Protective effect of hesperidin on oxidative and histological liver damage following carbon tetrachloride administration in Wistar rats

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Abstract

Introduction: In the current study, the protective effect of hesperidin (HP) on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats was investigated.
Material and methods: Twenty-eight rats were divided equally into four groups. The first group was kept as a control and given only vehicle. In the second, rats were orally administered 50 mg/kg/day HP for 10 days. Carbon tetrachloride was given in a single intraperitoneal injection at the dose of 2 ml/kg in the third group. In the fourth group, the rats were treated with equal doses of CCl_4 and HP.
Results: It was found that CCl_4 induced oxidative stress via a significant increase in the formation of thiobarbituric acid-reactive substances (TBARS) and caused a significant decline in the levels of glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in rats. In contrast, HP blocked these toxic effects induced by CCl_4, causing an increase in GSH, CAT and SOD levels and decreased formation of TBARS ($p < 0.01$). In addition, histopathological damage increased with CCl_4 treatment. In contrast, HP treatment eliminated the effects of CCl_4 and stimulated anti-apoptotic events, as characterized by reduced caspase-3 activation.
Conclusions: The current study demonstrated that CCl_4-induced hepatotoxicity can be prevented with HP treatment. Thus, co-administration of HP with CCl_4 may be useful for attenuating the negative effects of CCl_4 on the liver.

Key words: liver, hesperidin, carbon tetrachloride, hepatotoxicity.

Introduction

Carbon tetrachloride (CCl_4) is a potent hepatotoxic chemical that produces free radicals and is widely used to induce acute hepatic injury in experimental animal models [1]. Carbon tetrachloride-induced hepatic necrosis is caused by bioactivation of the microsomal cytochrome P450-dependent monooxygenase system, resulting in the formation of a trichloromethyl radical (CCl_3) and reactive oxygen species (ROS) [2]. Reactive oxygen species consist of free radicals or oxygen free-radical-generating agents, such as a superoxide anion (O_2^-), an hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) [3]. Metabolic processes are usually associated with the generation of free radicals, particularly oxy-

gen-derived radicals that oxidize and damage surrounding biomolecules [4]. The consequences of CCl_4-induced lipid peroxidation include membrane disintegration, loss of membrane-associated enzymes [5, 6] and necrosis.

Hesperidin (HP) is a bioflavonoid that plays a role in plant defense and is abundant in citrus species, such as grapefruit, lemon and orange. Hesperidin is used effectively as a supplemental agent in complementary therapy protocols, since it possesses biological and pharmacological properties as an effective antioxidant, anti-inflammatory, anti-carcinogenic, and anti-hypertensive agent with lipid-lowering activity [7–9]. The antioxidant properties of HP protect testicular function from cadmium toxicity, and HP regulates hepatic cholesterol synthesis by inhibiting the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase [10–12].

Hepatotoxicants, including CCl_4, lead to oxidative stress and histological damage in the liver. Therefore, antioxidant agents such as HP may prevent CCl_4-induced hepatotoxicity. In this study, we examined the biochemical and histological effects of HP on CCl_4-induced toxicity.

Material and methods

Chemicals

Hesperidin was obtained from Sigma Chemical Co. (St. Louis, MO). Carbon tetrachloride was given by İnonu University chemistry laboratory as a gift. All other chemicals for biochemical and histological analysis were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and treatment

A total of 28 healthy adult male Wistar albino rats (2–3 months of age, 250–300 g) were obtained from the Experimental Animal Research Institute (Malatya, Turkey). Animals were housed in sterilized polypropylene rat cages, under a 12/12-h light/dark cycle, at an ambient temperature of 21°C. Food and water were provided ad libitum. Experiments were performed in accordance with the animal ethics guidelines of the Institutional Animal Ethics Committee.

