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Research Article
Cinnamaldehyde Mitigates Carbon Tetrachloride-induced Acute Liver Injury in Rats Through Inhibition of Toll-like Receptor 4 Signaling Pathway

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Abstract

Background and Objective: Acute Hepatic Failure (AHF) is associated with a high mortality rate. Toll-like receptors (TLRs) have been implicated in different liver diseases, however, their role in the pathogenesis of carbon tetrachloride (CCl_4)-induced acute liver injury has not been extensively studied. Therefore, the present study was conducted to investigate the protective effect of cinnamaldehyde (CIN), a natural product that possesses anti-inflammatory and anti-oxidant properties, against acute hepatic injury. **Materials and Methods:** Liver injury was induced by CCl_4/olive oil mixture (1:1, v/v) at a dose of 280 µL/100 g b.wt., once daily for 3 days followed by a dose of 140 µL/100 g b.wt., once daily for the following 3 days by oral gavage. Rats were treated with CIN (10 mg kg^{-1} day^{-1}, p.o.) or silymarin (SIL, 100 mg kg^{-1} day^{-1}, p.o.) for 6 consecutive days starting from the first day. **Results:** Cinnamaldehyde significantly reversed CCl_4-induced elevation in serum alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase activities and bilirubin level ($p<0.05$). Further, CIN significantly reduced CCl_4-induced oxidative stress and inflammation mediated through TLR4 signaling pathway including myeloid differentiation factor (MyD) 88-dependent and toll/interleukin-1 receptor domain-containing adaptor inducing interferon-beta (TRIF)-dependent pathways, as well as the expression of downstream transcription factors such as nuclear factor-kappa B. The protective effect of CIN was comparable to that of SIL. **Conclusion:** The results show that CIN may be a therapeutic alternative to SIL for the protection of acute liver injury via inhibition of inflammation mediated through TLR4 signaling cascade. These results are promising, particularly with the increased demand to find new hepatoprotective therapies considering the shortage of liver transplants that are considered the only effective treatment for acute hepatic failure.

Key words: Acute hepatic injury, carbon tetrachloride, cinnamaldehyde, inflammation, myeloid differentiation factor 88, nuclear factor-kappa B, oxidative stress, toll-like receptor 4, tumor necrosis factor-alpha

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Acute Hepatic Failure (AHF) is associated with a high mortality rate reaching approximately 80-90%[1]. Different causes are implicated in developing AHF including viruses such as hepatitis A and E[2], some drugs such as acetaminophen[3], acute ischemic hepatocellular injury[4] and metabolic diseases such as Wilson's disease[5]. Serious systemic and neurological complications associated with AHF make management difficult[6]. Liver transplantation is considered the only effective treatment, however, the availability is hindered by shortage of donors. Therefore, there is a growing demand to evaluate new therapies for their hepatoprotective effect.

Carbon tetrachloride (CCl_4) is a solvent that has been frequently used in different studies for the induction of both acute and chronic liver injury[7]. Hepatotoxicity induced by CCl_4 is attributed to the formation of free radicals leading to lipid peroxidation[8,9] and apoptotic cellular death[10]. In addition, CCl_4 results in the release of proinflammatory cytokines from stimulated Kupffer cells leading to liver inflammation[11]. Different inflammatory mediators, such as tumor necrosis factor alpha (TNF-α) and nuclear factor-kappa B (NF-κB) are involved in CCl_4-induced hepatotoxicity.

Toll-like receptors (TLRs) have been implicated in different liver diseases such as acute and chronic hepatitis, alcoholic and non-alcoholic liver disease and hepatocellular carcinoma[12]. Theses receptors play a pivotal role in the activation of the innate immune system and inflammation. They recognize pathogen-associated molecular patterns (PAMPs) such as bacterial, viral, protozoal and fungal molecular motifs[13] and damage-associated molecular patterns (DAMPs) such as endogenous substances released in response to stress or tissue injury[14,15]. Signaling of TLRs involves two pathways, namely myeloid differentiation factor (MyD) 88-dependent pathway and MyD88-independent pathway[16]. Ji *et al.*[17] reported that TLRs are implicated in CCl_4-induced liver fibrosis, however, their role in the pathogenesis of CCl_4-induced acute liver injury has not been extensively studied.

