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# Antioxidant Role and Hepatoprotective Effects of Carob (*Ceratonia siliqua* L.) Seeds against Ethanol-Induced Oxidative Stress in Rats

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**Abstract** The purpose of this research was to determine the effects of carob seeds (CS) concerning with hepatoprotective and antioxidant role against ethanol-induced oxidative stress (OS) in rats. Experiment was conduted as control, 20% ethanol, 15% CS and 20% ethanol + 15% CS groups. At the end of the 50-day exposure period of test groups, the hepatopreventive and antioxidant capacity were assessed by measuring level of serum enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH); antioxidant defense systems, including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) activities, reduced glutathione (GSH) levels and malondialdehyde (MDA) contents in the tissues of rats. According to results, while the levels of the serum enzymes increased in ethanol group compared with the control group whereas decreased in ethanol + CS-treated group compared with the ethanol group. Administration of CS supplementation restored the ethanol induced imbalance between malondialdehyde and fluctuated antioxidant system towards near normal particularly in the tissues. Finally, it was concluded that CS has a hepatoprotective effect and antioxidant capacity in rats with ethanol toxicity, probably acting by promoting the antioxidative defense systems.

***Keywords:*** *carob seeds, serum biomarkers, antioxidant defense systems, malondialdehyde, rats*

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## 1. Introduction

The carob tree, an evergreen plant, is generally grown Mediterranean region. The carob includes pod and seed. Carob pods have been consumed generally as human and animal food. The importance of carob arises from its seed. Nowadays, carob seeds have been paid attention on and they have acted an important role economically since they are used as food additives (Locust bean gum is also known as carob gum, carob bean gum, carobin, E410), in pharmaceutical and cosmetic industries (Batista *et al*., 1996; Barracosa *et al*., 2007; Sidina *et al*., 2009). Carob seeds are a particularly rich source of complex polymers of flavonoids such as proanthocyanidin, ellagitannin, and gallotannin. These phytochemicals have been used in medicine for its pharmacological properties against numerous diseases and have free radical scavenging activities (Luthria, 2006).

Cells have plenty of protective mechanisms, called antioxidant defense system, against OS to prevent damaging effects (Cheeseman and Slater, 1993; De Zwart *et al*., 1999). Numerous studies have demonstrated that a great number of medicinal and aromatic herbs, as well as fruits and leaves of some berry plants, biosynthesis phytochemicals possessing antioxidant property and may be used as a natural source of free radical scavenging compounds (Yu *et al*., 2005; Sacchetti *et al*., 2005). The harmful effect of free radicals causing potential biological damage is termed OS and nitrosative stress (Kovacic and Jacintho, 2001; Ridnour *et al*., 2005; Valko *et al*., 2007; Gülçin, 2012; Gülçin and Beydemir, 2013). Ethanol-induced OS is related to the ethanol metabolism. Each metabolic pathway (alcohol dehydrogenase, microsomal ethanol oxidation system, and catalase) produces specific metabolic and toxic disturbances (Zima et al., 2001).

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, no studies have so far been reported on hepatoprotective role and antioxidant capacity of the CS suplementation used in this study. The objective of this study was to determine healthful potentials of CS against ethanol-induced oxidative stress by evaluating their *in vivo* hepatoprotective role and antioxidant role. Thus, in the present study, we have extensively studied the antioxidant activity of ethanol using in vivo models. For this aim, the

treatment of CS was done orally as food containing CS 15% because the effect of the functional plant represents a well characterized in nutrition and widely used as consumption by human in our country Turkish folk medicine. The serum biomaekers were chosen due to their importance as index of hepatotoxin. The antioxidant activity of ethanol on some phase II detoxification ADS such as SOD, GPx, GST, GR activities, GSH levels and MDA contents in the various tissues were evaluated 50 days during experiment.

## 2. Materials and Methods

*Chemicals:* Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), reduced glutathione (GSH), metphosphoric acid, 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB), trihydroxymethyl aminomethane (Tris), 1-chloro-2,4-dinitrobenzene (CDNB), oxidized glutathione (GSSG), β-Nicotinamide adenine dinucleotide phosphate (NADPH), potassium dihydrogen phosphate ($KH_2PO_4$), ethanol and sodium chloride (NaCl) of technical grade used in this study were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Kits for the analysis of antioxidant enzymes were provided by Randox Laboratories (Crumlin, County Antrim, UK).

