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Hepatoprotective and Antioxidant Activity of Linden (*Tilia platyphyllos* L.) Infusion Against Ethanol-Induced Oxidative Stress in Rats

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Abstract The present study was carried out to evaluate the hepatoprotective effect and antioxidant role of infusion prepared from linden flowers (LF) against ethanol-induced oxidative stress. The hepatoprotective and antioxidant role of the plant's infusion against ethanol-induced oxidative stress was evaluated by measuring liver damage serum biomarkers, aspartate aminotransferase (AST), alanine aminotransferase, lactate dehydrogenase (LDH), total protein, total albumin, and total cholesterol level; ADS such as GSH, GR, SOD, GST, CAT and GPx, and MDA contents in various tissues of rats. Rats were divided into four experimental groups: I (control), II (20 % ethanol), III (2 % LF), and IV (20 % ethanol + 2 % LF). According to the results, the level of serum marker enzymes, AST and LDH, was significantly increased in group alcohol and group LF as compared to control group, whereas decreased in group IV as compared to ethanol group. With regard to MDA content and ADS constituents, MDA contents of alcohol group in all tissues, except for erythrocytes and heart, and in brain, kidney, and spleen of LF group significantly increased compared to control group, whereas LF beverage extract supplementation did not restore the increased MDA towards close the control level. In addition, while ethanol caused fluctuation in antioxidant defense system constituents level as a result of oxidative stress condition in the rats, it could have not been determined the healing effects of the LF against these fluctuations. The results indicated that LF beverage extract could not be as important as diet-derived antioxidants in preventing oxidative damage in the tissues by reducing the lipid oxidation or inhibiting the production of ethanol-induced free radicals in rats.

Keywords Linden flowers · Serum biomarkers · Antioxidant defense system · Malondialdehyde · Rats

Abbreviation

LF	Linden flowers
AST	Aspartate aminotransferase
ALT	Alanin aminotransferase
LDH	Lactate dehydrogenase
TPRO	Total protein
TALB	Total albumin
TCHOL	Total cholesterol;
ADS	Antioxidant defense systems
GSH	Reduced glutathione
GR	Glutathione reductase
SOD	Superoxide dismutase
GST	Glutathione-S-transferase
CAT	Catalase
GPx	Glutathione peroxidase
MDA	Malondialdehyde

Introduction

Many naturally occurring compounds with antioxidative action are now known to protect cellular components from oxidative damage and prevent diseases (Gulcin 2009, 2010). A number of such compounds can activate the phase II detoxification enzymes, which can remove the toxic elements from our system. Intake of such phytochemicals is therefore beneficial to human health. In addition, many natural compounds are now known to have a modulator role on physiological functions and biotransformation

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reactions involved in the detoxification process, thereby affording protection from cytotoxic, genotoxic, and metabolic actions of environmental toxicants (Saha and Das 2003). Numerous studies demonstrate that a great number of medicinal and aromatic herbs, as well as fruits and leaves of some berry plants biosynthesize phytochemicals possessing antioxidant activity and may be used as a natural source of free radical scavenging compounds (Yu et al. 2005; Sacchetti et al. 2005). Also, a great number of spices and aromatic herbs contain chemical compounds exhibiting antioxidant properties. These properties are attributed to a variety of active phytochemicals including vitamins, carotenoids, terpenoids, alkaloids, flavonoids, lignans, simple phenols and phenolic acids, and so on (Liu and Ng 2000).

The Tiliaceae plant *Tilia argentea* (synonym: *T. tomentosa*; common names: linden or lime), a well-known Occidental herb, is cultivated in Bulgaria and Albania. The dried flowers of this plant, which are commonly called "silver linden flowers", have been widely used in herbal teas, and as a diuretic, stomachic, antineuralgic, and sedative in European countries. By bioassay-guided separation, six flavonol glycosides were isolated from the methanolic extract of linden. On the other hand, they found that the methanolic extract from the flowers of *T. argentea* (Linden) showed potent protective effects against d-galactosamine (d-GalN)/lipopolysaccharide-induced liver injury in mice and on d-GalN cytotoxicity in primary cultured mouse hepatocytes (Matsuda et al. 2002).

