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Ameliorative effects of humic acid on copper-induced liver injury via antioxidant and chelating mechanisms

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Abstract

The liver plays a critical role in metabolism, detoxification, and maintaining internal homeostasis. Excessive accumulation of copper (Cu), a metal with known toxic effects, can cause severe hepatic injury, particularly in genetic disorders such as Wilson's disease. This study aimed to investigate the chelating and antioxidant properties of humic acid (HA) in mitigating Cu-induced toxicity, oxidative damage, and apoptosis in an experimental model. Forty male rats were randomly assigned to four groups (n=10). Group I received a standard diet (Control); Group II received HA, 536 mg/kg/day, oral); Group III was administered copper sulfate ($CuSO_4$, 75 mg/kg/day, oral); Group IV received both HA and $CuSO_4$ for 14 days. Blood and liver tissue samples were collected post-euthanasia for biochemical, histopathological, and immunohistochemical analyses. Biochemical parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), copper (Cu), ceruloplasmin, malondialdehyde (MDA), oxidative stress index (OSI), total antioxidant status (TAS), total oxidant status (TOS), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Caspase-3 and 9 expressions were evaluated immunohistochemically. The $CuSO_4$ group showed significantly increased ALT, AST, and Cu levels, alongside elevated MDA and TOS, and reduced TAS, GSH, SOD, CAT, and GPx levels ($p<0.05$). HA treatment significantly reversed these alterations. Histopathologically, severe hepatic damage induced by $CuSO_4$ was markedly alleviated by HA. Additionally, caspase-3 and 9 expressions were significantly upregulated in the $CuSO_4$ group but notably reduced with HA administration. Humic acid exhibits both chelating and antioxidant effects in alleviating copper-induced hepatotoxicity. It reduces hepatic Cu accumulation, mitigates oxidative stress and apoptosis, and alleviates liver damage. Further clinical studies are required to determine its optimal dosage, formulation, and safety for human use.

Keywords: Humic acid, copper toxicity, oxidative stress, hepatotoxicity, chelation, apoptosis, rat model

Introduction

The liver is fundamentally involved in regulating metabolism, facilitating detoxification, and preserving the body's internal balance. Hepatotoxicity caused by heavy metals is a significant health concern. Modern medicine continues the search for effective and safe agents to treat liver diseases stemming from hepatotoxicity, and numerous studies investigate the effects of various natural compounds on liver injury. Copper (Cu) is an essential micronutrient required for the activity of multiple enzymes in biological systems [1]. Under normal conditions, Cu is protein bound; however, when it transitions to the free form, it can catalyze redox reactions generating highly reactive hydroxyl radicals. This may lead to cellular damage via DNA, protein, and lipid peroxidation [2]. In hereditary conditions like Wilson's dissease (WD) impaired Cu elimination leads to its initial buildup in the liver, which eventually extends to the brain, resulting in hepatic, neurological, and hematological complications. Therefore, maintaining Cu homeostasis and preventing excessive accumulation in tissues is crucial, and

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Cu chelators are used as first line therapeutic agents in clinical practice.

Humic acid (HA) is a compound characterized by its high molecular weigh containing phenolic, carboxyl, aromatic, and aliphatic functional groups. Due to its colloidal properties, HA can markedly reduce the toxic effects of heavy metals ingested via food and water by chelating them within the digestive system [3]. Additionally, HA exhibits various biological activities: it is capable of binding metal ions through its functional groups and supports the body's endogenous antioxidant capacity, mediated in part by enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase, which play vital roles in redox balance reduce pro inflammatory cytokines and protect tissues from oxidative damage. Its low toxicity and the absence of significant adverse effects even following long term use render HA a promising agent [4,5]. Experimental models have shown that HA administration reduces hepatic metal accumulation, normalizes antioxidant enzyme activities, and alleviates oxidative stress [2]. Consequently, HA is considered to have potential both as a therapeutic and a prophylactic agent.