Rats were randomly divided into four equal groups: control, CCl_4, HP, CCl_4 + HP (n = 7 per group). Carbon tetrachloride was diluted 1 : 1 with corn oil and administered in a single intraperitoneal (*i.p.*) dose of 2 ml/kg. Hesperidin was dissolved in 1% carboxymethyl cellulose (CMC) and administered orally at a dose of 50 mg/kg for 10 consecutive days. In the control group, rats were treated with the corn oil and 1% CMC vehicle. In the CCl_4 group, CCl_4 was administered in a single injection on day 2. Rats in the HP group were treated with HP for 10 days, and those in the CCl_4 + HP group were treated with CCl_4 and HP together. Tissue samples were collected on day 10 after the first HP treatment. The animals were euthanized under ether anesthesia, and tissue samples were removed immediately, dissected on ice-cold glass, and stored at –86°C until analysis.

Histological examination

For light microscopic evaluation, liver samples were fixed in 10% formalin and embedded in paraffin. The specimens were cut into 5-µm thick sections, mounted on slides and stained with hematoxylin and eosin (H + E). Tissue samples were examined using a Leica DFC280 light microscope and the Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

For immunohistochemical analysis, thick sections were mounted on polylysine-coated slides. After rehydrating, samples were transferred to citrate buffer (pH 7.6) and heated in a microwave oven for 20 min. After cooling for 20 min at room temperature, the sections were washed with phosphate-buffered saline (PBS). Then sections were kept in 0.3% H_2O_2 for 7 min and afterward washed with PBS. Sections were incubated with primary rabbit-polyclonal caspase-3 antibody (Abcam, Ab4051) for 2 h. They then were rinsed in PBS and incubated with biotinylated goat antipolyvalent for 10 min and streptavidin peroxidase for 10 min at room temperature. Staining was completed with chromogen + substrate for 15 min, and slides were counterstained with Mayer's hematoxylin for 1 min, rinsed in tap water, and dehydrated. The caspase-3 kit was used according to the manufacturer's instructions.

Biochemical assay

The levels of homogenized tissue TBARS, as an index of lipid peroxidation, were determined by thiobarbituric acid reaction using the method of Yagi [13]. The product was evaluated spectrophotometrically at 532 nm and results are expressed as nmol/g tissue. The glutathione (GSH) content of the liver homogenate was measured at 412 nm using the method of Sedlak and Lindsay [14]. The GSH level was expressed as nmol/ml. Superoxide dismutase (SOD) activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O_2^- generated by the xanthine/xanthine oxidase system [15]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The product was evaluated spectrophotometrically at 560 nm. Results are expressed as IU/mg protein. Catalase (CAT) activity of tissues was determined according to the method of Aebi [16]. The enzymatic decom-

position of H_2O_2 was followed directly by a decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity. Tissue protein content was determined according to the method developed by Lowry *et al.* [17] using bovine serum albumin as standard.

Statistical analysis

All values are presented as mean ± SD. Differences were considered to be significant at $p < 0.01$ for biochemical changes. The computer program SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For biochemical values, statistical analyses were performed using one-way ANOVA and post hoc Tukey's honestly significant difference test. For histological evaluation, the microscopic score of each tissue was calculated as the sum of the scores given for each criterion. Scores were given as absent (0), slight (1), moderate (2), and severe (3) for each criterion. Statistical analysis was performed with SPSS 13 and MedCalc programs. All groups were compared by the nonparametric Kruskal-Wallis test. Exact *p*-values were given where available, and $p < 0.0001$ was accepted as statistically significant. All results are expressed as means ± standard error (SE).

Results

Histological evaluation

All figures demonstrate the histological changes in the livers of rats of each group. In the control (Figure 1 A) and HP (Figure 1 B) groups, we observed normal liver architecture and hepatocytes with well-preserved cytoplasms and nuclei. In the CCl_4 (Figure 2) and CCl_4 + HP (Figures 3 A, B) groups, we observed distortion of the hepatic cords, hepatocellular necrosis, hemorrhage (Figures 2 A, C), mononuclear cell infiltration (Figures 2 B, D), vascular congestion (Figures 2 D), eosinophilic and pyknotic nuclei hepatocytes (Figures 2 C, E), as well as vacuolated hepatocytes (Figure 2 F), which were not as extensive as in the CCl_4 group, indicating an improved histological appearance in the liver tissue. The microscopic damage score for each group was determined in the histological section, and the results are given in Table I.