In folk medicine, the linden flower is used as a diuretic, stomachic, antispasmodic, and sedative agent (Wichtl 2004). The inflorescence of the linden contains between 3 and 10 % polysaccharides which are mucilaginous. Furthermore, condensed tannins (Behrens et al. 2003) (strong antioxidants), such as dimers of procyanidin (B-2), were identified in this raw material. Other phenolic compounds identified in linden flowers (LF) are flavonoids, mainly quercetin glycosides (rutin, quercitrin, and isoquercitrin), kaempferol glycosides (Toker et al. 2001), and phenolic acids (caffeic, p-coumaric, and chlorogenic acids) (Czygan 2007).

The reactive oxygen species (ROS) are known to play a major role in either the initiation or progression of carcinogenesis by inducing oxidative stress (Gulcin 2006). Peroxides and superoxide anion ($\cdot O_2^-$) produce cytotoxicity/genotoxicity in cellular system (Gulcin et al. 2008, 2010). ROS and nitrogen species are formed in the human body, and endogenous antioxidant defenses are not always sufficient to counteract them completely. Free radicals and ROS have been implicated in many human pathological conditions, including rheumatoid arthritis (Kunsch et al. 2005), hemorrhagic shock (Childs et al. 2002), cardiomyopathy (Akhileshwar et al. 2007), cystic fibrosis (Cowley and Linsdell 2002), and gastrointestinal ischemia (Halliwell and Gutteridge 1999; Henrotin et al. 2003; Hogg 1998). A high concentration of ROS leads to the destruction of cell membranes (Mishra 2004), proteins, and nucleic acids (Briganti and Picardo 2003; Bergamini et al. 2004), which is dangerous because it leads to carcinoma formation (Kang 2005). A large number of studies support the hypothesis that oxidative damage to DNA, lipids, and proteins may contribute to the development of cardiovascular disease, cancer, and neurodegenerative diseases (Halliwell 1996; Gulcin et al. 2006). Diet-derived antioxidants may therefore be particularly important in protecting against chronic diseases (Halliwell 1996; Vendemiale et al. 1999). Plant extracts rich in phenolic compounds with antiradical activity (Gohil and Packer 2002; Sastre et al. 2002; Celik et al. 2009), such as extracts from the linden, could be used as medicines or preventive agents protecting humans and other animals from the destructive action of free radicals and ROS.

Although some studies reported the major flavonol contents and antioxidant activities in linden varieties in the world, the literature lacks information on chemopreventive and antioxidant role of linden grown in Turkey. Therefore, the objective of this study was to determine hepatoprotective and antioxidant capacity in flowers of linden variety, which are widely grown in Çankırı province located in Inner Anatolia Region of Turkey.

There is a growing interest of natural products in human diet, both due to the possible negative effects of synthetic food additives on human health and the increased consumer perception of this problem in recent years. As far as our literature survey could ascertain, so far no studies have been reported on hepatoprotective role and antioxidant capacity of the LF beverage extract used in this study. The objective of this study was to determine healthful potentials of LF beverage extract against alcohol-induced oxidative stress by evaluating their in vivo hepatoprotective role and antioxidant capacity. Thus, in the present study, we have extensively studied the antioxidant activity of LF infusion using in vivo models. For this aim, the treatment of LF infusion was done orally as food containing 2 % powdered LF infusion because the effect of the functional plant represents a well characterized in nutrition and widely used as consumption by human in our country and worldwide. The serum enzymes were chosen due to their importance as index of hepatotoxin and hepatoprotective. The antioxidant activity of LF on some phase II detoxification ADS such as reduced glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT) and glutathione peroxidase (GPx), and malondialdehyde (MDA) contents in the various tissues were evaluated during experiment.

Materials and Methods

Chemicals

Thiobarbituric acid (TBA), butylated hydroxytoluene, trichloroacetic acid, ethylenediaminetetraacetic acid (EDTA), GSH, metaphosphoric acid, 5,5′dithiobis-(2-nitrobenzoic acid), trihydroxymethyl aminomethane (Tris), 1-chloro-2,4-dinitrobenzene (CDNB), oxidized glutathione, β-Nicotinamide adenine dinucleotide phosphate (NADPH), potassium dihydrogen phosphate (KH_2PO_4), and sodium chloride (NaCl) of technical grade used in this study were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Kits for antioxidant enzymes analysis were supplied by Randox Laboratories Ltd.