*Animals:* Female rats (*Wistar albino*) in the weight range of 200–250 g and 4 months of age were provided from the Experimental Animal Research Center, Yuzuncu Yil University (Van, Turkey), and were housed in four groups, each group containing six rats. The animals were housed at 20 ± 2°C with 12:12 h reverse light/dark cycle and given free access to standard laboratory chow for rodent with water 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 ethics 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 Yuzuncu Yıl University (no. B.30.2.YYU.0.05.06.00/300-335).

*Preparation of foods:* Briefly, CS were collected in Antalya, Turkey of Mediterranean region in summer term of 2010. Carob seeds were separated from fruits afterwards were ground into powder and then the amount of powdered CS was adjusted 15% of rat food (Yurt and Celik, 2011; Dogan and Celik, 2011).

***Experimental Design****:* The rats were randomly divided into six 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 administered orally (Aykac et al. 1985; Sonde et al. 2000; Yurt and Celik 2011; Celik et al. 2009; Dogan and Celik 2012).

*Group III (15%* carob seeds*)*: the rats received tab water and fed with 5% walnuts powder containing diet supplementation.

*Group IV (15%* carob seeds *+ 20% alcohol)*: the rats received %20 alcohol water and fed with 5% walnuts powder containing diet supplementation.

*Preparation of tissues supernatant and erythrocyte pellets:* After 50-days exposure, the rats were anaesthetised by an injection of ketamine (5 mg/100 g body weight) intra-peritoneally. The blood samples were obtained from a cardiac puncture, using injector for the determination of serum marker enzyme levels and biochemical analysis. The serum samples were obtained by centrifuging blood samples at 4000x*g* for 15 min at 4°C, and enzyme levels were measured from these serum samples. For biochemical analysis, blood samples were put immediately into disposable silicon glass tubes with EDTA as an anti-coagulant and were centrifuged at 4000x*g* for 15 min at 4°C and erythrocyte pellets were obtained. Then, the pellets were washed tree times with physiological saline (0.9% NaCl). The tissues of brain, kidney, spleen, heart and liver were dissected and put in Petri dishes. After washing the tissues with physiological saline (0.9% NaCl), the samples were taken and kept at -78°C during the analysis. The tissues were homogenised for 5 min in 50 mM ice-cold $KH_2PO_4$ solution (1:5 w/v) using stainless steel probe homogeniser (ultrasonic frequency 20 KHz; Jencons Scientific, Leighton Buzzard, Beds, UK) for 5 min and then centrifuged at 7000 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).

*Biochemical analysis:* The concentration of MDA in erythrocyte and tissues was measured using the method described by Jain *et al*. (1989), based on TBA reactivity. The concentration of GSH in erythrocyte and tissues was measured using the method described by Beutler *et al*. (1963). GST was assayed by following the conjugation of GSH with CDNB at 340 nm as described by Mannervik and Guthenberg (1981). Glutathione reductase (GR) activity was assayed as described by Carlberg and Mannervik (1975) as the decrease in absorbance of NADPH at 340 nm. GPx activity was assayed as described by Paglia and Valentine (1967) based on that of GPx catalyses and the oxidation of GSH by cumene hydroperoxide. Superoxide dismutase activity was measured at 505 nm by calculating inhibition percentage of formazan dye formation (McCord and Fridovich, 1969).

*Measurement of enzyme levels:* The serum marker enzymes, such as AST, ALT and LDH were measured by an auto analyser (BM/HITACHI–911; Boehringer Mannheim, Mannheim, Germany), using the kits.

*Statistical analysis:* All data were expressed as mean and standard deviation and analyzed using one-way ANOVA, followed by the Tukey test. Statistical significance was accepted when $p \leq 0.05$.

## 3. Results

Following the exposure of experimental groups, the effects of alcohol and the CS supplemented diet on liver damages index and antioxidative roles were evaluated as marker serum enzymes, ADS and MDA content of blood and various tissues samples from control and treated rats. The results of experiment showed that the treatment of

rats with alcohol and alcohol + CS containing diet supplementation caused changes in the level of serum enzymes, MDA content and ADS constituents in comparison with control rats. The effects of carob seeds administration alone or in combination serum activities, MDA and ADS parameters are presented in Table 1 and Table 2. The serum levels of ALT, AST and LDH were used as biochemical markers for the early acute hepatic damage. According to the results, levels of serum enzymes AST, ALT and LDH in ethanol-treated group were significantly increased compared with the control group whereas ALT and LDH levels of ethanol+CS-treated group resulted in marked decreases (Table 1).

