

Research Article



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The protective role of oleuropein against diethylnitrosamine and phenobarbital induced damage in rats

Sıçanlarda dietilnitrozamin ve fenobarbital ile oluşturulan hasara karşı oleuropeinin koruyucu rolü

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Abstract

Objective: Liver cancer is amongst the most lethal cancers worldwide. Diethylnitrosamine (DEN) and phenobarbital (PB) are common agents that form reactive oxygen species (ROS). Oleuropein (OLE) has efficient biological properties and used as a therapeutic agent. In this study, we aimed at investigating OLE against DEN + PB induced liver damage.

Methods: Adult Sprague-Dawley rats were divided into 5 groups (n=10): Control, DEN, DEN + PB, DEN + PB + OLE and OLE. DEN, DEN + PB, DEN + PB + OLE groups were administered a single dose of 150 mg/kg DEN. After two weeks, DEN + PB and DEN + PB + OLE groups received 500 ppm of PB. 10 mg/kg/day of OLE was orally administered to DEN + PB + OLE and OLE groups. Biochemical and histopathological changes evaluated after the 8 weeks study.

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Results: DEN and PB application with OLE treatment resulted significant differences, alone or combined. Although there was a significant difference among the groups in terms of liver GSH and MDA levels and CAT activities, there was no significant difference among the groups in SOD activity. In the liver sections of the DEN, DEN + PB and OLE groups, increase in some histopathological findings and TUNEL positive cells were increased compared to the control group.

Conclusion: OLE can be used as a protector against the effects of carcinogens causing liver damage.

Keywords: Carcinogen; Rat; Liver; Diethylnitrosamine; Phenobarbital; Oleuropein.

Öz

Amaç: Karaciğer kanseri dünya çapında görülen en ölümcül kanserlerdendir. Dietilnitrozamin (DEN) ve Fenobarbital (FB) reaktif oksijen türlerinin oluşumuna yol açan ajanlardır. Oleuropein (OLE) etkin biyolojik özellikleri olan ve tedavide kullanılan bir ajandır. Bu çalışmada DEN + FB ile indüklenen karaciğer hasarına karşı OLE'in etkisini araştırmak amaçlanmıştır.

Yöntemler: Yetişkin Sprague-Dawley sıçanlar 5 gruba ayrıldı (n=10): Kontrol, DEN, DEN + FB, DEN + FB + OLE, OLE. DEN, DEN + FB, DEN + FB + OLE gruplarına 150 mg/kg tek doz DEN uygulaması yapıldı. İki hafta sonrasında DEN + FB ve DEN + FB + OLE gruplarının içme sularına 500 ppm FB konuldu. DEN + FB + OLE ve OLE gruplarına oral yolla 10 mg/kg/gün dozunda OLE uygulandı. Biyokimyasal ve histopatolojik değerlendirmeler 8 haftalık çalışma sonunda yapıldı.

Bulgular: DEN ve FB uygulaması ile OLE tedavisi birlikte veya kombine uygulandığında belirgin değişimler görüldü. Karaciğerde GSH, MDA düzeyleri ile CAT aktivitesi açısından anlamlı fark olmasına rağmen SOD aktivitesi açısından gruplar arasında istatiki farka rastlanamamıştır. DEN, DEN + FB, OLE gruplarının karaciğer kesitlerinde kontrol grubuna kıyasla bazı histopatolojik bulgular ve TUNEL pozitif hücrelerde artışa rastlanmıştır.

Sonuç: OLE' in karaciğer hasarına sebep olabilen karsinogenlerin etkilerine karşı koruyucu olarak kullanılabileceği ortaya konmuştur.

Anahtar kelimeler: Karsinogen; Sıçan; Karaciğer; Dietilnitrozamin; Fenobarbital; Oleuropein.