This study aims to assess the chelating potential of HA and its antioxidant effectiveness against Cu-induced redox imbalance and toxicity in an experimental setting of hepatic Cu accumulation caused by oral Cu exposure.

Material and Methods

The present study was carried out at the Experimental Animal Research Center of İnönü University, involving 40 adult female Wistar albino rats with body weights ranging from 222.0 to 245.2 grams (average weight: 233.4 g; see Table 1). Ethical clearance for the experimental protocol was obtained in advance from the İnönü University Ethics Committee for Animal Experiments. (Date: 11/04/2022, Decision No: 2022/5-1, HAYBIS No: 14700). All experimental procedures adhered strictly to the ARRIVE 2.0 guidelines [6].

The rats were randomly assigned into four experimental groups, each consisting of 10 subjects. Throughout the study, animals were provided with standard commercial pellet feed and water ad libitum. During the experimental period, the animals had unrestricted access to standard commercial pellet feed and drinking water. Environmental parameters were maintained under controlled conditions, including ambient temperature set at 21 ± 2 °C, air moisture levels stabilized at 60 ± 5%, and a regulated photoperiod consisting of 12 hours of light followed by 12 hours of darkness

Copper sulfate ($CuSO_4$)was prepared by dissolving it in isotonic saline, which served as the carrier, and administered orally to the rats at a dose volume of 5 mL per kilogram of body weightThe experimental design was as follows: We divided our study into 4 groups, each consisting of 10 rats. The rats that were given a normal diet for a duration of 14 consecutive days were accepted as the control group (Group I, n=10). Rats that were given a normal diet along with HA 536 mg/kg/day orally obtained from Sigma Aldrich Fine Chemicals Biosciences, 100 g package) for 14 days were designated as HA (Group II, n=10). Rats receiving a standard diet in combination with $CuSO_4$ solution (98% purity, anhydrous; Thermo Scientific Chemicals) at a daily oral dose of 75 milligrams per kilogram of body weight over a 14-day period were assigned to Group III ($CuSO_4$, n=10). Group IV ($CuSO_4$+HA, n=10) consisted of rats administered both HA (536 milligrams per kilogram, orally in the evening) and $CuSO_4$ solution at the same dosage for two weeks, along with a standard diet.

On the 15th day, deep anesthesia was induced via intraperitoneal administration of urethane at a concentration of 1.2 grams per kilogram. Euthanasia was subsequently performed through exsanguination in line with institutional ethical standards. Following euthanasia, 4–5 mL of blood was collected through intracardiac puncture for biochemical evaluations. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), ceruloplasmin, and Cu in serum were quantitatively measured (table 2). Liver tissue samples were also harvested for both biochemical assessments and histopathological examinations (refer to table 3, figure 1, and figure 2).

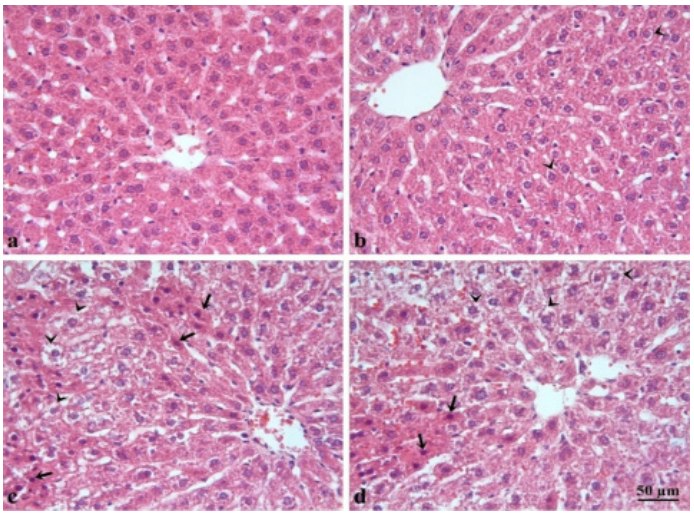

Figure 1. Panel (a) shows the control group with preserved normal liver histology. In the HA-treated group (b), the liver exhibits a structure largely comparable to the control, with only mild hydropic degeneration observed in some hepatocytes (arrowheads). In the Cu-treated group (c), notable pathological changes include hepatocytes displaying hydropic degeneration (arrowheads), along with pyknotic nuclei and condensed cytoplasm (arrows). Despite HA administration, histological signs of liver damage remain visible in the Cu+HA group (d). Staining: H&E; magnification: ×400.

