathione peroxidase) as well as total glutathione (GSH-content) in the liver of experimental groups

Groups	CAT (U/mg protein)	GPx (U/mg protein)	GSH (nmol/mg protein)
Control	172.6 ± 1 ^a	9.3 ± 1.5 ^a	0.67 ± 0.08 ^{a,b}
Ethanol	158.6 ± 2.2 ^a	10.4 ± 2.2 ^a	0.72 ± 0.04 ^a
Oleuropein	223.3 ± 1.7 ^b	5.3 ± 1 ^a	0.46 ± 0.04 ^b
Oleuropein and Ethanol	193.6 ± 2.6 ^a	7.9 ± 0.04 ^a	0.56 ± 0.07 ^{a,b}

^{a, b} Means in the same columns with different superscripts differ statistically (One-way ANOVA followed by Tukey's post hoc test). Data are expressed as mean ± SEM (n = 8 rats per group)

and GPx activity (in order compensatory) to suppress oxidative stress in the rat liver.

The present data indicated that oleuropein consumption suppresses oxidative stress as monitored by the elevation activity of the main anti-oxidative enzyme, catalase, and decreases lipid peroxidation products in the rat liver. Our results indicate that ethanol induces oxidative stress and enhances GSH content and GPx activity (in compensatory order). In this context, Masella *et al.* (2005) have expressed that “antioxidant responsive elements (AREs) are present in the promoter regions of many of the genes inducible by oxidative and chemical stresses”. Thus, it appears that in the present study consumption of ethanol (as oxidative inducing agent) was able to increase activity

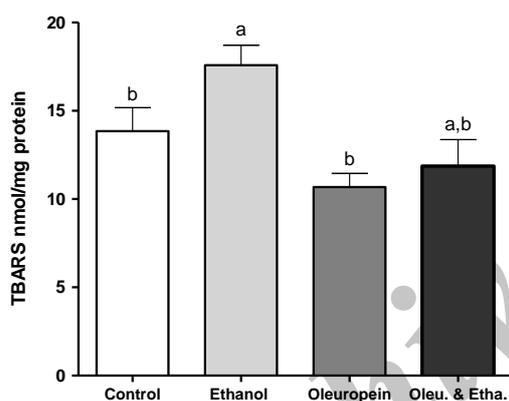


Fig. 1: Thiobarbituric acid reactive substances (TBARS) concentration in the control and treated rats. Data are expressed as Mean \pm SEM of TBARS (nmol/mg protein of liver tissue). Comparison among groups was made using one-way ANOVA followed by Tukey’s post hoc test ($P < 0.05$). ^{a, b} Means with different superscripts differ statistically ($n = 8$ rats per group)