Animals

Four months old Rats (Wistar albino) with an average weighing 200–250 g were provided from the Experimental Animal Research Center, Yuzuncu Yil University, and were housed in four groups, each group containing six rats. The animals were housed at 20 ± 2 °C in a daily light/dark cycle. All animals were fed a group wheat–soybean meal-based diet and water ad libitum in stainless cages, and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institutes of Health. The ethic regulations were followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments. This study was approved by The Ethic Committee of the Yüzüncü Yıl University.

Preparation of Foods

Briefly, the natural and organic LF were provided from a local herbalist in Çankırı, a major linden-producing province of Turkey. This variety was chosen because the remarkable total production of LF in the region comes from the variety.

Experimental Design

The rats were randomly divided into four groups each containing six rats.

Group I (Control): the rats received tab water and fed with standard pellet diet as ad libitum.
Group II (Alcohol): the rats received 20 % ethanol water and fed with standard pellet diet as ad libitum. Dose of ethanol was selected on the basis of a 20 % concentration at which caused oxidative stress when administered orally (Aykac et al. 1985; Sonde et al. 2000; Yurt and Celik 2011; Dogan and Celik 2012).
Group III (2 % LF): the rats received 2 % LF infusion and fed with standard pellet diet as ad libitum.
Group IV (2 % LF beverage extract + 20 % alcohol): the rats received 2 % LF infusion + 20 % alcohol water and fed with standard pellet diet as ad libitum.

Preparation of Tissues Supernatant and Erythrocyte Pellets

At the end of the 50 days experiments, the rats were anesthetized by injection of ketamine (5 mg/100 g body weight) intra-peritoneally. The blood samples were obtained from a cardiac puncture using syringe for the determination of serum marker enzyme levels and biochemical analysis. The serum samples were obtained by centrifuging blood samples at 4,000×*g* for 15 min at 4 °C, and enzyme levels were measured in these serum samples. For biochemical analysis, blood samples were put immediately into silicon disposable glass tubes with EDTA as an anticoagulant and were centrifuged at 4,000×*g* for 15 min at 4 °C and erythrocyte pellets were obtained. Then, the pellets were washed three times with physiological saline (0.9 % NaCl).

The tissues as brain, kidney, spleen, heart, and liver were dissected and put in petri dishes. After washing the tissues with physiological saline (0.9 % NaCl), samples were taken and kept at −78 °C during the analysis. The tissues were homogenized for 5 min in 50 mM ice-cold KH_2PO_4 solution (1:5 w/v) using stainless steel probe homogenizer (20 kHz frequency ultrasonic, Jencons Scientific Co.) for 5 min. and then centrifuged at 7,000×*g* for 15 min. All processes were carried out at 4 °C. Supernatants and erythrocyte pellets were used to determine ADS constituents and MDA contents (Celik et al. 2009; Yurt and Celik 2011; Dogan and Celik 2012).

Biochemical Analysis

The erythrocyte and tissues MDA concentration were determined using the method described by Jain et al. (1989) based on TBA reactivity. The erythrocyte and tissues GSH concentration were measured using the method described by Beutler et al. (1963). GST was assayed by following the conjugation of glutathione with CDNB at 340 nm as described by Mannervik and Guthenberg (1981). GR activity was assayed according to Carlberg and Mannervik (1975) as the decrease in absorbance of NADPH at 340 nm. GPx activity was assayed according to Paglia and Valentine (1967) based on that of GPx which catalyzes the oxidation of GSH by cumene hydroperoxide.

