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The protective effects of chrysin and flunixin meglumine against excess copper in male rats

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Abstract: Thirty-six Sprague Dawley male rats were housed in individual cages and randomly divided into six groups: control, copper sulfate (Cu; 500 ppm body weight (BW)/day), flunixin meglumine (FM; 2.2 ppm BW/day), chrysin (chrysin; 50 ppm BW/day), copper sulfate + FM (Cu + FM; 500 ppm BW/day of copper sulfate and 2.2 ppm BW/day of FM), and copper sulfate + chrysin (Cu + Chrysin; 500 ppm BW/day of copper sulfate and 50 ppm BW/day of chrysin). Feed intake (FI) in the Cu + Chrysin group significantly increased in comparison with that of the Cu group ($P < 0.01$). Cu excess significantly increased malondialdehyde, indicating oxidative stress. Chrysin and FM administration significantly decreased malondialdehyde levels and increased the superoxide dismutase and catalase activities in the liver and kidney tissues ($P < 0.001$). Serum TNF-α levels were significantly lower in the Cu + FM and Cu + Chrysin groups in comparison to the Cu group ($P < 0.001$). It was seen that FM and chrysin treatments alleviated degeneration, necrosis, and apoptosis in the liver and kidney tissues of the Cu-treated rats. Chrysin appeared to ameliorate the adverse effects on FI and liver and kidney tissues by scavenging where the free radicals are located and increasing the activity of antioxidants.

Key words: Antioxidant status, body weight change, chrysin, copper, flunixin meglumine, histological changes

1. Introduction

Continuous contamination with copper (Cu) is anticipated because copper and its salts are extensively used in many areas of industry and agriculture. Although copper is an element involved in a variety of biological processes in life, it can be toxic when it is in excess (1). Copper is related to numerous processes responsible for normal development and growth. It is the building block of cuproproteins, e.g., ceruloplasmin, tyrosinase, and superoxide dismutase (2,3). It supports the function of numerous cellular enzymes. Ions of Cu can be in different redox states, reduced Cu(I), or oxidized Cu(II), permitting the metal to play a basic role as a cofactor in cell physiology of the redox chemistry of enzymes, elastin cross-linking, mitochondrial respiration, and free radical scavenging (4). On the other hand, Cu homeostasis disorders or the accumulation of Cu in amounts that exceed metabolic requirements of the organism can lead to negative effects (5). Copper is a redox-active metal. The redox identity of copper contributes to its potential toxicity (6). Copper can induce oxidative stress by increasing the production of reactive oxygen species (ROS), which causes peroxidative degeneration of polyunsaturated fatty acids in membrane lipids, and biomolecular damage (7,8). The liver is the primary organ affected by copper-induced toxicity. Long-term use of Cu in rats may cause nephrotoxicity and renal dysfunction (9).

Flunixin meglumine is one of many nonsteroidal antiinflammatory drugs (NSAIDs) that are widely used and prescribed by veterinarians. Antiinflammatory drugs are effective in decreasing inflammation. Mouithys-Mickalad et al. (10) reported that NSAIDs had the capacity to fight against free radicals and had strong antioxidant effects. On the other hand, NSAID use has considerable adverse effects, including associated morbidity and mortality (11). In a previous study, it was reported that there was no clinical case report of flunixin meglumine overdoses, which indicates that it may be a safe drug (12).

Flavonoids are natural polyphenols existing in plants. Several studies showed that diets rich in flavonoids prevent various diseases such as oxidative damage (13), cancers (14), cardiovascular diseases (15), diabetes

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(16), and neurodegenerative diseases (17). Chrysin (5,7-dihydroxyflavone) is a flavonoid that is found in propolis, honey, and various plants (18,19). It has very important biological properties such as antioxidant (20), antiinflammatory (21), and anticancer (22) properties.

The present study was designed to examine the effects of flunixin meglumine when it is used as an antiinflammatory for the treatment of many diseases and of chrysin, having the flavonoid structure, on the oxidative stress induced by Cu in an animal model using male rats. Histopathological studies were also carried out to assess the effects of flunixin meglumine and chrysin on Cu damage to the rats' livers and kidneys.

2. Materials and methods

2.1. Drugs

Copper sulfate was purchased from Sigma. Chrysin (Aldrich) and flunixin meglumine (50 mg/mL injectable solution) were provided from commercial firms.

2.2. Animals, diet, and treatment

Thirty-six Sprague Dawley adult (250–300 g, 6–8 weeks old) male rats were used in the experiment. We obtained the rats from the Experimental Research Center of Fırat University and housed them in stainless steel cages (40%–60% humidity, 24 ± 3 °C, 12 h dark-light cycle). The rats were fed with standard pellet feed, containing 2650 kcal/kg metabolic energy and 23% crude protein (Elazığ Food Company, Turkey). Drinking water was given ad libitum. The present study was approved by the Animal Ethics Committee of Fırat University (08.01.2014/No:15).

Animals were randomly divided into 6 groups and housed in individual cages. Over a period of 21 days, rats in group 1 served as the control group; those in group 2 (Cu) were given copper sulfate at 500 ppm body weight (BW) per day by gavage; those in group 3 (FM) were given flunixin meglumine at 2.2 ppm BW per day by intraperitoneal (IP) administration; those in group 4 (Chrysin) were given chrysin at 50 ppm BW per day by gavage; those in group 5 (Cu + FM) were given 500 ppm BW per day of copper sulfate by gavage and 2.2 ppm BW per day of flunixin meglumine by IP administration; and those in group 6 (Cu + chrysin) were given 500 ppm BW per day of copper sulfate and 50 ppm BW per day of chrysin by gavage. Rats were individually weighed at the start of the study, and then weekly to monitor BW. Additionally, feed intake (FI) and body weight change (BWC) were determined on days 7, 14, and 21 of the experiment. Flunixin meglumine (23), copper sulfate (24,25), and chrysin (13) doses were determined based on previous studies. Subacute toxicity formed in the rats that were given copper sulfate.