Introduction

Cancer, characterized by excessive and uncontrolled cell proliferation, is a complex disease that may result in death if it is not cured [1]. Hepatocellular carcinoma, the third most common cause of the cancer deaths and having an increased frequency in the last decade, is the most common cancer type [2]. It causes deaths of 250,000 to 1 million people worldwide per year [3, 4].

Diethylnitrosamine (DEN) and phenobarbital (PB) are frequently used agents to induce two-step carcinogen protocol in cancer studies [5]. Nitrosamine compounds can be found in many foods such as meat and dairy products, alcoholic beverages [6, 7]. Moreover, nitrosamines can be formed in the body as a result of reaction with nitrites and amines [8, 9]. DEN causes the formation of reactive oxygen species (ROS) and hydrogen peroxide and thus causing the oxidation of DNA [10]. The accumulation of ROS, such as superoxide and hydroperoxide radicals, causes an increase in lipid peroxidation (LPO) and thus leading to cell membrane damage [11, 12].

PB accounts for an increase in peroxisomal fatty acid oxidation and microsomal hydroxylation activity by leading to growth and increased in numbers of hepatocytes [13]. It is obvious that there is an increased need for an antioxidant agent which can be found easily in daily consumed food and can function as a protector against ROS produced in our body.

The Mediterranean region is an important area for olive agriculture and olive leaves are one of the by-products of olive cultivation [14]. Olive leaves are a cheap material rich in phenolic materials [15]. Found in olive and its leaf, rich in phenolic substance, OLE is a compound which has important biological properties [16]. It is reported that OLE is used in the treatment and prevention of hepatocellular carcinoma [17].

It is well known that carcinogens, which we frequently encounter in daily life, cause damage to the liver, kidney and other organs [18]. OLE, which has antioxidant properties and can be produced from olive agronomic by-products, has the potential to exhibit protective properties against this damage [17, 19].

In this context, it was aimed to investigate the effect of OLE against liver damage induced by DEN and PB in rats.

Materials and methods

Experimental design

In this study, 50 male (220 ± 20 g), Sprague-Dawley rats were used. Animals were fed with standard pellet diet and tap water. Rats were divided into 5 groups and named as control group, DEN group, DEN+PB group, DEN+PB+OLE group and OLE group. The control group received ad libitum standard diet and drinking water for 8 weeks. DEN, DEN+PB, DEN+PB+OLE groups were administered intraperitoneally (i.p.) at a single dose of 150 mg/kg DEN [20]. Fifteen days after the DEN application, DEN+PB and DEN+PB+OLE groups received 500 ppm PB in drinking water for 6 weeks [21]. OLE administered to DEN+PB+OLE and OLE groups for 8 weeks by oral gavage at a dose of 10 mg/kg/day from the beginning of the experimental period [22]. At the end of the 8 week experimental period, the rats were decapitated, blood and liver tissues were taken.

Blood sample preparation

Blood was collected into biochemical tubes containing EDTA and allocated. A part of aliquots was stored for whole blood analyses. The other part of aliquots was centrifuged for 5 min at 3500 rpm, plasma was collected and stored at -20°C until the working day. The remaining red blood cell suspension were washed three times with 0.9% NaCl and stored at -20°C until the working day. Malondialdehyde (MDA), glutathione (GSH) levels and catalase (CAT), superoxide dismutase (SOD) activities were evaluated spectrophotometrically.

Liver sample preparation

A part of the liver tissues was washed with 0.9% NaCl for biochemical analysis and stored at -80°C deep freezer. Tissue samples were diluted 1/10 (weight/volume) in 1.15%

KCl, homogenized and centrifuged at 3500 rpm for 15 min and supernatant were collected. Malondialdehyde (MDA), glutathione (GSH) levels and catalase (CAT), superoxide dismutase (SOD) activities were evaluated spectrophotometrically as well.