Table 1 Determination hepatoprotective capacity of *Tilia platyphyllos* infusion against ethanol exposed in rats (Mean ± SD)

Parameters	Control X ± SD	20 % Ethanol X ± SD	2 % LF X ± SD	2 % LF + 20 % Ethanol X ± SD
AST (U/L)	146.3 ± 10.90	279.3 ± 22.80[a]	184.0 ± 18.30[a]	249.3 ± 24.30[a]
ALT (U/L)	46.2 ± 3.30	76,2 ± 4,60[a]	44.3 ± 3.30	61.7 ± 4.50[ab]
LDH (U/L)	1707.5 ± 12.00	2101.3 ± 127.10[a]	2158.8 ± 123.60[a]	1711.2 ± 155.00[b]
TPRO (g/dL)	6.8 ± 0.30	7.0 ± 0.30	7.0 ± 0.30	6.9 ± 0.20
TALB (g/dL)	4.3 ± 0.20	4.6 ± 0.30	4.5 ± 0.20	4.2 ± 0.10[b]
TCHOL (mg/dL)	58.3 ± 4.80	64.3 ± 3.80[a]	59.8 ± 4.70	47.2 ± 3.80[ab]

Each value represents the Mean ± SD

[a] Significantly different from control

[b] Significantly different from 20 % ethanol exposed rats at $p < 0.05$ (one way ANOVA)

SOD activity was measured at 505 nm by calculating inhibition percentage of formazan dye formation (McCord and Fridovich 1969). CAT activity was determined using the method described by Aebi (1974), based on that of the rate of H_2O_2 consumption and as the decrease in absorbance at 240 nm.

Measurement of Enzyme Levels

Serum marker enzyme activities such as AST, ALT, LDH, TPRO, TALB, and TCHOL were measured by an auto analyzer (BM/HITACHI-911), using the kits.

Analysis of Data

All data were expressed as mean ± standard deviation (SD). The statistical analyses were made using the Minitab 13 for windows packet program. Means and Standard deviations were calculated according to the standard methods for all parameters. One-way analysis of variance (ANOVA) statistical test was used to determine the differences between means of the experimental groups accepting the significance level at $p \leq 0.05$.

Results

At the end of 50 days of treatment, the hepatoprotective effect of linden infusion against ethanol-induced oxidative stress was evaluated by measuring liver damage via serum marker enzymes AST, ALT, and LDH enzyme levels and TPRO, TALB, and TCHOL levels. On the other hand, antioxidant role of the linden infusion was evaluated by measuring CAT, SOD, glutathione peroxidase (GSH-Px), GST, GR enzyme activities and GSH, and MDA contents in brain, kidney, spleen, erythrocyte, heart, and liver tissues of rats. The results of experiment showed that the treatment of rats with alcohol and alcohol + LF beverage extract containing diet supplementation caused changes in the level of serum biomarkers, MDA content and ADS constituents in comparison with control rats. As known, serum AST, ALT, and LDH levels are susceptible to hepatotoxin and serve as markers of liver damage that promote the release of such aminotransferases from hepatocytes into blood stream. According to the results, serum AST, ALT, LDH, and TCHOL levels of group II and serum AST, ALT, and LDH levels of group III were significantly increased compared with group I. Serum LDH and TCHOL levels of group IV significantly decreased compared with group II (Table 1). With regard to MDA content and ADS constituents, MDA contents of II group in all the tissues except for erythrocytes and heart and group III in brain, kidney, and spleen significantly increased compared with group I. On the other hand, while ethanol caused fluctuation in antioxidant defense system constituents level as a result of oxidative stress condition in the rats, it could have not been determined the healing effects of the LF against these fluctuations (Table 2).

Discussion

Overexposures to oxidative stress caused by environmental pollutants are thought to increase the risk of cancer. Also, it is known that alcoholic liver disease is a major medical complication of alcohol intake. Oxidative stress plays an important role in the development of alcohol-related liver disease (Gulcin 2007; Coban et al. 2008). Hence, efforts are needed to provide effective protection from the damaging agents and experimental studies have implicated the influence of a functional plant, LF in this regard. The first aim of this study was to investigate whether the LF infusion serum biomarkers could prevent hepatotoxicity of ethanol, and decrease content of the MDA and efficacy on the antioxidant defense system in rats.