For histopathological analysis, liver tissues were fixed in a 10% formalin solution buffered to a neutral(NBF) pH to ensure optimal histological preservation. while those intended for biochemical evaluation were promptly frozen in liquid nitrogen. After thawing at room temperature, oxidative stress and antioxidant system parameters were analyzed. In addition, immunohistochemical analyses for caspase 3 and 9, as well as dry Cu measurements, were performed on the tissue samples. All specimens were stored at -80 °C until analysis (table 4) .

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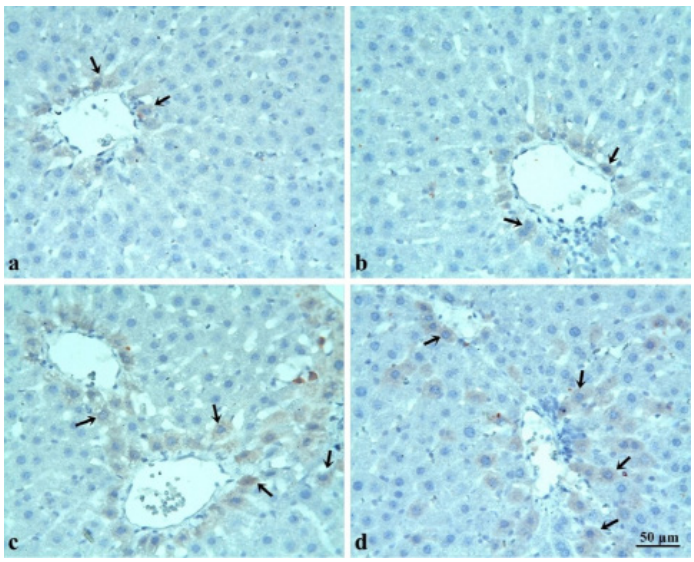

Figure 2. Immunohistochemical detection of caspase-3 expression in liver sections from each experimental group. Caspase-3-positive hepatocytes are indicated by arrows. Minimal caspase-3 immunoreactivity is evident in the control (a) and HA (b) groups. In contrast, a pronounced increase in caspase-3-positive cell density is observed in both the Cu (c) and Cu+HA (d) groups. Immunostaining for caspase-3; magnification ×400.

Tissue weights were documented prior to processing. Tissue samples were processed in a Tris-HCl buffer solution (pH 7.4) for homogenization. Post-homogenization, centrifugation was applied, and supernatant fractions were collected for further analysis. Spectrophotometric readings were performed using a UV-visible spectrophotometer and a multi-mode microplate reader. The quantification of Malondialdehyde (MDA) was carried out in accordance with the procedure established by Uchiyama and Mihara, and the results were calculated via a standard curve, expressed in nanomoles per gram of tissue (nmol/g) [7]. The protein concentration, which served as the reference for evaluating enzymatic and oxidative markers, was quantified according to a modified lowry method and reported in micrograms per milliliter (µg/mL) [8]. The enzymatic activity of SOD was assessed by its capacity to inhibit the reduction of nitroblue tetrazolium (NBT), and the outcomes were normalized to protein content and expressed as units per milligram of protein (U/mg protein). [9]. CAT performance was assessed according to Aebi's method, with enzymatic efficiency reported relative to protein content (k/g protein) [10].