2.3. Sample collection

One day after the last administration, the rats were anesthetized by inhalation of diethyl ether and killed by decapitation. For biochemical analysis, we took 1.5 mL of blood sample and liver and kidney tissues from each rat. The samples were stored at −20 °C until the analyses were completed.

2.4. Lipid peroxidation (LPO) and antioxidant analyses

2.4.1. Lipid peroxidation

The LPO levels were spectrophotometrically measured at 532 nm (26) according to the concentrations of thiobarbituric acid reactive substances and the amount of malondialdehyde (MDA) produced was used as index of LPO. 1,1,3,3-Tetraethoxypropane was used as standard. The levels of MDA were expressed as nmol/mL homogenate.

2.4.2. Reduced glutathione (GSH)

The GSH level was spectrophotometrically measured at 412 nm (27). The contents of protein in the liver and kidney were determined using bovine serum albumin as the standard (28). Results were expressed as nmol/mg protein.

2.4.3. Catalase (CAT)

The CAT activities of the liver and kidney were detected using Aebi's method (29) that is based on the determination of the rate constant (k) for the hydrogen peroxide separation rate at 240 nm. Results of the CAT activity were expressed as k/g protein.

2.4.4. Superoxide dismutase (SOD)

Measurements were performed using Sun et al.'s method. (30). This method is based on the reduction of xanthine oxidase, which produces nitro blue tetrazolium. The results were spectrophotometrically evaluated at 560 nm and expressed as percent inhibition/mg protein.

2.5. Serum tumor necrosis factor alpha (TNF-α)

Serum TNF-α was determined by enzyme-linked immunosorbent assay using a commercial kit (ELISA, Cat. No. E-EL-R0019). The samples were read using the automated Triturus enzyme immunoassay analyzer at 450 nm (Grifols-Quest, Miami, FL, USA). The cytokine quantities of samples were detected from standard curves of recombinant cytokines according to the linear regression method.

2.6. Histopathological and immunohistochemical (IHC) analyses

For histological assessments, the kidney and liver tissues were fixed in 10% formalin solution, dehydrated in a graduated alcohol series, and then embedded in paraffin. The tissue samples that were embedded in paraffin were cut at a thickness of 5 µm, mounted on 1/4 shaven slides, and stained with hematoxylin and eosin (H&E) for histological examination according to the standard procedure (31). The cells that underwent apoptosis in the tissues were evaluated immunohistochemically by using the caspase

method. The samples were mounted on polylysine-coated slides for analysis. After deparaffinization and rehydration, the samples were transferred to citrate buffer at pH 6.0 and were kept in a microwave oven for 20 min. The samples were then washed with phosphate buffered saline (PBS) after they were cooled for 20 min at room temperature. They were immersed in 0.3% hydrogen peroxide for 5 min, washed with PBS, incubated with Ultra V block for 5 min at room temperature, and then incubated with a primary rabbit-polyclonal active caspase-3 antibody (Thermo Scientific, CPP32, Ab-4, UK) for 1 h in a humidified chamber at 37 °C. The sections were rinsed in PBS before incubation with biotinylated goat antipolyvalent reagent for 30 min at room temperature and were washed with PBS. Then they were incubated in streptavidin peroxidase for 30 min at room temperature and were washed with PBS. Staining was completed after the substrate was incubated with a 3.3'-diamino-benzidine-tetrahydrochloride (DAB) chromogen (Thermo Scientific, Ultra Vision Detection System Anti-polyvalent, HRP/DAB, UK) for 5–15 min and then the slides were washed for 5 min with distilled water. The slides were counterstained with Mayer's hematoxylin for 30 s, rinsed in tap water for 5 min and dehydrated, and then mounted using an aqueous mounting medium. The active caspase-3 kit was used according to the manufacturer's instructions. The routine H&E tissue sections and the active caspase-3 activities of the liver and kidney tissues were examined under a binocular-headed light microscope with the feature of photomicroscope (Olympus BX-51, Olympus Optical Co., Ltd., Tokyo, Japan) and photographs were taken. The active caspase-3 positive cells in the tissue sections were stained a brown color and were evaluated by looking at the staining intensity (–, none; +, slight; ++, moderate; +++, strong) with a semiquantitative scoring system.

2.7. Statistical analysis

All data were expressed as means ± SEM. Using one-way analysis of variance (ANOVA) and the post hoc Duncan test, SPSS 21 for PC was used to determine whether there were any statistically significant differences between the group means (IBM Corp., USA). The results were considered statistically significant when $P < 0.05$.

3. Results

In the present study, FI and BW of rats treated with Cu were significantly decreased in comparison with those of the control, Cu + FM, and Cu + chrysin groups during the course of the experiment ($P < 0.01$; Table 1).

MDA, indicating oxidative stress, significantly increased in the kidney and liver tissues of the group that was given copper ($P < 0.001$; Tables 2 and 3). Chrysin and flunixin meglumine administration significantly decreased the MDA levels in the kidneys and liver ($P < 0.001$). The GSH levels in the liver ($P < 0.001$) and kidney ($P < 0.01$) tissues of the Cu-administrated group were found to be significantly lower than those of the rats in the control, flunixin meglumine, and chrysin groups ($P < 0.01$). Flunixin meglumine and chrysin supplementations to Cu groups significantly increased the GSH levels to be similar to those of the control group ($P < 0.01$; Tables 2 and 3). Superoxide dismutase and CAT activities of the kidney and liver tissues in the Cu group were found to be

Table 1. Effects of flunixin meglumine (FM) and chrysin on body weight (BW, g), body weight change (BWC, g/rat daily), and feed intake (FI, g/rat daily) of the experimental group members.