Cinnamaldehyde (CIN) occurs naturally in various species of the genus *Cinnamomum* and is used in preparing beverages, medicinal products, perfumes and cosmetics. Cinnamaldehyde possesses anti-oxidant and anti-inflammatory properties and has been shown to exert beneficial therapeutic effects in different experimental models including antihyperglycemic and antihyperlipidemic effects[18,19], protection against high glucose-induced endothelial dysfunction in rat aorta[20] and protection of cultured rat dorsal root ganglion neurons against high glucose-induced damage[21]. In addition, CIN has protective effects against cerebral ischemia injury in mice via inhibiting inflammation, which is partly mediated by inhibiting TLR4/NF-κB pathway[22]. Therefore, in the present study, we aimed to assess the protective effect of CIN against CCl_4-induced acute hepatic injury in a rat model and to disclose the possible underlying mechanisms including the role of TLR4/NF-κB signaling pathway. In addition, we compared the effects of CIN with silymarin (SIL), which is isolated from *Silybum marianum* and has been used as a reference hepatoprotective drug in different experiments of liver injury[23,24] induced by CCl_4. Furthermore, SIL is used clinically for the treatment of different liver diseases[25,26].

MATERIALS AND METHODS

Animals: Adult male Wistar rats (180-250 g) were used in the current study. Animals were obtained from the Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were acclimatized for 1 week prior to experiments. The animals were kept at controlled temperature (23±2°C), humidity (60±10%) and light/dark cycle (12/12 h).

Ethical statement: All procedures performed in studies involving animals were approved by the local authorities, Ethical Committee for Animal Handling at Zagazig University (ECAHZU), at the Faculty of Pharmacy, Zagazig University, Egypt in accordance with the recommendations of the Weatherall report. Every effort was done to minimize the number of animals used and their suffering during experiments.

Drugs and chemicals: Carbon tetrachloride and CIN were purchased from El-Gomhouria Company for Trading Chemicals (Cairo, Egypt). Silymarin (SIL) was provided by SEDICO Pharmaceuticals Company (Giza, Egypt). All other chemicals were of analytical grade. Carbon tetrachloride was dissolved in olive oil at a ratio of 1:1 (v/v), CIN was dissolved in olive oil at a concentration of 0.4% and SIL was suspended in 0.9% NaCl solution at a concentration of 40% immediately before use.

Experimental design: Rats were randomly divided into four groups (6 rats each). Group I (Control): Rats received the vehicles, olive oil (280 μL/100 g b.wt., once daily for 3 days followed by 140 μL/100 g b.wt., once daily for the following 3 days, p.o.) plus 0.9% NaCl (25 μL/100 g b.wt., once daily for 6 days, p.o.). Group II (CCl_4): Rats received CCl_4/olive oil mixture (1:1, v/v) at a dose of 280 μL/100 g b.wt., once daily for 3 days followed by a dose of 140 μL/100 g b.wt., once daily for the following 3 days by oral gavage plus 0.9% NaCl

(25 µL/100 g b.wt., once daily for 6 days, p.o.). Group III (CIN): Received CCl_4/olive oil mixture (1:1, v/v) and 0.9% NaCl as described in group II plus CIN (10 mg kg^{-1} once daily for 6 days, p.o.). Group IV (SIL): Received CCl_4/olive oil mixture (1:1, v/v) as described in group II plus SIL (100 mg kg^{-1} once daily for 6 days, p.o.). Rats in group III and IV received drug treatment 1 h before the administration of CCl_4. Dosing of drugs or vehicles were carried out between 10:00 and 11:00 am. The animals had free access to water and normal chow diet during the experimental period.

Rationale of CCl_4 and drug dosing: Although, CCl_4 is a widely used animal model of liver injury, however, it is poorly reproducible[27]. Therefore, it was prudent to perform a pilot study aiming to try different doses described in the literature to establish our own model before commencing the experiments. We tried first the administration of a single oral dose (150 µL/100 g) of CCl_4/olive oil mixture (1:1, v/v) as described by Lee *et al.*[28] or a single oral dose (500 µL/100 g) of CCl_4/olive oil mixture (1:1, v/v) modified from the method described by Nardo *et al.*[29]. Both doses were unsuccessful in inducing liver injury, which was indicated by the non-significant changes observed in liver function test parameters that were measured 24 h after CCl_4 administration compared to control rats. We made another attempt using a single oral dose (1000 µL/100 g) of CCl_4/olive oil mixture (1:1, v/v) modified from the method described by Yan *et al.*[30], however, this high dose resulted in 100% mortality after 2 h of administration. Therefore, the dose of CCl_4 was chosen based on the data described in the literature[23], albeit with some modifications. With this model the mortality rate was only 10-20% with detectable liver damage. The doses of CIN and SIL were chosen based on previous data described in the literature[19,31], respectively.

