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Research Article

Mechanistic Insights into the Mitigating Role of Beta-Caryophyllene on Cadmium-Induced Liver Injury

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Abstract

Cadmium (Cd) is a toxic heavy metal that can cause severe hepatotoxicity in the body. This study investigates the hepatoprotective effects of Beta-Caryophyllene (BCP), a natural sesquiterpene, against Cd-induced liver injury. In the study, the protective effects of BCP on biological processes such as oxidative stress, inflammation and apoptosis were investigated through TLR4/NF-κB, SIRT1/KEAP1/Nrf2/HO-1 and Bax/Bcl-2/Caspase3 signaling pathways. For this purpose, 50 male rats were divided into 5 groups: Control, Cd, BCP100+Cd, BCP200+Cd and BCP200, 10 in each group. At the end of the study, it was determined that Cd exposure caused damage to cells by increasing lipid peroxidation, oxidative stress and inflammation in liver tissue. However, BCP treatment was found to reduce oxidative damage by increasing antioxidant enzyme activities (SOD, GSH, CAT) and reducing lipid peroxidation (MDA). Furthermore, it was determined that BCP lowered proinflammatory cytokine levels (TNF-α, IL-1β) by inhibiting TLR4/NF-κB signaling activity, while also increasing anti-inflammatory IL-10 levels. It was also observed that BCP inhibits the suppression of Nrf2 through KEAP1 by activating the Nrf2 signaling pathway, resulting in elevated levels of SIRT and HO-1. In the analyses of apoptosis, it was determined that BCP inhibited Caspase3 activity and reduced apoptosis in liver cells by balancing the Bax/Bcl-2 ratio. These findings suggest that BCP provides potent protection against Cd-induced liver toxicity by regulating various signaling pathways and could potentially be used as a hepatoprotective agent.

Keywords: Apoptosis, Beta Caryophyllene, Cadmium, Hepatotoxicity, Inflammation, Oxidative stress

Introduction

Cadmium (Cd) is one of the heavy metals that has no physiological function, such as arsenic, lead, mercury and chromium, and is considered toxic to human health [1-4]. Smokers are 3-4 times more exposed to Cd than non-smokers. The amount of Cd ingested through food usually varies between 10-25 µg, but this value can be much higher in contaminated areas [5-7]. The accumulation of this metal in the body, the production of free radicals, leads to peroxidation in cell membranes, disruption of DNA repair mechanisms and cellular changes, causing serious damage to the body [8,9]. Cd triggers harmful biological processes in liver cells, such as oxidative stress, inflammation, and apoptosis. Furthermore, Cd has been shown to suppress antioxidant defense mechanisms by increasing the production of reactive oxygen species (ROS), trigger inflammation by activating the TLR4/NF-κB signaling pathway, and weaken cellular protection mechanisms by inhibiting the Nrf2/HO-1/SIRT1 signaling pathway via KEAP1 [10,11].

Beta-caryophyllene (BCP) is an herbal sesquiterpene noted for its powerful antioxidant, anti-inflammatory, and antiapoptotic properties [12,13]. BCP, a variety of herbal it is abundant in springs, especially in herbs such as black pepper, cloves, lavender, thyme and rosemary. In particular, CB2 is known to exert anti-inflammatory and antioxidant effects by interacting with cannabinoid receptors [14]. BCP has been shown to have the potential to protect cells by reducing oxidative stress and suppressing inflammatory signaling pathways [13].

It is thought that BCP may have a protective effect against oxidative stress, inflammation and apoptosis that occur in Cd-induced liver damage via TLR4/NF-κB, KEAP1/Nrf2/HO-1/SIRT1 and Bax/Bcl-2/Cas3 pathways.

Materials and Methods

Ethical Approval

Ethics committee approval for this study was obtained from Atatürk University Animal Experiments Local Ethics Committee (Ethical No: 2025/57).

Chemicals

CD (≥99%) (CAS No: 7440-43-9) and BCP (≥80%) (CAS No: 87-44-5) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). ELISA kits were obtained from SunRed.

Animals

The Sprague Dawley rats used in this study were provided by Atatürk University Experimental Research and Application Center (ATADEM). The average weight of each of the 50 male rats selected for the study was between 200-250 g. A power analysis program was used to determine the number of animals per group (G-Power 3.1.9.7). A minimum of 10 rats per group and a total of 50 rats were calculated to require 99% power (Type II error, β) with an error of 0.05 (Type I, α). Data from a previous study were used for this analysis *(Fig. 1)* [15].