Caspase-3-stained cells were not observed in the control (Figure 4 A) or HP (Figure 4 B) groups but were abundant in the CCl_4 group (Figure 4 C). The density of caspase-3-positive cells was decreased in the CCl_4 + HP group (Figure 4 D).

Biochemical evaluation

Carbon tetrachloride administration led to a significant increase in thiobarbituric acid-reactive substance (TBARS) levels compared with the other groups. Moreover, HP treatment caused a significant decrease in elevated TBARS levels when administered together with CCl_4, compared with the CCl_4 group (Table II). Glutathione, CAT and SOD levels were decreased significantly by CCl_4 treatment compared with the other experimental groups, and these parameters were elevated significantly by HP treatment when compared with the CCl_4 group (Table II). There were no significant differences between the control and HP groups, except for the CAT values, which were decreased significantly by HP treatment compared with the other groups.

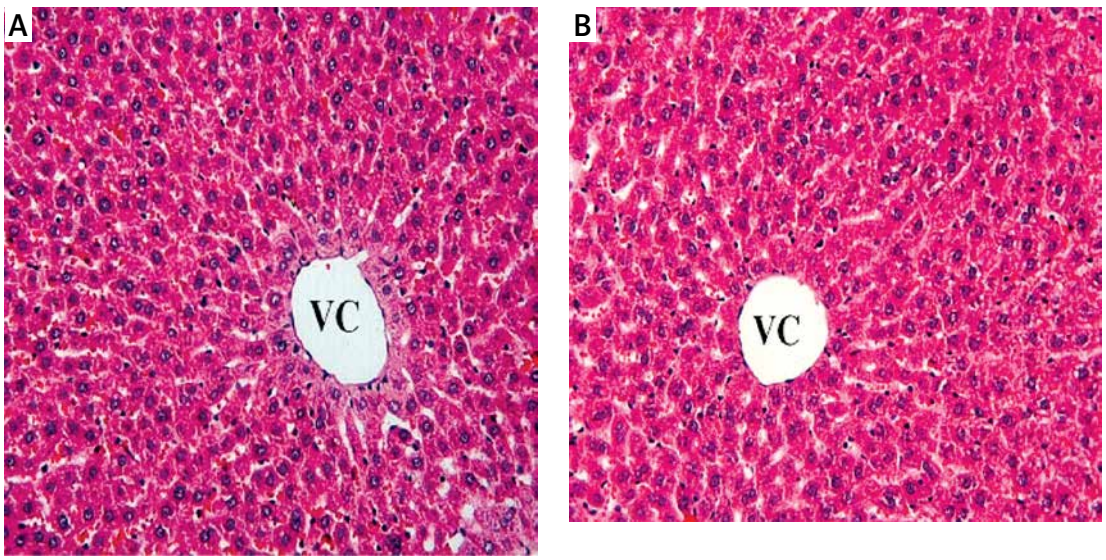

Figure 1. In the liver, a normal histological appearance was observed following hematoxylin and eosin staining of the (**A**) control and (**B**) hesperidin (HP) groups

VC – vena centralis; 20×.