	Days	Control	Cu	FM	Chrysin	Cu + FM	Cu + chrysin	P
BW	IW	251 ± 13.4	249.5 ± 16.8	249 ± 14.3	249.5 ± 17	248.8 ± 14.9	250.5 ± 8.3	NS
	7	275.4 ± 14	265.3 ± 15.7	271.3 ± 17.6	272.7 ± 15.5	266.7 ± 14.3	269.4 ± 8.3	NS
	14	291.5 ± 12.7	274.9 ± 17.6	286.6 ± 16.4	288.5 ± 14.5	277.5 ± 13.7	280.8 ± 4.3	NS
	21	312.8 ± 13.9	285.4 ± 17.9	306.7 ± 16.4	309 ± 13.8	302.7 ± 15.2	300.4 ± 4.5	NS
BWC	1–7	4.07 ± 0.35	2.64 ± 0.5	3.72 ± 0.59	3.86 ± 0.36	2.98 ± 0.69	3.15 ± 0.42	NS
	8–14	2.29 ± 0.37	1.37 ± 0.32	2.18 ± 0.24	2.26 ± 0.42	1.54 ± 0.55	1.62 ± 0.58	NS
	15–21	3.04 ± 0.32^{a}	1.5 ± 0.12^{b}	2.89 ± 0.17^{a}	2.93 ± 0.25^{a}	2.73 ± 0.14^{a}	2.80 ± 0.09^{a}	**
	1–21	3.09 ± 0.07^{a}	1.79 ± 0.21^{b}	2.89 ± 0.16^{ab}	2.97 ± 0.26^{a}	2.26 ± 0.33^{ab}	2.49 ± 0.19^{ab}	**
FI	1–7	22.74 ± 1.46	19.93 ± 1.08	22.56 ± 0.85	22.68 ± 0.67	20.40 ± 1.83	21.78 ± 1.06	NS
	8–14	23.33 ± 0.29^{a}	17.41 ± 1.15^{c}	22.56 ± 0.53^{a}	22.92 ± 0.67^{a}	19.2 ± 0.63^{bc}	19.80 ± 0.79^{b}	**
	15–21	22.92 ± 0.84^{a}	16.83 ± 1.37^{c}	22.38 ± 1.06^{ab}	22.44 ± 1.05^{ab}	18.66 ± 0.55^{bc}	18.54 ± 2.17^{bc}	**
	1–21	22.99 ± 0.64^{a}	18.06 ± 0.90^{c}	22.50 ± 0.48^{a}	22.68 ± 0.69^{a}	$19.42 \pm 0,71^{bc}$	20.04 ± 1.04^{ab}	**

IW: Initial weight; $^{a, b, c}$: mean values with different superscripts within a row differ significantly; NS: nonsignificant; **: $P < 0.01$

significantly lower than those of all the other groups (P < 0.001; Tables 2 and 3).

The serum TNF-α level of the Cu group was significantly higher (P < 0.001) than those of other groups. Serum TNF-α levels were significantly lower in the Cu + FM and Cu + Chrysin groups in comparison to the Cu group (P < 0.001; Table 4).

The liver and kidney samples of the control rats and those of the rats that were treated with flunixin meglumine alone and chrysin alone showed no abnormalities (Figures 1 and 2). However, the livers of the Cu-supplemented rats showed hepatocellular degeneration and necrosis. Karyolysis and karyomegaly were observed in several hepatocyte cells. Moreover, it was determined that Cu exposure caused vasocongestion and dilatation in most sinusoids, mononuclear inflammatory cell infiltrations in periportal areas, dilatation with hyperemia and hyaline residue in some central veins, disorganization in some hepatic cord areas, and biliary duct proliferation in some portal areas. The flunixin meglumine and chrysin treatments alleviated these Cu-induced degenerative and necrotic changes. In addition, regenerative changes such as a double nuclei in many hepatocytes were observed (Figure 1). The kidneys from the Cu-treated rats also demonstrated degeneration and necrosis of mostly the proximal and minority distal tubules in the cortex. Swelling, karyomegaly, and karyolysis were observed in epithelial cells of the proximal tubules. Epithelial cell desquamation and hyaline cylinders in the lumen of some of those as well as congestion were found in some proximal tubules. Shrinkage, congestion, and thickening in the basal membrane of some glomeruli located in the cortex were also observed. Congestion and focal mononuclear cell infiltration were found in blood vessels in interstitial tissue of the cortex while dilatation and degeneration were seen in Bowman's capsules. The flunixin meglumine and chrysin treatments decreased tubular degeneration and necrosis and proved that the majority of changes in the

Table 2. Effects of flunixin meglumine (FM) and chrysin on MDA (nmol/mL homogenate), GSH (nmol/mg protein), SOD (%inhibition/mg protein), and CAT (k/g protein) activities in the livers of the experimental group members.

		Control	Cu	FM	Chrysin	Cu + FM	Cu + chrysin	P
Liver	MDA	2.42 ± 0.13^{c}	3.84 ± 0.16^{a}	2.23 ± 0.16^{c}	2.15 ± 0.21^{c}	2.94 ± 0.14^{b}	3.05 ± 0.19^{b}	***
	GSH	19.07 ± 0.57^{a}	9.80 ± 0.75^{c}	18.45 ± 1.85^{a}	17.82 ± 1.32^{a}	14.48 ± 1.13^{b}	13.90 ± 0.97^{b}	***
	SOD	0.24 ± 0.01^{a}	0.16 ± 0.01^{c}	0.27 ± 0.02^{a}	0.26 ± 0.02^{a}	0.19 ± 0.01^{bc}	0.20 ± 0.01^{ab}	***
	CAT	0.77 ± 0.02^{a}	0.18 ± 0.01^{d}	0.73 ± 0.02 ^{ab}	0.70 ± 0.03^{b}	0.30 ± 0.01^{c}	0.27 ± 0.01^{c}	***

$^{a, b, c, d}$: Mean values with different superscripts within a row differ significantly; ***: P < 0.001.

Table 3. Effects of flunixin meglumine (FM) and chrysin on MDA (nmol/mL homogenate), GSH (nmol/mg protein), SOD (%inhibition/mg protein), and CAT (k/g protein) activities in the kidneys of the experimental group members.