Experimental Design

Prior to the start of the experiment, all rats were weighed and randomly assigned to one of five groups: Group I (Control): Intragastric (IG) administration of 1 mL ddH_2O for 7 days. Group II (CD): Intraperitoneal (IP) administration of Cd at a dose of 6.5 mg/kg [15] for 7 days. Group III (BCP100+Cd): Administration of BCP at a dose of 100 mg/kg [16,17] IG, followed by administration of Cd at the 6.5 mg/kg dose for 7 days. Group IV (BCP200+Cd): Administration of BCP at a dose of 200 mg/kg [16,17] IG, followed by administration of Cd at the 6.5 mg/kg dose for 7 days. Group V (BCP200): IG administration of BCP at a dose of 200 mg/kg/day for 7 days. For Groups III and IV, BCP was administered 30 min before Cd each day. On the 8th day, the rats were weighed again and then euthanized under sevoflurane anesthesia. Liver tissues were harvested, weighed, rinsed with physiological saline, and stored at -80°C for subsequent analyses

Oxidative Parameters and Antioxidant Enzymes Analysis

Oxidative parameters and antioxidant enzyme activities were analyzed with an ELISA plate reader (Bio-Tek, Winooski, VT, USA) capable of measuring absorbance at a wavelength of 450 nm. Using previously obtained supernatants, malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels were determined in accordance with the guidelines of the relevant ELISA kits [18].

Inflammation Markers Analysis

Interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), nuclear factor kappa-B (NF-κB) and Toll-like receptor 4 (TLR4) levels in supernatants were evaluated in accordance with the protocols of the relevant ELISA kits.

Histopathological Examination

When the study was completed, the liver tissues were fixed in 10% buffered formalin solution. The tissues were then followed with routine tissue and embedded in paraffin. Five μm thick sections were taken from the paraffin blocks. The sections were stained with Hematoxylin Eosin (H&E) and Masson's Trichrome (MT). The stained sections under a light microscope were examined and visualized (Leica, Flexacam i5, Germany). The levels of degeneration and necrosis in hepatocytes were examined in H&E staining, while the level of fibrosis in the tissue was determined in MT staining. The findings were evaluated as absent (No cells were affected) (0), mild (1-5 cells affected) (+1), moderate (6-15 cells affected) (+2) and severe (15< cells affected) (+3) [19].

Immunohistochemical Examination

After routine rehydration and deparaffinization of the sections taken on adhesive slides for immunohistochemical examinations, endogenous peroxidase was inactivated by

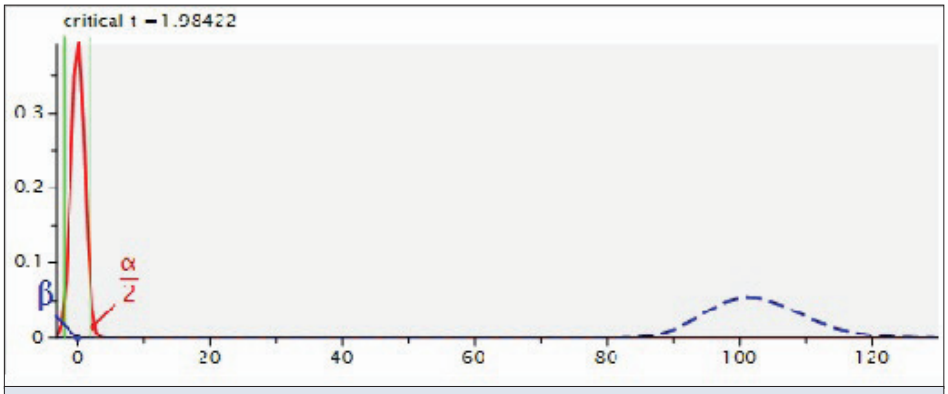

Fig 1. G-Power analysis findings to determine the number of animals in groups

keeping the tissues in 3% H_2O_2 for 10 min. The tissues were then boiled in 1% antigen retrieval solution (TRIS EDTA buffer (pH+6.1) 100X). Sections were incubated with protein block for 5 min to prevent nonspecific background staining. The tissues were then incubated with primary antibody (SIRT1 Cat No: BT-AP07980, Reconstitution Ratio: 1/100; Keap1 Cat No: E-AB-60460, Reconstitution: 1/200, US; Bcl2 Cat No: sc-7382, Reconstitution Ratio: 1/100, US) were added and incubated according to the instructions for use. 3-Amino-9-Ethylcarbazole (AEC) chromogen was used as chromogen in the tissues. Stained sections were examined by light microscopy (Leica, Flexacam i5, Germany). ImageJ analysis software was used to determine the intensity of immunopositivity in immunohistochemical analysis [20].

