RESEARCH ARTICLE

Kaempferol prevents cadmium chloride-induced liver damage by upregulating Nrf2 and suppressing NF-κB and keap1

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Abstract

This study evaluated the protective effect of kaempferol, a natural flavonoid, against cadmium chloride ($CdCl_2$)-induced liver damage and examined the possible anti-inflammatory and antioxidant mechanisms of protection. Adult male rats were divided into 4 groups (each of 8 rats) as control, kaempferol (50 mg/kg/day orally), $CdCl_2$ (15 ppm/day), and $CdCl_2$ (15 ppm/day) + kaempferol (50 mg/kg/day). All treatments were given for 30 days. With no effect on attenuating the reduced food intake, kaempferol significantly increased body weight and lowered serum levels of liver injury markers including bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase 1 (γ-GTT1) in the $CdCl_2$-treated rats. It also restored normal liver architectures, prevented hepatocyte, loss, and swelling and reduced inflammatory cell infiltration. These effects were associated with a reduction in mitochondrial permeability transition pore, as well as in the expression of cytochrome-c and cleaved caspase-3, markers of mitochondrial damage, and intrinsic cell death. In both the control positive and $CdCl_2$-treated rats, kaempferol significantly lowered the hepatic levels of reactive oxygen species, malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), Interleukine-6 (IL-6), and the nuclear activity and localization of NF-κB p65. Besides, kaempferol significantly increased the hepatic total and nuclear levels of the nuclear factor erythroid 2–related factor 2 (Nrf2) and heme oxygenase-1, as well as levels of superoxide dismutase (SOD) and reduced glutathione (GSH) but reduced the cytoplasmic protein levels of keap1. In conclusion, the protective effect of kaempferol against $CdCl_2$-induced hepatic damage is mediated by antioxidant and anti-inflammatory effects driven by upregulating Nrf2/HO-1 axis and suppressing the NF-κB p65 and keap1.

Keywords Cadmium; Hepatotoxicity · NF-κB; Kaempferol · Nrf2 · Oxidative stress

Introduction

Cadmium (Cd) is one of the most toxic non-essential heavy metals on the earth crust and is associated with renal, hepatic, reproductive, neural, and pulmonary toxicities in both humans and experimental animals (Rani et al. 2014; Go et al. 2015; Rana et al. 2020). Cd-induced hepatotoxicity is well established in both humans and animals and is a leading cause for the development of liver damage, steatosis, fibrosis, and failure (Ağır and Eraslan 2019; Arroyo et al. 2012; Hyder et al. 2013; Rana et al. 2020; Toppo et al. 2015). Currently, several studies have investigated the mechanism underlying Cd ions-induced liver damage where most of them pointed out the emerging role of oxidative stress (Abarikwu et al. 2017; Eybl et al. 2004; Rani et al. 2014; Sanjeev et al. 2019).

Indeed, overproduction of reactive oxygen species (ROS) and subsequent induction of oxidative stress, inflammation, and apoptosis are mechanisms that underlie the hepatotoxic effect of Cd. Within this view, Cd ions stimulate the production of ROS in the livers of mammals through several mechanisms including impairing metals hemostasis (i.e., Cu, Zn, Fe), binding and depleting the sulfhydryl groups containing

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proteins and thiols (i.e., glutathione/GSH and metallothionein/MTT), downregulating of antioxidant enzymes, and uncoupling the mitochondria oxidative phosphorylation (Arroyo et al. 2012; Rani et al. 2014; Rikans and Yamano 2000). Besides, Cd ions induce hepatic inflammation and increase the production of ROS by direct activation of Kuepfer cells (Yamano et al. 2000). Also, Cd ions activate the intrinsic (mitochondria-mediated) hepatocytes apoptosis by increasing intracellular Ca^{+2} levels, activating DNA damage, suppressing DNA repair, activating caspases, and promoting the opening of membrane permeability transition pore (MTP) (Arroyo et al. 2012; Liu et al. 2019; Rani et al. 2014).

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a natural flavonoid that is abundantly found in grapes, broccoli, tea, tomato, spinach, and beans (Devi et al. 2015). Studies have shown that dietary supplementation of kaempferol protects against several oxidants and inflammatory disorders including cancer and diabetes mellitus (DM), as well as other pulmonary, renal, hepatic, and cardiac disorders (Kim and Park 2020). The health beneficial effects of kaempferol were attributed to its potent antioxidant and anti-inflammatory properties mediated by scavenging ROS and suppressing NF-κB and apoptosis (Chen et al. 2013; Kim and Park 2020; Park et al. 2011; Tsai et al. 2018; Wang et al. 2020). Concerning its hepatoprotective effect, kaempferol also ameliorated liver injury in rodent's models of carbon tetrachloride (CCL$_4$), propacetamol; alcohol, D-galactosamine, and lipopolysaccharide (LPS)-induced liver damage by acting through several mechanisms including scavenging ROS, upregulating cellular antioxidants, inhibiting NF-κB and inflammatory cytokines production, and suppressing hepatic fibrosis, mitochondria damage, endoplasmic reticulum (ER) stress, and intrinsic cell apoptosis (Dong et al. 2017; Wang et al. 2019; Wang et al. 2015; Wu et al. 2020; Xu et al. 2019; Zang et al. 2017).

Whether kaempferol could prevent hepatic damage in Cd-treated rats is still not investigated. Of note, El-Kott et al. (El-kott et al. 2020a) have shown that kaempferol mitigates CdCl$_2$-induced hippocampal damage and memory loss in CdCl$_2$-treated rats, mainly by suppressing ROS generation, inhibiting NF-κB, and stimulating antioxidant levels. These data were very encouraging to us. Therefore, this study was designed to evaluate the protective effect of kaempferol against CdCl$_2$-induced liver damage in rats and to investigate the possible underlying mechanisms.

Materials and methods

Animals

Healthy adult male rats (Wistar strain) (150 ± 10 g, 7 weeks old) were supplied from the animal house of the College of Science, King Khalid University, Abha. During the experimental period, all rats were housed under a controlled condition (temperature of 21°C, humidity of 50–60%, and 12 h/dark/light cycle) and had always free access to their water and diet. All procedures included in this study were approved by the animal ethical committee at the College of Science (Ethical number ECM#2020-1701) which followed the guidelines of the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Experimental design

All rats were randomly selected and divided into 4 groups (n=8 rats/each and 1 rat/cage) as (1) control rats: orally administered an equivalent volume of the vehicle (2% 2-methyl cellulose (Cat. No. M0512-100G, Sigma Aldrich, St. Louis, MO, USA) for 30 consecutive days; (2) kaempferol-treated rats: orally (intragastrically) administered kaempferol (Cat. No. 60010, Sigma Aldrich, St. Louis, MO, USA) dissolved in 2% 2-methyl cellulose to a final concentration of 50 mg/kg/day orally for 30 days (El-Kott et al. 2020a); (3) CdCl$_2$-treated rats: administered CdCl$_2$ (Cat. No. 202908 Sigma Aldrich, St. Louis, MO, USA) dissolved in drinking water (15 ppm) for 30 days (Bilgen et al. 2003; Koyu et al. 2006; Yazıhan et al. 2011); and (4) CdCl$_2$ + kaempferol-treated rat: treated with CdCl$_2$ in the drinking water and received a concomitant daily oral dose of kaempferol (50 mg/kg) for 30 days. Body weights and food intake were determined weekly. We have placed the rats in their cages individually to make sure to consume the whole water volume (20–25 ml) containing the daily dose of CdCl$_2$.