Table 1. Body weight of rats

Groups (n = 40)	Pre-experimental weight (g)	Post-experimental weight (g)
Control	234.4000 ± 13.23463	245.1000 ± 14.74562
HA	221.6667 ± 26.97684	229.1111 ± 22.01388
CU	230.8889 ± 19.19925	229.7778 ± 17.88000
CU+ HA	224.2500 ± 12.34909	222.7500 ± 16.26346

Pre-experimental and post-experimental body weights (g) of rats in each group. Values are presented as mean ± standard deviation (SD). No statistically significant differences were observed between the groups ($p > 0.05$).

Table 2. Comparison of the serum biochemical parameters among the study groups

Groups (n = 40)	AST (U/L)	ALT (U/L)	Cu (ppm)	Ceruloplasmın (g/L)
Control	137 (121-183)	40 (32-54)	1.21 (0.50-1.93)	0.41 (0.32-0.52)
Cu	155 (116-201)	54 (39-64)	1.74 (1.33-3.25)	0.29 (0.21-0.39)
HA	127 (89-168)	38 (31-45)	1.43 (0.92-2.08)	0.36 (0.25-0.54)
HA + Cu	122 (107-156)	35 (32-51)	1.12 (0.56-2.17)	0.35 (0.23-0.42)
p	0.070	0.017	0.070	0.038

Comparison of serum biochemical parameters AST, ALT,Cu and ceruloplasmin among the experimental groups. Data are expressed as median (minimum–maximum). Statistical significance was assessed using the Kruskal-Wallis test followed by post hoc analysis. A p-value < 0.05 was considered statistically significant.

Table 3. Biochemical and oxidative stress parameters in study groups

Study Groups (n=40)	MDA (nmol/g tissue)	GSH (µmol/g tissue)	SOD (U/mg tissue)	CAT (k/g protein)	GPx (U/mg protein)	TAS (expressed in Trolox equivalents/L	TOS (µmol/g tissue)	OSI (AU)
Control	34.50 (23.64–50.6)	53.7 (46.93–73.5)	1.60 ± 0.09	434.5 ± 41.47	51.7 (44.1–76.15)	1.10 ± 0.15b	0.10 ± 0.11	0.90 ± 0.17
Cu	41.40 (36.23–99.41)	39.0 (36.04–50.8)	1.50 ± 0.05	332.0 ± 60.03	44.00 (25.97–56.39)a	0.81 ± 0.16a	1.70 ± 0.53	2.30 ± 1.07
HA	36.30 (30.84–48.24)	54.20 (45.87–58.2)	1.60± 0.03	417.20 ± 48.20	49.80 (40.17–64.51)	0.97 ± 0.19	0.78 ± 0.24	0.86 ± 0.42
HA+Cu	40.50 (31.91–48.26)	46.80 (38.93–0.57)	1.5 ± 0.05	379.60 ± 38.80	53.80 (30.95–70.44)	0.97 ± 0.14	1.50 ± 0.52	1.01 ± 0.34

Data are presented as mean ± standard deviation or median (min–max), depending on distribution. Parameters include MDA, GSH, SOD, CAT, GPx, TAS, TOS, and OSI. Statistical analysis was performed using one-way ANOVA or Kruskal-Wallis test where appropriate. Superscript letters indicate significant differences compared to the control group (a: $p<0.05$, b: $p>0.05$)

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Table 4. Histological findings and apoptotic marker expression in liver tissues

Groups (n = 40)	Hepatocyte degeneration score Median (Min–Max)	Caspase-3 positive hepatocyte score Median (Min–Max)
Control	0 (0–1)	0 (0–2)
Cu	2 (0–3)	0 (0–6)
HA	0 (0–2)	0 (0–2)
HA + Cu	2 (0–3)	0 (0–6)

Hepatocyte degeneration scores and Caspase-3 positive hepatocyte scores are presented as median (minimum–maximum) values for each group.

GPx activity was determined using the method developed by Paglia and Valentine, and values were given in U/mg protein [11]. Glutathione (GSH) concentrations were measured by the Ellman technique and reported in micromoles per gram of tissue (µmol/g), based on a standard calibration curve [12].

