Protective effect of *Amaranthus lividus* L. on carbon tetrachloride induced hepatotoxicity in rats

**Abstract**

Objective: *Amaranthus lividus* is consumed as popular vegetable in West Black Sea Region of Turkey. In this study, we aimed to evaluate the protective and antioxidant effects of *A. lividus* on carbon tetrachloride (CCl₄) induced oxidative stress and acute liver injury in rats.

Methods: Male albino Wistar rats were divided into 7 groups: Normal control, *A. lividus* control, silymarin control, CCl₄, *A. lividus* (250 mg/kg)+CCl₄, *A. lividus* (500 mg/kg)+CCl₄, silymarin+CCl₄. Rats were orally pretreated with *A. lividus* (250 and 500 mg/kg) or silymarin (25 mg/kg) daily for 9 days before administration of CCl₄ (1.5 mL/kg, 1:1 in olive oil, i.p.).

Results: Pretreatment of rats with *A. lividus*, significantly prevented the CCl₄ induced elevation in the levels of serum alanine aminotransferase, aspartate aminotransferase, bilirubin and hepatic lipid peroxidation and myeloperoxidase. In addition, pretreatment with *A. lividus* significantly prevented the CCl₄ induced depletion in the activities of antioxidant enzymes such as catalase, glutathione-S-transferase, glutathione peroxidase, glutathione reductase, superoxide dismutase and glutathione level in liver. It has been observed that the hepatoprotective effect of *A. lividus* was comparable to that of silymarin, a standard drug. Histopathological evaluation of the liver also revealed that *A. lividus* at 250 mg/kg dose partially suppressed the CCl₄ induced liver damage in rats.

Conclusion: Our results indicated that *A. lividus* has a protective effect against CCl₄ induced acute hepatotoxicity in rats, and this effect might be related to its antioxidant activity.

Key Words: *Amaranthus lividus*, antioxidant enzymes, carbon tetrachloride, hepatotoxicity, alanine aminotransferase, aspartate aminotransferase, silymarin

Conflict of Interest: The authors have no conflict of interest.

**Özet**

Amaç: *Amaranthus lividus*, Türkiye’nin Batı Karadeniz Bölgesinde sebze olarak yaygın şekilde tüketilen bir bitkidir. Bu çalışmadı *A. lividus’un* karbon tetraklorür (CCl₄) ile sıçanlarda oluşturulan oksidatif stres ve akut karaciğer hasarı üzerine koruyucu ve antioksidan etkilerinin ölçülmemesini amaçladık.

Metod: Erkek albino Wistar cinsi sıçanlar 7 gruba ayrıldı: Normal kontrol, *A. lividus* kontrol, silymarin kontrol, CCl₄, *A. lividus* (250 mg/kg)+CCl₄, *A. lividus* (500 mg/kg)+CCl₄, silymarin+CCl₄. Rats were orally pretreated with *A. lividus* (250 ve 500 mg/kg) or silymarin (25 mg/kg)’in ön uygulaması yapıldı.

Bulgular: Sıçanlara *A. lividus* ile ön uygulama yapılmasının, serumdaki alanin aminotransferaz, aspartat aminotransferaz, bilirubin ve karaciğerdeki lipit peroksidadyonu ve miyeloperoxidaz düzeylerinde CCl₄’un neden olduğu artışa anlamlı şekilde önledi. İlave olarak *A. lividus* ile ön uygulama yapılmasının, karaciğerdeki katalaz, glutatyon-S-transferaz, glutatyon peroksidadyon, glutatyon redüktaz ve süperoksit dismutaz gibi antioksidan enzim aktivitelerinde ve glutatyon düzeyinde CCl₄’un neden olduğu azalışa anlamlı şekilde önledi. *A. lividus* ve standart ilaçlar kullanlan silymarinin karaciğer üzerinde benzer koruyucu etkiler gösterdiğini gözlemlemiştik. Ayrıca *A. lividus*’un histopatolojik olarak değerlendirilmesi, 250 mg/kg dozda *A. lividus* ve CCl₄ nedeni ile sıçanların karaciğerinde oluşan hasarı kısmen baskılama etkisi gösterdiğini görmüşтарıktaydı.