Immunofluoresence Examination

For immunofluorescence examination, tissue sections taken on adhesive slides were deparaffinized and rehydrated. The tissues were then boiled in 1% antigen retrieval solution (TRIS EDTA buffer (pH+6.1) 100X). Sections were incubated with protein block for 5 min to prevent nonspecific background staining. The tissues were then treated with primary antibody (Nrf2 Cat. No: E-AB-68254, Reconstitution Ratio: 1/200, US; HO-1 Cat No: E-AB-66079, Reconstitution: 1/200, US; BAX Cat No: sc-7480, Reconstitution Ratio: 1/100, US; Caspase 3 Cat No: sc-56053, Reconstitution Ratio: 1/100, US) were added and incubated according to the instructions for use. Immunofluorescence secondary antibody was used as secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/1000) and kept in the dark for 45 minutes. Then, DAPI with mounting medium (Cat no: D1306, Diluent Ratio: 1/200 UK) was added to the sections and kept in the dark for 5 min and the sections were covered with coverslips. The stained tissues were examined under a fluorescence attachment microscope (Zeiss AXIO, Germany). ImageJ analysis software was used to determine the intensity of immunopositivity in immunoforesence analysis [21].

Statistical Analysis

Statistical analyses of the study data were calculated in GraphPad Prism 8.0.2 software and $P<0.05$ was considered significant. In histopathologic analyses, nonparametric Kruskal-Wallis test was used to determine group interaction and Mann Whitney U test was used to determine the differences between groups

Results

Effect of Cd and BCP Applications on Starting, Ending and Liver Weights of Rats

At the outset of the study, there were no discernible differences in initial body weights among the experimental groups. Upon completion of the experiment, a reduction in body weight was observed across all groups subjected to Cd intoxication and subsequent treatment. The animals in the control group specifically exhibited significantly greater body weights compared to those in the Cd-treated groups. In terms of liver weight, the Cd group exhibited the highest liver weight, followed by the BCP100+Cd group, which also showed a relatively higher liver weight compared to the other three groups. The liver weights in the remaining groups were comparatively lower, though this disparity did not achieve statistical significance *(Table 1)*.

Table 1. *Effects of Cd and BCP on the starting, ending, and liver weights of rats. Weight of organ in g/kg body weight in the experimental groups (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Weight of Liver (g/kg body weight)
Control	0.47±0.02^{b}
CD	0.58±0.01^{a}
BCP100+CD	0.53±0.01^{c}
BCP200+CD	0.49±0.03^{bc}
BCP200	0.44±0.02^{b}

a, b, c: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Oxidative Stress Parameters in Liver Tissue

MDA levels were significantly increased in the Cd group compared to the control group. However, it was found that this increase was significantly suppressed in the BCP200+Cd group and MDA levels were found to be close to the control group. SOD, GSH and CAT activity was significantly decreased in the Cd group compared to the control group, but a statistically significant increase was observed in the BCP200+Cd group *(Table 2)*.

Parameters Related to Inflammation in Liver Tissue

TNF-α, IL-1β, NF-κB and TLR4 levels showed a significant increase between the Cd group and the control group. However, the BCP200+Cd group significantly suppressed this increase by significantly lowering levels.