		Control	Cu	FM	Chrysin	Cu + FM	Cu + chrysin	P
Kidneys	MDA	6.06 ± 0.31^{c}	11.23 ± 0.27^{a}	6.71 ± 0.44^{c}	6.69 ± 0.44^{c}	8.51 ± 0.20^{b}	8.64 ± 0.26^{b}	***
	GSH	25.48 ± 2.39^{a}	14.84 ± 0.86^{c}	22.13 ± 1.59^{ab}	22.36 ± 1.79^{ab}	17.62 ± 1.48^{bc}	15.39 ± 1.91^{c}	**
	SOD	0.27 ± 0.03^{a}	0.10 ± 0.01^{c}	0.25 ± 0.01^{a}	0.23 ± 0.01^{a}	0.17 ± 0.02^{b}	0.16 ± 0.02^{b}	***
	CAT	0.68 ± 0.01^{a}	0.14 ± 0.01^{c}	0.62 ± 0.01^{a}	0.65 ± 0.03^{a}	0.30 ± 0.01^{b}	0.25 ± 0.01^{b}	***

$^{a, b, c}$: Mean values with different superscripts within a row differ significantly; **: P < 0.01; ***: P <0.001.

Table 4. Effects of flunixin meglumine (FM) and chrysin on TNF-α (pg/mL) levels of experimental groups.

	Control	Cu	FM	Chrysin	Cu + FM	Cu + chrysin	P
TNF-α	106.22 ± 2.65^{c}	164.32 ± 5.26^{a}	114.43 ± 3.32^{c}	114.44 ± 3.08^{c}	129.62 ± 3.38^{b}	131.25 ± 2.49^{b}	***

$^{a, b, c}$: Mean values with different superscripts within a row differ significantly; ***: P < 0.001.

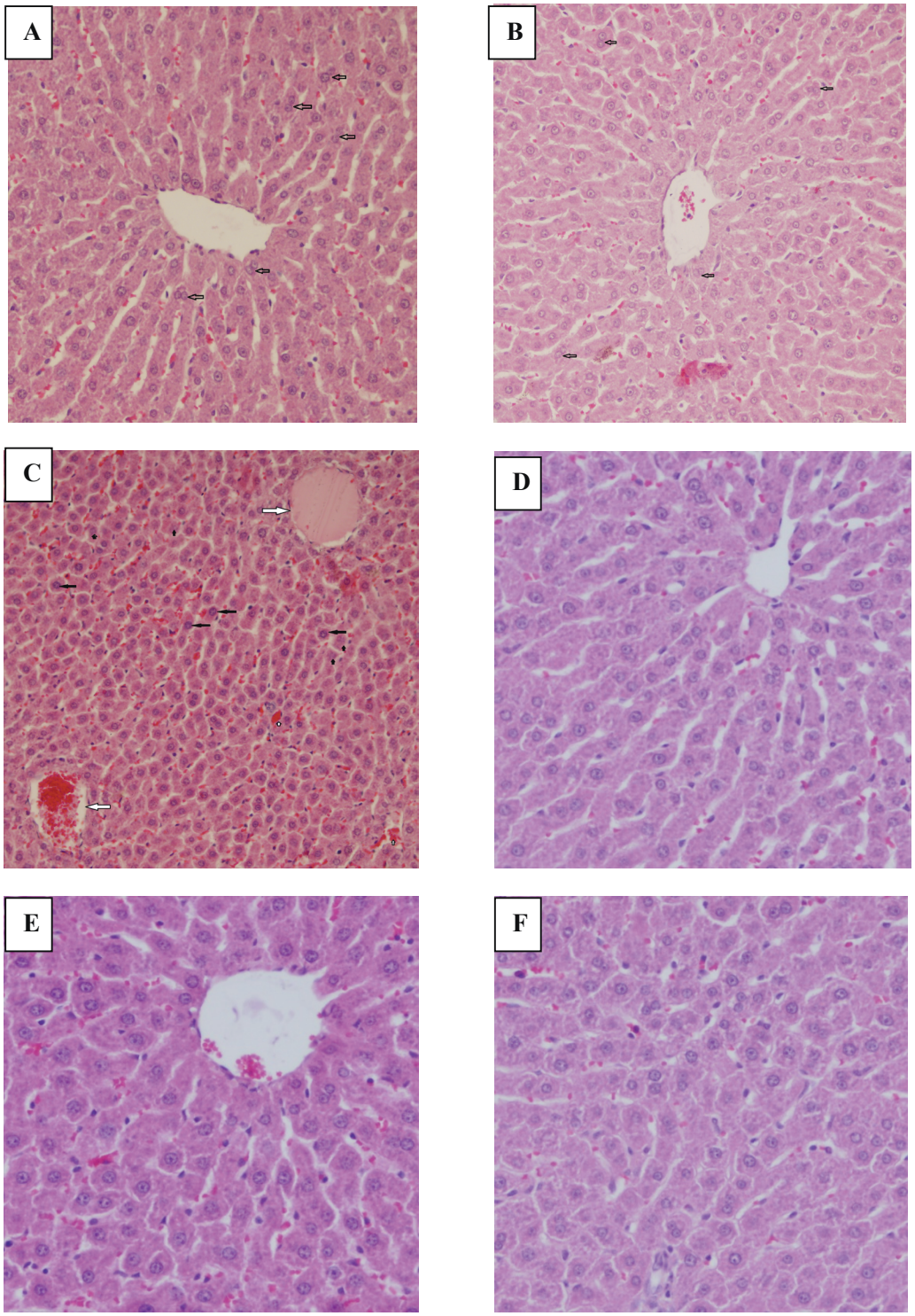

Figure 1. Histopathological findings in the livers of the experimental group members. (A) Cu + FM treatment; double nuclei in many hepatocytes (arrows) (H&E staining, magnification 100×). (B) Cu + chrysin treatment; double nuclei in many hepatocytes (arrows) (H&E staining, magnification 100×). (C) Cu treatment alone; karyomegaly in hepatocyte (black arrows), karyolysis in hepatocyte (black arrowheads), vasocongestion and dilatation in sinusoids (white arrowheads), dilatation with hyperemia and hyaline residue in central veins (white arrows) (H&E staining, magnification 100×). (D) Control; normal liver histology (H&E staining, magnification 200×). (E) Flunixin meglumine treatment alone; normal liver histology (H&E staining, magnification 200×). (F) Chrysin treatment alone; normal liver histology (H&E staining, magnification 200×).