Sonuç: *A. lividus’un* karaciğer toksisitesine karşı *A. lividus’un* koruyucu etkisi tıpkı silymarin için olduğu gibi ön uygulaması sonucunda, *A. lividus’un* antioksidan ve karaciğer koruyucu etkilerine sahip olduğu sinyalli göstermiştir.

Anahat Kelimeler: *Amaranthus lividus*, antioksidan enzimler, karbon tetraklorür, karaciğer toksisitesi, alanin aminotransferaz, aspartat aminotransferaz, silymarin

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.
Introduction

Liver regulates many important metabolic functions. Therefore, maintenance of liver functions and protection to the hepatic cells from the damage are vital to overall health and well being. Plants are used in traditional medicine for the treatment of liver disorders, as they may serve as potential sources for new therapeutic agents that could be applied in the prevention of hepatic injuries. Plants, rich in different phytochemical derivatives such as triterpenes, flavonoids or polyphenols, have been reported to exhibit antihepatotoxic effects on experimental liver injury models [1-3].

Carbon tetrachloride (CCl4) induced liver injury has been widely used as a model for the screening of the hepatoprotective effects of drugs and plant extracts. CCl4 induced hepatotoxicity results from the toxic metabolites of CCl4 that impair crucial cellular processes and cause centrilobular hepatic necrosis and steatosis [4,5]. Oxidative stress has been reported to play an important role in the pathogenesis of liver damage due to CCl4 administration [5].

Amaranthus lividus L. (Amaranthaceae) locally called as “dari mancari” in Turkish, is consumed as popular vegetable in the West Black Sea Region of Turkey also used as vegetable and cultivated in Southern and Central Europe, India and Malaysia [6,7]. Ozsoy et al. [8] showed in vitro antioxidant potential of A. lividus. But, the possible hepatoprotective activity which might be due to its antioxidant activity of A. lividus has not been reported so far. Therefore, in this study we aimed to evaluate whether A. lividus has a protective effect against CCl4 induced oxidative stress and hepatotoxicity or not by using acute liver injury model in rats. In this study the effect of A. lividus on acute liver injury was compared to that of silymarin, a well known hepatoprotective drug.

Materials and Methods

Chemicals

CCl4 was purcashed from Merck KGaA (Darmstadt, Germany). Silymarin was obtained from Sigma Chemicals Co. (St. Louis, Mo, USA). All other chemicals used in this study were analytical grade.

Plant material

A. lividus was collected from Bartin in the West Black Sea Region of Turkey and identified by Prof. Dr. Asuman Baytop. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE); herbarium code number: ISTE 83401. Plant material was washed in running tap water and shade dried. The dried stems with leaves and flowers of A. lividus were manually comminuted well before extraction.

Preparation of A. lividus extract

A crude water extract was prepared by heating comminuted A. lividus (100 g) in a flask with distilled water (1 L) for 30 min while stirring [8]. The extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator (Buchi R210, Switzerland). The water extract of A. lividus was yielded a dark-brown solid residue, weighing 26 g (26%, w/w) which was stored at -20°C. A. lividus is generally cooked with water before being consumed, for this reason water extract of A. lividus was used in this study.

Animals

Male albino Wistar rats (180-280 g) were obtained from the Institute of Experimental Medicine of Istanbul University and acclimatized to their environment for one week prior to experimentation. The animals were housed in an air-conditioned room with a 12 h light/dark cycle at controlled temperature and humidity conditions and supplied with standard laboratory diet and tap water ad libitum. The experimental procedure used in this study was approved by the Animal Assays Ethics Committee of Istanbul University (No:18527/16.07.2007).