With regard to MDA content and ADS constituents, the results of the present experiment showed that MDA, as a product of lipid peroxidation, increased significantly in erythrocytes, brain, liver, spleen and kidney exposure of ethanol group. However, MDA decreased significantly in the ethanol+CS-treated group's erythrocytes, brain, spleen and kidney (Table 2). Compared to the ethanol+CS-treated group, ethanol group GSH levels decreased significantly in the heart and liver. Antioxidant enzymes such as GPx activities increased in ethanol+CS-treated group's brain and kidney. Increased activity of GST was observed erythrocytes, spleen and kidney in the ethanol group, whereas decreased activity the same tissues in the ethanol+CS-treated group. Ethanol exposure revealed significantly decrease SOD activities in the erythrocytes, heart and liver, but an increase in only liver ethanol+CS-treated animals.

**Table 1. Effect of carobseed supplementation on liver damage marker enzymes in serum of rats**

| Groups / Serum | Control | Ethanol (20%) | CS (15%) | Ethanol (20%) + CS (15%) |
|---|---|---|---|---|
| AST (U/l) | 102,0±17,7 | 143,8± 22,9[a] | 108,2±21,3 | 123,2±29,3 |
| ALT (U/l) | 52,7±4,5 | 78,7±5,4[a] | 52,7±9,1[b] | 61,0±4,8[b] |
| LDH (U/l) | 659,8±65,5 | 844,7±83,3[a] | 653,0±54,0[b] | 575,8±121,0[b] |

a Mean± standard deviation values were significantly different from control ($P \leq 0.05$, one-way ANOVA followed by Tukey).
b Mean±standard deviation values were significantly different from the ethanol group ($P \leq 0.05$, one-way ANOVA followed by Tukey).

**Table 2. Effect of carob seeds supplementation on antioxidant status and malondialdehyde (MDA) content in various tissues of rats**