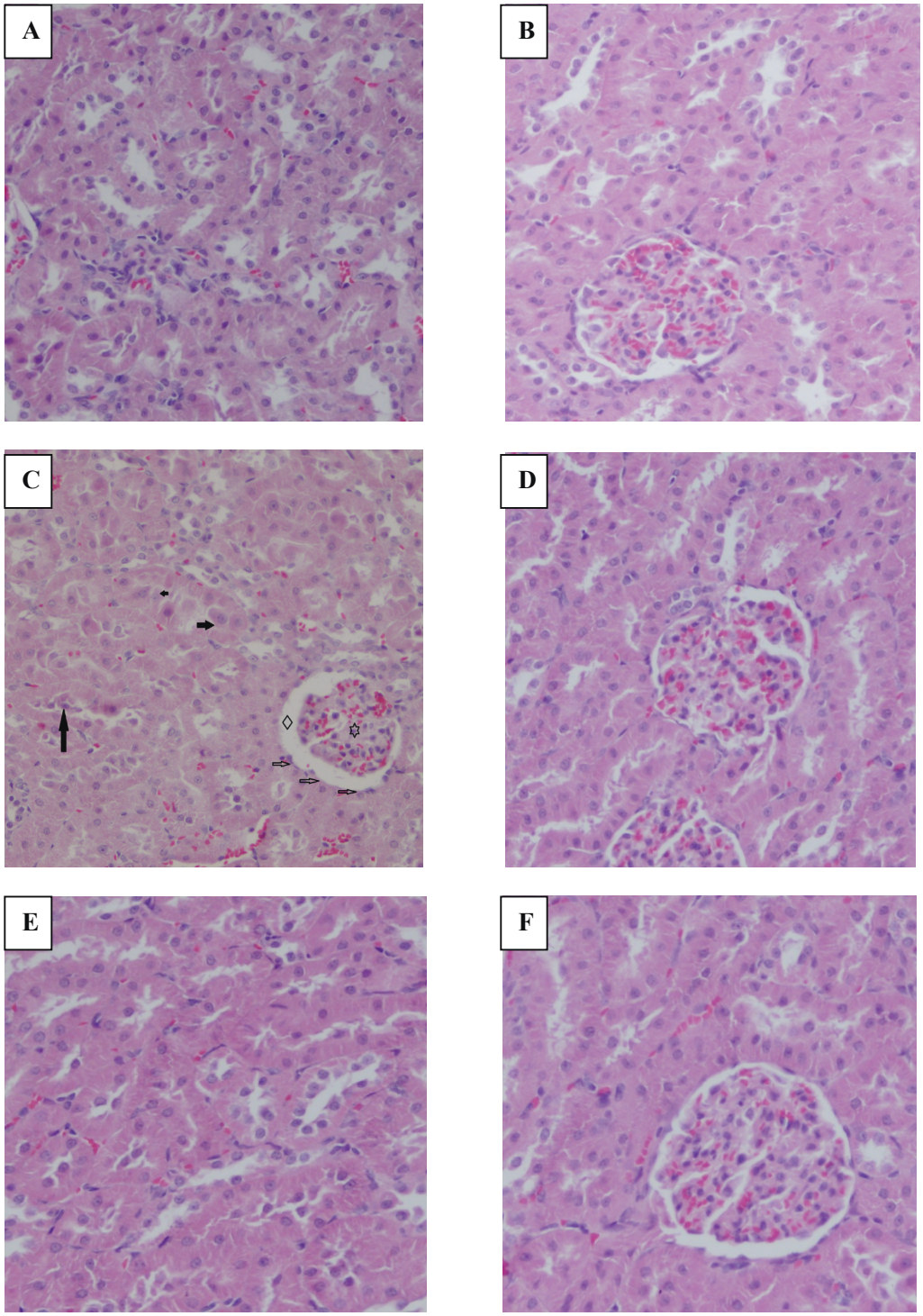

Figure 2. Histopathological findings in the kidneys of the experimental group members. (A) Cu + FM treatment; decreased degenerative and necrotic changes (H&E staining, magnification 200×). (B) Cu + chrysin treatment; decreased degenerative and necrotic changes (H&E staining, magnification 200×). (C) Cu treatment alone; karyomegaly (small black arrow) and karyolysis (black arrowhead) in epithelial cells of proximal tubules, epithelial cell desquamation in lumen of proximal tubule (big black arrow), shrinkage and congestion in glomeruli (star), thickening in basal membrane of glomeruli (arrows), dilatation and degeneration in Bowman's capsule (diamond) (H&E staining, magnification 100×). (D) Control; normal kidney histology (H&E staining, magnification 200×). (E) Flunixin meglumine treatment alone; normal kidney histology (H&E staining, magnification 200×). (F) Chrysin treatment alone; normal kidney histology (H&E staining, magnification 200×).

cortex had been caused by the Cu supplements and could be reasonably counteracted with flunixin meglumine and chrysin supplements (Figure 2). Apoptotic cells showing strong positive reactions in the hepatocytes with mostly the proximal and minority distal tubules when compared to the control, flunixin meglumine alone, and chrysin alone groups were encountered with Cu exposure. Although the flunixin meglumine treatment reduced the Cu-induced apoptotic cells and the staining intensities of these apoptotic cells were moderate, the chrysin treatment still increased the apoptotic cells (Figures 3 and 4) in the liver and kidney tissues.