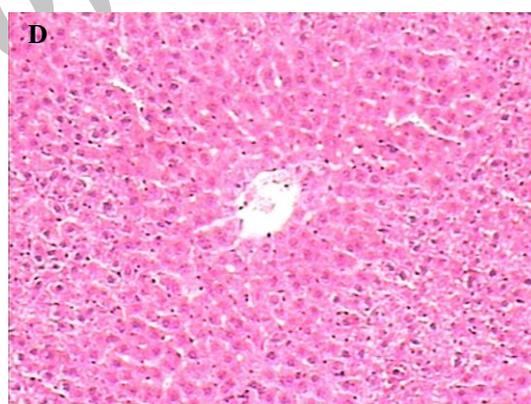
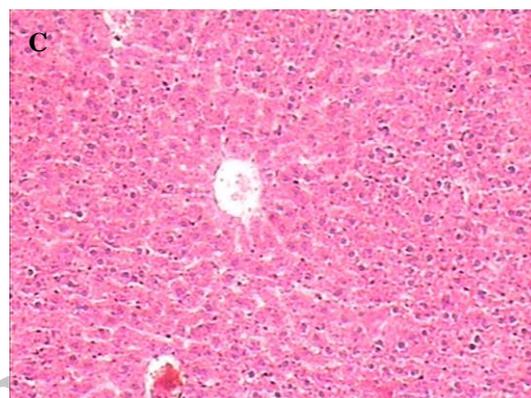
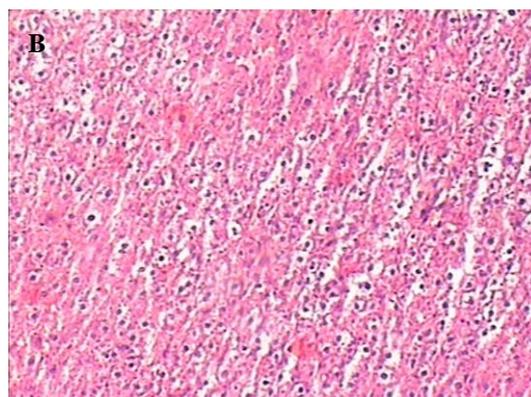
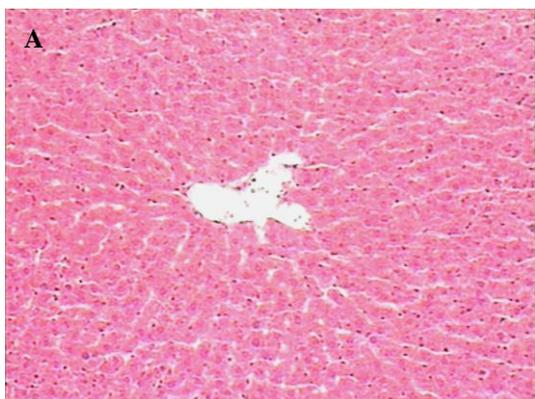


Fig. 2: Histopathological features of liver tissue in normal (A), ethanol-treated (B), oleuropein-treated (C) and oleuropein plus ethanol-treated rats (D), (H&E, $\times 100$). Hepatocytes have normal appearance in control liver (A). Intracytoplasmic accumulation of glycogen or lipid droplets in most hepatocytes is formed, in which their nuclei is located in the center or pushed into one pole of the cytoplasm. The lesion is principally initiated from the central vein and expanded to the periphery in the ethanol-treated rats (B). The normal hepatic cells are completely intact (C). Only a small number of hepatocytes adjacent to the central vein or midzonal area show fatty changes or glycogen depositions in their cytoplasm, whereas the majority of cells show a normal appearance with reversal of ethanol-induced changes (D)

of the antioxidant enzymes such as GPx by the compensatory mechanism via AREs. In this regard, previous studies showed that ethanol could enhance GSH content in the developing cerebellum of neonatal rats (Smith *et al.*, 2005), and GPx activity in the kidney (Dinu *et al.*, 2005) as well as cerebellum of rats (Alirezaei *et al.*, 2011). While in contrast, chronic feeding of ethanol (8 weeks) due to depletion of GSH decreased activity of antioxidant enzymes such as GPx, and SOD (Taati *et al.*, 2011). Thus, our results support and extend previous reports suggesting that ethanol intoxication generally impairs the liver antioxidant defense system and induces lipid peroxidation in experimental animals (Kasdallah-Grissa *et al.*, 2008).

The principal active component in olive leaf extract is oleuropein. Its concentration in olive leaf extract was high compared to the other phenolic compounds (Jemai *et al.*, 2008). Oleuropein is a phenolic compound, which has been shown to possess diverse healing properties for its vasodilatory, hypotensive, anti-inflammatory, anti-rheumatic, diuretic, anti-atherogenic, antipyretic and antioxidant effects (Visioli *et al.*, 1998; Diaz *et al.*, 2000; Visioli *et al.*, 2000; Khayyal *et al.*, 2002; Visioli *et al.*, 2002; Vissers *et al.*, 2002; Al-Azzawie and Alhamdani, 2006). It seems that many of these pharmacologic features of oleuropein are due to its potent antioxidant actions (Manna *et al.*, 2002; Manna *et al.*, 2004; Andreadou *et al.*, 2006). It has been shown that oleuropein is rapidly absorbed from the intestine with t_{max} of 2 h reaching a peak of 200 ng/ml of plasma after administration of 20 mg/kg oleuropein in rats (Del Boccio *et al.*, 2003). In addition, oleuropein has been detected in plasma only in its glycoside form, suggesting that it is absorbed intact from the intestine (Al-Azzawie and Alhamdani, 2006). Hence, the high availability of oleuropein in its active form *in vivo* may explain the positive impact on the enzymatic and non-enzymatic (GSH-content) antioxidants observed in our study.