Treatments

The water extracts of A. lividus at 250 and 500 mg/kg [9] and the standard hepatoprotective drug silymarin (25 mg/kg) [10] were administered orally as a fine suspension in carboxymethyl cellulose (CMC, 0.1%, w/v). These solutions were freshly prepared at each day of process. Liver damage was induced by intraperitoneal (i.p.) administration of a single dose of an equal mixture of CCl4 and olive oil (1.5 mL/kg) [11]. The CCl4 mixture was prepared immediately before treatment.

Male albino Wistar rats were randomly divided into 7 groups. Group I, II and III served as control groups (n=5) which received orally CMC (4 mL/kg), A. lividus (500 mg/kg) and silymarin (25 mg/kg), respectively, daily for 9 days. Group IV, V, VI and VII served as experimental groups (n=7) which received orally CMC (4 mL/kg), A. lividus (250 mg/kg), A. lividus (500 mg/kg) and silymarin (25 mg/kg), respectively, daily for 9 days. On the 10th day, rats in the experimental groups (IV-VII) were treated with CCl4 (1.5 mL/kg, 1:1 in olive oil, i.p.) while rats in the control groups (I-III) were treated with olive oil (1.5 mL/kg, i.p.).

24 h after CCl4 administration, the animals were anesthetized with diethyl ether and sacrificed by collecting blood via cardiac puncture. Blood was allowed to coagulate for 30 min and serum was separated by centrifugation at 1,016 x g for 5 min at 4°C. Serum was kept at -85°C until it was used in further biochemical assays. Livers were quickly excised and washed in 0.9% NaCl to remove as much blood as possible and then tissue samples were immediately frozen at -85°C for later use. The right upper lobe of the liver of each animal was used for the biochemical and histopathological analysis.

Serum biochemical assay

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed in the serum using a spectrophotometric method (UV 1201, Shimadzu, Japan) and expressed as U/L.
by the methods of Bergmeyer [12] and Bergmeyer et al. [13], respectively. One unit of ALT or AST activity was defined as μmol β-nicotinamide adenine dinucleotide reduced (NADH) oxidized per minute. Serum total bilirubin (TBil) content was measured colorimetrically by the diazo method of Jendrassik and Gröf [14]. A spectrophotometer (Shimadzu UV-1800, Japan) was used for all biochemical measurements.

Antioxidant and oxidant parameters in liver

The liver tissues were homogenized (10%, w/v) in ice cold phosphate buffer (5 mM, containing 0.15 M NaCl, pH 7.4) using a homogenizer (Art-MICCRA D-1, Germany) and the homogenates were used for the estimation of lipid peroxidation (LPO) and glutathione (GSH) levels. LPO level was assayed by measuring the concentration of thiobarbituric acid reactive substances on the basis of malondialdehyde (MDA), an end product of LPO [15]. GSH level was determined colorimetrically at 412 nm using 5,5'-dithiobis (2-nitrobenzoic acid) in the de-proteinized supernatant of the liver homogenate [16].

The liver homogenates (10%, w/v) as mentioned above were centrifuged (Heraeus Biofuge-Stratus, Germany) at 19,083 g for 5 min at 4°C and the postmitochondrial supernatants were used for the estimation of the activities of antioxidant enzymes such as catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD). Total protein content was determined by the method of Lowry et al. [17] using bovine serum albumin as standard.

CAT activity was measured according to the method of Aebi [18] following the decomposition of hydrogen peroxide (H₂O₂) and the enzyme activity was expressed as nmol H₂O₂ consumed/min/mg protein. GST activity was measured by determining the rate of conjugate formation between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) [19] and the enzyme activity was expressed as nmol CDNB conjugate formed/min/mg protein. GPx activity was assayed by the Lawrence and Burk method [20] using H₂O₂ as a substrate and the enzyme activity was monitored by recording the oxidation of β-nicotinamide adenine dinucleotide phosphate reduced (NADPH). GR activity was estimated following the oxidation rate of NADPH in the presence of glutathione oxidized (GSSG) according to the method described by Carlberg and Mannervik [21]. GPx and GR activities were expressed as nmol NADPH oxidized/min/mg protein. SOD activity was assayed by its ability to increase the rate of riboflavin-sensitized photo-oxidation of o-dianisidine [22]. The enzyme activity was calculated using the SOD standard and expressed as units/mg protein.