Liver and blood sample analyzes

MDA assay were conducted on the collected plasma and supernatant. The MDA assay was performed according to the method modified by Placer et al. [23]. This method is based on the reaction of thiobarbituric acid with MDA which is one of the aldehyde products of LPO [23]. The whole blood was diluted with purified water for GSH analyzes. GSH levels were determined according to the method of Chavan et al. in the blood homogenates and liver tissues supernatant [24]. The liver tissues supernatant and washed erythrocytes were diluted with deionized water and used for SOD, CAT activity determinations. SOD activity measurement is based on color formation which occurred by reducing nitrobluetetrazolium with the activity of superoxide generated in xanthine-xanthine oxidase method [25]. CAT activity was measured in diluted erythrocytes and liver tissues supernatant according to Aebi method [26].

Histopathological evaluation

The other part of the liver tissues was removed and fixed in 10% formaldehyde for about 24 h for histological analyzes. After adequate fixation, the tissues were processed by using routine paraffin techniques, embedded in paraffin blocks and the blocks were cut into 5–6 µm thick sections. Tissue samples of all groups were stained by using standard Hematoxylin & Eosin (H&E) and Masson's Trichrome (MT) techniques [27].

TUNEL assay

In the 5–6 µm thickness paraffin block sections were taken to slides with poly-L-lysine. ApopTagPlus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) was used for detection of apoptotic cells in accordance with the manufacturer's instructions for use. Liver tissue was used for positive control. Reaction Buffer was used instead of the TdT enzyme for negative control. Harris hematoxylin was used for contrast-staining. Preparations were reviewed, evaluated and photographed with a research microscope (Novel N-800 M, Ningbo, China). In

the evaluation of TUNEL staining, cells with blue nuclei were normal, while brown nuclear staining was admitted as representing apoptotic cells. At least 500 cells were counted on each field. Apoptotic index was calculated as a ratio of the TUNEL – positive cell number to the total cell number (normal + apoptotic cells).

Statistical analyzes

In statistical evaluation of the data, Windows compatible SPSS package programs (Kruskal Wallis and Mann-Whitney U tests) were used. p-Values <0.05 were considered statistically significant.

Results

Biochemistry

The application of DEN separately and with PB, and OLE for treatment resulted in a statistically significant difference in blood MDA, GSH levels, and CAT, SOD activities. There was a statistically significant increase in blood MDA levels of the DEN + PB group compared to the control. OLE treatment caused a significant reduction in OLE group MDA levels compared to those of control, just as MDA levels of DEN + PB + OLE group compared to those of DEN + PB group. Blood CAT activity indicated a significant increase in the OLE group compared to the DEN + PB group. Moreover, it showed a significant increase in DEN + PB + OLE group compared to those of DEN + PB group. Upon blood GSH levels were compared, there was no statistically significant difference between the DEN + PB + OLE group and the control group, while DEN and DEN + PB groups showed a significant decrease compared to the control group. GSH levels in the OLE group were significantly higher than those of control. There was no difference in the blood SOD levels of OLE and DEN + PB + OLE groups compared to the control whereas these levels significantly decreased in the DEN + PB group compared to the control group (Table 1).

Although there was a significant difference among the groups in terms of liver GSH and MDA levels and CAT activities, there was no significant difference among the groups in SOD activity. There was a significant decrease in liver GSH levels in the DEN and DEN + PB groups compared to the control group while there was no statistically significant difference between the OLE, and DEN + PB + OLE groups and the control group. In terms of CAT activity of the liver, a significant decrease was observed in the OLE

Table 1: Effects of OLE on blood/serum/plasma antioxidants enzymes and malondialdehyde (MDA) levels in DEN + PB induced liver damage in rats.