4. Discussion

Prolonged exposure to elevated concentrations of copper may have detrimental effects (8). The results of the present study demonstrate that a dose of 500 ppm BW Cu intake can have toxic effects from day 21. Kornegay et al. (32) determined that feeding 200 mg/kg Cu increased the growth rate and FI ($P < 0.01$) during 5 weeks of tests; 400 mg/kg Cu depressed growth and FI after week 2 in weanling crossbred pigs (n = 216; 6.9 kg initially). In another study (33), Chiou et al. found that increasing dietary Cu supplements of 400, 500, and 600 ppm significantly lowered egg production and FI after 4 weeks in laying hens ($P < 0.05$). The daily FI of increased Cu groups in their study (400, 500, and 600 ppm dietary Cu) was 109, 107, and 97.8 g/hen, respectively ($P < 0.004$). These results agree with the findings of the present study. BW of the animals steadily exposed to Cu decreased, which can be a predictor of poor general health (8). Treatment of rats with antioxidant compounds partially alleviated the negative effects of oxidative stress induced by Cu (8). The effects of amelioration on performance of chrysin in mice were connected to the flavonoids in its structure (34).

The present study demonstrated that BW and BWC statistically increased when flunixin meglumine and chrysin supplementation was administered to the rats that were given Cu ($P < 0.01$). A similar study (35) found that the BW of cyclosporin-A (CsA)-treated animals was lower than that of the control group. The decrease in BW was certainly due to a parallel decrease in FI, which followed CsA implementation. These results agree with those of the present study. However, as earlier studies (8,36,37) have shown that flavonoids protect against Cu toxicity, an excess of Cu in rats may cause LPO and damage of cellular membranes. In the present study, the decrease in BWC of the Cu + chrysin group could be connected with the flavonoid content of chrysin. It could also be due to the fact that flavonoids show characteristics of antioxidants, which also were chelating with radicals or trace elements (38).

Excessive copper in diet may cause damage to the membranes and LPO in rats. Mladenović et al. (8) reported that LPO increased after incubation of erythrocyte suspension with Cu^{2+} ions. The Cu^{+} ion was generated by the reduction of Cu^{2+} in the presence of the superoxide anion ($O^{2\bullet-}$), which catalyzed the formation of hydroxyl radicals (OH) that readily initiate further chemical reactions. Ozcelik et al. (39) reported that the plasma MDA levels in rats given water containing copper (100 µg/mL) were higher than those in the control group, and the GSH levels in their livers were lower. Mladenović et al. (8) noticed that flavonoids can block LPO owing to their structure, thus enabling them to scavenge ROS as electron- or hydrogen-donating compounds or to chelate metal ions. Thus, they can be reformative in situations of oxidative stress or increased metal concentrations (8). Similar to our study, Sultana et al. (40) reported increases in LPO of the cisplatin-treated group compared to that of the control group, and also dose-dependent inhibition of LPO by chrysin. They claimed that chrysin protected the kidney from free radical damage by cleaning free radicals produced by cisplatin toxicity. Flunixin meglumine and chrysin supplementations in Cu administration increased the GSH levels significantly to be like that of the control group ($P < 0.01$; Tables 2 and 3). Superoxide dismutase and CAT activities of kidney and liver tissues in the Cu group were significantly lower than those of all the other groups ($P < 0.001$). SOD, a cellular antioxidant, is one of the several important antioxidant enzymes. It reacts rapidly and is removed by CAT as it converts O_2^- into H_2O_2. It has been reported that excessive Cu accumulation in the liver decreases SOD activity and causes increased MDA levels in liver homogenates and serum (41). Chrysin and flunixin meglumine treatments partially ameliorate the Cu-induced imbalance in the oxidant–antioxidant systems of the liver and kidney tissues. Konyalioglu et al. (23) reported that flunixin meglumine had antioxidant properties. The cause of its ameliorative effects on the antioxidant status in cases of Cu toxicities can be connected to its antioxidant properties (23). Chrysin supplementation in rats treated with Cu caused significant increases of CAT and SOD ($P < 0.001$; Tables 2 and 3).

Ciftci et al. (42) suggested that the serum TNF-α levels in the group that was given 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were significantly higher ($P < 0.05$) when compared to those in other groups on day 15. The TNF-α levels of the curcumin + TCDD group (60.81 pg/mL) decreased significantly in comparison to those of the TCDD group (104.0 pg/mL). These results were similar to ours. Lee et al. (43) noted that TNF-α induced the activation of many transcriptional factors, which had long been considered a prototypical proinflammatory signaling pathway, through TNF-α receptor-dependent signaling pathways. Flavonoids are widely used to inhibit the activation of NF-κB, and they inhibit the expression