Previously, we have shown that administration of either drinking water or 2% 2 methylcelluloses has no effect on body weight, food intake, and hippocampus structure, and function in rats (El-Kott et al. 2020a, 2020b). Also, in our preliminary experiments, we have found that administration of either drinking water or 2% 2 methylcelluloses to control rats did not affect the liver structure, liver enzymes, and hepatic markers of oxidative stress (data not shown). For this reason, we have omitted the control + drinking water from the experimental design for simplicity.

Serum and tissue collection

By the end of day 30, all rats of all groups were anesthetized by an intraperitoneal dose of sodium pentobarbital (55 mg/kg). Blood samples were directly collected from the heart into plain tubes and centrifuged (1200 × g/10 min/room temperature) to isolate the serum. All serum samples were stored at −20°C for further biochemical analysis. The animals were killed by cervical dislocation, and their livers were rapidly isolated on ice, weighed, and washed with ice-cold phosphate buffer saline (pH=7.4). The livers were then cut into smaller

pieces. Parts of the livers were rapidly snap-frozen in liquid nitrogen and stored at −80°C until use. Other parts were rapidly fixed in 10% buffered formalin for 24 h and forwarded to the pathology laboratory at the College of Medicine, King Khalid University, and subjected to routine staining with hematoxylin and eosin.

Serum analysis

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyltransferase 1 (γ-GTT1) were assessed by rats' ELISA kits (Cat. No. MBS2540581, Cat. No. MBS2540582, and Cat. No. MBS923419, respectively (MyBioSource CA, USA). All measurements were determined for n-8 rats/group and per the manufacturer's instructions.

Preparation of tissue homogenates

Parts of the frozen liver samples (50 mg) were homogenized in 1 ml ice-cold 1X phosphate buffer saline containing a protease/phosphatase inhibitor cocktail (Cat. No. ab201119, Abcam, Cambridge, UK). The homogenates were centrifuged at $10,000 \times g$ for 15 min at 4°C and the supernatants were isolated to new tubes. The tubes were kept at −20°C until further biochemical analysis. To prepare tissue homogenates for western blotting, frozen liver samples (40 mg) were humanized in 0.5 ml 1× radioimmunoprecipitation assay (RIPA) buffer (Cat. No. 91116, Abcam, Cambridge, UK) containing protease/phosphatase inhibitor, centrifuged ($11,000 \times g$/4°C/10 min), and supernatants were isolated. Protein levels in all samples were determined using a commercially available kit (Cat. No. 704002, Caymen Chemicals, MI, USA).

Biochemical measurements in the liver homogenates

Hepatic levels of total free radicals (ROS&RNS) were measured using a fluorometric assay kit (Cat. No. E-BC-K138-F, Elabscience, USA). Levels of malondialdehyde (MDA), total glutathione (GSH), superoxide dismutase (SOD), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) were measured using special rats' ELISA kits (Cat. No. MBS738685, Cat No. MBS265966, Cat. No: MBS036924, Cat. No. MBS2507393 Cat. No. MBS175908, respectively) (MyBioSource, CA, USA). All procedures were done for $n=8$ samples/group and per each kit instruction.

Measurements of mitochondrial permeability transition pore (mtPTP)

The cytoplasmic/mitochondria fraction from frozen livers was prepared using a commercially available kit (Cat. No. Ab110168, Abcam, Cambridge, UK) as per the manufacturer's

instructions. To determine the mtPTP, we followed the procedures established by Adhihetty et al. (Adhihetty et al. 2005) and Kavazis et al. (Kavazis et al. 2017) who have shown that treating the mitochondria with CaCl$_2$ or specific ROS-generating compound such as tert-butyl hydroperoxide (t-BuOOH) causes mitochondria swelling and parallel damage of the external membrane through the opening of the mtPTP. In the test, the increase in the decline of the absorbance (V_{max}) with a concomitant decrease in the time required to reach V_{max} indicates higher mtPTP values. Accordingly, freshly isolated mitochondria from each sample were suspended in 0.5 ml buffer containing 215 mM mannitol, 71 mM sucrose, 3 mM HEPES, and 5 mM succinate to a final concentration of 1 mg/ml. Then, each sample was incubated with CaCl$_2$ (400 μM) and t-BuOOH (75 μM), and the decline in the absorbance was monitored over 10 min at an absorbance of 540 nm.

Determination of NF-κB activation in the nuclear extract

The cytoplasm/nuclear fractions of the frozen livers of all samples were prepared using a special kit (Cat. No. 78833). Protein levels in the nuclear extract were determined using the provided kit (Cat. No. 704002, Caymen Chemicals, MI, USA). The activity of NF-κB p65 was determined using a specific assay kit using 20 μg of the isolated nuclear proteins (Cat. No. 31102, Active Motif, Tokyo, Japan). A standard recombinant NF-κB p65 was used to generate the standard curve used to determine the nuclear levels of NF-κB p65. All procedures were done for $n=8$ sample/group as per the manufacturer's instructions

Western blotting

Protein levels were measured using all supernatants of the liver RIPA-homogenates using the commercial kit. The proteins were prepared in the 2× Laemmli buffer at a final concentration of 3 μg/μl and then boiled for 5 min. Equal proteins from each sample (40 μg) were separated on different percentages of SDS polyacrylamide gel (100 v for 2h). Membranes were then transferred on nitrocellulose membranes (100 v for 2 h) and blocked 5% w/v non-fat dry milk. The membranes were then washed for 3 times with 1×TBST incubated with the primary antibody against cleaved caspase-3 (Asp175) (Cat. No. 9664, 17/19 kDa), cytochrome-c (Cat. No. 11940, 14 kDa, 1:1000), NF-κB p65 antibody (Cat. No. 3034, 65 kDa, 1:1000), Nrf2 (cat. No. 17212, 100 kDa, 1:1000), HO-1 (Cat. No. 70081, 28 kDa), β-actin (Cat. No. 4970, 45 kDa), keap 1 (Cat. No. 60 kDa) (cell signaling technology), and Lamin A (Cat. No. sc-293162, 69 kDa, 1:1000) (Santa Cruz Biotechnology). The incubation with the 1st and 2nd antibodies was done at room temperature for 2 h with continuous shaking. Washing between steps and preparation of the

skimmed milk and antibodies were done in 1× tris-buffered saline tween 20 (TBST) buffer. Membranes were stripped up to 3 times in which the phosphorylated proteins were detected first. Bands were visualized using a price ECL substrate (Cat. No. 32109, ThrmoFisher Scientific). All bands were scanned and analyzed using the LI-COR C-DiGit scanner (USA). Normalization between the stripped gels was done using an internally known sample.

Histological evaluation

The protocol of the histological evaluation was done as previously shown by others (Fischer et al. 2008; Grosset et al. 2019). In brief, parts of the freshly collected liver samples were fixed in 10% buffered formalin for the next 24 h and then dehydrated with ascending alcohol (70–100%) and cleared in xylene. All tissues were then embedded in paraffin wax, cut at a thickness of 3 μM, and then stained with hematoxylin and eosin (H&E). The alterations in the liver architectures were done by a histologist who is unaware of the experimental groups. All photos were examined and photographed under a magnification of 200× using a light microscope (Nikon Eclipse, model ME600).