TAS was assessed according to the method proposed by Erel and expressed as Trolox equivalent per liter (mmol Trolox eq./L). Total oxidant status (TOS) was quantified using Erel's automated colorimetric approach. The oxidative stress index (OSI) was calculated by dividing TOS by TAS and expressed in arbitrary units (AU) [13; see table 3].

Liver tissues were initially fixed with 10% NBF and then dehydrated through a stepwise ethanol gradient ranging from 70% to 99% the specimens were then cleared using xylene and embedded in paraffin wax. From the resulting paraffin blocks, Sections of 4 µm thickness were obtained from the paraffin blocks, placed onto microscope slides, and subsequently stained with Hematoxylin and Eosin (H&E) for histological analysis. Hepatocellular degeneration was assessed semi-quantitatively across 10 randomly selected microscopic fields using A semi-quantitative scoring scale was employed: 0 indicating no alteration, 1 for mild, 2 for moderate, and 3 for severe changes [14].

Immunohistochemical Analyses In paraffin deparaffinized sections, antigen retrieval was conducted in citrate buffer. Endogenous peroxidase activity was inhibited prior to incubation of the tissue sections with caspase-3 primary antibody (Santa Cruz Biotechnology, Inc.). Next, labeling was performed with biotinylated secondary antibody, streptavidin peroxidase, and AEC substrate. After counterstaining with hematoxylin, slides were evaluated. Cells exhibiting positive immunoreactivity for caspase-3 were identified by brown cytoplasmic staining Immunoreactivity scoring was based on cell density (% 0–100) and staining intensity (0–3); the total score was the product of these two parameters [15].

Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 26.0 (SPSS Inc., Chicago, IL, USA). Normality of the quantitative data was evaluated via the Kolmogorov–Smirnov and Shapiro–Wilk tests. For datasets exhibiting normal distribution, intergroup comparisons were performed through one-way ANOVA. In cases requiring post hoc analysis, Tukey's HSD or Tamhane's T2 test was utilized, depending on the homogeneity of variances.

In cases where data did not meet normality criteria, the Kruskal-Wallis H test was applied to examine differences between groups, followed by Bonferroni correction. The Mann-Whitney U test was used for pairwise comparisons. Parametric data are expressed as mean±standard deviation, and nonparametric results are expressed as medians with interquartile ranges.

Results

As illustrated in Table 2, serum ALT and AST concentrations were significantly elevated in the $CuSO_4$-treated group, exhibiting a marked difference when compared with the control group ($p < 0.05$). However, in the $CuSO_4$ + HA group, these elevations were markedly reduced. The HA group showed values comparable to the control. Likewise, serum Cu concentrations showed a marked increase in the $CuSO_4$-treated group ($p < 0.01$), whereas they were noticeably diminished in the $CuSO_4$ + HA group ($p < 0.05$), suggesting a potential chelating effect of HA. Ceruloplasmin levels experienced a significant decline following $CuSO_4$ administration but demonstrated partial recovery upon HA co-treatment. Hepatic dry Cu accumulation was most pronounced in the $CuSO_4$ group and was substantially alleviated in rats receiving both $CuSO_4$ and HA($p < 0.05$, table 2).

Oxidative stress markers such as MDA and TOS were markedly elevated in the $CuSO_4$-treated group ($p < 0.01$), reflecting increased oxidative burden. In contrast, antioxidant indicators including TAS, GSH, SOD, CAT, and GPx exhibited a significant reduction ($p < 0.01$), as presented in table 3 and graphic 1. HA treatment normalized these parameters in the $CuSO_4$ + HA group, supporting its antioxidant role. These alterations are visually presented in Figure 1, demonstrating group-wise differences across oxidative stress markers.

In the H&E-stained liver sections, the control group exhibited normal hepatic architecture (figure 1a). The hepatic cords displayed an orderly arrangement, with hepatocytes exhibiting distinct morphological features, including eosinophilic cytoplasm and euchromatic nuclei. The HA-treated group showed a histological appearance largely comparable to the control group, with only mild alterations such as occasional hydropic changes in hepatocytes (figure 1b).