Oleuropein is able to chelate metal ions, such as Cu^{2+} and Fe^{2+} , which catalyse free radical generation reactions (Andrikopoulos *et al.*, 2002). Oleuropein and its metabolite, hydroxytyrosol, both possess the structural

requirement (a catechol group) needed for optimum antioxidant and/or scavenging activity (Al-Azzawie and Alhamdani, 2006). Therefore, oleuropein attenuates ethanol-induced oxidative stress and lipid peroxidation in this study possibly by two pathways; First, by rapid conversion of H_2O_2 to H_2O and preventing H_2O_2 accumulation and second, by quenching the hydroxyl radicals in which trapping HO° leads to oxidative breakdown of the phenolic compounds (Al-Reza *et al.*, 2009). Indeed, both oleuropein and hydroxytyrosol have been shown to be scavengers of superoxide anions, and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals (Al-Azzawie and Alhamdani, 2006). Therefore, it was concluded that oleuropein protects the membrane of liver cells against ethanol-induced oxidative damage and appears to be a good candidate in the prevention of ethanol-induced injuries in the liver.

Ethanol-induced liver injury associated with increased oxidative stress and free radical-mediated tissue damages are widely demonstrated in rats and in humans (Lieber, 1994; Manna *et al.*, 2002). Several studies have demonstrated the ability of olive oil to inhibit oxidative stress in the liver through various mechanisms (De La Cruz *et al.*, 2000; Aguilera *et al.*, 2003; Kasdallah-Grissa *et al.*, 2008). The present work showed that purified oleuropein from olive leaves, is able to prevent lipid peroxidation when tested on rat liver. Similar results with olive oil compounds preventing lipid peroxidation were found in the liver of rats chronically exposed to ethanol (Kasdallah-Grissa *et al.*, 2008). Thus, our data suggest that the potent antioxidant effect exerted by phenolic constituent of olive leaves, oleuropein, might contribute to protecting the structure of phospholipids containing polyunsaturated fatty acids present in rat liver membranes from oxidation.

It is well known that oxidative stress is one of the main factors that links hypercholesterolemia with atherosclerosis (Young and Eneny, 2001). Hypercholesterolemia leads to increased cholesterol accumulation in cells thereby activating the production of reactive oxygen species (ROS), and coronary vessels are

extremely vulnerable to these oxidative challenges and hypercholesterolemia (Kay *et al.*, 1991). In this investigation, administration of oleuropein resulted in reductions of total cholesterol concentration and transaminases activity in serum of the oleuropein group compared to ethanol-treated rats. Similarly, the phenolics extract of olive leaves decreased the concentration of TG and LDL-cholesterol and improved the level of HDL-cholesterol that has been linked to a lower risk of coronary heart disease (Jemai *et al.*, 2008).

Data have been collected indicating that the elevated phenolic antioxidant content of the components of the Mediterranean diet, together with antioxidant vitamins, greatly contributes to the health-beneficial effects of this diet (Manna *et al.*, 2002; Manna *et al.*, 2004; Al-Azzawie and Alhamdani, 2006; Andreadou *et al.*, 2006). In this respect, our results exhibit significant antioxidant effects in oleuropein-treated rats. Our findings and those in the literature confirm the nutritional benefit of olive leaf extract.

In conclusion, the findings of the present study reveal that oleuropein administration during ethanol consumption in rats reduces ethanol-induced toxicity in the liver by improving antioxidant defense system and reducing the levels of lipid peroxides.

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