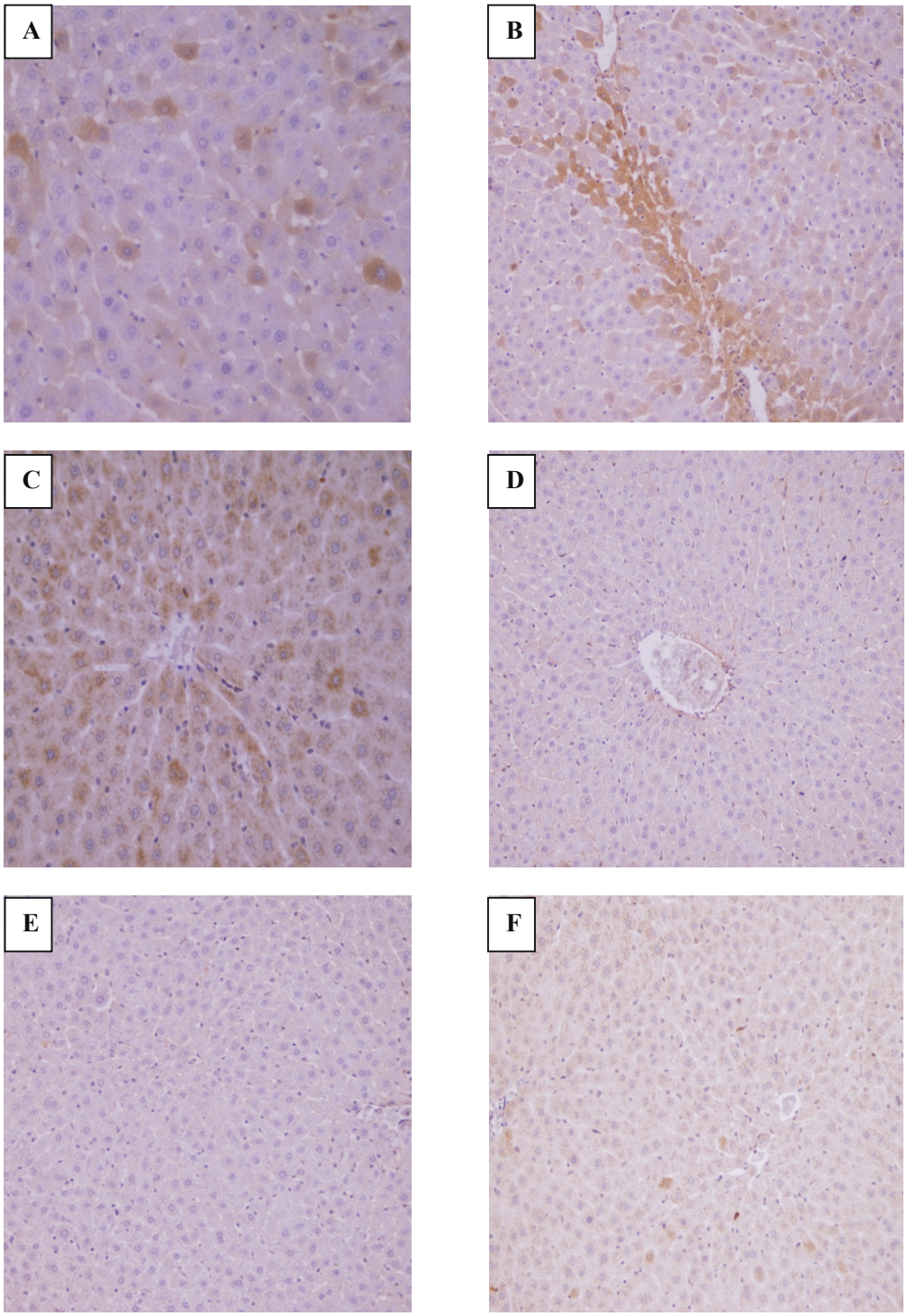

Figure 3. Immunohistochemical findings in the livers of the experimental group members. (A) Cu + FM treatment; decreased numbers of strong immunopositive cells for active caspase-3 with the moderate staining intensities (IHC, Mayer's hematoxylin counterstain, magnification 200×). (B) Cu + chrysin treatment; many apoptotic cells showing strong immunoreaction for active caspase-3 (IHC, Mayer's hematoxylin counterstain, magnification 100×). (C) Cu treatment alone; many apoptotic cells showing strong immunoreaction for active caspase-3 (IHC, Mayer's hematoxylin counterstain, magnification 200×). (D) Control; no immunoreactivity for active caspase-3 antibody (IHC, Mayer's hematoxylin counterstain, magnification 100×). (E) Flunixin meglumine treatment alone; no immunoreactivity for active caspase-3 antibody (IHC, Mayer's hematoxylin counterstain, magnification 100×). (F) Chrysin treatment alone; a few active caspase-3 positive apoptotic cells (IHC, Mayer's hematoxylin counterstain, magnification 100×).