The results of the present study demonstrated, for the first time, that the treatment of rat with LF effectively did not protect the rat against alcohol-induced hepatotoxicity,

Table 2 Determination antioxidant capacity of *Tilia platyphyllo* infusion against ethanol exposed in rats

Tissue	Parameters	Control X ± SD	20 % Ethanol X ± SD	2 % LF X ± SD	2 % LF + 20 % Ethanol X ± SD
Erythrocyte	GSH (mg/ml)	4.15 ± 0.30	4.79 ± 0.26[a]	5.19 ± 0.13[a]	6.81 ± 0.23[ab]
	MDA (nmol/ml)	1.68 ± 0.2	1.76 ± 0.23	1.63 ± 0.15	2.01 ± 0.21[*a]
	GST (U/ml)	84.75 ± 0.79	92.32 ± 1.56[**a**]	146.92 ± 3.02[a]	100.05 ± 3.96[ab]
	GPx (U/ml)	464.09 ± 10.54	446.15 ± 14.73[a]	422.00 ± 13.10[a]	431.42 ± 11.40[a]
	GR (U/ml)	7.95 ± 0.32	5.48 ± 0.61[a]	6.77 ± 0.62[a]	5.79 ± 0.54[a]
	SOD (U/ml)	2150.20 ± 56.87	2128.36 ± 28.41	2112.71 ± 27.44	2124.55 ± 53.53
	CAT (U/ml)	2484.77 ± 131.33	2585.28 ± 156.31	2568.53 ± 156.66	2267.01 ± 187.93[b]
Liver	GSH (mg/g)	21.77 ± 1.37	38.13 ± 0.61[a]	38.18 ± 1.47[a]	36.94 ± 0.90[ab]
	MDA (nmol/g)	40.48 ± 2.34	47.81 ± 3.24[**a**]	42.74 ± 1.16	45.55 ± 2.08[a]
	GST (U/g)	166.68 ± 8.60	156.33 ± 7.32[a]	123.63 ± 6.03[a]	200.74 ± 8.98[ab]
	GPx (U/g)	194.65 ± 4.15	158.98 ± 4.41[a]	168.25 ± 6.94[a]	169.34 ± 6.67[ab]
	GR (U/g)	3.0 ± 0.42	4.13 ± 0.22	4.28 ± 0.64[a]	3.60 ± 0.60[ab]
	SOD (U/g)	2232.12 ± 37.21	2065.00 ± 30.15[a]	1901.83 ± 57.98[a]	1942.90 ± 22.75[ab]
	CAT (U/g)	1831.47 ± 150.59	2233.50 ± 143.50[a]	2095.03 ± 186.93[a]	2348.53 ± 161.91[a]
Brain	GSH (mg/g)	43.37 ± 1.94	58.78 ± 2.18[a]	50.67 ± 1.52[a]	47.68 ± 0.94[ab]
	MDA (nmol/g)	20.51 ± 0.78	32.49 ± 1.04[a]	29.90 ± 0.95[a]	14.25 ± 0.92[ab]
	GST (U/g)	59.65 ± 1.71	60.74 ± 1.83	65.76 ± 1.41[a]	74.52 ± 1.40[ab]
	GPx (U/g)	205.15 ± 4.64	199.84 ± 3.35[a]	203.43 ± 6.50	221.45 ± 1.36[ab]
	GR (U/g)	0.77 ± 0.09	0.89 ± 0.11	0.85 ± 0.12	0.82 ± 0.12
	SOD (U/g)	1967.23 ± 67.94	1703.61 ± 150.62[a]	1433.54 ± 120.34[a]	1650.69 ± 94.62[a]
	CAT (U/g)	234.52 ± 36.70	234.52 ± 56.06	301.52 ± 36.70[a]	402.03 ± 59.93[ab]
Kidney	GSH (mg/g)	17.77 ± 0.75	19.71 ± 0.88[a]	16.12 ± 0.73[a]	17.70 ± 0.74[a]
	MDA (nmol/g)	181.01 ± 17.18	233.15 ± 16.56[a]	211.35 ± 15.21[a]	128.66 ± 13.51[ab]
	GST (U/g)	68.92 ± 1.67	62.56 ± 1.89[a]	58.99 ± 1.49[a]	51.95 ± 1.43[ab]
	GPx (U/g)	388.50 ± 6.69	377.13 ± 8.93[a]	400.09 ± 9.47[a]	404.35 ± 7.55[ab]
	GR (U/g)	1.84 ± 0.28	1.53 ± 0.17[a]	1.76 ± 0.28	1.97 ± 0.20[a]
	SOD (U/g)	1886.08 ± 79.28	1784.53 ± 74.13[a]	1744.33 ± 132.98[a]	1684.05 ± 72.82[ab]
	CAT U/g	1719.80 ± 85.77	1738.78 ± 132.10	2065.99 ± 69.46[a]	2017.97 ± 129.75[ab]
Spleen	GSH (mg/g)	51.81 ± 2.48	44.32 ± 3.14[a]	44.60 ± 3.48[a]	44.81 ± 3.71[a]
	MDA (nmol/g)	82.68 ± 7.15	106.10 ± 6.14[a]	125.64 ± 7.55[a]	89.70 ± 7.77[b]
	GST (U/g)	41.24 ± 1.23	51.29 ± 1.83[**a**]	45.26 ± 1.53[a]	42.99 ± 0.99[ab]
	GPx (U/g)	327.41 ± 9.52	351.71 ± 8.76[a]	344.16 ± 9.43[a]	342.21 ± 6.87[a]
	GR (U/g)	3.54 ± 0.45	3.83 ± 0.56	2.68 ± 0.54[a]	2.89 ± 0.50[ab]
	SOD (U/g)	2118.70 ± 41.90	2040.02 ± 60.06[a]	1997.69 ± 49.22[a]	1937.72 ± 87.31[ab]
	CAT (U/g)	661.12 ± 53.04	484.67 ± 50.97[a]	651.07 ± 71.22	734.82 ± 75.41[b]
Heart	GSH (mg/g)	18.30 ± 0.71	20.17 ± 1.31[a]	22.18 ± 1.52[a]	21.04 ± 1.23[a]
	MDA (nmol/g)	58.29 ± 2.87	60.55 ± 2.41	59.69 ± 2.70	48.14 ± 4.07[ab]
	GST (U/g)	27.14 ± 1.80	34.83 ± 1.90[a]	40.23 ± 1.57[a]	39.01 ± 1.88[ab]
	GPx (U/g)	192.85 ± 3.12	191.81 ± 4.60	203.40 ± 5.18[a]	207.77 ± 1.81[ab]
	GR (U/g)	0.90 ± 0.13	1.15 ± 0.21[a]	0.87 ± 0.07	1.04 ± 0.17
	SOD (U/g)	2108.39 ± 80.16	2007.39 ± 66.58[a]	1982.44 ± 86.55[a]	1969.94 ± 69.12[a]
	CAT (U/g)	208.83 ± 37.29	276.95 ± 22.29[a]	422.13 ± 42.59[**a**]	422.13 ± 52.76[ab]