Methods

Blood sampling and serum preparation: Twenty four hours following the administration of the last dose of CCl_4, blood samples were collected from the retro-orbital sinus of rats using heparinized microcapillary tubes. Serum was prepared by centrifugation (Hermle Z230, Gosheim, Germany) at 3000 rpm for 30 min. Serum was stored at -20°C for further determination of different biochemical parameters.

Tissue sampling: Rats were euthanized by cervical dislocation under anesthesia with thiopental sodium (120 mg kg^{-1}) for tissue specimen collection. Livers were removed and rinsed thoroughly with saline and divided into two parts. The first part was kept in 10% neutral buffered formalin at room temperature for histopathological examination and immunohistochemical measurements, while the other part was snap frozen in liquid nitrogen and kept at -80°C for further determination of biological parameters. Tissue homogenates were prepared using Polytron PT1200E disperser (Kinematica AG, Luzern, Switzerland) in ice-cold phosphate-buffered saline (PBS, pH 7.2). Homogenates were centrifuged at 12,000×g for 10 min and supernatants were used for measurements.

Liver function tests: Serum activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) as well as albumin and bilirubin levels were determined colorimetrically using commercial kits supplied by Biodiagnostic (Giza, Egypt).

Hepatic oxidative stress markers: Malondialdehyde (MDA) level was measured in the supernatants from liver homogenates by a colorimetric method using a kit supplied by Diamond Diagnostics (Cairo, Egypt). The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was measured using a kit supplied by BioVision, Inc. (Milpitas, CA, USA).

Quantitative real-time PCR for the expression of TLR4, MyD88 and toll/interleukin-1 receptor domain-containing adaptor inducing interferon-beta (TRIF) in liver tissues: Total RNA was extracted from liver using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA pellet was resuspended in DEPC-treated water. The quality and concentration of the RNA were assessed using the OD 260/280 ratio and only samples with ratios above 1.5 were used in the experiments. Total RNA was reverse transcribed using revert aid premium reverse transcriptase-kit (Fermentas International Inc., Burlington, Canada). Briefly, revert aid H Minus MMuLV reverse transcriptase was added to dNTP mix (10 mM), 5x reaction buffer and random hexamer primers; the mixture was subjected to cDNA synthesis cycling conditions at 37°C for 30 min and at 85°C for 5 min. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using ABI PRISM 7500 sequence detector system (Applied Biosystems, Foster city, CA, USA), using the Maxima SYBR green qPCR kit (Fermentas International Inc., Burlington, Canada). Primer sequences (5'-3') were as follows: TLR4, forward: CTACCTCGAGTGGGAGGACA, reverse: TGCTACTTCCTT GTGCCCTG; MyD88, forward: TGTCTCCCCTGACATGCCTA, reverse: TTTTGTGTGTCCGCTGCTTG; TRIF, forward: TGGAT ATCTCCCCTTCGCCT, reverse: TGGAGGTGAGACAGACCCTT and β-actin, forward: TA TCCTGGCCTCACTGTCCA, reverse: AACGCA GCTCAGTAACAGTC. Reaction mixtures contained 5 pmol μL^{-1} of each primer, 25 µL Maxima SYBR mix and 22.5 µL nuclease-free water. An amount of 0.5 µL of template cDNA was added to each reaction mix. The thermal cycling protocol

consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec and a final cycle at 72°C for 10 min. Data from real-time assays were calculated using Sequence detection Software (PE Biosystems, Foster city, CA, USA). Relative expression of studied genes was calculated using the comparative C_t method. All values were normalized to the β-actin gene expression and reported as fold change over background levels detected in diseased group[32].

Hepatic TNF-α level: Tumor necrosis factor-alpha (TNF-α) level was measured by ELISA technique using a kit supplied by R and D systems (Minneapolis, MN, USA).