| Tissues | Parameters | Control | Ethanol 20% | Carob seeds 15% | Ethanol 20% + CS 15% |
|---|---|---|---|---|---|
| Erythrocytes | GSH (mg/g) | 1,78±0,08 | 1,84±0,14 | 1,58±0,04[a,b] | 1,7±0,05 |
| | MDA (nmol/g) | 1,52±0,3 | 3,05±0,7[a] | 1,42±0,34[b] | 1,54±0,27[b] |
| | GPx (U/g) | 1194±18 | 546±14[a] | 534±5[a] | 533±3[a] |
| | GR (U/g) | 2,14±0,63 | 1,8±0,79 | 2,43±0,41 | 2,01±0,47 |
| | SOD (U/g) | 2171±27 | 2072±38[a] | 2161±15[b] | 2105±32[a,c] |
| | GST (U/g) | 7,02±0,35 | 10,18±0,55[a] | 7,2±0,67[b] | 8,77±0,34[a,b,c] |
| Brain | GSH (mg/g) | 34,14±5,13 | 28,83±0,8 | 37,22±4,18[b] | 40,35±2,96[a,b] |
| | MDA (nmol/g) | 42,09±2,56 | 47,49±3,86[a] | 40,9±3,7[b] | 41,01±3,86[b] |
| | GPx (U/g) | 140±7 | 135±13 | 141±10 | 162±7[a,b,c] |
| | GR (U/g) | 1,42±0,23 | 1,57±0,2 | 1,52±0,24 | 1,54±0,21 |
| | SOD (U/g) | 1692±37 | 1740±28 | 1710±119 | 1749±24 |
| | GST (U/g) | 1,25±0,51 | 1,42±0,23 | 1,69±0,41 | 1,33±0,13 |
| Liver | GSH (mg/g) | 90,64±4,02 | 100,1±3,48[a] | 84,1±1,1[b] | 88,29±7,67[b] |
| | MDA (nmol/g) | 34±5,32 | 51,37±4,92[a] | 34,64±5,39[b] | 46,62±2,52[a,c] |
| | GPx (U/g) | 2207±12 | 2365±9[a] | 2145±12[a,b] | 2322±9[a,b,c] |
| | GR (U/g) | 4,45±0,35 | 5,9±0,57[a] | 5,23±0,25 | 5,91±0,8[a] |
| | SOD (U/g) | 1795±51 | 1411±26[a] | 1742±55[b] | 1639±51[a,b,c] |
| | GST (U/g) | 4,28±0,44 | 13,61±0,75[a] | 4,36±0,13[b] | 12,65±0,52[a,b,c] |
| Spleen | GSH (mg/g) | 94,62±2,09 | 99,72±3,18[a] | 102,52±1,16[a] | 98,98±3,22[a] |
| | MDA (nmol/g) | 44,03±6,4 | 90,45±8,33[a] | 52,02±2,52[b] | 58,5±4,51[a,b] |
| | GPx (U/g) | 406±5 | 395±4[a] | 411±9[b] | 395±3[a,c] |
| | GR (U/g) | 2,35±0,14 | 2,07±0,26 | 2,03±0,37 | 2,03±0,13 |
| | SOD (U/g) | 2024±29 | 1905±46[a] | 2020±55[b] | 1982±58 |
| | GST (U/g) | 0,86±0,07 | 1,16±0,06[a] | 0,77±0,21[b] | 0,82±0,12[b] |
| Kidney | GSH (mg/g) | 71,22±4,7 | 76,22±5,52 | 71,53±8,09 | 70,24±2,66 |
| | MDA (nmol/g) | 93,69±6,01 | 106,21±8,25[a] | 86,02±5,02[b] | 95,74±2,17[b,c] |
| | GPx (U/g) | 375±8 | 402±4[a] | 395±3[a] | 406±4[a,c] |
| | GR (U/g) | 7,08±0,49 | 7,36±0,33 | 6,15±0,15[a,b] | 7,36±0,19[c] |
| | SOD (U/g) | 1768±144 | 1581±49 | 1732±204 | 1676±42 |
| | GST (U/g) | 1,1±0,29 | 1,21±0,28 | 1,36±0,12 | 1,21±0,25 |
| Heart | GSH (mg/g) | 78,91±7,26 | 82,93±6,07 | 78,85±7,64 | 68,11±6,26[b] |
| | MDA (nmol/g) | 15,21±2,49 | 20,83±4,15 | 14,03±3,31[b] | 15,43±3,17 |
| | GPx (U/g) | 371±4 | 411±5[a] | 414±5[a] | 409±4[a] |
| | GR (U/g) | 0,44±0,08 | 0,45±0,06 | 0,38±0,11 | 0,46±0,06 |
| | SOD (U/g) | 1981±44 | 1765±48[a] | 1945±42[b] | 1812±33[a,c] |
| | GST (U/g) | 0,71±0,07 | 0,81±0,1 | 0,72±0,08 | 0,79±0,07 |

a Mean±standard deviation values were significantly different from control ($P \leq 0.05$, one-way ANOVA followed by Tukey).
b Mean±standard deviation values were significantly different from the ethanol group ($P \leq 0.05$, one-way ANOVA followed by Tukey).
c Mean±standard deviation values were significantly different from the carobseeds group ($P \leq 0.05$, one-way ANOVA followed by Tukey).

# 4. Discussion

This is the first report investigating the effects of CS upon rats' serum enzymes and ADS against OS *in vivo* in the literature as guidance for many scientists. Acute and chronic alcohol abuse is one of the leading reasons of liver diseases in many countries and has becoming a social problem (Çoban et al., 2008). Ethanol-induced tissue damage is partly related to nutritional deficiencies and direct toxic effects, linked to the metabolism of ethanol (Zima et al., 2001). The reason of this damage can also be stated in a way that excessive ethanol consumption activates several systems generating ROS and reactive

aldehyde species (Yurt and Celik, 2011). Long-term ethanol intake experiments could change the oxidative damage parameters as well as antioxidant enzyme activity, improving the effects of different treatments. In order to reflect the protective effect of CS, we established the ethanol-induced subchronic liver injury model in experiment. Our results are confirmed by the findings of the study of Nishimura and Teschke (1982), Teschke et al. (1987) and Dogan and Celik (2011) stating that the serum transaminase enzymes (AST and ALT) and LDH levels increased in ethanol group whereas ALT and LDH decreased by possibility of the hepatoprotective effect of CS in the ethanol+CS-treated group.