Table 2. *Illustrates the levels of oksidan and antioksidant mediators MDA, SOD, GSH, and CAT in liver tissues (n = 10). One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	MDA	SOD	GSH	CAT
Control	17.45±1.37^{b}	28.51±1.76^{b}	391.15±13.56^{b}	34.25±2.05^{b}
CD	32.07±2.22^{a}	16.51±1.21^{a}	302.95±11.24^{a}	13.79±2.94^{a}
BCP100+CD	28.24±1.71^{a}	20.14±1.36^{a}	331.29±7.79^{c}	20.51±1.47^{c}
BCP200+CD	20.93±1.4^{c}	26.17±2.22^{b}	360.87±11.24^{d}	29.59±2.77^{b}
BCP200	16.8±1.05^{b}	30.01±0.93^{b}	395.04±10.34^{b}	34.64±2.95^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Table 3. *Illustrates the levels of inflammatory mediators TNF-α, IL-1β, IL-10, NF-κB, and TLR4 in liver tissues. One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	TNF-α	IL-1β	IL-10	NF-κB	TLR4
Control	229.4±20.66^{b}	960.33±54.31^{b}	111.35±4.71^{b}	1.76±0.34^{b}	1.85±0.42^{b}
CD	457.4±31.88^{a}	1662.33±71.85^{a}	79.51±2.46^{a}	3.53±0.18^{a}	4.22±0.55^{a}
BCP100+CD	386.88±25.08^{c}	1383.66±87.12^{c}	91.78±3.83^{c}	3.05±0.2^{c}	3.27±0.26^{c}
BCP200+CD	304.17±32.61^{d}	1137±39.59^{d}	107.57±4.86^{b}	2.19±0.25^{b}	2.21±0.68^{d}
BCP200	216.57±24.12^{b}	997.66±82.49^{b}	115,24±4.78^{b}	1.85±0.15^{b}	1.81±0.35^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

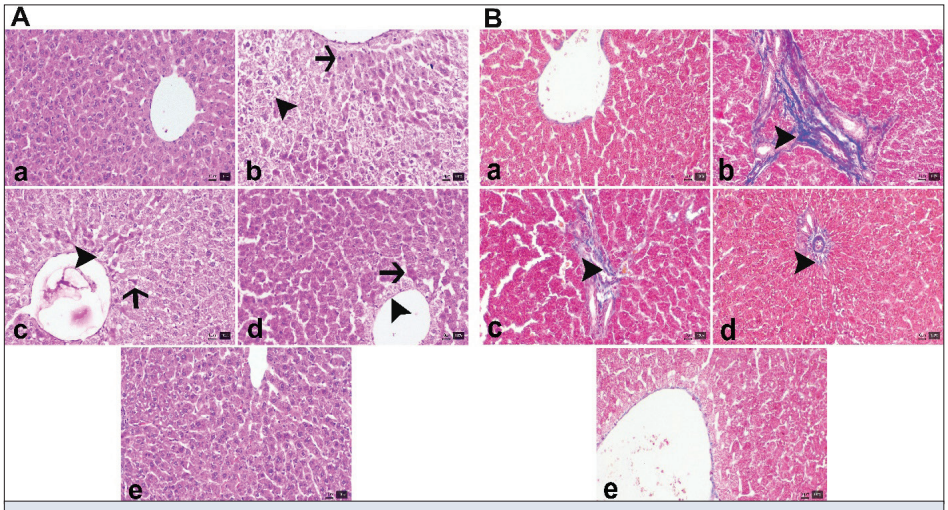

Fig 2. Liver tissue. **A)** control (a), Cd (b), BCP100+Cd (c), BCP200+Cd (d) and BCP200 (e). Degeneration in hepatocytes is shown by *arrowheads* and necrosis by *arrows* (H&E); **B)** Control (a), Cd (b), BCP100+Cd (c), BCP200+Cd (d) and BCP200 (e). Fibrosis is shown by arrowheads. MT, Bar:10μm, Objective:20X, Zoom:100%

Table 4. *Scoring of degeneration and necrosis observed in hepatocytes and statistical analysis data. Scoring of fibrosis determined in liver tissue and statistical analysis data. Kruskal-Wallis and Mann Whitney U test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Degeneration	Necrosis	Fibrosis
Control	0.4±0.48^{b}	0±0^{b}	0±0^{b}
CD	3±0^{a}	2.8±0.4^{a}	2.6±0.49^{a}
BCP100+CD	2.2±1.4^{ab}	1.6±0.49^{c}	1.2±0.4^{c}
BCP200+CD	1.4±0.49^{c}	0.4±0.49^{d}	0.6±0.49^{d}
BCP200	0.2±0.4^{b}	0±0^{b}	0±0^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

A significant decrease in IL-10 levels was observed in the Cd group compared to the control group, but BCP200+Cd administration significantly reversed this decrease and increased IL-10 levels *(Table 3)*.