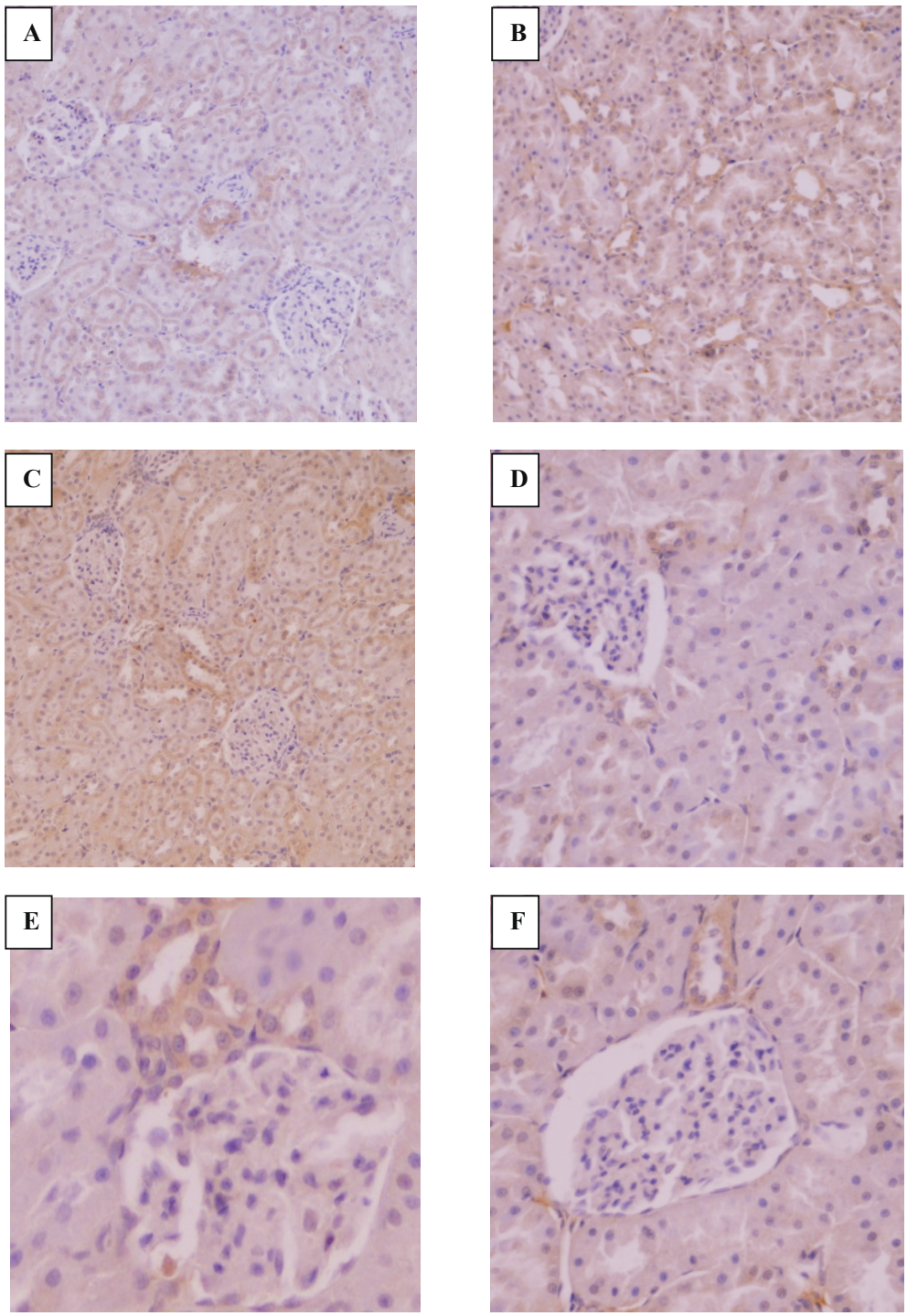

Figure 4. Immunohistochemical findings in the kidneys of the experimental group members. (A) Cu + FM treatment; decreased numbers of cells showing strong positive immunoreaction in tubules for active caspase-3 with moderate staining intensities (IHC, Mayer's hematoxylin counterstain, magnification 100×). (B) Cu + chrysin treatment; many apoptotic cells showing strong immunoreaction in a large number of tubules for active caspase-3 (IHC, Mayer's hematoxylin counterstain, magnification 100×). (C) Cu treatment alone; many apoptotic cells showing strong immunoreaction in a large number of tubules for active caspase-3 (IHC, Mayer's hematoxylin counterstain, magnification 100×). (D) Control; no immunoreactivity for active caspase-3 antibody (IHC, Mayer's hematoxylin counterstain, magnification 200×). (E) Flunixin meglumine treatment alone; a few active caspase-3 positive apoptotic cells in tubules (IHC, Mayer's hematoxylin counterstain, magnification 200×). (F) Chrysin treatment alone; a few active caspase-3 positive apoptotic cells in tubules (IHC, Mayer's hematoxylin counterstain, magnification 200×).

of proinflammatory genes in response to inflammatory mediators such as TNF-α. Flunixin meglumine and chrysin administration to rats that were given Cu decreased the TNF-α level (Table 4). This may be due to the flavonoid content structure of chrysin and the antiinflammatory properties of flunixin meglumine that decreased the TNF-α levels (43–45).

The histopathological changes induced by Cu treatment include confirmed antioxidant status and TNF-α levels. Similarly, Abu-Zinadah et al. (6) noted that •OH, which is produced through the biochemical reaction of excess Cu, was believed to be responsible for devastating cellular damage, including LPO. Copper excess causes damage to liver and kidney tissues. Babaknejad et al. (9) reported that Cu excess toxicity was closely correlated to renal dysfunction. They also stated that tubular necrosis and cellular pleomorphic adenoma were reported in rats that had received excessive amounts of Cu. One of the most toxic effects of Cu on kidneys is proteinuria (46). Moreover, the liver damage induced by Cu decreases serum protein (47). The livers from the Cu-supplemented rats showed hepatocellular degeneration and necrosis. The flunixin meglumine and chrysin treatments alleviated the Cu-induced degenerative, necrotic, and hemorrhagic changes (Figure 1). Moreover, the kidneys from the Cu-treated rats demonstrated degeneration and necrosis of mostly proximal and minority distal tubules in the cortex. The flunixin meglumine and chrysin treatments decreased tubular degeneration and necrosis and proved that the majority of changes in the cortex had been caused by the Cu supplements and could be reasonably counteracted with flunixin meglumine and chrysin supplements (Figure 2). Apoptotic cells showing strong positive reactions in the hepatocytes with mostly proximal and minority distal tubules when compared to the control, flunixin meglumine alone, and chrysin alone groups were encountered with Cu exposure. Although the flunixin meglumine treatment reduced the number of Cu-induced apoptotic cells, and the staining intensities of these apoptotic cells were moderate, the chrysin treatment still increased the apoptotic cells (Figures 3 and 4) in the liver and kidney tissues. Rana (48) reported that, when administrating Cu, ROS may be produced by the liver. The liver toxicity might be associated with the LPO formation and induction of oxidative damage. In some studies (38,48), lipid peroxidation of mitochondrial membranes of liver cells and MDA in the livers of Cu-loaded rats were reported. In another study (49), apoptosis and necrosis were found in the kidney and liver tissues in experimental Cu toxicity in rats, as in our study. Treatments of chrysin and flunixin meglumine modulated the toxic effects of Cu in histopathological examinations of the liver and kidney tissues (Figures 1 and 2). The reason for this may simply be the strong antioxidant properties of chrysin (50) and flunixin meglumine (23). Xuan et al. (50), determined that chrysin in anticancer treatment significantly activated caspase-3, which showed that chrysin-induced apoptosis inhibits the proliferation of MCF-7 cells. Similarly, Kasala et al. (34) reported the ability of chrysin to inhibit cell proliferation, inflammation, and tumor development. These findings confirm the chemopreventive potential of chrysin against Cu toxicity in rats, as shown in our study (Figures 3 and 4). We determined that chrysin, which is rich in antioxidants (51,52), could be used to prevent the adverse effects on the liver and kidney tissues resulting from the toxicity of Cu.

In conclusion, chrysin, a natural bee product, appeared to ameliorate adverse effects on feed intake and liver and kidney tissues caused by Cu toxicity by increasing the antioxidant activities and scavenging the free radicals. Flunixin meglumine is a product that is not natural and has side effects. Chrysin supplementation instead of flunixin meglumine may be used as a good option to diminish the negative effects of Cu on rats. Chrysin may be used for treatment of some diseases that cause necrosis in the liver and kidneys. This subject calls for further research.

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