Myeloperoxidase (MPO) activity was determined in the liver tissue according to the methods of Hillegass et al. [23] and Singbartl et al. [24]. The liver tissues were homogenized in potassium phosphate buffer (50 mM, pH 6.0) and the homogenates were centrifuged at 11,292 x g for 15 min at 4°C. Supernatants were decanted and pellets were resuspended in 0.5% hexadecyltrimethyl ammonium bromide. After three freeze-thaw cycles with sonication between cycles, the samples were centrifuged at 11,292 x g for 15 min at 4°C. MPO activity was determined in the supernatant by measuring the H₂O₂ dependent oxidation of o-dianisidine. One unit of the enzyme activity was defined as the amount of MPO required to decompose 1 μmole of H₂O₂ in 1 min.

Histopathological analysis

Pieces of liver from the right upper lobe were fixed with Bouin’s solution, embedded in paraffin and sliced in 5 μm sections. The sections were stained with Haematoxylin-Eosin (H&E) and evaluated under light microscope (Olympus CX-41, Japan). Liver damage was evaluated from central vein to portal areas according to following degenerative changes: The presence of necrotic cells and areas, liver cells including vacuoles called foamy cells, dark eosinophilic cells, hypertrophic hepatocytes, rupturings in endothelium of central vein, sinusoidal expansions and mononuclear cell infiltrations.

Statistical analysis

The results were evaluated using an unpaired t-test and ANOVA variance analysis with the NCSS statistical computer package and expressed as means±SD. The differences were considered statistically significant at p<0.05.
Results

No significant alteration was found for all biochemical parameters investigated in control groups which received A. lividus or silymarin alone (Groups II and III) compared to the normal control group (Group I) (Table 1-2). High standard deviation values obtained in some biochemical parameters, may be due to marked individual differences in response to toxicity among the rats.

Effect of A. lividus on serum ALT, AST and TBil levels

The effect of A. lividus pretreatment on CCl₄ induced alterations in the serum biochemical parameters are presented in Table 1. Administration of a single dose of CCl₄ (Group IV) developed severe liver damage in the rats, as evidenced by the significant \((p<0.05)\) elevations of ALT and AST activities and TBil level in serum compared to the normal control group. Pretreatment with A. lividus at 250 and 500 mg/kg doses (Groups V and VI) and silymarin (Group VII) prevented the CCl₄ induced elevations in the levels of ALT and AST significantly \((p<0.05)\) compared to the CCl₄ group. Both doses of A. lividus showed similar preventive effects against increase of ALT and AST activities in serum.

However, pretreatment with A. lividus (250 and 500 mg/kg) and silymarin did not significantly prevent the CCl₄ caused increase in the level of TBil.

Effect of A. lividus on hepatic antioxidant parameters

As shown in Table 2, CCl₄ treatment caused significant \((p<0.05)\) decreases in the activities of antioxidant enzymes; CAT, GST, GPx, GR and the level of GSH compared to the normal control group. Also, MPO activity significantly \((p<0.05)\) increased after CCl₄ treatment in the liver compared to the normal control group, however there was no significant difference for SOD activity and MDA level. Pretreatment with both doses of A. lividus (250 and 500 mg/kg) and silymarin significantly \((p<0.05)\) prevented the depletion of CAT, GST, GPx, GR, SOD activities and GSH level compared with the CCl₄ group. Also, pretreatment with A. lividus (250 and 500 mg/kg) and silymarin significantly \((p<0.05)\) prevented the elevation in the levels of hepatic MDA and MPO compared to the CCl₄ group.