Liver histopathology: Liver specimens were fixed using 10% neutral buffered formalin. After proper fixation, the specimens were dehydrated in ethyl alcohol of increasing concentrations (70, 90 and 100%), cleared in xylol, impregnated and then embedded in paraffin wax. Sections, 5 μm thick were cut using a rotatory microtome. The sections were then stained with H and E stain and examined under light microscope. A morphometric analysis for the percentage of damaged area was performed using Leica Qwin 500 image analyzer (Leica Microsystems GmbH, Wetzlar, Germany) as described by Li *et al.*[33].

Immunohistochemical staining: Immunohistochemical staining was performed on formalin-fixed paraffin embedded liver sections, 5 μm thick. The sections were washed with PBS (pH 7.4) and treated with 0.3% H_2O_2 for 20 min to block endogenous peroxidase activity. After three washes with PBS, the sections were incubated with the primary antibody (α-smooth muscle actin "α-SMA" or NF-κB, 1:100, Thermo Fisher Scientific, Fremont, CA, USA) in a humidified chamber at 37°C for 3 h. A biotinylated secondary antibody and streptavidin peroxidase complex were consecutively applied at 37°C for 15 min each and three PBS washes were performed between applications. Antibody binding was visualized by color development using 3,3 -diaminobenzidine (DAB) supplied by Sigma-Aldrich (St., Louis, MO, USA). The nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich, St., Louis, MO, USA) and the slides were mounted and examined under a light microscope. The optical density of positive staining of NF-κB and α-SMA in each photograph was measured and the average of all photographs in each group was calculated using Leica Qwin 500 image analyzer (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis: All data are expressed as Mean±Standard Error of the mean (SEM). Statistical analysis was performed using Graphpad prism software version 6 (GraphPad Software Inc., La Jolla, CA, USA). The statistical significance of differences between groups was tested using one-way analysis of variance (ANOVA) followed by Tukey's post-test. A significant difference was assumed for values of $p<0.05$.

RESULTS

Effect on liver function tests: Table 1 shows that CCl_4 resulted in significant increases in the serum activities of ALT (882±66 vs. 49±1.6 U L^{-1}, $p<0.05$), AST (1719±197 vs. 152±2.9 U L^{-1}, $p<0.05$) and LDH (2195±88 vs. 1000±17 U L^{-1}, $p<0.05$) and bilirubin level (1.84±0.06 vs. 0.56±0.02 mg dL^{-1}, $p<0.05$), whereas it caused a significant reduction in serum albumin level (2.9±0.05 vs. 4±0.04 g dL^{-1}, $p<0.05$) compared to the control group. The administration of both CIN and SIL significantly ($p<0.05$) reduced the serum activities of ALT (by -53 and -42%, respectively), AST (by -46 and -45%, respectively) and LDH (by -46 and -40%, respectively) and the serum bilirubin level (by -52 and -59%, respectively) compared to CCl_4-treated rats. On the other hand, CIN and SIL resulted in significant increases in serum albumin level (by +30%, $p<0.05$) compared to CCl_4-treated rats. The effects of CIN and SIL were comparable regarding liver function tests except for LDH activity that was significantly lower in CIN-treated rats.

Pathological changes: Liver sections of control rats showed normal histological structures with hepatic cords radiating from the central vein forming anastomosing plates separated by blood sinusoids. Hepatocytes appeared polyhedral with eosinophilic cytoplasm having central rounded and vesicular nuclei (Fig. 1a). On the other hand, liver sections of CCl_4-treated rats showed few hepatocytes with normal histological structures, whereas many hepatocytes had fatty degeneration in which the cells are distended with large fat globule pushing the nuclei toward the periphery. In addition, they showed dilated and congested blood vessels (Fig. 1b, c). The administration of CIN restored the normal histological structures of the liver tissue. We observed mononuclear cellular infiltrations within the portal tract area with congested

Table 1: Effect of cinnamaldehyde on liver function in CCl_4-treated rats

Parameters	Control	CCl_4	CIN	SIL
ALT (U L^{-1})	49±1.6	882±66[a]	415±14[ab]	513±13[ab]
AST (U L^{-1})	152±2.9	1719±197[a]	932±50[ab]	952±20[ab]
LDH (U L^{-1})	1000±17	2195±88[a]	1190±104[b]	1327±51[ab]
BIL (mg dL^{-1})	0.56±0.02	1.84±0.06[a]	0.88±0.04[ab]	0.75±0.09[ab]
ALB (g dL^{-1})	4±0.05	2.9±0.05[a]	3.7±0.05[ab]	3.7±0.09[ab]