Statistical analysis

All data were analyzed using Graphpad Prism (V8, Australia). The differences in all measured parameters among all the study group parameters were analyzed using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. Data were presented as mean ± standard deviation (SD). Values were considered significantly different at $P < 0.05$.

Results

Kaempferol prevents CdCl$_2$-induced liver damage

Final body weights and average daily food intake were significantly decreased in the CdCl$_2$-treated rats as compared to control rats ($P = 0.002$ and $P < 0.0001$, respectively) (Figure 1A, B). However, no significant variations in body weights and food intake were observed when kaempferol or CdCl2+ kaempferol-treated rats were compared with the control- or CdCl$_2$-treated rats, respectively ($P > 0.05$) (Figure 1A, B). However, serum levels of ALT, AST, γ-GTT1, and bilirubin were not significantly different in kaempferol-treated rats ($P > 0.05$) but were significantly increased in the serum of CdCl$_2$-treated rats, as compared to control rats ($P < 0.0001$, $P = 0.004$, and $P < 0.0001$, respectively) (Figure 1C–F). Besides, while normal liver architectures were observed in the control or kaempferol-treated rats (Figure 2A, B), livers from CdCl$_2$-treated rats showed severe

loss of hepatocytes, hepatocyte swelling, and damaged cell membranes with increased fatty cells, dilated central vein, and increased infiltration of inflammatory cells (Figures 2C and 3D). On the other hand, the livers obtained from CdCl$_2$ + kaempferol-treated rats showed normal serum levels of all measured enzymes with a concomitant decrease in fatty cells and macrophage infiltration, as well as an obvious improvement in hepatocyte and liver structure (Figures 1A–D and 3E, F).
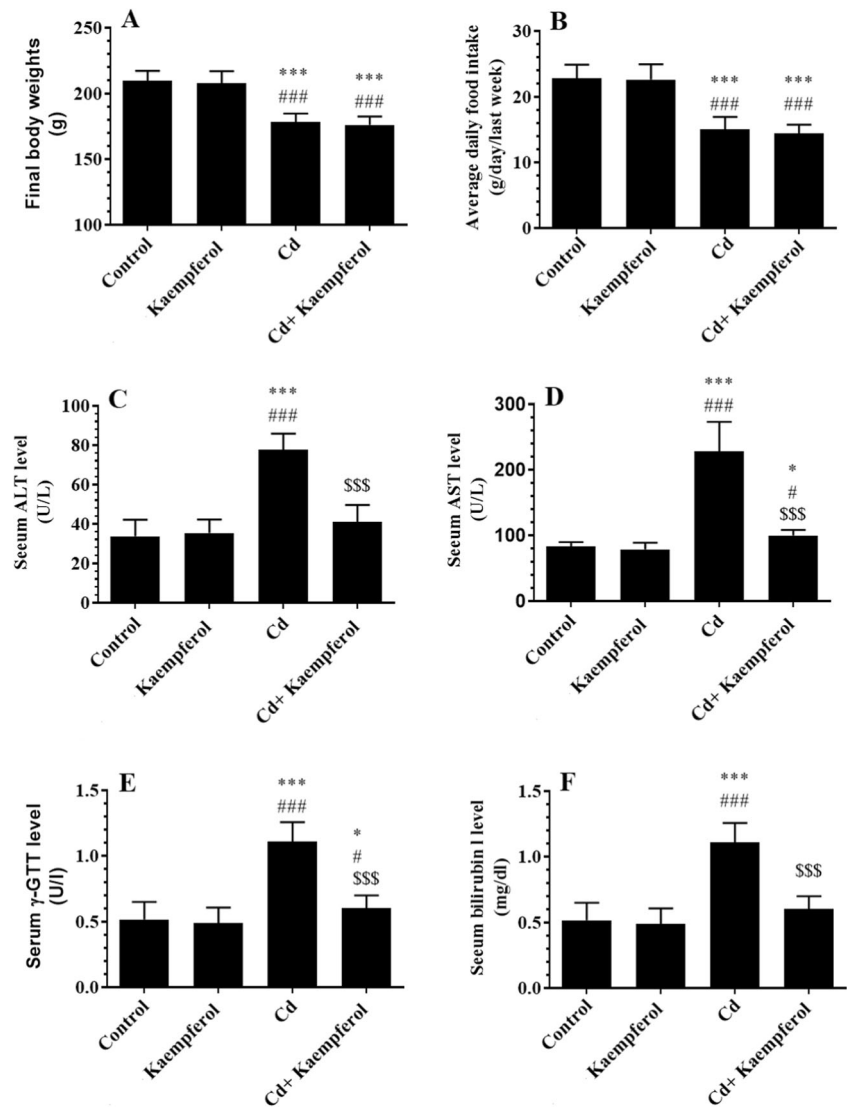
Kaempferol suppresses oxidative stress and upregulates Nrf2 in the livers of both the control- and CdCl$_2$-treated rats by suppressing keap1

Levels of ROS and MDA were significantly increased but the levels of SOD and GSH, as well as the total protein levels of HO-1 and Nrf2, were significantly decreased in the livers of CdCl$_2$-treated rats as compared to control rats ($P < 0.0001$ for all) (Figures 3A–D and 4A, B). Besides, nuclear protein levels of Nrf2 were significantly reduced ($P < 0.0001$) but the total cytoplasmic levels of keap1 were significantly increased ($P < 0.0001$) in the livers of CdCl$_2$-treated rats as compared to control rats (Figure 4C, D). However, a significant reduction in the levels of ROS and MDA coincided with a significant increase in the levels of SOD and GSH, and protein levels of HO-1 and Nrf2 were seen in the livers of the kaempferol and CdCl$_2$ + kaempferol-treated rats as compared to the control or CdCl$_2$-treated rats ($P < 0.0001$ and $P = 0.0021$ for HO-1 and $P < 0.0001$ and $P = 0.0013$ for Nrf2, respectively) (Figures 3A–D and 4A, B). Concomitantly, nuclear protein levels of Nrf2 were significantly increased but cytoplasmic levels of keap1 were significantly reduced in the livers of both kaempferol- and CdCl$_2$ + kaempferol-treated rats as compared to their the control group or CdCl$_2$-treated rats, respectively ($P = 0.0012$ and $P < 0.0001$ for Nrf2, and $P = 0.0214$ and $P < 0.0001$ for keap1, respectively) for HO-1 and $P < 0.0001$ and $P = 0.0013$ for Nrf2, respectively (Figure 4C, D).

Kaempferol reduced mTPTP and inhibits apoptosis in the livers of CdCl$_2$-treated rats

The value of V_{max} and time to reach V_{max} (markers of mTPTP), as well as total protein levels of cleaved caspase-3 were not significantly different between the control- and kaempferol-treated rats ($P > 0.05$) (Figure 5A–D). However, the value of V_{max} was significantly increased ($P < 0.0001$), and the time needed to reach V_{max} was significantly decreased ($P = 0.0002$) in the isolated mitochondria of CdCl$_2$-treated rats, as compared to control rats, thus indicating an increase in mtPTP (Figure 5A, B). Besides, total protein levels of cleaved caspase-3 and cytoplasmic protein levels of cytochrome-c were significantly increased ($P < 0.0001$) in the livers of CdCl$_2$-treated rats as compared to control rats (Figure 5C, D). CdCl$_2$ + kaempferol-treated rats showed a

Fig. 1 Final body weights (**A**), average daily food intake (**B**), and serum levels of hepatic markers (**C**–**F**) in all experimental groups of rats. Data are expressed as mean ± SD of 8 rats/group. Values are significantly different at $P < 0.05$. *,***Significantly different as compared to control rats at $P < 0.05$ and 0.001, respectively. #,###Significantly different as compared to kaempferol-treated rats at $P < 0.05$ and 0.001, respectively. $$$Significantly different as compared to CdCl$_2$ (cd)-treated rats at $P < 0.001$

significant reduction in the value of V_{max} ($P < 0.0001$), as well as in the protein levels of cleaved caspase-3 ($P < 0.0001$) and cytochrome-c ($P < 0.0001$) with a concomitant increase in the time to reach V_{max} ($P < 0.0007$) as compared to CdCl$_2$-treated rats (Figure 5C, D).