In contrast, animals exposed to Cu exhibited a marked rise in hepatocytes showing hydropic degeneration, evidenced by cellular enlargement, pale-staining cytoplasm, and a finely granular appearance. Additionally, numerous hepatocytes

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in this group displayed pyknotic nuclei accompanied by intensely eosinophilic and condensed cytoplasm. Hepatic cord disorganization or complete disruption was also evident (Figure 1c). In the $CuSO_4$+HA group, although slight amelioration was observed in some cases, histopathological changes remained largely consistent with those seen in the Cu-only group (Figure 1d).

Immunohistochemical Findings. Apoptotic activity was evaluated through immunohistochemical staining using an antibody specific for cleaved caspase-3. Similar histological features were observed in the control and HA groups only a small number of caspase-3-positive hepatocytes were identified, primarily located near the central veins (Figures 2a and 2b). Conversely, the Cu-treated group showed a significant increase in caspase-3-positive hepatocytes relative to both the control and HA-treated groups (Figure 2c). Similarly, the $CuSO_4$ and HA group demonstrated a comparable pattern of caspase-3 immunoreactivity to that observed in the Cu group (Figure 2d). A detailed summary of the histopathological findings across all groups is presented in Table 4.

Discussion

Hepatotoxicity caused by heavy metals has emerged as a significant public health issue, particularly due to the rapid advancement of industrial technologies. Modern medicine has focused on identifying effective and safe agents to treat liver diseases caused by hepatotoxicity, which remains a significant concern, and numerous studies have explored the hepatoprotective potential of natural compounds, yielding encouraging outcomes. Cu is a vital trace mineral involved in a variety of physiological functions such as the synthesis of connective tissue, melanin production, neurotransmitter formation, and maintenance of antioxidant defense systems. WD is an inherited disorder caused by mutations in the ATP7B gene, impairing hepatic copper excretion through bile and resulting in pathological copper accumulation within the body. This results in increased levels of free Cu, accompanied by reductions in glutathione and other antioxidants leading to elevated ROS and LPO [16-18].

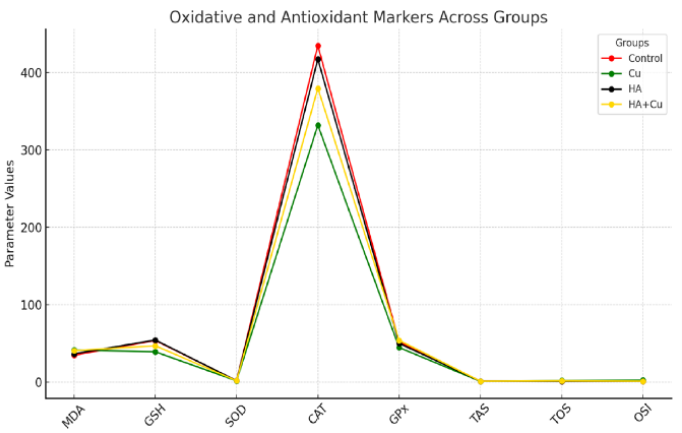
The current therapeutic management of WD centers on the use of Cu-excreting compounds, such as D-penicillamine and trientine, aimed at decreasing serum Cu levels. However, these agents are not suitable for all patients due to high cost, limited accessibility, and potential adverse effects [19]. As a result, the need for safer and more effective alternative chelating agents has become increasingly evident.

In an experimental murine model, Kumar et al. explored the correlation between the dosage and exposure period of high-concentration $CuSO_4$, and found that the liver exhibited the greatest accumulation of Cu compared to other organs [20]. Similar animal models have linked Cu exposure with hepatotoxicity, oxidative stress, lipid peroxidation, and mitochondrial dysfunction, contributing not only to our understanding of orally induced Cu toxicity but also to the cellular damage mechanisms in inherited disorders like WD [21].