Values are expressed as Mean±SEM (n = 6), ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, LDH: Lactate dehydrogenase, BIL: Bilirubin, ALB: Albumin. Statistical analysis was performed using ordinary one-way ANOVA, followed by Tukey's post-test. [a]$p<0.05$ vs. control, [b]$p<0.05$ vs. CCl_4

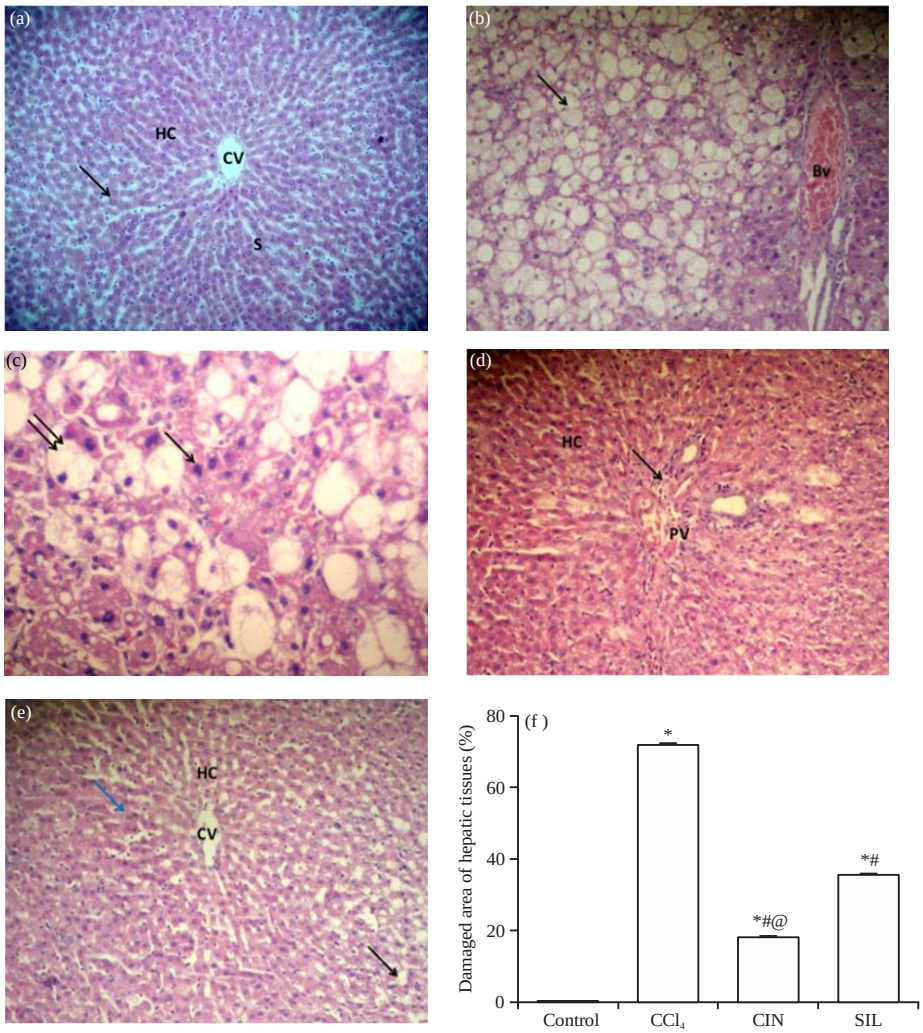

Fig. 1(a-f): Histopathological examination of liver tissue. Representative photomicrographs are depicted from (a) Control rats showing normal histological structures with Hepatic Cords (HC) radiating from the Central Vein (CV) forming anastomosing plates separated by blood sinusoids (S). Hepatocytes are polyhedral with eosinophilic cytoplasm having central rounded and vesicular nuclei (arrow) (H and E x200), (b) CCl_4-treated rats showing fatty degeneration (arrow) with dilated and congested blood vessels (Bv) (H and E x200), (c) CCl_4-treated rats showing distended cells with large fat globule pushing the nuclei toward the periphery (double arrows) with few hepatocytes appearing normal (arrow) (H and E x400), (d) Cinnamaldehyde (CIN)-treated rats showing the restoration of the normal histological structure of the liver with mononuclear cellular infiltration within the portal tract area (arrow) and congested Portal Vein (PV) (H and E x200), (e) Silymarin (SIL)-treated rats showing partial improvement in the histological structure of the liver with some hepatocytes having eosinophilic cytoplasm and central rounded and vesicular nuclei (blue arrow), as well as other cells showing fatty degeneration (black arrow) (H and E x200) and (f) Quantitative morphometric analysis showing the percentage of the damaged liver area, data are expressed as Mean±SEM (n = 5), statistical analysis was performed using ordinary one-way ANOVA, followed by Tukey's post-test, *$p<0.05$ vs. control, $^{\#}p<0.05$ vs. CCl_4 and $^{@}p<0.05$ vs. SIL