Each value represents the Mean ± SD

[a] Significantly different from control

[b] Significantly different from ethanol exposed rats at $p < 0.05$ (one way ANOVA)

as evidenced by unchanged serum biomarkers level. In this study, experimental alcoholosis was induced in rats by feeding them with a diet containing 20 % as drinking water for 50 days. The known biochemical findings of the ethanol toxicity in rats were also observed in the present study. The hepatotoxic effects of alcohol have been well documented in a variety of animal species. ROS and consequent peroxidative damage caused by alcohol are considered to be the main mechanisms leading to hepatotoxicity (Aykac et al. 1985; Sonde et al. 2000; Kolankaya et al. 2002; Yurt and Celik 2011; Dogan and Celik 2012).

As shown in Table 1, alcohol caused a significant elevation in the levels of AST, ALT, and LDH in comparison to those of control rats and LF infusion caused a significant elevation in the levels of AST and LDH too, whereas 2 % LF + 20 % alcohol group decrease the serum marker enzymes in comparison to those of alcohol treated rats. The reasons for such effect of the alcohol and the LF supplementation are not understood at present certainly. However, it is known that several soluble enzymes in blood serum such as these enzymes have been considered as indicators of the hepatic dysfunction and damage. Also, the increase in the activities of AST and ALT in plasma of rats treated with ethyl alcohol is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (Navarro et al. 1993). Further, ALT and AST levels are also of value indicating the existence of liver diseases, as this enzyme is present in large quantities in the liver. ALT increases in serum when cellular degeneration or destruction occurs in this organ (Hassoun and Stohs 1995). Any interference in these enzymes leads to biochemical impairment and lesions of the tissue and cellular function (Khan et al. 2001). Yousef et al. (2007) reported that the changes in the activities of these enzymes in $SnCl_2$-treated rats were regarded as the biochemical manifestation of the toxic action of inorganic tin. On the other hand, phosphatases and dehydrogenases are important and critical enzymes in biological processes too. They are responsible for detoxification, metabolism, and biosynthesis of energetic macromolecules for different essential functions. The increase in plasma LDH activity may be due to the hepatocellular necrosis leading to leakage of the enzyme to the blood stream (Wang and Zhai 1988). Thus, when alcohol may lead to the release of these enzymes into plasma as a result of autolytic breakdown or cellular necrosis, the LF infusion impart protection against alcohol-induced oxidative injury that may result in development of liver damage.