Kaempferol inhibits NF-κB p65 and inflammatory cytokines in the livers of both the control- and CdCl$_2$-treated rats

Levels of TNF-α and IL-6, as well as the nuclear activity and protein levels of NF-κB p65, were significantly decreased ($P = 0.0108$, $P = 0.027$, $P = 0.0189$, and $P = 0.0014$, respectively) in the livers of kaempferol-treated rats as compared to control rats (Figure 6A–D). However, levels of TNF-α and IL-6, as well as the nuclear activity and protein levels of NF-κB p65 were significantly increased ($P < 0.0001$ for all) in the livers of CdCl$_2$-treated rats as compared to control rats

(Figure 6A–D). On the contrary, levels of all these markers were significantly reduced in the livers of CdCl$_2$ + kaempferol-treated rats when compared to CdCl$_2$-treated rats ($P < 0.0001$ for all) (Figure 6A–D).

Discussion

The findings of this study confirm the previously reported data which have shown that administration of CdCl$_2$ contributes significantly to liver damage in rodents (Ağır and Eraslan 2019; Arroyo et al. 2012; Hyder et al. 2013; Rana et al. 2020; Toppo et al. 2015). Besides, our data uniquely show that the concomitant administration of kaempferol is an excellent preventative strategy to alleviate CdCl$_2$-induced hepatotoxicity. Accordingly, the administration of kaempferol along with CdCl$_2$ preserved the rat's liver structure and prevented hepatocyte damage, loss, and apoptosis. Mechanistically, our
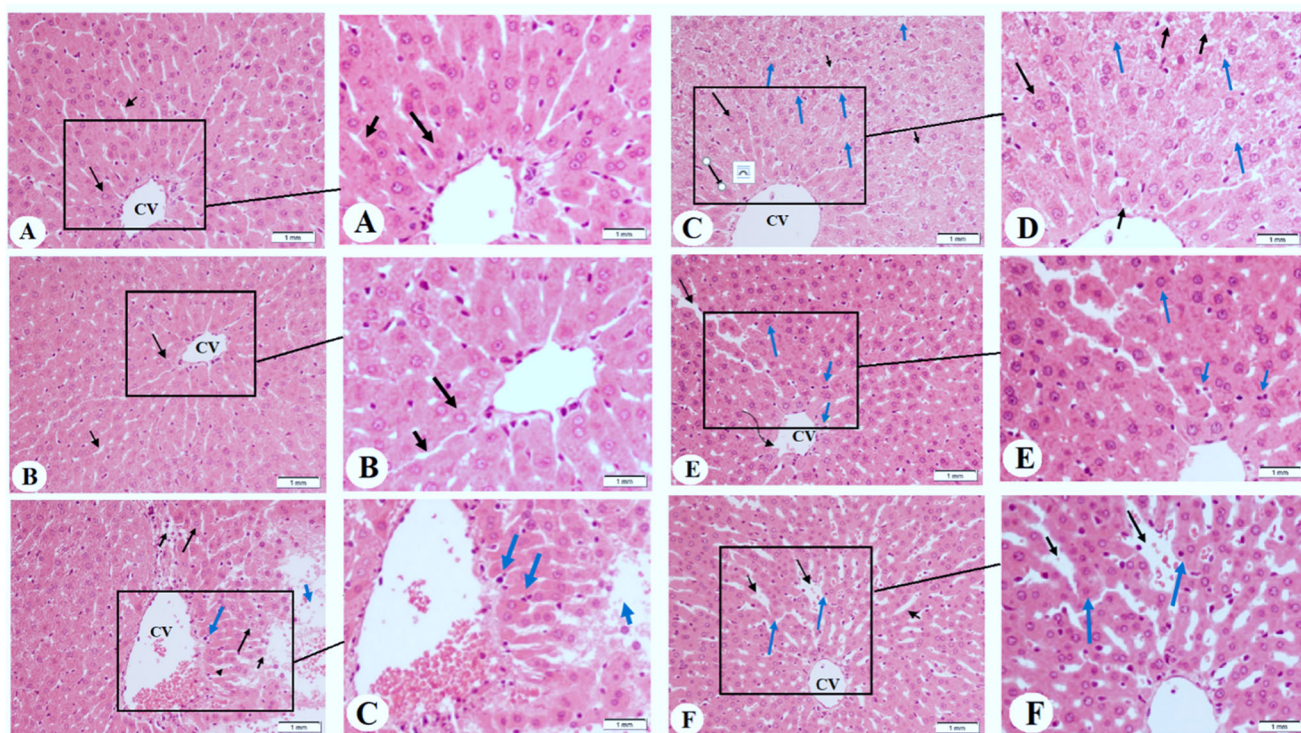
Fig. 2 Photomicrographs of liver sections of some groups of rats after staining with hematoxylin and eosin (H&E). **A,B** Taken from control- and kaempferol-treated rats, respectively, and showed normal liver structure with normally sized central vein (CV) and hepatocytes (long arrow) and sinusoids (short arrow). **C,D** Taken from $CdCl_2$-treated rats and showed dilated CV, severe loss of the hepatocyte parenchyma (short blue arrow), hepatocytes swelling (long black arrow), damage of hepatocytes membrane (short black arrow), dilated sinusoids (arrowhead), increased infiltration of inflammatory cells (thick long blue arrow). **E,F** Taken from $CdCl_2$ + kaempferol-treated rats and showed improvement in hepatocytes structure and organization with a concomitant reduction in the diameter of the CV, amount of inflammatory cells, and hepatocytes loss. Most of the hepatocytes have intact membranes. However, some inflammatory cells (short blue arrow), hepatocytes damage (long black arrow), cell swelling (long blue arrow), damage in the CV (curved black arrow) were still observed

data suggest that such protective effect of kaempferol is attributed mainly to its ability to antioxidant and anti-inflammatory effects mediated by reducing the generation of ROS, Nrf2/HO-1-dependent increase in the levels of GSH and SOD, preventing mitochondria mtPTP, and suppressing the activation of NF-κB and the generation of some inflammatory cytokines. A graphical abstract demonstrating the basic suggested mechanism of protection of kaempferol is shown in Figure 7.