portal vein (Fig. 1d). Silymarin (SIL) administration resulted in a partial improvement in the histological structures of the liver tissue. Some hepatocytes have eosinophilic cytoplasm with central rounded and vesicular nuclei, while other hepatocytes still show fatty degeneration (Fig. 1e).

As depicted in Fig. 1f, a morphometric analysis for the damaged area of the hepatic tissues disclosed a significant, substantial increase in the percentage of the damaged area in rats treated with CCl_4 (72%) compared with control rats (0.2%). Treatment of rats with CIN or SIL significantly abrogated the

damage induced by CCl_4 showing 18 and 36% damage, respectively. Interestingly, the protective effect exerted by CIN was significantly higher than that of SIL ($p<0.05$).

Effect on hepatic oxidative stress markers: In order to evaluate the influence of CIN on oxidative stress in the liver tissues, we measured the level of MDA, a product of lipid peroxidation, which is used frequently as a marker of oxidative stress. Table 2 shows that CCl_4 significantly increased MDA levels (11.8 ± 0.75 vs. 1.6 ± 0.15 nmol g^{-1} tissue, $p<0.05$) compared to control group. Administration of CIN and SIL significantly diminished CCl_4-induced elevation in MDA level by -54 and -58%, respectively. Moreover, the effect of CIN on hepatic oxidative stress was verified by measuring the ratio of GSH-GSSG, which is another sensitive marker of oxidative stress. Compared to the control group, CCl_4 significantly lowered GSH level and the ratio of GSH/GSSG by -34 and -79%, respectively. The treatment of rats with either CIN or SIL was effective in reversing the effects of CCl_4 that was observed as significant elevations in GSH level by approximately 18% (for both drugs) and the ratio of GSH/GSSG by 123 and 92%, respectively. Although non-significant, CIN produced apparently better improvement in the ratio of GSH/GSSG compared to SIL. This effect is attributed to the greater reduction in GSSG level rather than increase in GSH level.

Effect on the expression of TLR4 and toll/interleukin-1 receptor (TIR)-domain-containing adaptors: Figure 2 shows that treatment of rats with CCl_4 upregulated the hepatic

Table 2: Effect of CIN on hepatic oxidative stress markers in CCl_4-treated rats

Parameters	Control	CCl_4	CIN	SIL
MDA (nmol g^{-1} protein)	1.6 ± 0.15	11.8 ± 0.75^a	5.4 ± 0.34^{ab}	5 ± 0.003^{ab}
GSH (μmol g^{-1} protein)	58 ± 0.65	38 ± 1.2^a	45 ± 1.05^{ab}	45 ± 2.4^{ab}
GSSG (μmol g^{-1} protein)	17.3 ± 0.27	53.7 ± 1.9^a	28.4 ± 0.05^{ab}	33.2 ± 0.9^{ab}
GSH/GSSG ratio	3.41 ± 0.02	0.71 ± 0.04^a	1.58 ± 0.05^{ab}	1.36 ± 0.11^{ab}

Values are expressed as Mean±SEM (n = 3), MDA: Malondialdehyde, GSH: Reduced glutathione, GSSG: Oxidized glutathione. Statistical analysis was performed using ordinary one-way ANOVA, followed by Tukey's post-test. [a]$p<0.05$ vs. control, [b]$p<0.05$ vs. CCl_4