Liver Cu accumulation significantly elevates levels of lipid peroxidation products (LPO) such as MDA, which is clearly observable in both serum and liver tissue homogenates [22]. Experimental studies by Kumar et al. demonstrated that $CuSO_4$ exposure increases hepatic oxidative stress by reducing GSH and total antioxidant capacity (TAC), while elevating MDA levels [20]. The most substantial decline in TAC and GSH levels was observed in hepatic tissues. Notably, hepatic TAC and GSH levels demonstrated an inverse correlation with serum transaminases, bilirubin, and tissue-free copper levels, while showing a positive association with MDA concentrations.

These findings suggest that Cu accumulation in WD may promote liver damage by triggering redox imbalance and enhancing the release of pro-inflammatory cytokines and excitatory neurotransmitters such as glutamate. Moreover, excessive Cu levels have been shown to diminish the activity of critical antioxidant enzymes, including SOD, GPx, and CAT, which in turn leads to elevated intracellular ROS and intensified oxidative injury [23]. This impairment undermines the cellular redox balance and negatively impacts liver functions.

In the present study, the $CuSO_4$-treated group exhibited significantly elevated serum ALT and AST levels, indicating hepatic injury (Group I, Table 2), along with elevated MDA and TOS in liver tissues. Conversely, total TAS, GSH, SOD, CAT, and GPx levels (Table 3, Figure 1, Graphic 1). These were significantly decreased in alignment with literature reporting the oxidative stress-related harmful effects of Cu. These findings are consistent with those reported by Kumar et al and Fuentealba et al [20,24].



Graphic 1. Colored line graph showing group-wise variation in liver tissue markers

Mitochondrial dysfunction is recognized as a key consequence of copper toxicity. A recently identified form of cell death, known as cuproptosis, involves the binding of copper ions to mitochondrial tricarboxylic acid cycle enzymes, leading to

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proteotoxic stress and direct disruption of mitochondrial activity [25]. Caspase-3 and 9 are key mediators of the mitochondrial apoptotic pathway, and their upregulation indicates the activation of apoptosis triggered by Cu toxicity. Moreover, Cu accumulation inhibits elements of the mitochondrial respiratory chain, leading to reduced ATP production, collapse of membrane potential, and severe disruption in energy metabolism [26].

These mechanisms align with previously reported effects of Cu on apoptosis [27]. Stated that Cu enhances free radical production, promoting lipid peroxidation and activating apoptotic signaling. Oe S, et al. found that Cu exposure in hepatocytes induces endoplasmic reticulum stress, triggering the caspase cascade[28]. Similarly, showed that 28-day $CuSO_4$ administration in mice significantly increased caspase-9 and caspase-3 mRNA expression and enzymatic activity, confirming mitochondrial apoptosis involvement[29].

In our study, $CuSO_4$ exposure significantly upregulated caspase-3 and caspase-9 expression in liver tissues (Table 4, Figure 2). Whereas co-administration of HA notably reduced these levels. These results suggest that HA may have not only antioxidant but also anti-apoptotic regulatory potential. The elevated density of caspase-3-positive hepatocytes observed in the Cu group was markedly reduced following HA administration. Considering the role of ATP7B protein in Cu excretion, it is plausible that ATP7B-associated mechanisms may also contribute to these apoptotic effects.

Indicators of oxidative stress, including MDA levels, the activities of SOD, CAT, and GPx, as well as GSH concentrations, serve as crucial biomarkers for evaluating redox status of the antioxidant defense system, that preserve cell membrane integrity. During oxidative stress, the oxidant-antioxidant balance is disrupted. SOD functions as a primary antioxidant defense by transforming superoxide radicals into hydrogen peroxide (H_2O_2), whereas GPx is crucial in further detoxifying H_2O_2 into harmless compounds and is regarded as more efficient than CAT in this detoxification pathway [30]. GSH, the most vital non-enzymatic intracellular antioxidant, also functions as a substrate for several enzymes including GPX. A decrease in GSH levels exacerbates oxidative stress [31].