Cadmium ions accumulate at varying degrees in many tissues including the brain, liver, kidneys, and testes, leading to tissue damage and organ failure (Go et al. 2015; Rani et al. 2014). In vivo evidence suggests that Cd ions accumulate at the highest levels in the livers of rats at all measured intervals (4, 8, and 12 weeks after exposure) (Haouem and El Hani 2013), thus confirming that hepatotoxicity is the earliest damage seen after intoxication with Cd ions (Kawagoe et al. 2005; Pari and Murugavel 2005). Weight and appetite loss are two major symptoms associated with Cd toxicity in both humans and animals and are attributed to the Cd ions-induced systemic toxicity and the reduction in appetite due to abnormal neurotransmitters signaling in the hypothalamus (Amara et al. 2008; El-kott et al. 2020a; Hwang and Wang 2001; Nwokocha et al.

2012). This has been also confirmed in this study in $CdCl_2$-treated rats where we have also found a significant reduction in rat's final body weights of $CdCl_2$-treated rats.

On the other hand, kaempferol prevented the loss of body weight without altering the rat's food intake of the $CdCl_2$-treated rats. These data support our previous data in the same animal model (El-kott et al. 2020a; El-Kott et al. 2020b). Based on these data, we concluded that the protective effect of kaempferol on rat's body weights is not related to altering food intake but could be possibly due to its chelating properties or its protective effect on the liver and other peripheral organs. Unfortunately, we could not measure the levels of Cd ions in the livers and other tissues to confirm this.

Nevertheless, oxidative stress and inflammation are the best-known mechanisms mediating the hepatotoxic effect of Cd (Arroyo et al. 2012; Liu et al. 2019; Rani et al. 2014). Cadmium-induced liver damage is associated with an increase in the serum levels of ALT, AST, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-GTT, and bilirubin, all of which are considered conventional biomarkers of loss of cell integrity (Ağır and Eraslan 2019; Renugadevi and Prabu 2010). Also, Cd ions can induce severe pathological

Fig. 3 Levels of oxidative stress markers in the livers of all groups of rats (**A**–**D**). Data are expressed as mean ± SD of 8 rats/group. Values are significantly different at $P < 0.05$. *,***Significantly different as compared to control rats at $P < 0.05$ and 0.001, respectively. #,###Significantly different as compared to kaempferol-treated rats at $P < 0.05$ and 0.001, respectively. $$$Significantly different as compared to CdCl$_2$ (cd)-treated rats at $P < 0.001$
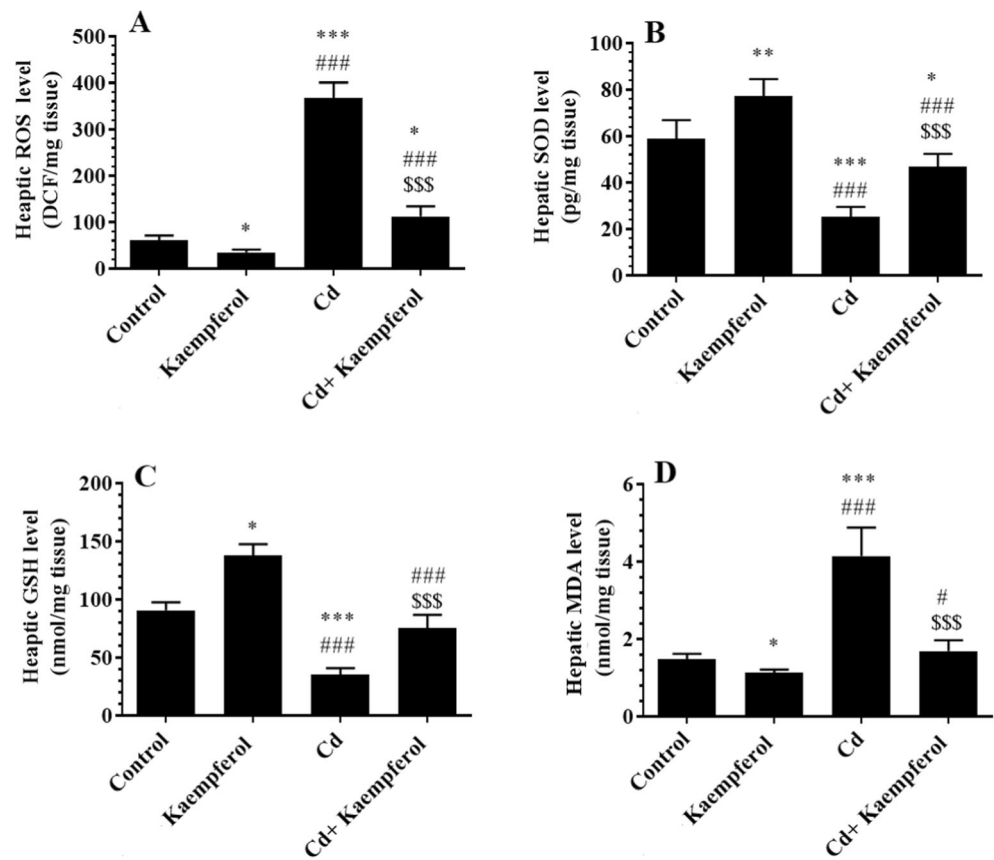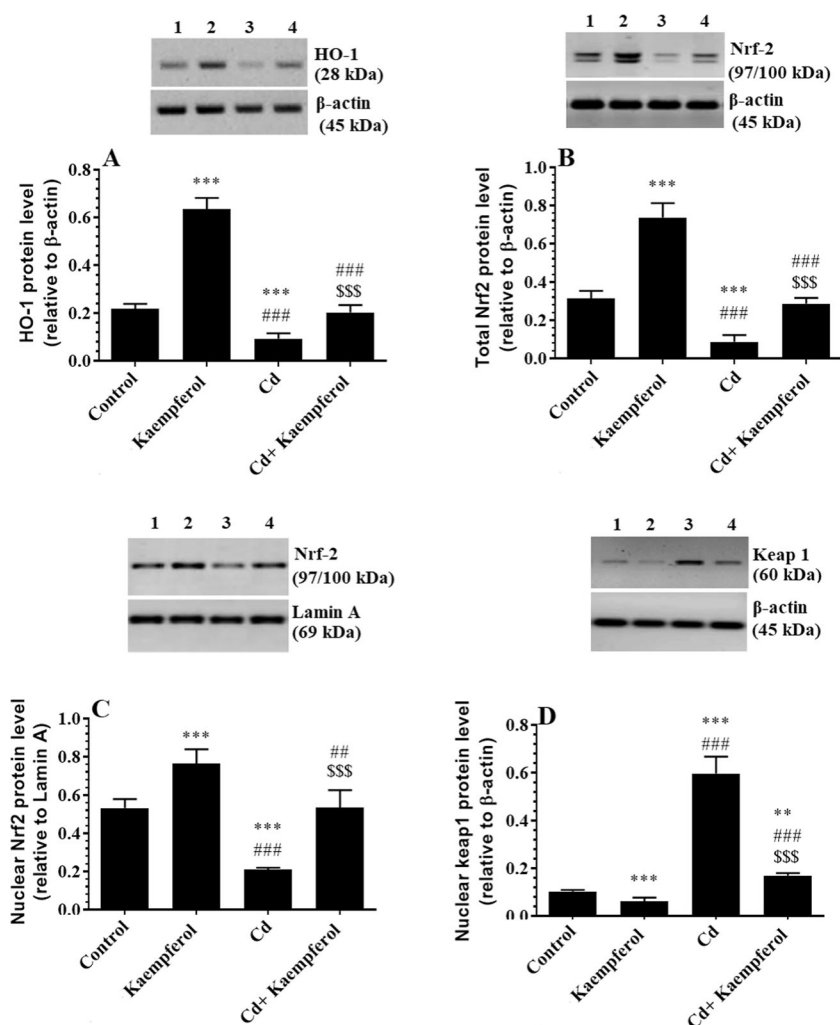

alterations, including hepatocytes damage, necrosis, apoptosis, and inflammatory cell infiltration (Ağır and Eraslan 2019; Rana et al. 2020). Besides, it is currently well-accepted that Cd ions are an independent cause for the development of NAFLD and non-alcoholic steatohepatitis (NASH) in both humans and animals by suppressing fatty acids (FAs) oxidation, stimulating FAs-synthesis, and induction of oxidative stress, inflammation, mitochondria damage, and endoplasmic reticulum (ER) stress (Go et al. 2015).