Items	Groups				
	Control	DEN	DEN + PB	OLE	DEN + PB + OLE
MDA (nmol/mL)	94.45 ± 4.69 ^b	98.69 ± 6.35 ^b	123.99 ± 6.16 ^a	60.71 ± 2.63 ^c	101.84 ± 2.17 ^b
CAT (k/g Hb)	58.83 ± 2.69 ^a	66.14 ± 3.91 ^a	41.91 ± 3.21 ^b	69.59 ± 1.62 ^a	58.74 ± 3.71 ^a
GSH (μmol/g Hb)	61.80 ± 4.21 ^b	40.36 ± 3.16 ^c	46.07 ± 2.50 ^c	77.48 ± 3.75 ^a	49.79 ± 2.53 ^{b,c}
SOD (U/g Hb)	94.77 ± 11.97 ^a	77.84 ± 6.91 ^{a,b}	57.35 ± 4.02 ^b	83.49 ± 9.50 ^{a,b}	79.44 ± 6.71 ^{a,b}

The data presented as means and standard error.

^{a,b,c}Values with different letters in the rows indicates statistically difference between groups ($p < 0.05$).

Table 2: Effects of OLE on liver antioxidants enzymes and malondialdehyde (MDA) levels in DEN + PB induced liver damage in rats.

Items	Groups				
	Control	DEN	DEN + PB	OLE	DEN + PB + OLE
Liver MDA (nmol/g)	21.92 ± 0.93 ^c	39.06 ± 3.25 ^b	61.30 ± 3.95 ^a	55.44 ± 4.87 ^a	33.98 ± 3.26 ^{b,c}
Liver CAT (k/g protein)	189.40 ± 8.82 ^{a,b}	208.71 ± 5.59 ^a	208.14 ± 3.20 ^a	167.98 ± 3.09 ^b	191.82 ± 5.27 ^a
Liver GSH (μmol/g protein)	600.95 ± 17.44 ^a	485.13 ± 36.22 ^b	473.13 ± 13.41 ^b	611.81 ± 27.57 ^a	540.36 ± 14.70 ^{a,b}
Liver SOD (U/g protein)	115.10 ± 3.48	110.11 ± 3.91	119.40 ± 3.74	118.17 ± 4.13	117.14 ± 4.98

The data presented as means and standard error.

^{a,b,c}Values with different letters in the rows indicates statistically difference between groups ($p < 0.05$).

group compared to the DEN, DEN + PB, DEN + PB + OLE groups. DEN + PB group liver MDA levels were significantly lower than DEN + PB + OLE group liver MDA levels. There was no difference among groups in terms of liver SOD levels (Table 2).

Histopathology

The histological examination by light microscope of rat liver tissues showed normal hepatocytes, sinusoids, sinusoidal cells, portal and periportal areas in control group. In DEN group, there were increases in the number of double nucleated hepatocytes, mild congestion, pronounced inflammatory cell infiltration in the periportal area and sinusoidal dilatation. Moreover, in the sections of the DEN + PB group, increase in all histopathological findings and large nucleated hepatocytes were observed when compared to the DEN group. In the OLE group sinusoidal dilatation was common, as well as increased inflammatory cells in the periportal area, compared with the control group (Figure 1).

Evaluation of apoptosis in liver tissues

A significant increase was detected in TUNEL positive cells number in DEN, DEN + PB, OLE groups compared

to the control group. The number of TUNEL positive cells was evaluated in DEN + PB + OLE group similar to control group (Figure 2). The results of the apoptotic index are shown in Table 3.

Discussion

It is well known that DEN and PB are carcinogenic and the most used agents in experimental hepatocarcinoma studies. One of the most crucial effects of their carcinogenesis is related to increased production of ROS leading to a damage on cellular macromolecules. ROS takes hydrogens from the membrane lipids and causes increased levels of MDA [28]. Moreover, Banakar et al. are reported that MDA levels increased in rats exposed to DEN and PB as a result of ROS formation in liver tissues [13]. In this study, also there was a significant increase in blood and liver MDA levels of the DEN + PB groups.