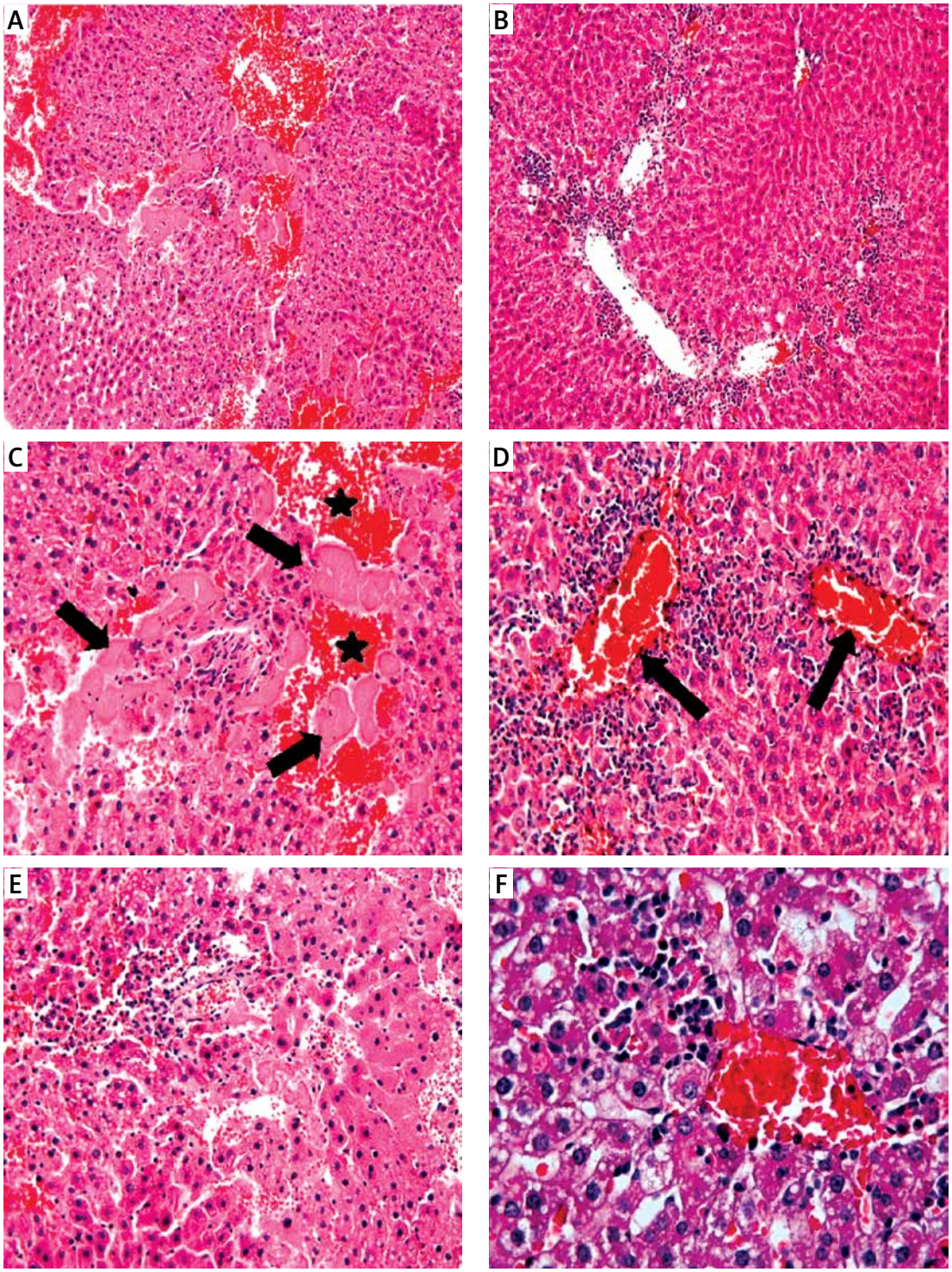

Figure 2. In the CCl_4 group, we observed (**A**) distortion of the hepatocyte radial arrangement, hemorrhage and necrosis, (**B**) cell infiltration, (**C**) necrosis and hemorrhage, (**D**) vascular congestion and infiltration, (**E**) eosinophilic and pyknotic nuclei, and (**F**) vacuolization and congestion (A, B: H + E; 10×, C, D, E: H + E; 20×, F: H + E; 40×)

Discussion

Carbon tetrachloride is a well-established hepatotoxic agent that causes severe liver damage and produces liver fibrosis and biochemical patterns that resemble human liver cirrhosis. The present study was designed to establish the protective effects of HP, a citrus bioflavonoid, on CCl_4-induced liver damage. The results demonstrated that HP ameliorated biochemical and histological evidence of CCl_4-induced liver damage.