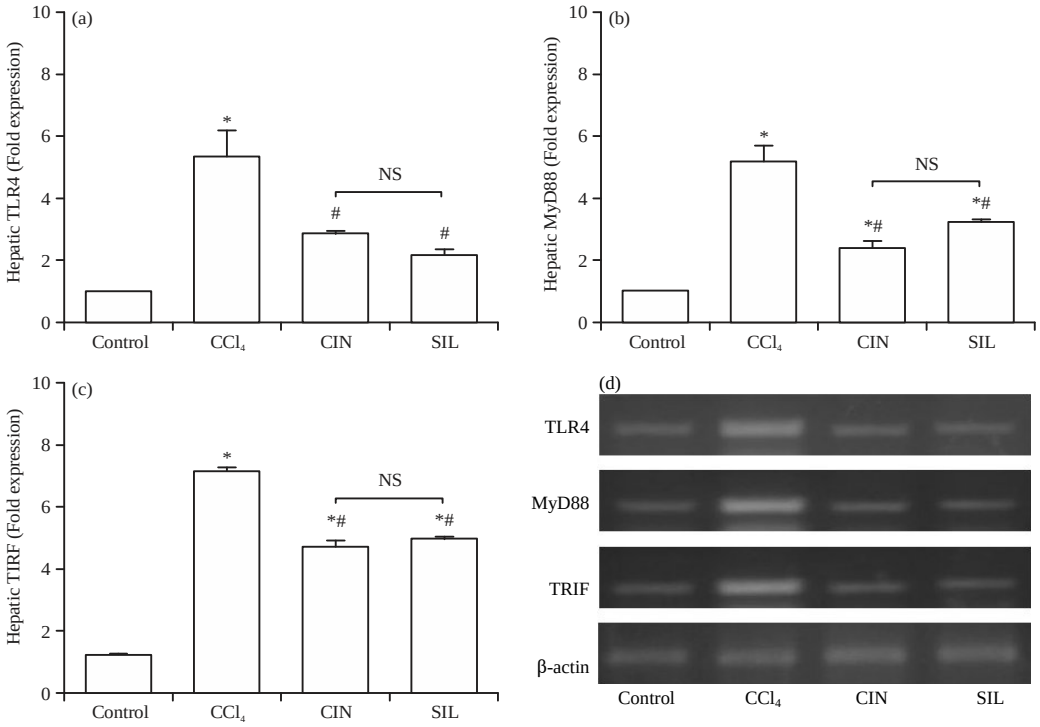

Fig. 2(a-d): Effect of CIN on the hepatic expression of (a) TLR4, (b) MyD88, (c) TRIF in CCl_4-treated rats and (d) A representative agarose gel electrophoresis showing PCR products in different groups. Data are expressed as Mean±SEM (n = 3). Control: Rats received olive oil (280 μL/100 g b.wt., once daily for 3 days followed by 140 μL/100 g b.wt., once daily for the following 3 days, p.o.) plus 0.9% NaCl (25 μL/100 g b.wt., once daily for 6 days, p.o.), CCl_4: Rats received CCl_4/olive oil mixture (1:1, v/v) at a dose of 280 μL/100 g b.wt., once daily for 3 days followed by a dose of 140 μL/100 g b.wt., once daily for the following 3 days by oral gavage plus 0.9% NaCl (25 μL/100 g b.wt., once daily for 6 days, p.o.), CIN: Rats received CCl_4/olive oil mixture and 0.9% NaCl plus cinnamaldehyde (10 mg kg^{-1} once daily for 6 days, p.o.), SIL: Rats received CCl_4/olive oil mixture plus silymarin (100 mg kg^{-1} day^{-1}, p.o.). Statistical analysis was performed using ordinary one-way ANOVA, followed by Tukey's post-test, *$p<0.05$ vs. control, #$p<0.05$ vs. CCl_4

expression of TLR4, as well as the adaptors, MyD88 and TRIF, that mediate its signaling pathways. Compared to the control group, the expression of TLR4, TRIF and MyD88 were increased significantly by 5.4, 7.2 and 5.2 fold ($p<0.0.5$), respectively. The administration of CIN or SIL ameliorated the elevated expression induced by CCl_4. Compared to the CCl_4 group, treatment of rats with CIN or SIL significantly lowered the expression of TLR4 by -48 or -59%, TRIF by -35 or -32% and MyD88 by -54 or 38%, respectively.

Effect on hepatic NF-κB and α-SMA expression and TNF-α level: Treatment of rats with CCl_4 caused significant ($p<0.05$) elevations of NF-κB and α-SMA expression and TNF-α level (108 ± 5.3 vs. 29 ± 0.4 pg g^{-1} protein) in hepatic tissues compared to the control group. The administration of either CIN or SIL significantly reduced the expression of NF-κB and α-SMA compared to rats treated with CCl_4 alone. Cinnamaldehyde-induced reduction in NF-κB expression was significantly higher than that produced by SIL. On the other hand, there was no significant difference in the expression of α-SMA between the two groups. Similarly, treatment of rats with either CIN or SIL significantly lowered TNF-α level by -43 and -45%, respectively, compared to rats treated with CCl_4 alone (Fig. 3, 4).