It is reported that DEN administration causes decrease in GSH levels in rats [28, 29]. In our study, there was a decrease in liver and blood GSH levels in DEN and DEN + PB groups (Table 1). These findings are accordance with previous studies. Some researchers explain this decrease with the metabolic dysfunction such as the dysfunction in the pentose phosphate pathway, which occurs due to the activity of ROS [28, 29].

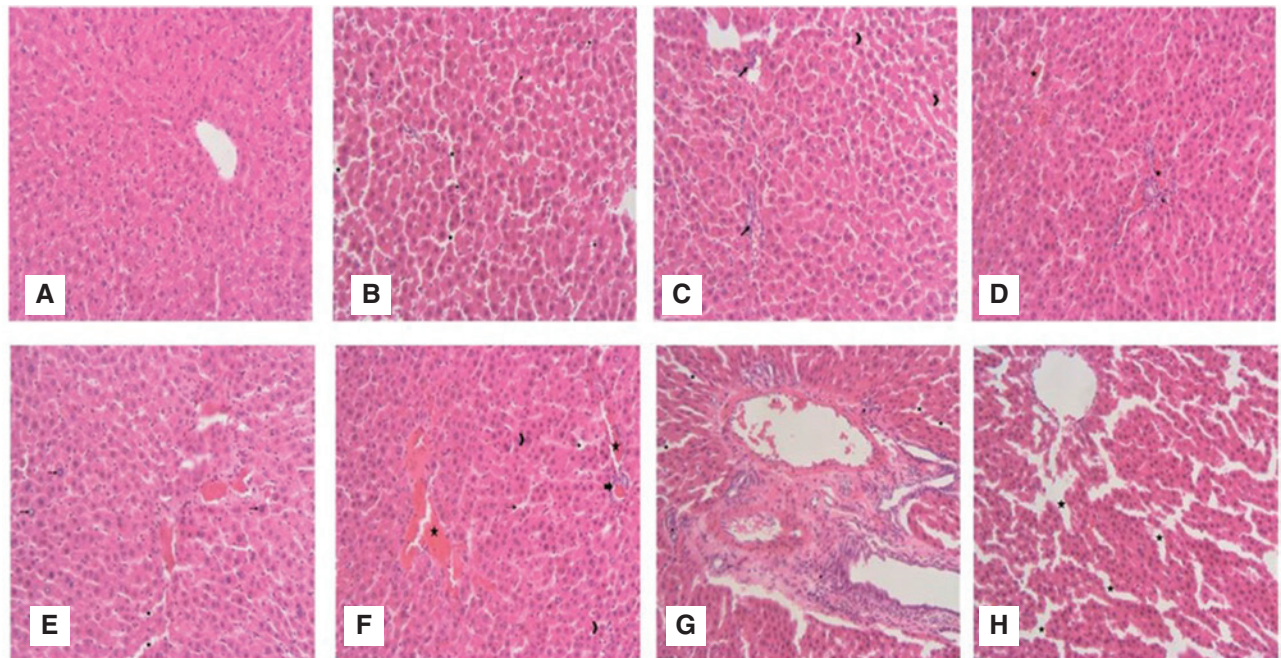


Figure 1: Oleuropein Effect on Liver Histopathology of diethylnitrosamine and phenobarbital induced damage in rats.

(A) Control group. Normal-looking hepatocytes and sinusoids. (B) DEN group. Sinusoidal dilation (star) and dual nucleated hepatocytes (arrow). (C) The DEN group. Inflammatory cell growth (arrow) and mild congestion areas (arrowhead). (D) DEN + PB group. Inflammatory cell growth (arrow) and congestion areas (star). (E) DEN + PB group. Sinusoidal dilation (star) and large nucleated hepatocytes (arrow). (F) DEN + PB + OLE group. Conjunction (star) and sinusoidal dilation (arrow), inflammatory cell increase (thick arrow) and dual nucleated hepatocytes (arrowhead). (G) The OLE group. Sinusoidal dilation (star), inflammatory cell increase (arrow). (H) OLE group. Sinusoidal dilation (star). H & E x200.