Oxidative stress is caused by an imbalance between free radicals, such as TBARS, and the

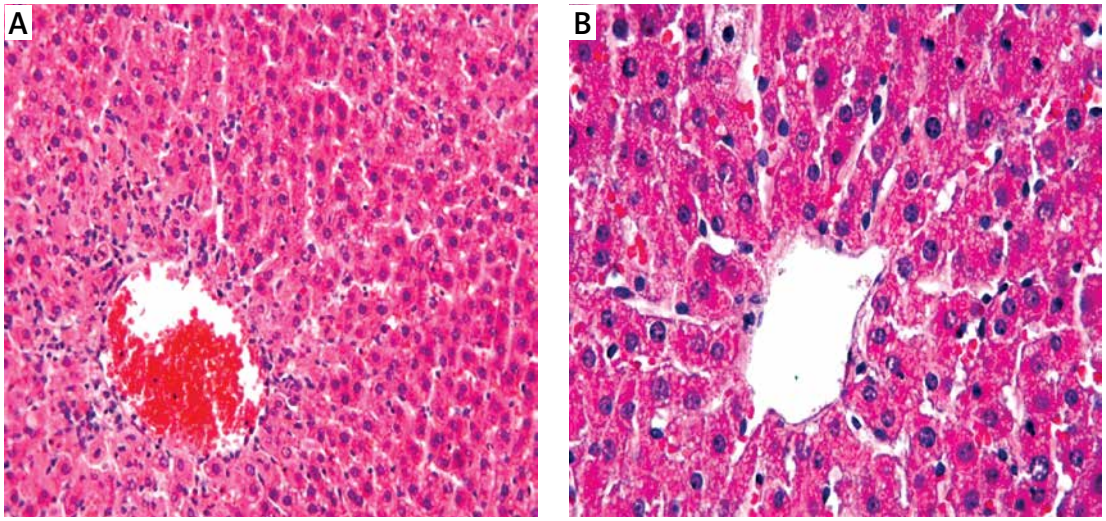

Figure 3. Histological findings were decreased in the CCl_4 + hesperidin (HP) group (**A**: H + E; 20×, **B**: H + E; 40×)

Table I. Comparison of the effect of HP on microscopic damage caused by CCl_4 in liver

Groups	Microscopic damage (mean ± SD)
1 Control	0.39 ±0.49[a]
2 CCl_4	2.13 ±0.74[b]
3 HP	0.70 ±1.29[a]
4 CCl_4 + HP	1.64 ±0.70[c]

The differences between the mean values bearing different superscript letters within the same column are statistically significant ($p \leq 0.0001$). SE – standard deviation.

activity of the antioxidant defense system, including SOD, CAT, and GSH levels, which leads to lipid peroxidation and enzymatic inactivation [18]. TBARS are the final metabolites of peroxidized polyunsaturated fatty acids and are considered a late biomarker of oxidative stress [19]. Carbon tetrachloride treatment in rats markedly changed antioxidant enzyme activities, which was prevented by the co-administration of rutin, supporting a role for oxidative stress in CCl_4-induced liver damage [20].

The liver contains many drug metabolizing enzymes that metabolize toxic chemicals in the liver. Carbon tetrachloride is metabolized by a cytochrome P450 enzyme to produce highly toxic CCl_3 and CCl_3O_2 free radicals that damage hepatocytes [21–24]. Both CCl_3 and CCl_3O_2 bind to proteins or lipids and extract a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage. Therefore, increased TBARS in CCl_4-treated rats may result from enhanced membrane lipid peroxidation by free radicals and the failure of antioxidant defense mechanisms that prevent formation of excessive free radicals [25, 26]. Similarly, we found that CCl_4 significantly induced oxidative damage, increased TBARS levels, and decreased GSH levels and the activities of antioxidant enzymes, including SOD and CAT, in the liver. Another study showed that the balance between ROS production and antioxidant defenses mediates oxidative stress during CCl_4-induced hepatotoxicity. In addition, decreased SOD and CAT activities in the livers of CCl_4-treated rats may be due to free radicals generated by CCl_4 or inactivation of the antioxidant enzymes [27]. Another study demonstrated that administration of CCl_4 to rats caused oxidative stress in the liver and was associated with significantly lower antioxidant activities of GSH, CAT and SOD. Therefore, the available literature confirms our results [28–30].

