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The effect of black mulberry (*Morus nigra*) extract on carbon tetrachloride-induced liver damage

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Abstract: In this study, the effect of *Morus nigra* (*M. nigra*) on carbon tetrachloride (CCI_4)-induced hepatic injury in the rat was investigated. A hepatotoxic rat model was developed by the injection of CCI_4 dissolved in soybean oil (1 mL/kg/twice a week, intraperitoneal (i.p.) injection). Following the formation of hepatic injury, extracts of *M. nigra* at doses ranging from 150 to 300 mg/kg were administered to rats by i.p. injection for eight weeks. At the end of administration, rat livers were excised by dissection. The activities of liver enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were detected in the serum, and the activities of antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), were established in the liver. Histological changes and immunohistochemical localization of caspase-3 and 8-oxo-2'-deoxyguanosine (8-OhdG) were performed by hematoxylin-eosin (H&E) staining of liver sections and caspase-3 and 8-OhdG immunohistochemical staining. The results showed that the *M. nigra* extract prevented protein oxidation generated by CCI_4. The extracts demonstrated the ability to modulate the activity of SOD and GPx, and also prevented the CCI_4-induced increase in AST and GGT levels. These results indicate that *M. nigra* extracts provided significant protection against CCl_4-induced hepatic injury and might also present a novel approach for the treatment of some liver diseases.

Key words: *Morus nigra*; hepatic injury; antioxidant enzymes; caspase-3; 8-hydroxydeoxyguanosine

INTRODUCTION

The liver is the body's largest gland and a vital organ that supports nearly every other organ in some way. Major functions of the liver include carbohydrate, protein and fat metabolism, as well as secretion of bile. Many chemicals, drugs, xenobiotics, infections and alcoholism induce hepatic dysfunctions [1]. Hepatic disturbances lead to cirrhosis, hepatitis, hypertrophy, carcinoma and liver failure [2,3]. The therapy for hepatic diseases has been extensively explored with remarkable progress in the last few decades; however, the outcomes are still not desirable, mostly due to complications incurred and the relatively high cost of treatment [4]. Chemical compounds such as CCl_4, thioacetamide and dimethylnitrosamine injure hepatocytes, trigger secondary inflammatory reaction and induce liver fibrosis and cirrhosis [5]. Acute administration of a large dose of CCl_4 causes severe liver necrosis, while chronic administration of lower doses is frequently used to induce hepatic fibrosis [6]. As oxidative stress is known to play a role in the pathogenesis of different diseases, the contribution of dietary polyphenols to their prevention has been widely investigated [7]. Dietary fatty acid substances have been extensively studied because of their antioxidant activity. Mulberry fruits were found to be rich in linoleic acid, which is an essential fatty acid. The fatty acid composition of mulberries highlights the nutritional and health benefits of their consumption [8].

Black mulberries (*Morus nigra*) are thought to have originated in the mountainous areas of Mesopotamia and Persia and are now widespread throughout Iraq,

Iran, Pakistan and Turkey. Many studies have found that black mulberry is rich in polyphenols, flavonoids, and anthocyanins [9,10]. They are important for humans not only because they contribute to plant color but also because they show many biological and pharmacological activities, including antioxidative, antiinflammatory and antiviral effects [11]. *M. nigra* flavonoids possess strong antioxidant activity. Its berries are used in medicine against inflammation and to stop bleeding, the bark is used for toothache, and the leaves for treating snakebites and as an antidote to poisoning [12,13]. Another study showed that black mulberry possesses prokinetic, laxative and antidiarrheal effects, putatively mediated through cholinomimetic, antimuscarinic, and Ca^{2+} antagonist mechanisms, respectively [14]. *M. nigra* is used in ethnomedicine for the prevention and treatment of several diseases. Therefore, this work aimed to study the effect of black mulberry extract on liver in CCl_4-induced hepatic damage in rats as an initial step in the further elucidation of the therapeutic potential of this plant product.