Histopathological observations

Distinct severe degenerative changes were usually observed around of central veins in the liver tissues of the CCl₄ treated rats (Fig. 1b, c) compared to the control groups (Fig. 1a). This damage was usually spreading from the central vein to portal areas in some individuals of this experimental group. A lot of foamy cells and dark eosinophilic cells were observed around of the central veins in the CCl₄ treated group. Other findings such as an increase in vacuolization and hypertrophy in hepatocytes, rupturings in endothelium of central vein, sinusoidal expansion, necrotic cells and areas, mononuclear cell infiltration were noticed in liver tissues of the CCl₄ treated rats (Fig. 1b, c).
of CCl₄ and stage of toxicity in liver [26,27]. Hepatic cells consist of high concentrations of ALT and AST in cytoplasm and AST exists particular in mitochondria. Injury to the hepatocytes alters their membrane permeability and a variety of enzymes located normally in cytosol are released into blood [28]. Elevated levels of serum enzymes, ALT and AST are indicative of cellular leakage and loss of functional integrity of cellular membrane in liver [29]. Our results showed that a single i.p. dose of CCl₄ at 1.5 mg/kg administration caused very severe acute liver damage in rats, demonstrated by excessive elevation in serum ALT and AST activities as well as histopathological findings. This elevation of the activities of ALT and AST are consistent with the findings of Mehmetçik et al. [26] which is similar acute CCl₄ toxicity study. Pretreatment with A. lividus at 250 and 500 mg/kg and silymarin for 9 days prior to the CCl₄ administration, efficiently prevented the CCl₄ induced elevation in the serum ALT and AST activities and TBil levels which suggest that A. lividus maintains the stabilization of plasma membrane and thus protects the liver against CCl₄ induced damage. This improvement of the serum transaminase levels was not found consistent with the histopathological results, which revealed that pretreatment with A. lividus (250 and 500 mg/kg) and silymarin did not efficiently prevent the CCl₄ induced elevation in serum ALT and AST activities and TBil levels.

Discussion

CCl₄ is a well known hepatotoxic agent used to screen the antihepatotoxic/hepatoprotective effect of drugs. CCl₄ metabolism begins with formation of trichloromethyl free radical (CCl₃•) by the cytochrome P450 system in liver microsomes. This radical reacts with cellular molecules (nucleic acid, protein, lipid) and impairing crucial cellular processes [5]. In the presence of oxygen, CCl₃• is converted to the more reactive trichloromethylperoxy radical (CCl₃OO•). CCl₃OO• attacks and destroys polyunsaturated fatty acids, thereby initiates the chain reaction of LPO. The primary toxic consequences of LPO are related to disruption of cellular membranes, resulting in loss of membrane integrity which eventually leads to liver damage [5,25]. In literature, there have been several studies regarding CCl₄ induced toxicity in liver. However, data obtained show some different results depending on dose of CCl₄ and stage of toxicity in liver [26,27].

Hepatic cells consist of high concentrations of ALT and AST in cytoplasm and AST exists particular in mitochondria. Injury to the hepatocytes alters their membrane permeability and a variety of enzymes located normally in cytosol are released into blood [28]. Elevated levels of serum enzymes, ALT and AST are indicative of cellular leakage and loss of functional integrity of cellular membrane in liver [29]. Our results showed that a single i.p. dose of CCl₄ at 1.5 mg/kg administration caused very severe acute liver damage in rats, demonstrated by excessive elevation in serum ALT and AST activities and TBil levels which suggest that A. lividus maintains the stabilization of plasma membrane and thus protects the liver against CCl₄ induced damage. This improvement of the serum transaminase levels was not found consistent with the histopathological results, which revealed that pretreatment with A. lividus (250 and 500 mg/kg) and silymarin did not efficiently prevent the CCl₄ induced elevation in serum ALT and AST activities and TBil levels.
suppressed the acute hepatic damage. Preventive effects of the extract as well as silymarin could not been clearly observed histologically due to widespread liver damage. Nevertheless, partial protection was observed at 250 mg/kg dose of *A. lividus* histologically. Similarly, it was stated that sometimes there could not be strict correlation between histological findings and serum transaminase values and that entire histologic spectrum of liver disease can be seen in individuals with normal ALT values [30,31].