Genetic models have shown that oxidative stress is a principal pathophysiological feature of Cu toxicity[32]. A marked reduction in GSH levels alongside elevated MDA concentrations was reported in LEC rats and toxic milk mice, reflecting oxidative stress triggered by Cu accumulation [20]

In the present study, the Cu-treated group demonstrated a significant rise in MDA and TOS levels, accompanied by marked reductions in TAS, SOD, CAT, GPx, and GSH levels, providing strong evidence for the induction of oxidative stress. These changes in antioxidant parameters confirmed the disruption of redox balance. Furthermore, in the group receiving HA along with Cu, these parameters approached normal levels, demonstrating HA's role in supporting antioxidant defense. A significant reduction in both serum and tissue Cu levels was also observed in this group, reinforcing HA's Cu chelating potential. These results align with earlier research indicating the hepatoprotective potential of HA [33].

HA is a naturally derived, non-toxic organic macromolecule that exists abundantly in soil and aquatic environments. Due to its carboxyl and phenolic groups, it exhibits strong metal ion binding and chelating capabilities [34,35]. Its antioxidant capacity varies with pH conditions reported that HA can reduce Cu^{2+} and Fe^{3+} ions and scavenge reactive oxygen species [36]. According to Klein et al., the antioxidant properties of HA are attributed to the combined contributions of its phenolic and non-phenolic components [37]. Additionally, HA's antibacterial, antiviral, antitoxic, and anti-inflammatory properties have been documented [2]. Our findings confirm that HA mitigates Cu-induced oxidative stress through its dual antioxidant and chelating functions. In the group treated with Cu and HA, liver oxidative stress caused by Cu-induced lipid peroxidation was significantly reduced, supporting HA's antioxidant efficacy [38]. Declines in the enzymatic activities of SOD, GPx, and CAT are likely attributable to excessive ROS production and impaired antioxidant capacity due to Cu-related tissue damage.

Histopathological evaluation remains widely regarded as the definitive method for evaluating tissue injury caused by toxic agents. Cu exposure led to a pronounced increase in hepatocytes exhibiting hydropic degeneration. In the Cu-treated group, hepatic cells displayed pyknotic nuclei, dense and deeply eosinophilic cytoplasm, and disrupted hepatic cord architecture. Similar hepatotoxic changes were prominently observed in the study by Kumar V et al. [20].

Our histopathological findings align with the literature: while hydropic degeneration and caspase-3-positive cells were increased in the Cu group, co-treatment with HA significantly reduced both serum and tissue Cu levels, decreased oxidative stress markers, enhanced antioxidant defense parameters, and alleviated histopathological damage. Caspase expression was also significantly downregulated. These results can be attributed to HA's strong Cu-complexing capacity and its potent antioxidant effects [39,40].

Conclusion

In our experimental model of Cu-induced liver toxicity in mice, the potential chelating and antioxidant properties of HA in mitigating copper accumulation were examined. The potential chelating and antioxidant properties of HA in mitigating copper accumulation were examined. The study demonstrated that oxidative stress caused by Cu was confirmed through both biochemical and histopathological analyses. HA administration significantly reduced Cu accumulation in liver tissues, ameliorated oxidative stress and apoptotic markers, and notably decreased caspase expression levels.

Overall, HAs are considered a promising option in models of

intoxication due to their protective and therapeutic effects via antioxidant, adsorptive/chelating, and anti-inflammatory mechanisms. However, especially for human health applications, Additional studies are required to clarify the optimal dosage, formulation, source, and safety profile of HA for therapeutic use. These findings should be supported by clinical trials, and existing reviews emphasize the importance of further studies in this area.

Conflict of Interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

This study was carried out with the support of İnönü University Scientific Research Projects Coordination Office.

Ethical Approval

The study was approved by the Inonu University Experimental Animal Ethics Committee (Date: 11/04/2022, Decision No: 2022/5-1, HAYBIS No: 14700).

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