MATERIALS AND METHODS

Preparation of lyophilized extract of *M. nigra*

M. nigra was collected from Gümüşhane and Erzurum provinces in Turkey during the summer of 2013. The plant was identified and authenticated by experts from the Botanical Department (Atatürk University, Erzurum, Turkey). One hundred g of air-dried *M. nigra* were weighed and cut with a blender prior to each treatment. The samples were divided into two parts, and 100 mL of purified water were added to first part. The sample was extracted at room temperature overnight. It was then filtered, and the operation was repeated. Alcohol was added to the second part and the sample was extracted, filtered and removed by an evaporator. After the extracts were combined, they were filtered through filter paper and the filtrates were frozen. The frozen extracts were lyophilized under 50 mm Hg until it dry in a lyophilizer. The extracts were incubated at 4°C until use.

Animals

Forty-nine adult male Sprague-Dawley rats (weighing 230-270 g) obtained from the Medical Experimental Application and Research Center, Atatürk University, were used. The animals were housed inside polycarbonate cages in an air-conditioned room (22°C±2°C) with a 12-h light/dark cycle. Standard rat feed and water were provided *at libitum*. The rats were allowed to acclimatize to the laboratory environment for 7 days before the start of the experiment. Liver damage was induced by i.p. injection of 30% CCI_4 diluted with soybean oil at a dose of 1 mL per 100 g of body weight twice a week for eight weeks. At the end of the experiment, all animals were weighed and killed. All procedures were performed in conformity with the Institutional Ethical Committee for Animal Care and Use at Atatürk University (protocol number: 36643897-188/13) and the Guide for the Care and Use of Laboratory Animals.

Measuring the body weight

The body weight of the animals was weighed to examine the effects of *M. nigra* on weight change of CCl_4 intoxicated rats.

Experimental design

The rats were randomly divided into seven groups, each containing seven rats. Group 1 (C), the control rats, were intraperitoneally injected with physiological saline solution; Group 2 (SOC) rats were i.p. injected with soybean oil without CCl_4 (1 mg/kg body weight (bw), twice a week for eight weeks); Group 3 (MN-150) or nonhepatotoxic rats were treated with *M. nigra* (MN) at a dose of 150 mg/kg bw; Group 4 (MN-300) or nonhepatotoxic rats were treated with 300 mg/kg bw of *M. nigra*, Group 5 (CCI_4) rats were i.p. injected with 30% CCl_4 mixed with soybean oil (1 mL/kg bw); Group 6 (CCI_4+MN-150) or hepatotoxic rats were treated with 150 mg/kg bw of *M. nigra*; Group 7 (CCI_4+ MN-300) or hepatotoxic rats were treated with 300 mg/kg bw of *M. nigra*. *M. nigra* extracts were administered i.p. daily for eight weeks.

At the end of the 8th week, the rats were anesthetized with ether. Blood samples were collected from the interior vena cava, centrifuged at 3000 rpm for 30 min at 4°C and the serum was kept at -70°C for liver function tests. The liver tissues were immediately removed, washed in ice-cold saline, dried on filter paper and weighed. The tissue specimens were taken from

the right lobe of the liver of each rat, fixed in 10% phosphate buffered formaldehyde, routinely processed and blocked into paraffin for detecting the collagen content by biochemical methods and image analysis, while others were snap-frozen in liquid nitrogen and stored at -70°C.

Histopathological examinations and assessments

Liver sections were fixed in 10% formalin and embedded in paraffin to form blocks. The samples were then serially-sectioned (5-µm thick) using a Leica RM2135 microtome (Leica, Berlin, Germany), mounted on glass slides and stained using H&E solution. Pathological changes were assessed and photographed with a light microscope (Nikon Eclipse E600, Olympus) at 10 and 20 magnifications and photographed.