In the same line, these effects were also shown in this study where there was a significant increase in the serum levels of ALT, AST, γ-GTT, and bilirubin with increased hepatocyte damage, inflammatory cell infiltration, and accumulation of fat droplets in the CdCl$_2$-treated rats. Therefore, we can strongly argue that CdCl$_2$-intoxication is a leading cause of liver damage and idiopathic hepatic steatosis. However, the first evidence supporting the hepatic protective role of kaempferol in CdCl$_2$-induced rats was observed by the obvious attenuation in the serum levels of all these markers and the improvement in the liver architectures of the treated rats. This is expected given the well-known previously reported hepatoprotective effects of kaempferol in several animal models of hepatic injury, including those induced by treatment with CCL4, paracetamol; alcohol, D-galactosamine, and lipopolysaccharide (Dong et al. 2017; Wang et al. 2019; Wang et al. 2015; Wu et al. 2020; Zang et al. 2017).

On the other hand, although Cd ions cannot generate ROS by themselves, they indirectly generate high levels of superoxide, hydrogen peroxide, and hydroxyl radical by several mechanisms (Arroyo et al. 2012). Within this view, it has been demonstrated that Cd ions bind to sulfhydryl groups-containing cellular components such as GSH and reduce their availability (Ağır and Eraslan 2019; Arroyo et al. 2012; Gebhardt 2009; Rani et al. 2014). Besides, Cd ions can suppress and downregulate several endogenous antioxidant enzymes such as GPx, SOD, and CAT by direct binding to their active sites or by increasing their consumption to detoxify higher levels of ROS (Koyu et al. 2006; Newairy et al. 2007). Also, Cd ions stimulate ROS by accumulating in the hepatocytes mitochondria, a critical event that leads to uncoupling oxidative phosphorylation, altering membrane fluidity, dissipation of transmembrane electrical potential, and induction of mitochondrial permeability transition (Belyaeva et al. 2011; Diep et al. 2005; Zhang et al. 2011). In this study, CdCl$_2$-induced hepatotoxicity was also associated with higher hepatic levels of ROS and MDA and lower levels of GSH, SOD, and GPx. Besides, the administration of CdCl$_2$ induced mtPTP as noticed by the increase in the value of V_{max} and the parallel decrease in the time which is required to reach V_{max}. These data support the above-mentioned studies and confirm that CdCl$_2$-induced hepatic damage is an oxidative-stress-dependent mechanism that involves the depletion of antioxidant and mitochondria damage.

Fig. 4 Total protein levels of heme oxygenase-1 (HO-1) (**A**) and nuclear factor erythroid 2-related factor 2 (Nrf2) (**B**), as well as the nuclear levels of Nrf-2 (**C**) and the cytoplasmic levels of keap1 (**D**) in the livers of all groups of rats. Data are expressed as mean ± SD of 8 rats/group. Values are significantly different at $P < 0.05$. **,***Significantly different as compared to control rats at $P < 0.01$ and 0.001, respectively. ##,###Significantly different as compared to kaempferol-treated rats at $P < 0.01$ and 0.001, respectively. $$$Significantly different as compared to CdCl$_2$ (cd)-treated rats at $P < 0.001$

However, Nrf-2 and HO-1 are major transcriptional factors responsible for increasing GSH and antioxidant levels within the majority of cells including the hepatocytes (Luo et al. 2018). Nonetheless, the transactivation of Nrf2 is mainly regulated by the levels of keap1 which bind to it in the transcription factor thus impedes its nuclear translocation or stimulates its cytoplasmic degradation. Previous studies have shown that Cd ions could suppress the antioxidant potential by downregulating Nrf2 and /or stimulating its cytoplasmic degradation by upregulating keap1 (Liu et al. 2019). Similar to these data, our study also revealed a significant reduction in the total and nuclear levels of Nrf2 and its downstream target, HO-1, with a concomitant increase in the cytoplasmic levels of keap1 in the livers of CdCl$_2$-treated rats. These data suggest that CdCl2 is able not only to downregulate Nrf2 but also to stimulate its cytoplasmic degradation. Therefore, we can strongly argue that CdCl2 shifts the redox environment of the hepatocytes toward an oxidative stress one by increasing the production of ROS, overwhelming endogenous antioxidants, impairing the mitochondria function, downregulating Nrf2, and suppressing the transactivation of Nrf2 by upregulating keap1. These data

may suggest that Cd ions may suppress GSH and antioxidant enzymes in the livers of rats by downregulating Nrf2.

However, Cd-ions-derived ROS can also stimulate haptic inflammation by inducing NLRP3 inflammasome assembly and subsequently activate NF-κB p65 (Arroyo et al. 2012; Horiguchi et al. 2000; Liu et al. 2019; Rzepecka et al. 2015; Xu et al. 2019). Interestingly, a negative cross-talk between Nrf2 and components of NF-κB has been also established (Wardyn et al. 2015). In this context, pharmacological activation of Nrf2 protected the livers from Cd ions-induced injury by increasing antioxidants and inhibiting NF-κB P65 (Wu et al. 2012). On the contrary, the higher activity of NF-κB p65 reduced the expression and activation of Nrf2 by reducing its heterodimer formation and inhibiting its transcriptional activity, mainly by increasing the keap1 nuclear translocation and promoting the dissociation of Nrf2 from its transcriptional co-activator, the CREB-binding protein (Wardyn et al. 2015). Associated with CdCl$_2$-induced liver damage, ROS, and suppression of Nrf2, we have also found an increase in the inflammatory cell infiltration with a concomitant increase in the nuclear level/activity of NF-κB p65 and levels of TNF-α and