Our study further demonstrated that HP treatment reversed the oxidative effects of CCl_4 via a significant reduction in elevated TBARS levels and induction of the antioxidant defense system. Only one other study has described the effects of HP against CCl_4 toxicity, but that study did not address any histological changes [1]. That group concluded that HP could prevent CCl_4 toxicity, which is in agreement with our results. There are a few studies describing the protective effects of HP on general liver injury [31, 32]. For example, Bentli *et al.* [33] determined that HP protected the liver against dioxin toxicity and claimed that it can be used to prevent liver injury. In addition to those findings, Chen *et al.* determined that HP reduced indicators of oxidative stress, such as ROS and lipid peroxidation, in a dose-dependent manner [34]. Heffner and Repine [35] suggested that HP offers protection by terminating lipid peroxidation side chains rather than scavenging extracellular non-lipid radicals that initiate lipid peroxidation. This supports our conclusion that HP protects liver tissue against many toxic agents, such as CCl_4, and these effects may be due to HP's antioxidant and radical scavenging properties.

Upon histological evaluation, we determined that CCl_4 treatment caused severe histological

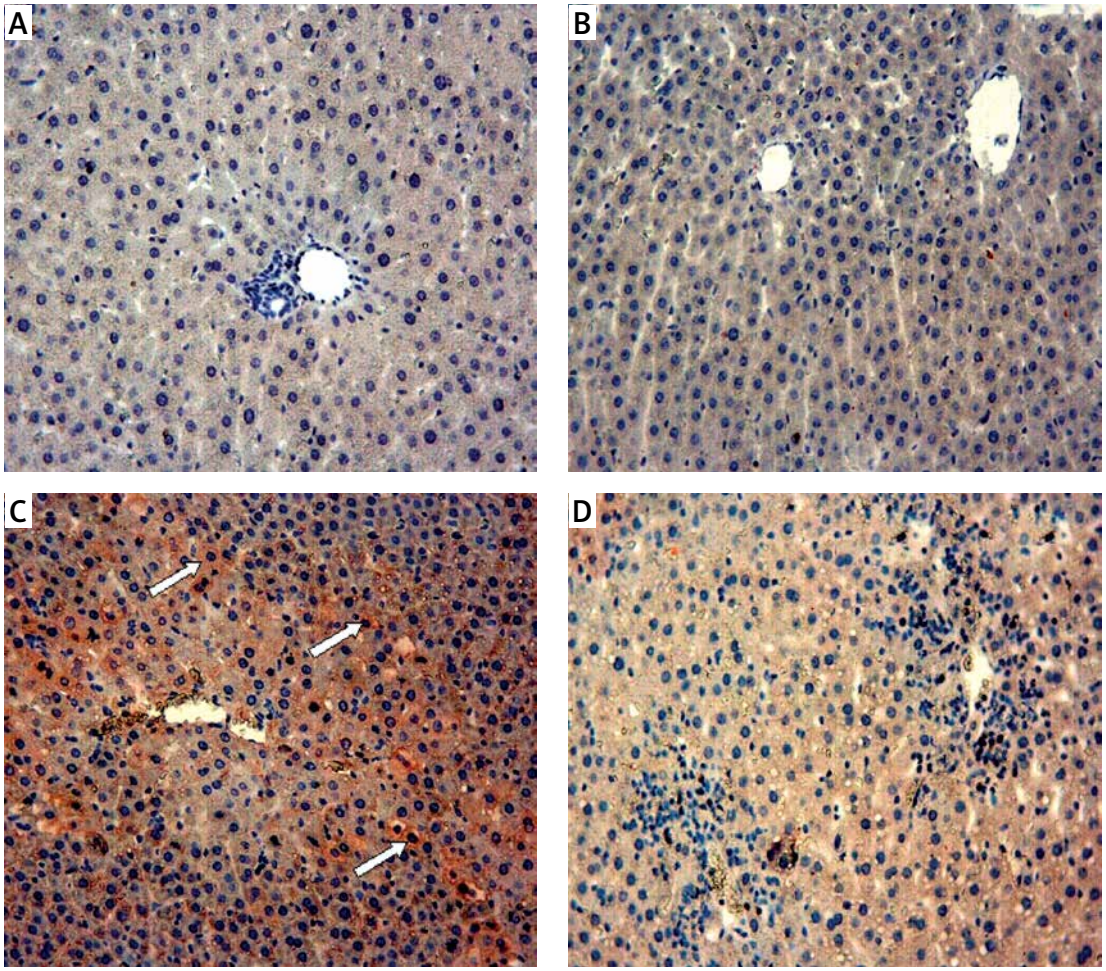

Figure 4. Immunohistochemical expression of caspase-3 in the (**A**) control, (**B**) hesperidin (HP), (**C**) CCl_4 and (**D**) CCl_4 + HP groups. The number of positively stained cells decreased in the CCl_4 + HP group. Positively stained caspase-3 cells are indicated by the arrows; 20×

Table II. Levels of SOD, CAT, GSH and TBARS in liver tissue (mean ± SD)

Group	TBARS [nmol/g tissue]	Reduced GSH [nmol/ml]	CAT [kU/mg protein]	SOD [U/mg protein]
Control	7.54 ±0.39[a]	181.6 ±22.9[a]	0.93 ±0.11[a]	15.2 ±2.25[a]
CCl_4	11.9 ±0.87[b]	112.3 ±14.1[b]	0.42 ±0.09[b]	9.41 ±1.04[b]
HP	8.08 ±1.40[a]	197.1 ±36.5[c]	0.93 ±0.09[a]	16.7 ±1.90[ac]
CCl_4 + HP	10.1 ±0.93[c]	158.5 ±17.5[a]	0.75 ±0.07[c]	14.2 ±2.27[a]

Means bearing different superscripts within same column are significantly different ($p < 0.01$).