Immunohistochemical examination

Immunohistochemical staining for caspase-3 and 8-OHdG proteins was performed by an automated method on the VENTANA BenchMark GX System (Ventana Medical Systems) with an Ultra View Universal DAB Detection Kit, on 4-µ-thick sections from a representative block in each rat. After deparaffinization, the antigenic determinant sites for caspase-3 and 8-OHdG were unmasked in citrate buffer with steam for 60 min. The primary antibody caspase-3 (Abcam-Ab4051) was used at a dilution of 1:1000 for 32 min at 37°C, and 8-OHdG (Santa Cruz sc-393871) was used at a dilution of 1:300 for 32 min at 37°C. The slides were then incubated with the diluted antibody, followed by application of the Ultraview Universal DAB detection kit (Ventana Medical Systems). DAB was used as a chromogen and hematoxylin as a counterstain. The specificity of staining was confirmed by the inclusion of negative control slides processed in the absence of primary antibody on tissue from the same animal.

Biochemical analyses

Estimation of serum biochemical parameters

Blood samples from animals were collected in gel-activated tubes for the assessment of specific liver markers. The gel-activated tubes were allowed to clot, and were then centrifuged at 4000 × g for 10 min at 4°C. The serum samples were collected for measuring liver markers, ALT, AST, GGT and total bilirubin (T-BIL) using commercial kits, on a Beckman Coulter AU5811 device (Japan).

Determination of GPx activity

The tissue was homogenized in 5-10 mL of cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM dithiothreitol) per tissue. Then, it was centrifuged at 10000 × g for 15 min at 4°C. The supernatant was removed after centrifugation. The serum was removed. GPx activity was measured in liver tissue. GPx activity was determined using a GPx assay kit (Cayman Chemical Co., Cat No. 703102 Ann Arbor, MI, USA) in an ELISA microplate reader (Bio-Tek PowerWave XS, USA). GPx activity was measured indirectly by a coupled reaction with glutathione reductase. The oxidized glutathione was produced upon reduction of hydroperoxide by GPx. The results were expressed as units per mg protein (U/mg) of liver tissue. The dynamic range of the assay was only limited by the accuracy of the absorbance measurement.

Determination of SOD activity

The tissue was homogenized at 16000 rpm on ice in HEPES buffer (20 mM, pH 7.2). The mixture was centrifuged at 1500 × g for 5 min at 4°C. The serum was diluted in the ratio 1:5 with sample buffer. SOD activity was measured in the supernatant using an assay kit (Cayman Chemical Co., Cat No.706002 Ann Arbor, MI, USA) in an ELISA microplate reader (Bio-Tek PowerWave XS, USA). One unit of SOD activity is defined as the amount of enzyme required to produce 50% dismutation of the superoxide radical. SOD activity was measured using the linear regression equation from the standard curve. The results were expressed as units per mg protein (U/mg) of liver tissue.

Statistical analysis

Data recording and analysis were performed on SPSS 20.0 for Windows (SPSS Inc., IL, USA) software. Descriptive data were expressed as the means±standard deviation. Compatibility with normal distribution of SOD and GPx levels and serum AST, ALT, GGT and T-BIL results were assessed using the Kolmogorov-

Smirnov test. Since all results were normally distributed, their comparisons in groups were performed using parametric one-way ANOVA, while the degree of significance of differences between the groups was determined using the post hoc least significant difference (LSD) test. Correlations between results were assessed using Pearson's correlation analysis. P<0.05 was regarded as significant.

RESULTS

Effect of the experimental treatments on body weight

The body weight of each rat was recorded every day during the experiment, and changes were noted for all groups. Administration of CCl_4 and treatment of the CCl_4-intoxicated group with *M. nigra* did not alter the weights of the rats (Table 1).

Table 1. The effect of *M. nigra* on body weight after treatment with CCL_4.

Treatment	Body weights (g)		
	Initial day	Sacrifice day	Difference (Δ, %)
Control	231±22	373±22	142 g, 61%
SOC	238± 17	389±28	151 g, 63%
MN-150	242±19	386±32	144 g, 59%
MN-300	249±25	396±35	147 g, 59%
CCI_4	269±21 ^{a1,b1}	355±12 ^{b1}	86 g, 31%
CCI_4**+MN-150**	270±26 ^{a1,b1}	361±15 ^{b1}	91 g, 33%
CCI_4**+MN-300**	271±24 ^{a1,b1}	366±17	95 g, 35%

Result expressed as means±SD (n=7).
^{a1} – significant differences with respect to the control at p<0.05, ^{b1} – significant differences with respect to the SOC group at p<0.05.