Histopathological and Masson's Trichrome Findings

In histopathological and MT analysis of liver tissues, mild degeneration of hepatocytes was observed in Control and BCP200. In only Cd group, severe degeneration and necrosis in hepatocytes and severe fibrosis were detected in the centrilobular region of the liver. These findings were significantly decreased in BCP100+Cd and BCP200+Cd groups compared to Cd group at dose-dependent level. Histopathologic findings are shown in *Fig. 2-A*, MT findings in *Fig. 2-B* and scoring of these findings and statistical analysis data are presented in *Table 4*.

Immunohistochemical Findings

Immunohistochemical analysis revealed severe Bcl-2 and SIRT1 expression in hepatocytes of Control and BCP200 groups, while very mild Keap1 expression was detected. In Cd, Bcl-2 and SIRT1 expressions were observed at mild level, while Keap 1 expressions were detected at severe level. These values were found to be significantly closer to normal levels in BCP100+Cd and BCP200+Cd groups compared to Cd group. SIRT1 expression is shown in

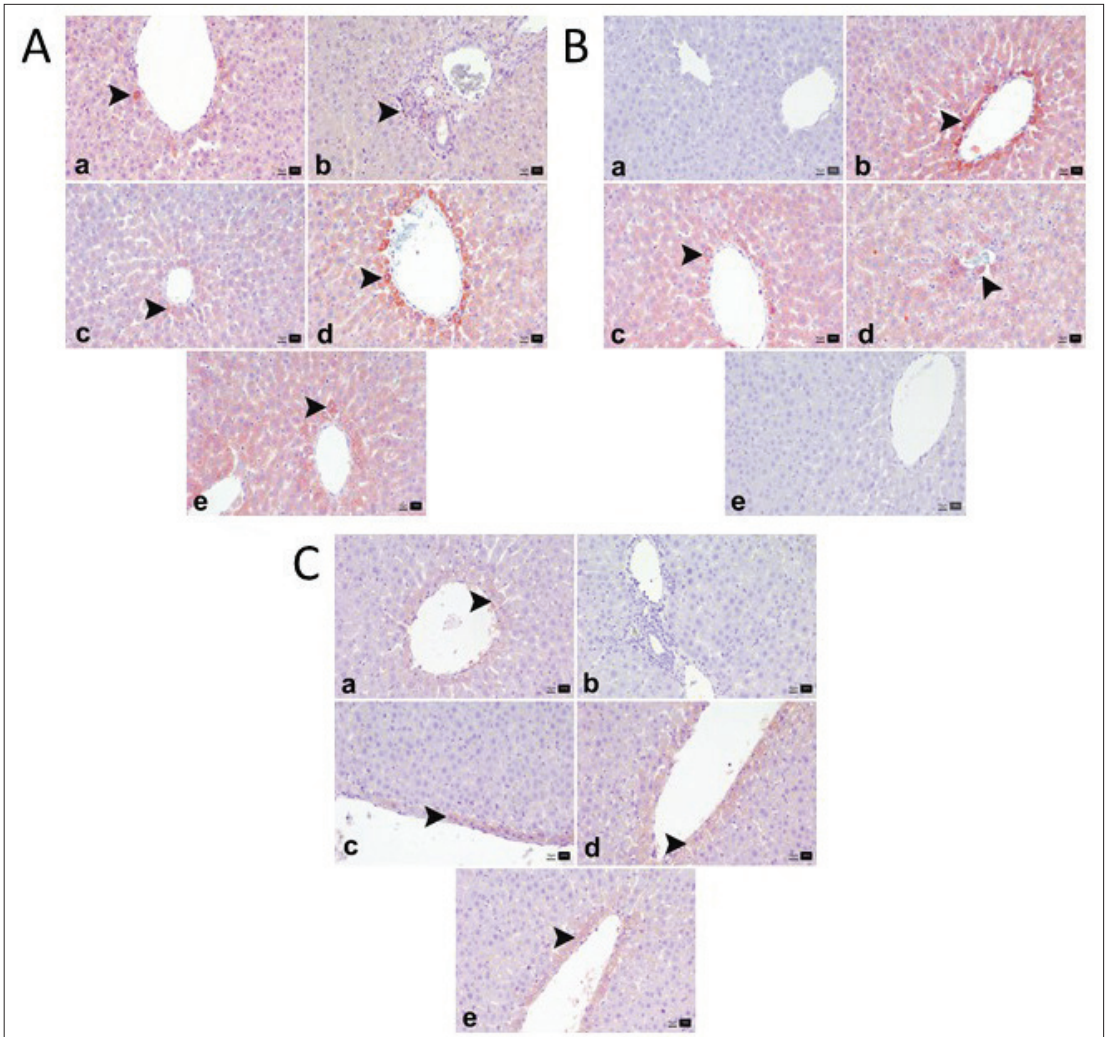

Fig 3. Liver tissue. **A)** control (a), Cd (b), BCP100+Cd (c), BCP200+Cd (d) and BCP200 (e). SIRT1 expressions are shown

Table 5. *Immunohistochemical staining results and statistical analysis data. One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Bcl-2	Sırt1	Keap1