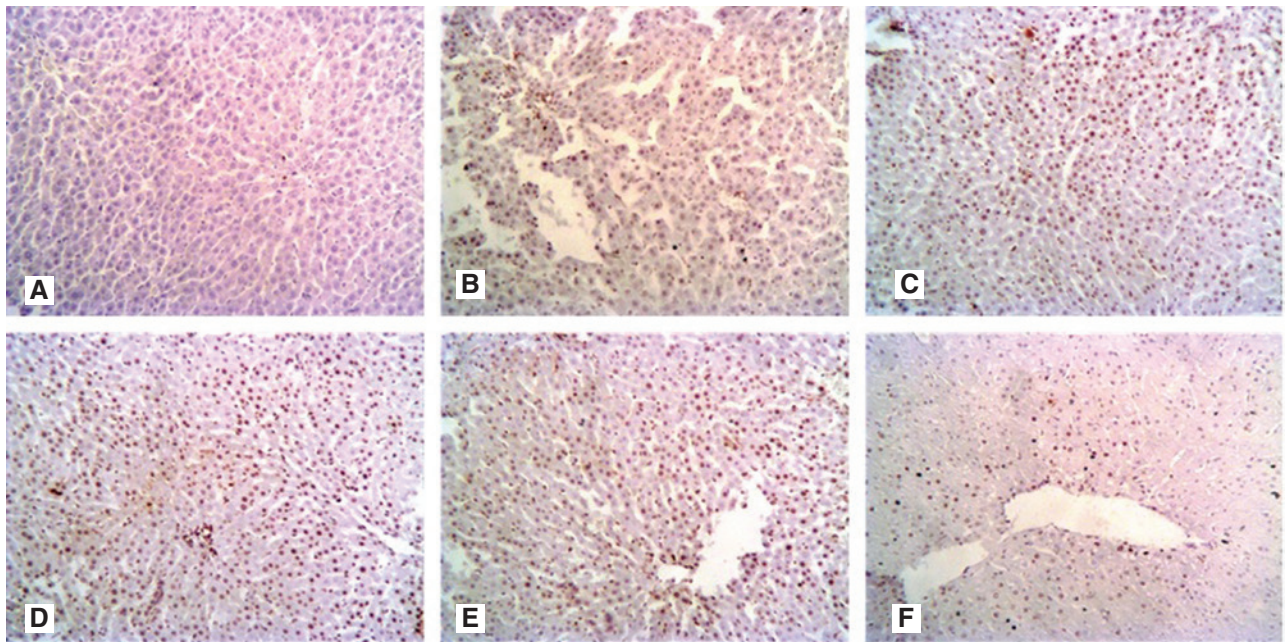


Figure 2: Oleuropein Effect on Liver Apoptosis Index of diethylnitrosamine and phenobarbital induced damage in rats.

Comparison of TUNEL positivity (brown nucleus) between groups. Control (A), OLE (B), DEN (C), DEN + FB (D,E) and DEN + FB + OLE (F).

Table 3: Effects of OLE on liver apoptotic index (%) in DEN + PB induced liver damage in rats.

	Groups				
	Control	OLE	DEN	DEN + PB	DEN + PB + OLE
Apoptotic index (%)	4.66 ± 2.73	11.33 ± 3.55 ^{a,b}	17.16 ± 3.18 ^{a,b}	24 ± 4.6 ^a	10.83 ± 2.48 ^{a,b}

Values are means ± standard deviation. ^ap < 0.05 Control vs. all group. ^bp < 0.05 DEN + PB vs. all group.