Effect of *M. nigra* on the biochemical parameters of liver functioning

Biochemical measurement of liver functions revealed that the treatment with CCl_4 induced a significant increase in the serum levels of AST, ALT, GGT and T-BIL (p<0.001 for all) compared with control rats. However, *M. nigra* (150 and 300 mg/kg) caused significant dose-dependent reductions in the CCl_4-induced elevation of ALT, AST and GGT activities and T-BIL concentration (p<0.001). Recovery in the group treated with the higher dose of the plant extract was more pronounced (Table 2).

Oxidative stress markers and antioxidant enzymes

To determine changes in the response to oxidative stress, we examined the activities of SOD and GPx in liver homogenates (Fig. 1). Treatment of animals with *M. nigra* alone significantly increased the activities of GPx and SOD when compared to the control group (p<0.001 for both treatment doses). However, SOD (p<0.001) and GPx (p=0.003) activities were higher in the high-dose *M. nigra* group than in the low-dose *M. nigra* group. CCl_4 rats showed lower concentrations of GPx and SOD in liver homogenates compared to the control group (p<0.0001). Administration of low and high doses of *M. nigra* extract to the CCl_4-treated animals significantly increased the activities of GPx and SOD as compared to the CCl_4 group (Table 3).

Table 2. Effect of *M. nigra* on serum biochemical parameters after treatment with CCL_4.

Treatment	AST U/L	ALT U/L	GGT U/L	T-BIL mg/dL
Control	44.64± 0.86	15.96 ± 0.34	6.33±0.36	2.7±0.24
SOC	41.36± 0.57^{a3}	14.03± 0.45^{a3}	5.4±0.56^{a2}	2.63±0.32^{a3}
MN-150	39.27± 0.55^{a3}	13.25± 0.51	5.21±0.31^{a2}	2.51±0.21^{a3}
MN-300	38.06± 0.34^{a3}	11.07± 0.59^{a3}	5.07±0.71^{a3}	2.55±0.13^{a3}
CCI_4	138.48±0.43^{a3,b3,c3,d3}	55.38±0.86^{a3,b3,c3,d3}	40.23±0.56^{a3,b3,c3,d3}	4.83±0.45^{a3,b3,c3,d3}
CCI_4+MN-150	65.2±0.46^{a3,b3,c3,d3,e3}	20.11±0.74^{a3,b3,c3,d3,e3}	32.51±0.76^{a3,b3,c3,d3,e3}	3.77±0.39^{a3,b3,c3,d3,e3}
CCI_4+MN-300	57.61±0.37^{a3,b3,c3,d3,e3,f3}	18.26±0.39^{a3,b3,c3,d3,e3,f3}	27.26±0.95^{a3,b3,c3,d3,e3,f3}	3,3±0.19^{a3,b3,c3,d3,e3,f3}

Result are expressed as means±SD (n=7).
^{a2} and ^{a3} – significant differences with respect to the control at p<0.01 and p<0.001.
^{b3} – significant differences with respect to the SOC group at p<0.001. ^{c3} – significant differences with respect to the MN-150 group at p<0.001.
^{d3} – significant differences with respect to the MN-300 group at p<0.001. ^{e3} – significant differences with respect to the cirrhotic group at p<0.001.
^{f3} – significant differences with respect to the cirrhotic group+MN-150 group at p<0.001