As shown in Table 2, the present study demonstrated that the LF could have not antioxidative role in rats. This was obvious from our observation that, by the consequence of LF infusion treatment in vivo, the concentration of MDA in the tissues did not differ from that of alcohol-exposed group. According to the obtained results, while MDA concentrations appreciable increased in the tissues of rats treated with alcohol and LF infusion, the tissue MDA contents significantly decreased in the 20 % alcohol + 2 % LF infusion supplementation group compared to that of alcohol and 2 % LF group. The reasons for such effect of alcohol and the LF infusion are not understood at the present. But, increased MDA content might have resulted from an increase of ROS as a result of stress condition in the rats with ethyl alcohol intoxication. Studies have shown that alcohol consumption may result in increased oxidative stress with formation of lipid peroxides and free radicals (Aykac et al. 1985; Sonde et al. 2000; Kolankaya et al. 2002; Yurt and Celik 2011; Dogan and Celik 2012; Nordmann et al. 1992; Bjomeboe and Bjorneboe 1993; El-Sokkary et al. 1999; Gentry-Nielsen et al. 2004). Alcohol-induced oxidative stress is linked to the metabolism of ethanol (Zima et al. 2001). On the other hand, it is known that the elevation of lipid peroxidation after the consumption of some xenobiotics and following superoxide overproduction which produce dismutation singlet oxygen and H_2O_2, can be easily converted later into the reactive ·OH. Both single oxygen and OH radical have a high potential to initiate free radicals chain reactions of lipid peroxidation. Further, it is known that ·OH can initiate lipid peroxidation in tissues (Kang 2002) and MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation (Freeman and Crapo 1981).

Meanwhile, SOD, GR, GPX, CAT, and GST activities and GSH levels were fluctuated at appreciable level in the alcohol-treated rats. However, the efficacy of the LF infusion against these fluctuations could have not been determined. The reasons for such effect of functional plant's supplemented are not understood at present. However, oxidative stress can affect the activities of protective enzymatic antioxidants in organisms exposed to alcohol. The fluctuation of ADS constituents may reflect an adaptive change against ethanol-induced lipid peroxide toxicity (Aykac et al. 1985). However, the fluctuation of ADS is known to serve as protective responses to eliminate xenobiotics (Smith and Litwack 1980). Thus, the existence of an inducible antioxidant system may reflect an adaptation of organisms. So far, no study examining the preventive role of grape seeds-supplemented food in vivo has been made on rat serum biomarker levels, antioxidant defense systems and MDA content as a containing diet supplementation. Therefore, we had no chance to compare our results with the previous ones. In addition, because of high variability in analyzing serum enzymes–chemicals interaction in vitro and in vivo, and inconsistent factors like treatment time and manner, the setting of studies and species tissue differences etc., it is difficult to compare the present data to different studies regarding the chemopreventive properties.

However, earlier studies have demonstrated the potent free radical scavenger ability of LF flavonoids, mainly quercetin glycosides (rutin, quercitrin and isoquercitrin), kaempferol glycosides (Toker et al. 2001), and phenolic acids (caffeic, p-coumaric, and chlorogenic acids) (Czygan 1997).

As a conclusion, the observations presented here led us to conclude that while administration of subchronic ethyl alcohol promotes MDA concentration fluctuations in the antioxidative systems and elevates liver damage serum biomarkers, but the LF infusion supplement could not impart protection against alcohol induced liver injury and oxidative stress. The observations, along with changes, also might suggest that such a test will also be of value in chemopreventive studies, and be of interest to understand molecular basis of refractoriness of LF protective role. In addition, we wish to study alcohol's destructive effects and the healing effects of the LF against alcohol before coming to any conclusion.

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