Relationship between alcohol abuse and liver fibrosis becomes clear via oxidation of polyunsaturated membrane lipids formed by ethanol metabolism (Kamimura *et al*. 1992; Poli, 2000). Findings obtained from our recent studies have demonstrated that malondialdehyde, a product of lipid peroxidation, while MDA levels of the ethanol group increased compared to the control group in erythrocytes, brain, liver, spleen and kidney, it's levels decreased in the ethanol+CS-treated group compared with the ethanol group in erythrocytes, brain, spleen and kidney. The present study's finding related to elevated MDA levels is consistent with the findings of some previous research. In these research, it was found that ethanol metabolism escalated MDA levels on the primer hepatocyte rat culture (Sergent *et al*., 1995), formation of hepatic microsomal MDA increased at the rats owing to exposed to ethanol (Wisniewska-Knyp and Wronska-Nofer, 1994) and the increase of sensitivity at the level of lipid peroxidation was determined at the rats given ethanol (Lindi et al., 1998). Glutathione ($_{L}$-γ-glutamyl-$_{L}$-cysteinylglycine; GSH) is a tripeptide and composed of glutamic acid, cysteine and glycine, and also an important reductant by aid of the thiol (–SH) of cysteine residue (Meister and Larsson, 1989). Most of the cellular GSH is mainly present reduced form in the cytosol and readily oxidized to GSSG during OS, and reverted to the reduced GSH by the NADPH-dependent GR (Wu *et al*., 2004; Pastore *et al*., 2003; Chai *et al*., 1994). GSH may cause an increase in ethanol-treated group in order to form antioxidant defense against OS formed by ethanol. On the other hand, the decrease was observed on GSH level of brain compared to other organs since brain has some disadvantages in the consideration of the generation and the detoxification of ROS. This decrease might be explained in a way that even if the weight of the brain is 2% of the weight of the body, brain cells consume 20% of the oxygen utilized by the body (Dringen, 2000; Clarke and Sokoloff, 1999). This situation points out the potential generation of a high amount of ROS in the course of oxidative phosphorylation in brain. Moreover, catalyzed generation of ROS results from a high content of iron in some brain areas (Dringen, 2000; Gerlach *et al*., 1994). Also, brain is the target for lipid peroxidation because of its richness for unsaturated fatty acids (Dringen, 2000; Porter, 1984; Halliwell, 1992). GST acts an important role at cell defense against xenobiotic and carcinogenic (Mannervik *et al*., 1992; Hayes and Pulford, 1995) so the increase of GST activity in the ethanol-treated group can be thought as followed adaptation for the mechanism of the defense against OS (Agrawal *et al*., 1991; Banerjee *et al*., 1999). However, the decrease of GST activity in the ethanol+CS-treated group can be considered that the carob seeds might have protective effect against xenobiotics. SOD activity, the first mechanism ADS, decreased in the ethanol-treated and the ethanol+CS-treated groups (in addition, the less decrease was observed in ethanol+CS-treated groups than ethanol-treated groups) in order to eliminate the excessive amount of increase of ROS due to ethanol. Also, GPx activity increased to neutralize $H_2O_2$ formed by SOD activity and this means that ADS works (Gaeta et al., 2002). Moreover, GR activity increased so as to provide GSH, which is oxidized by GPx, and to become reduced again. This mechanism of the enzyme system is similar to the finding of the previous research belonged to Sun (1990). In the meantime, antioxidant enzymes activity and GSH levels fluctuated at satisfactory level in the ethanol-treated rats. However, the efficacy of the different treated and contentful CS against these fluctuations have not been determined. The reasons for such effect of functional plant's supplemented are not understood properly at the present. However, OS can have effect on the activities of protective enzymatic antioxidants in organisms exposed to ethanol.

## 5. Conclusion

In conclusion, this study demonstrated that ethanol exposure gave rise to lipid peroxidation via inducing the OS and had different effects on various tissues of rats on their antioxidant defense enzymes systems. This can result from different qualities of cell physiology adaptation according to different tissues. In this respect, it might be stated that carob seeds can possibly be beneficial to defend against OS formed by ethanol owing to its antioxidant property but there are no reports about OS parameters carob seeds supplementation in the literature. Therefore, we had no chance to compare our results with the previous ones.

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## Conflict of Interest

The authors have declared that there is no conflict of interest.

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