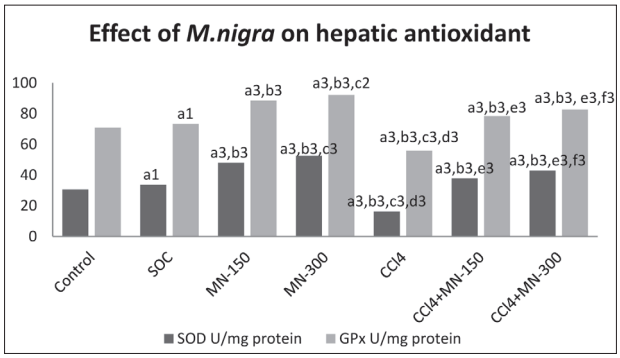

Fig 1. Effect of *M .nigra* extract on the hepatic antioxidant profile in the rat. Results of the study are expressed as means±SD (n=7). [a1], [a2] and [a3] – significant differences with respect to the control at p<0.05, p<0.01 and p<0.001, respectively. [b3] – significant differences with respect to the SOC group at p<0.001. [c2] and [c3] – significant differences with respect to the MN-150 group at p<0.01 and p<0.001, respectively. [d3] – significant differences with respect to the MN-300 group at p<0.001. [e3] – significant differences with respect to the CCI_4-treated group at p<0.001. [f3] – significant differences with respect to the CCI_4+MN-150 group at p<0.001.

Pathological changes of the liver tissues

H&E staining revealed that hepatocytes were arranged in cords projecting from the central veins to the portal area, with intact hepatic lobules in the normal control group (Fig. 2A). The histological appearance of the soybean oil-administered group and rats treated with both doses of *M. nigra* extracts were similar to the control group (data not shown). In the CCl_4 group, the lobular architecture of liver tissue was deformed, presenting evidence of extensive pericentral vein necrosis and fatty changes with ballooning of hepatocytes, vacuolization and increased numbers of Kupffer cells (Fig. 2B). Histopathological examination of liver tissue showed that the damaged or impaired liver was repaired by the treatment with *M. nigra* extracts in a dose-dependent manner; however, there was vacuolization at the high dose of *M. nigra* (Fig. 2C and 2D).

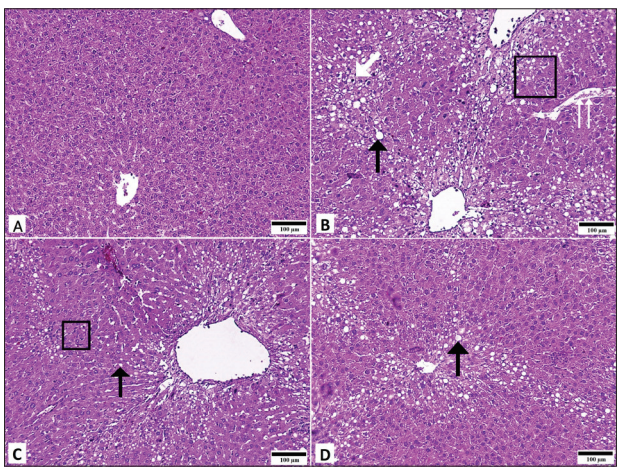

Fig. 2. Representative photographs from the liver showing the protective effect of *M. nigra* on CCI_4-induced hepatic injury in rats. **A** – Control. **B** – CCI_4; note the macrovesicular steatosis (black arrow), microvesicular steatosis (white arrows), pericentral vein necrosis (double arrow), Kupffer cells (inside the delineated square). **C** – CCI_4+MN-150; note the macrovesicular steatosis (black arrow), Kupffer cells (inside the delineated square). **D** – CCI_4+MN-300; note the macrovesicular steatosis (black arrow). Original magnification was ×10.

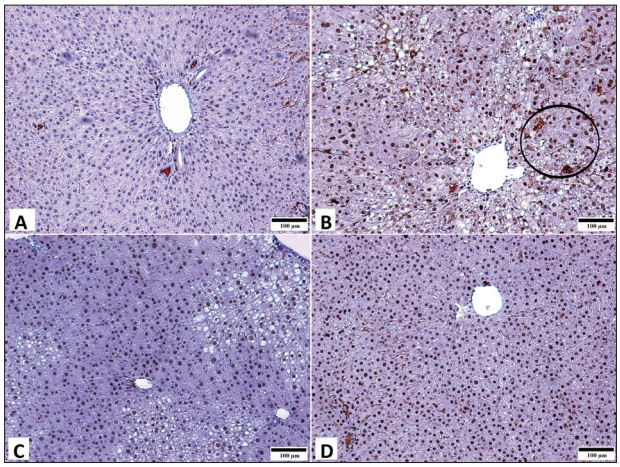

Fig. 3. Caspase-3 immunohistochemical staining in rat liver tissue. The immunohistochemical localization of caspase-3 appears as a brown staining. **A** – Control. **B** – CCI_4; the caspase-3-positive area is delineated by the circle. **C** – CCI_4+MN-150. **D** – CCI_4+MN-300. Original magnification was ×10.