Control	8.37±0.84^{b}	6.48±0.42^{b}	0.38±0.12^{b}
CD	0.71±0.56^{a}	0.94±0.26^{a}	20.16±2.27^{a}
BCP100+ CD	2.92±0.35^{c}	2.75±0.25^{c}	10.9±1.46^{c}
BCP200+ CD	6.16±0.66^{d}	7±0.39^{d}	4.46±1.31^{d}
BCP200	8.39±0.58^{b}	10.12±0.55^{b}	0.41±0.17^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Fig. 3-A, Keap1 expression in *Fig. 3-B* and Bcl2 expression in *Fig. 3C*. Immunohistochemical staining results and statistical analysis data are presented in *Table 5*.

Immunofluoresence Findings

In immunofluorescence analysis, severe Nrf2 and HO-1 expressions were observed in hepatocytes of Control and BCP200 groups, while very mild Bcl-2 and Caspase 3 expressions were detected. In Cd, mild Nrf2 and HO-1 expressions were observed, while severe Bcl-2 and Caspase 3 expressions were detected. These values were found to be significantly closer to normal levels in BCP100+Cd and BCP200+Cd groups compared to Cd group. Nrf2 expression is shown in *Fig. 4-A*, HO-1 expression in *Fig. 4-B*, BAX expression in *Fig. 5-A* and Caspase 3 expression in *Fig. 5-B*. Immunofluorescence staining results and statistical analysis data are presented in *Table 6*.

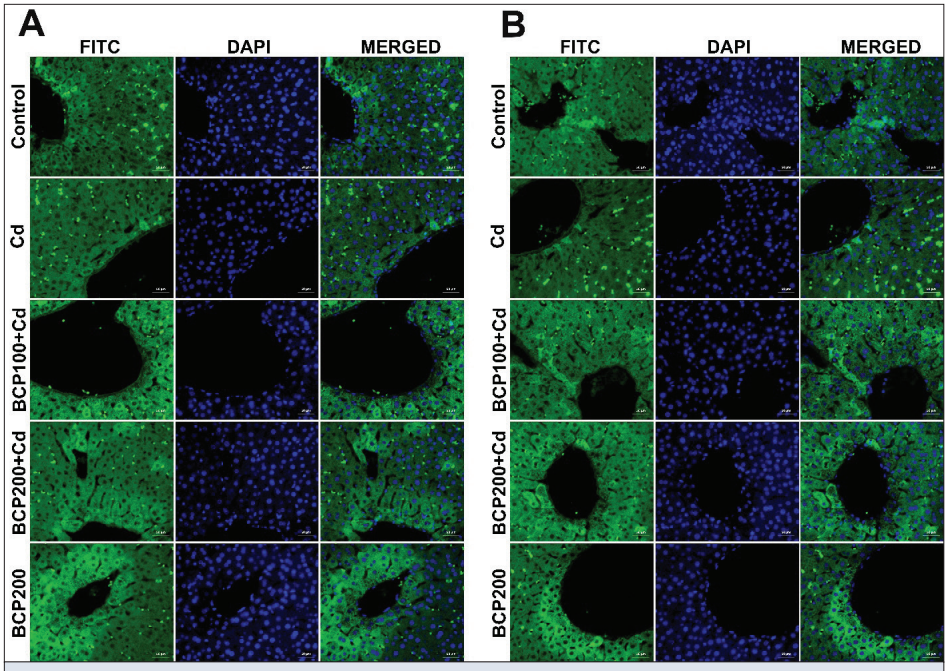

Fig 4. Liver tissue. **A)** Nrf2 expressions in hepatocytes (FITC); **B)** HO-1 expressions in hepatocytes (FITC). DAPI: nuclear staining. MERGED: Merge of images. IF, Bar: 50 µm