Fig. 5 Mitochondria membrane transition pores (mtMTP) function (**A**, **B**), protein levels of cleaved caspase-3 (**B**), and cyto-plasmic protein cytochrome-c (**C**) in the livers of all groups of rats. Data are expressed as mean ± SD of 8 rats/group. Values are significantly different at $P < 0.05$. [***]Significantly different as compared to control rats at 0.001. [###]Significantly different as compared to kaempferol-treated rats at $P\ 0.001$. [$$$]Significantly different as compared to $CdCl_2$ (cd)-treated rats at $P < 0.001$. V_{max}, the maximum decline in the absorbance of the isolated mitochondria after treatment with $CaCl_2$ and tert-butyl hydroperoxide (t-BuOOH) (a reactive oxygen species generating molecule)
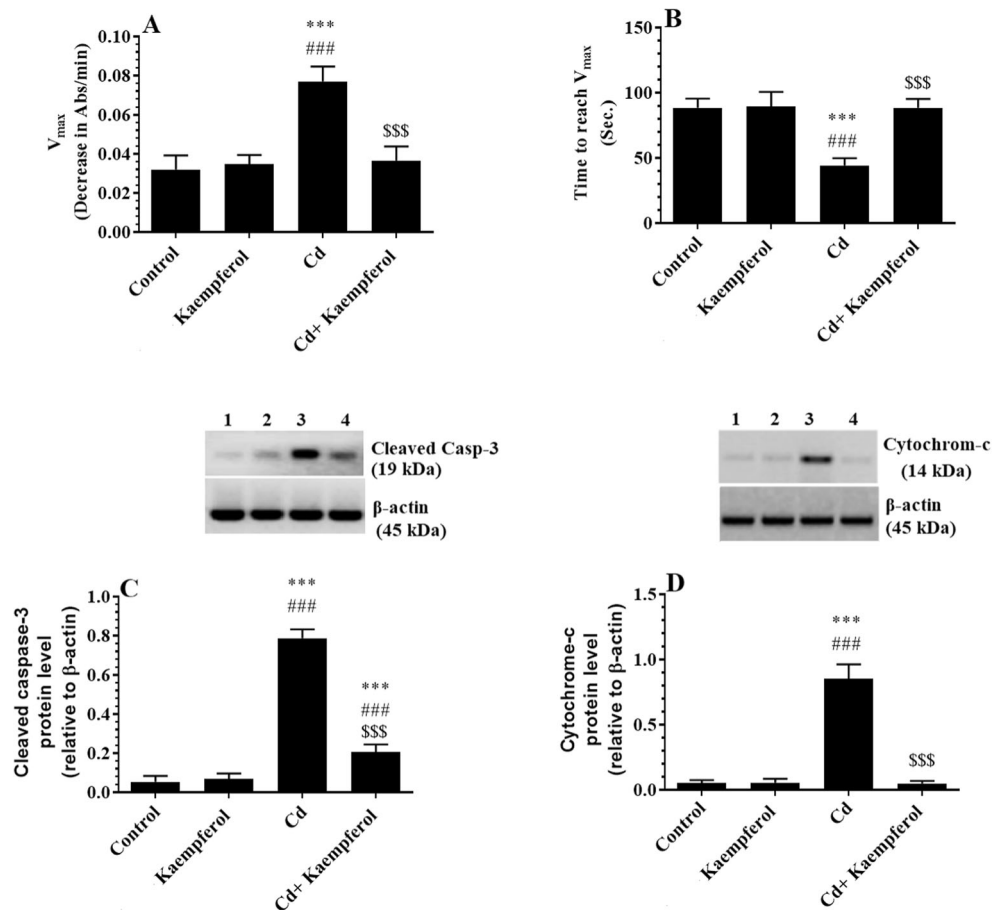
IL-6 in the livers of $CdCl_2$-treated rats. These data suggest a vicious cycle between ROS, Nrf2, and NF-κB P65 in the process of $CdCl_2$-induced hepatic damage. Besides, these data may indicate that CdCl2-induced upregulation of keap1 is mediated via the activation of NF-κB. However, further studies are required to precisely identify the major trigger responsible for all these connected events, which seems to be mainly induced by Cd-derived ROS.

Although the above discussion remains confirmatory to many previous studies, the novelist findings of this study are that the protective effect of kaempferol, as well its possible mechanism of protection against $CdCl_2$-induced liver damage is the first to be shown. Herein, kaempferol significantly attenuated the increase in ROS and lipid peroxidation, increased levels of GSH and SOD, upregulated Nrf2 and HO-1, suppressed the nuclear translocation and activation of NF-κB p65, lowered the levels of TNF-α and IL-6, and downregulated keap1 not only in the livers of $CdCl_2$-treated rats but also in the livers of the control-treated rats. Besides, kaempferol administration to $CdCl_2$-treated rats significantly suppressed mtPTP, the release of cytochrome-c, and activation of caspase-3. Based on these data, it seems logical that the mechanism of protection of kaempferol is attributed to antioxidant and anti-inflammatory mechanisms mediated by scavenging

ROS, suppression of NF-κB and keap1, and upregulation of Nrf-2 and HO-1, and upregulation of the cellular antioxidants.

Supporting our findings, the protective role of kaempferol against serval cardiovascular, renal, hepatic, and inflammatory disorders is well-reported in many well-designed studies, where these effects were attributed to its potent antioxidant, anti-inflammatory, and anti-apoptotic effects (Imran et al. 2019; Kim and Park 2020). Indeed, kaempferol attenuated doxorubicin (DOX)-induced mitochondria damage by reducing ROS generation, increasing GSH levels, caspase-3 inactivation, and preventing mtPTP (Wang et al. 2020). Also, kaempferol prevented CCL_4-induced hepatic damage by increasing GSH, SOD, and CAT (Zang et al. 2017). Besides, kaempferol protected against $CdCl_2$-induced memory loss and hippocampal damage by suppressing TNF-α and IL-6 release and upregulation of MnSOD and GSH, in a SIRT1-dependent mechanism (El-kott et al. 2020a). Similar effects were also observed in the livers of streptozotocin (STZ)-induced diabetic rats. In the same line, kaempferol prevented memory deficits and prevented brain cell apoptosis induced by chlorpyrifos, at least by, activating Nrf2 (Hussein et al. 2018). Also, Saw et al. (Saw et al. 2014) have confirmed that the potent antioxidant potential of kaempferol is related to its ability to activate/upregulate Nrf2.