Immunohistochemical staining of caspase-3 and 8-OHdG

Representative images from one of seven different tissue samples are shown (Fig. 3). Caspase-3 activation, a marker of the apoptotic protease cascade, was measured by immunohistochemistry. Cells expressing activated caspases were labeled using immunohistochemistry on thin sections of tissue taken from the control, CCI_4, CCI_4+MN-150 and CCI_4+MN-300 groups. Controls showed very poor or slight expression of caspase-3 (Fig. 3A), the CCI_4 treatment group showed a significant increase in hepatic caspase-3 activity (Fig. 3B), CCI_4-administered rats after treatment with MN-150 (Fig. 3C) and MN-300 (Fig. 3D) showed reduced activation of caspase-3.

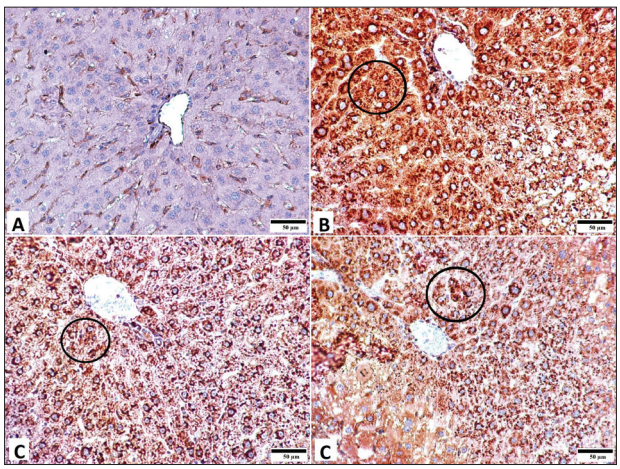

Fig. 4. 8-OHdG immunohistochemical staining in rat liver tissue. The immunohistochemical localization of 8-OHdG appears as brown staining **A** – Control. **B** – CCI_4; the 8-OHdG-positive area is inside the circle. **C** – CCI_4+MN-150; the 8-OHdG-positive area is delineated by the circle. (**D**) **D** – CCI_4+MN-300; the 8-OHdG-positive area is delineated by the circle. Original magnification was ×20.

Representative images from one of seven different tissue samples are shown in Fig. 4. 8-OHdG is a ROS-induced DNA damage marker. Expression of 8-OHdG was significantly higher in administered rats (Fig. 4B) than in CCI_4-untreated rats (Fig. 4A). 8-OHdG staining was much denser and mainly detected in CCI_4-treated liver cells (Fig. 4B). Positive 8-OHdG staining was decreased in the hepatocytes of both CCI_4+MN-150 (Fig. 4C) and CCI_4+MN-300 (Fig. 4D) groups when compared to the CCI_4-treated group. In CCI_4+MN-150 we observed a milder decrease in 8-OHdG accumulation when compared to CCI_4+MN-300.