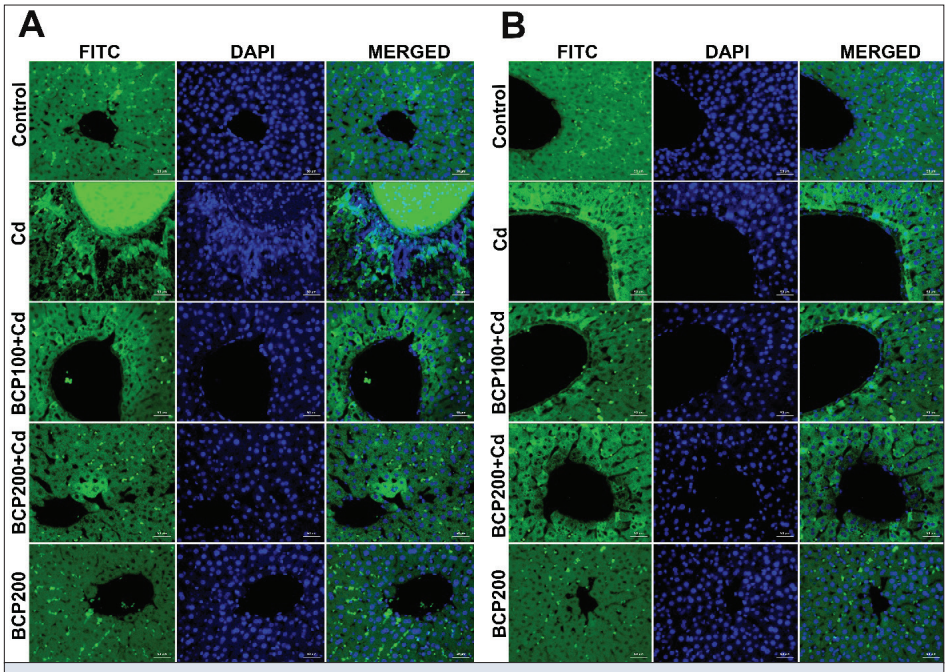

Fig 5. Liver tissue. **A)** BAX expressions in hepatocytes (FITC); **B)** Caspase 3 expressions in hepatocytes (FITC). DAPI: nuclear staining. MERGED: Merge of images. IF, Bar: 50 µm

Table 6. *Immunofluorescence staining results and statistical analysis data. One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Nrf2	HO-1	BAX	Caspase 3
Control	38.18±2.86[b]	3.59±3.13[b]	1.84±0.32[b]	2.24±0.51[b]
CD	8.74±1.1[a]	6.27±1.63[a]	71.81±3.28[a]	61.5±3.65[a]
BCP100+ CD	22.06±2.66[c]	24.57±3.33[c]	59.76±5.16[c]	37.81±4.07[c]
BCP200+ CD	31.92±2.58[d]	33.75±2.33[d]	30.03±3.87[d]	16.3±2.02[d]
BCP200	51.51±2.1[e]	45.39±3.42[e]	1.89±0.22[b]	1.8±0.31[b]

a, b, c, d, e: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Discussion

Cd an extremely toxic heavy metal, enters the body, primarily through inhalation and ingestion, leading to serious health problems [22]. Since the metabolism and accumulation of Cd occurs mainly in the liver and kidneys, these organs are directly affected by toxicity [23]. In this study, the protective effects of BCP, a biologically active compound, against Cd-induced liver toxicity were investigated.