MDA is a reactive aldehyde that released during peroxidation of membrane phospholipids. Therefore, hepatic MDA levels are used as an indicator of liver damage. LPO which induced by free radical derivatives of CCl₄ is the main cause of hepatic damage [4]. In our study elevated hepatic MDA level was observed in the CCl₄ treated rats, suggests enhanced LPO. Pretreatment with *A. lividus* caused a significant decrease in the MDA levels which may be explained by free radical scavenging properties of the plant. This finding is in accordance with Al-Dosari [32] and Ashok Kumar et al. [33] who reported that *Amaranthus* (*A. tricolor* and *A. caudatus*, respectively) treatment decreased hepatic MDA levels in liver injury.

Mammalian cells are equipped with both enzymatic (CAT, GST, GPx, GR and SOD) and non-enzymatic (GSH) antioxidant defense systems to prevent formation of reactive oxygen species (ROS) and their damaging effects. GSH effectively scavenges free radicals and other ROS and oxidized to form GSSG, then GR recycles GSSG to GSH. In addition, GSH reacts with various electrophiles, physiological metabolites and xenobiotics to form mercapturates, which are catalyzed by GST (a family of Phase II detoxification enzymes) [34]. SOD catalyze the dismutation of superoxide radicals to H₂O₂ and CAT/GPx decomposes H₂O₂ to water. GPx not only decomposes H₂O₂ but also lipid peroxides [35]. In the present study, significant decreases in the CAT, GST, GPx, GR and SOD activities and the GSH levels were observed after the CCl₄ treatment, suggesting increased oxidative damage in the liver. A reduction in antioxidant enzyme activity is related to an increase in free radical production in CCl₄ toxicity [36,37]. Our results showed that pretreatment with *A. lividus* effectively protected the rats against CCl₄ induced oxidative stress, as evidenced by increased levels of antioxidant enzyme and GSH in the liver. This elevation of antioxidative capacity suggests that *A. lividus* promotes the scavenging of reactive free radicals and improve the hepatic antioxidant enzyme activities. This suggestion is supported by the findings of Ozsoy et al. [8] that *A. lividus* exhibited antioxidant activity in the *in vitro* radical scavenging methods. These findings are consistent with the other studies which reported that some *Amaranthus* species demonstrated antioxidant effects by enhancing the antioxidant enzyme activities [9,33,38].

MPO is a heme peroxidase released by polymorphonuclear neutrophils which catalyzes the formation of numerous ROS and thus has strong proinflammatory and pro-oxidative properties [39]. MPO changes H₂O₂ to hypochlorous acid, a powerful oxidant, in the presence of Cl⁻ [40]. In the CCl₄ intoxicated rats, MPO activity significantly increased, an index of hepatic neutrophil infiltration [41]. *A. lividus* pretreatment significantly decreased the CCl₄ induced elevation in hepatic MPO activity, demonstrating prevention of infiltration of neutrophils into the damaged tissue. Similarly, several previous studies showed that some agents such as curcumin and melatonin decreased hepatic MPO activity in liver injury in addition to antioxidant effects [42,43].

In this study, *A. lividus* did not exhibit distinct dose dependent activity. Both doses of extract (250 and 500 mg/kg) showed similar preventive effect to that of silymarin against CCl₄ induced hepatotoxicity and oxidative stress, suggesting that low dose (250 mg/kg) is adequate for maximum protection. Even though harmful effect was not seen for all biochemical parameters with control group which received *A. lividus* (500 mg/kg) alone, the fact that 250 mg/kg extract dose provides the highest protection, is advantageous in view of possible adverse effects due to herb-drug interactions that could be observed at higher extract concentrations.

In conclusion, this study showed that *A. lividus* can be proposed to protect the liver against CCl₄ induced oxidative damage in rats and the hepatoprotective effect might be correlated with both increase of antioxidative defence system activity and the inhibition of LPO.

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**Conflict of Interest**

There are no conflicts of interest among the authors.

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