DISCUSSION

The liver plays a vital role in detoxification, and it is capable of regenerating most of its cells when they become damaged. However, if injury to the liver is too severe or long lasting, regeneration is incomplete, and the liver creates scar tissue, which blocks the portal flow of blood through the organ, disturbing normal hepatic functioning [15]. Scarring of the liver, also called fibrosis, may lead to cirrhosis [16]. Cirrhosis resulted in 1.2 million deaths in 2013, up from 0.8 million deaths in 1990 [17]. Cirrhosis affects the immune system, making people with cirrhosis more likely to develop an infection than healthy individuals. CCl_4 metabolism in the liver results in the stimulation of lipid peroxidation and the production of free radicals, which cause necrosis of hepatocytes, induce inflammation and further promote the progression of hepatic fibrogenesis [16]. Numerous ethnomedicinal studies have shown that the use of *M. nigra* by indigenous communities against a wide range of ailments is normal practice throughout the world. Almost all the parts of the tree are used for pharmacological actions. Experimental studies have demonstrated antiinflammatory, analgesic, hypoglycemic, laxative and antidiarrheal effects of black mulberry extract [13,14,18]. The aim of the current study was to determine whether *M. nigra* could improve pathological changes in the liver induced by CCl_4. The marked release of AST, ALT and ALP into the circulation indicates severe damage to hepatic tissue during CCl_4 intoxication [19]. Administration of *M. nigra* appeared to significantly reduce the serum levels of AST, ALT, GGT and T-BIL in our CCl_4-induced rat model of hepatic damage. The reduction of the levels of these enzymes indicated that *M. nigra* prevented protein loss. Lipid peroxidation and necrosis are significantly suppressed in the livers of animals supplemented with antioxidants, such as flavonoids, silymarin or vitamin E [20]. It has been reported that *M. nigra* is a plant rich in fatty acids, such as linoleic, stearic and oleic acids, free acids (mainly malic acid) and other molecules that possess antioxidant, antiinflammatory and analgesic effects [12,18], and is an appreciable source of flavonoids, which are considered to have possible protective effects on human health [21]. Blackberry consumption may exert beneficial effects on apolipoproteins, blood pressure and inflammatory markers in individuals with lipid disorders [22]. *M. nigra* fruits are taken internally for treating colds, influenza, eye infections and nosebleeds. Previous studies have indicated that the aqueous methanolic extract of *M. nigra* has hepatoprotective activity against paracetamol-induced liver injury in mice [23]. In addition, *M. nigra* leaf extract administration has been shown to protect liver tissue against the hepatotoxic effect of the antirheumatic drug methotrexate [24]. In recent studies, the extract of *M. nigr*a leaf was shown to be capable of reducing oxidative stress and complications in the kidneys and liver of diabetic rats [25,26]. Our results are in accordance with previous studies that reported the hepatoprotective effect of *M. nigra* leaf extract.

As a biomarker of oxidative damage to DNA, 8-OHdG is related to a number of disorders [27]. 8-OHdG is widely used as a marker for oxidative stress [28]. We investigated the role of caspase-3, which is closely related to cell apoptosis. Experimental results showed that the expression of caspase-3 was significantly higher in CCl_4-treated rats than in normal rats, and the administration of *M. nigra* extract inhibited the expression of caspase-3. Well-known macro- and microscopic lesions associated with fibrosis and cirrhosis were confirmed in rats that were intraperitoneally injected with CCl_4 [29]. *M. nigra* exerted a hepatoprotective effect, observed as increased antioxidant capacity, reduced 8-OHdG and apoptosis prevention.

In our study, the liver was examined histopathologically. CCl_4 treatment was characterized by apoptosis induction, sinusoidal dilatations, congestion, necrosis and fatty changes, with ballooning of hepatocytes and infiltration of Kupffer cells. Previous studies have suggested that CCl_4 induces hepatocellular damage, hepatic fibrosis and apoptosis, primarily by interfering with endothelial functioning [29,30], and endothelial cell injury can be caused by environmental stresses, manifesting as oxidative stress [31]. However, endothelial cell dysfunction causes diminished release of reactive oxygen species (ROS). High concentrations of ROS can lead to endothelial cell apoptosis or sudden death of endothelial cells [32]. Our histological results showed that *M. nigra* inhibited the occurrence and progression of hepatocellular damage. According to our results, liver damage could be prevented by the antioxidant activity of *M. Nigra*.

In conclusion, our study showed that apoptosis was reduced, and histopathological improvement was achieved with *M. nigra* treatment in the CCl_4-induced hepatotoxic rat model. The concentration of 300 mg/kg was found to be more effective than 150 mg/kg. The hepatoprotective effect of *M. nigra* extract on the injurious effects of CCl_4 is probably due to the inhibition of DNA damage and stimulation of antioxidant enzyme activities. The results of this study support future exploration of *M. nigra* as a novel preventive and therapeutic agent for the treatment of oxidative stress-induced liver injury.

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