damage including distortion of hepatic cords, necrosis, vascular congestion, vacuolated hepatocytes, hepatocellular necrosis, eosinophilic and pyknotic nuclei, as well as mononuclear cell infiltration in liver tissues of rats. We also found a significantly larger number of caspase-3-stained cells, which were indicative of liver apoptosis, in the CCl_4 group compared with the HP + CCl_4 group. This demonstrates that HP protected the liver against cell death. Ebaid *et al.* [36] reported that an increased number of mitotic figures, vacuolated hepatocytes, eosinophilic hepatocytes and collagen deposition were observed in the histological sections of the CCl_4-challenged group. In addition to those findings, Cui *et al.* [37] reported that in CCl_4-injured mice, the cytoplasm was significantly reduced and the nuclei became atrophic, suggesting that CCl_4 induced severe liver cell injury. Another study showed that CCl_4 causes hepatic injury, including hepatocytic necrosis, steatosis, and inflammation [38]. These findings paralleled and confirmed our results describing histological damage. Moreover, our observations indicate that histopathological damage was ameliorated by HP

treatment. A previous study by Bentli *et al.* (2013), which described the effect of HP treatment against liver injury, confirmed our findings, since they reported that HP treatment protects the liver against dioxin toxicities. Das Neves *et al.* also found that HP and lipoic acid exhibit protective effects against sodium arsenite-induced acute toxicity in the liver and kidneys of mice [39]. The histological effects of CCl_4 on liver tissue were correlated with and caused by oxidative stress. Therefore, strong antioxidant agents such as HP can protect the liver by scavenging free radicals.

In conclusion, in the current study, we confirmed that a single dose of 2 ml/mg CCl_4 is toxic to rats, causing increased oxidative stress and histological changes indicative of liver damage. Also, we found that the use of HP at the dose of 50 mg/kg/day for 10 consecutive days in combination with CCl_4 minimized its hepatotoxicity, which was evident from decreasing TBARS levels, histological changes in tissue and increasing antioxidant enzyme activities (SOD, CAT) and GSH levels. The beneficial effects of HP against CCl_4-induced liver damage may be due to its antioxidant, anti-inflammatory and free radical scavenging properties. Therefore, it appears that HP, a citrus flavonoid, can prevent and protect against many toxicological situations including CCl_4 toxicity caused oxidative stress. In this context, it is suggested that HP may be clinically used in human health as a radical scavenger agent.

Conflict of interest

The authors declare no conflict of interest.

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