The superoxide radical is converted to H_2O_2 by a reaction occurring spontaneously or SOD-dependent mechanism [30]. Conversion of Superoxide Radicals to H_2O_2 and then to H_2O and oxygen with the activity of CAT or Glutathione peroxidase, in the presence of reduced GSH and NADPH, is the most important defense system to protect cellular macromolecules from oxidative damage [31]. Shaban et al. are stated that DEN administration causes a reduction in SOD activity [32]. In our study blood SOD activity decreased significantly in the DEN + PB group (Table 1). However, there was no statistically significant difference between the groups in terms of liver SOD activity (Table 2). This result indicates that there is an increased presence of superoxide in blood and in this period of time liver cells can maintain antioxidant mechanism.

Fernandez et al. reported that CAT expression decreased when tumors were developed by inducing with DEN and PB [33]. Lam et al. is reported that GPx has a higher activity to catalyze H_2O_2 than CAT [34]. In agreement with other studies in the current study, a significant decrease in blood CAT activity was observed in the DEN + PB group (Table 1). However, DEN and DEN + PB groups did not show any difference in liver CAT levels (Table 2), suggesting that H_2O_2 is converted to water and oxygen by consuming GSH in other enzymatic way. The decrease in liver GSH levels confirms this assessment. As to blood, decreased GSH levels, CAT, and SOD activity may suggest that blood oxidative stress levels are far more out of the liver. This difference in liver and blood enzyme levels suggests that oxidative stress in the liver is still at an early stage and may have similar effects with blood in later periods.

In the present study biochemistry findings were supported with histopathology and tunel examination. Existence of apoptotic cells as well as double nucleated hepatocytes, mild congestion, pronounced inflammatory cell infiltration in the periportal area and sinusoidal dilatation in DEN applied groups indicate the presence of oxidative stress. Despite being an antioxidant OLE when administrated alone causes a defect in liver cells (Figures 1 and 2).

OLE is obtained from olive fruit and leaf. OLE, which surrounds cancerous cells irreversibly, prevents these cells from dividing, spreading to other organs [35]. It is reported that OLE is the main component responsible for anti-tumor activity among antioxidants found in olive and olive oil [36]. It is documented that as an antioxidant, OLE protects cells from tumor development and as an antiangiogenic prevents the spread of tumor cells [36]. In our study, the levels of liver MDA in the OLE group increased (Table 2). According to Katsoulis et al. OLE related increased MDA production may occur due to oxidation of OLE by ROS and forms peroxide from itself [37]. On the other hand, in case of damage induced by DEN and PB, the OLE application has reduced MDA levels (Table 1). This suggests that OLE may be effective against liver damage. Blood MDA levels were reduced in OLE-administered groups (Table 1). These results are compatible with the previous studies findings claiming that OLE has radical scavenging effects [38].

It has been reported that SOD activity and GSH levels decreased in liver damage induced by carbon tetrachloride in mice, while OLE application increased SOD activity and GSH levels in a dose-dependent manner [39]. On the other hand Santos et al. reported that SOD activity of mice exposed to DEN increased but these increase was not statistically significant [40]. In agreement with these study, there was no difference among the groups in liver SOD levels in our study (Table 2). While the levels of GSH decreased in groups applied with DEN and PB in the liver, OLE administration increased the levels of GSH (Table 2). These results are consistent with previous work. When the blood SOD levels were compared, it was observed that the lowest activity was in the DEN + PB group (Table 1). OLE therapy increased the GSH levels but this increase was not statistically significant (Table 1). These results show that H_2O_2 formation may have occurred with the SOD-independent mechanism.

It has been reported that OLE increases CAT levels in the presence of H_2O_2 [40]. There was no statistically significant difference between the groups that received OLE and the control group (Tables 1 and 2). These results indicate that OLE administration decreases the oxidative stress induced by DEN and PB.

Conclusion

OLE application is effective when there is a liver damage, on the other hand, if there is no damage it increases MDA levels. In this study, it has been demonstrated that OLE can be used as a protector against the effects of carcinogens causing damage to the liver and other organs. It is important to study more about OLE, which has low production costs and is used against oxidative damage in countries where olive farming is done.

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