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Fig. 6 Levels of inflammatory markers (**A**, **B**) and nuclear activity and levels of NF-κB p65 (**C**, **D**) in the livers of all groups of rats. Data are expressed as mean ± SD of 8 rats/group. Values are significantly different at $P <$ 0.05.*,**,***Significantly different as compared to control rats at $P <$ 0.05, 0.01, and 0.001, respectively. ##,###Significantly different as compared to kaempferol-treated rats at $P < 0.01$ and 0.001, respectively. $$$Significantly different as compared to CdCl$_2$ (cd)-treated rats at $P < 0.001$
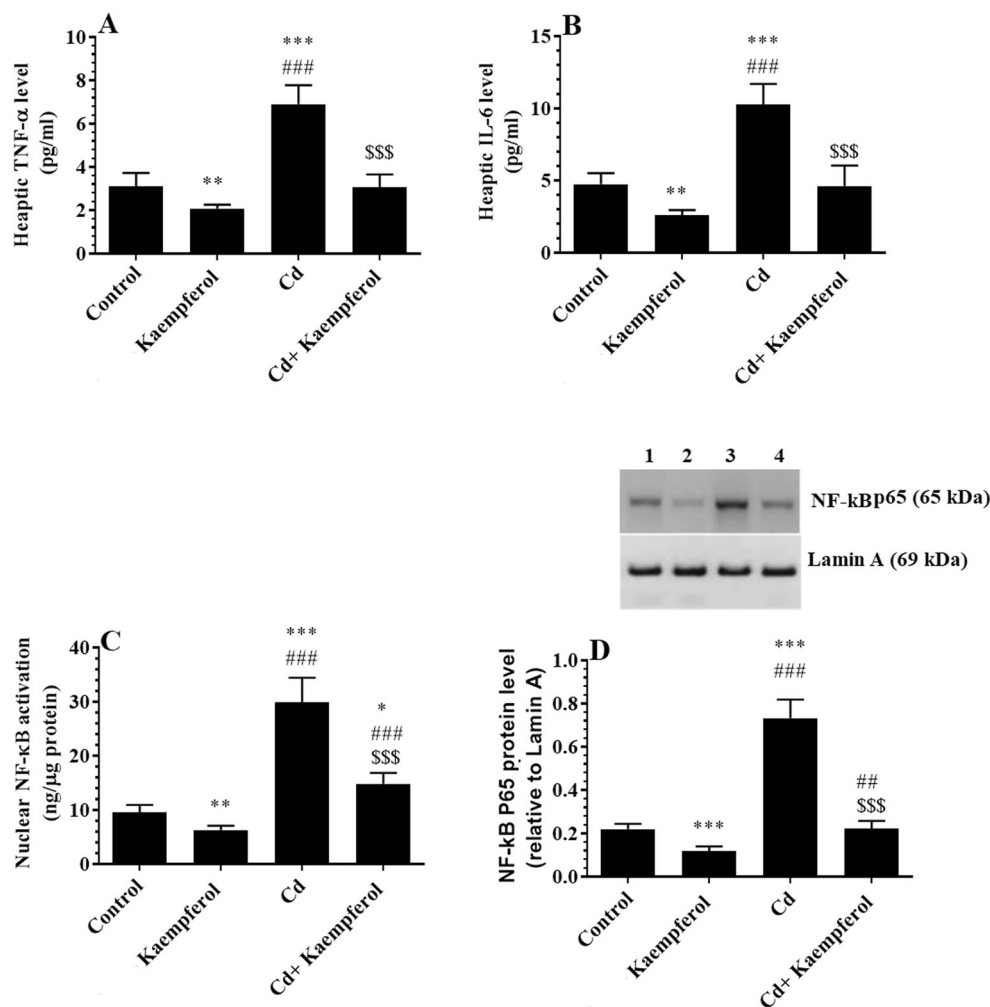
Nonetheless, kaempferol prevented cardiac damage in isoproterenol-induced heart failure in diabetic rats by suppressing NF-κB and stimulating Nrf2/antioxidants axis (Zhang et al. 2019). In a mouse model of vascular injury, as well as oxidative stress-induced umbilical vein endothelial cells (HUVECs) injury, kaempferol protective effect was mediated by upregulation of the Nrf2/HO-1 axis, increasing the expression of the antioxidants, and inhibition of NF-κB, TNF-α, and IL-6 (Yao et al. 2020). The inhibitory effect of kaempferol on NF-κB and inflammatory cytokines production has been also demonstrated in lipopolysaccharides (LPS)-induced human aortic endothelial cells (HAECs) injury, low-density lipoprotein (ox-LDL)-induced apoptosis in human aortic endothelial cells chondrocytes, and H9N2 virus-induced inflammation and lung injury (Cui et al. 2019; Dong et al. 2017; Zang et al. 2017).

Despite these findings, this study still has some limitations. Although the protective effect of kaempferol in this study is mediated by acting through increasing the expression of Nrf2 and suppression of NF-κB, we still unable to determine the

major target of kaempferol given the negative cross-talk between the two. Besides, the precise upstream signaling pathways regulating such effects were not studied yet. Also, kaempferol attenuated the fatty changes in the livers of the CdCl$_2$-treated rats in this study. Similar to these findings, kaempferol also prevented dyslipidemia and hepatic lipid accumulation in high-fat diet-fed rats by increasing fatty acid oxidation through activating PPARα and suppressing fatty acid synthesis by inhibiting SREBP1 (Chang et al. 2011; Wang et al. 2020). Yet, we did not investigate the effect of kaempferol on lipid synthesis/oxidation pathways in the livers of CdCl$_2$-treated rats which requires further investigation to illustrate if this effect is secondary to suppression of oxidative stress or a direct effect on these pathways.

In conclusion, the data in our hands support the hepatoprotective effect of kaempferol and add such protection in an animal model of CdCl$_2$ intoxication. It also supports the potent antioxidant and anti-inflammatory effect of this flavonoid and demonstrates that the mechanism of protection is mediated by the upregulation of Nrf2 and the concomitant

Fig. 7 A graphical abstract showing the protective effect of kaempferol against CdCl2-induced hepatic damage. In the graph, CdCl2 stimulates hepatic oxidative stress and inflammation by suppressing Nrf2 and activating NF-κB in the liver of rats. The inhibition of Nrf2 leads to overproduction of reactive oxygen species (ROS) and oxidative stress by decreasing the expression of heme-oxygenase-1 (HO-1) and subsequent decrease in the levels of some antioxidant markers such as glutathione (GSH) and superoxide dismutase (SOD). On the other hand, CdCl2-induced activation of NF-κB stimulates hepatic inflammation by increasing the expression of interleukine-6(Il-6) and tumor necrosis factor (TNF-α). Kaempferol prevents these events by activating Nrf2 and inhibiting NF-κB
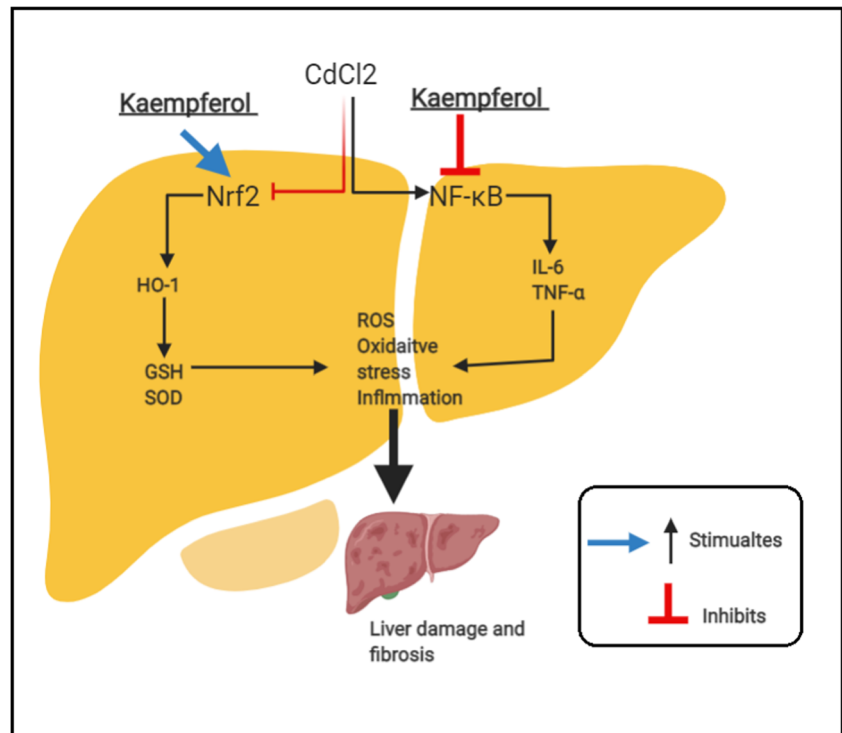
suppression of NF-κB p65. Our recommendation is to further study these effects in more in vivo and clinical trials in patients with liver disorders.

Declarations

Ethics approval and consent to participate All applicable international, national, and/or institutional guidelines for the care and use of animals and cell lines were followed and approved by the ethics committee at the King Khalid University (Ethical number ECM#2020-1701). All authors equally participate in the study.

Consent for publication All authors allow the publication of the paper.

Conflict of interest The authors declare no competing interests.

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