Cd increases the production of reactive oxygen species (ROS) inside the cell, causing lipid peroxidation, protein oxidation, and DNA damage. These processes can disrupt the structural and functional integrity of hepatocytes, triggering cellular death and inflammation [24-28]. It has been shown by various studies that Cd disrupts the oxidative stress balance by increasing the production of ROS and causes cellular damage [29,30]. In addition, BCP has been reported to alleviate oxidative damage, reduce lipid peroxidation, and increase antioxidant enzyme activities (SOD, GSH, CAT) thanks to its strong antioxidant properties. It has been noted that these effects of BCP reduce oxidative stress and strengthen cellular defense mechanisms by activating Nrf2 signaling pathways [31]. In addition, it has been demonstrated by various studies that it exerts hepatoprotective effects by preserving mitochondrial functions and lowering inflammatory cytokine levels [32]. In our study, it was observed that lipid peroxidation (MDA levels) and oxidative stress increased with cadmium exposure. In addition, BCP has been found to reduce oxidative stress and provide significant improvements in antioxidant enzyme activities by alleviating these processes. Our findings support the potential protective effect of BCP against cadmium-induced oxidative stress and liver damage.

Cd initiates inflammatory processes in the liver by activating immune cells and increasing the production of pro-inflammatory cytokines [33]. In particular, when Cd enters the body, it activates the TLR4/NF-κB signaling pathway in the immune system, leading it to initiate inflammatory processes. Activation of NF-κB triggers the expression of cytokines such as IL-1β, TNF-α, and IL-6, which increase the inflammatory response, which exacerbates hepatic inflammation [34]. Previous studies show that BCP has the capacity to suppress inflammatory processes and plays a role in the regulation of the inflammatory response [35]. It has been reported that BCP may suppress oxidative stress-induced inflammatory responses by activating the Nrf2 signaling pathway, one of the important regulators of inflammation, and inhibiting overactivation of NF-κB [33,36-38]. In our study, we observed that BCP reduces inflammation by inhibiting the NF-κB/TLR4 pathway.

Cd exposure activates mitochondrial pathways to initiate the process of apoptosis in cells. This process is usually associated with a destabilization of the ratio between BAX/BCL-2 and caspase activation [38,39]. Increased BAX/BCL-2 ratio as a result of cadmium exposure increases mitochondrial cytochrome C release, causing the apoptotic pathway to progress. As a result, cellular lysis and necrotic changes in liver cells are observed with the activation of effector caspases such as Caspase3 [40]. Previous studies indicate that BCP has effects that improve BAX/BCL-2 balance and reduce the cellular death process by inhibiting caspase activation [41,42]. In our study, we found that BCP protects liver cells against the toxic effects of cadmium by regulating these apoptotic mechanisms. In particular, BCP administration was observed to lower the BAX/BCL-2 ratio and reduce cellular apoptosis by inhibiting Caspase3 activity.

Nrf2 is one of the key transcription factors that govern cellular antioxidant defenses, providing cellular protection by regulating the expression of HO-1 (Heme Oxygenase-1), SOD, GSH and other detoxification enzymes [43,44]. But heavy metals such as cadmium suppress this defense mechanism by increasing the ubiquitination and proteasomal degradation of Nrf2 via KEAP1, causing cells to become more vulnerable to toxic effects [45,46]. Previous studies show that BCP plays an important role in regulating oxidative stress by activating the Nrf2 signaling pathway and increases cellular antioxidant capacity by preventing the suppressive action of KEAP1 [31]. It has also been noted that SIRT1 eliminates the suppressive effect of KEAP1 by activating Nrf2 and provides cellular protection by increasing HO-1 levels [47,48]. In our study, we observed that BCP strengthens the antioxidant response by activating the Nrf2 signaling pathway. In particular, it was determined that BCP administration caused a significant increase in HO-1 levels by increasing the translocation of Nrf2 to the nucleus and activated cellular defense mechanisms.

The results of this study show that BCP has a significant hepatoprotective effect against cadmium-induced liver toxicity. BCP provides effective protection against cadmium-induced biological processes such as oxidative stress, inflammation and apoptosis, and reduces hepatotoxicity. These effects are related to the effects of BCP on important biochemical pathways TLR4/NF-κB, KEAP1/Nrf2/HO-1/SIRT1 and BAX/BCL-2/CASP3. The findings suggest that BCP can be used as a potential treatment option to prevent the toxic effects of cadmium on the liver.

Declarations

Availability of Data and Materials: Data and Materials are available from the corresponding author (İ. Bolat).

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Competing Interests: The authors declare no competing of interest.

Declaration of Generative Artificial Intelligence (AI): The article and tables and figures were not written/created by AI and AI assisted technologies.

Authors' Contributions: Conceptualization, MB and IB; methodology, MB, IB, ST, and TY; software, MB and IB; resources, IB; writing - original draft preparation, MB; writing - review and editing, IB.

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