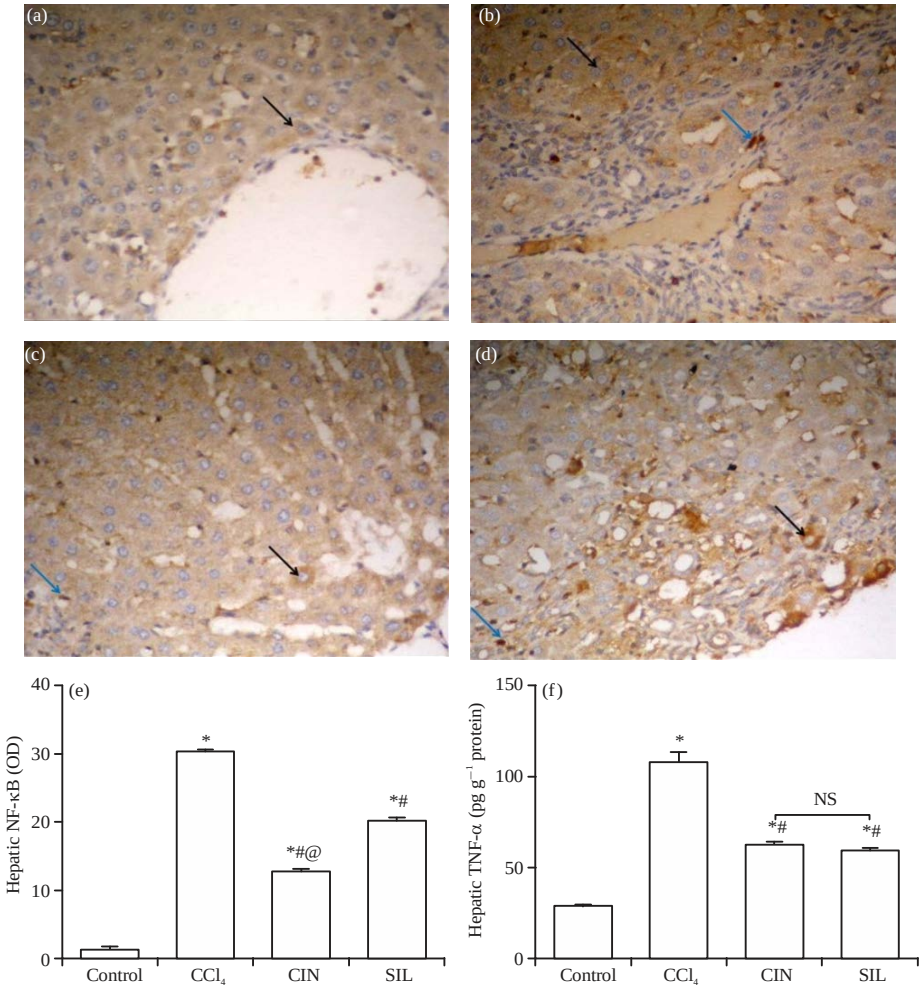

Fig. 3(a-f): Effect on hepatic NF-κB expression and TNF-α level. Immunohistochemical staining of hepatic NF-κB (Streptavidin-biotin peroxidase stain with Mayer's hematoxylin counterstain x400) from (a) Control rats showing very weak immunoreaction (arrow), (b) CCl_4-treated rats showing strong positive cytoplasmic (black arrow) and nuclear (blue arrow) immunoreactions, (c) Cinnamaldehyde (CIN)-treated rats showing mild cytoplasmic (black arrow) and nuclear (blue arrow) immunoreactions, (d) Silymarin (SIL)-treated rats showing moderate cytoplasmic (black arrow) and nuclear (blue arrow) immunoreactions, (e) Quantitative analysis for the Optical Density (OD) of positive staining and (f) Effect on TNF-α level. Data are expressed as Mean±SEM (n = 3-5), statistical analysis was performed using ordinary one-way ANOVA, followed by Tukey's post-test, *$p<0.05$ vs. control, $^{\#}p<0.05$ vs. CCl_4, $^{@}p<0.05$ vs. SIL

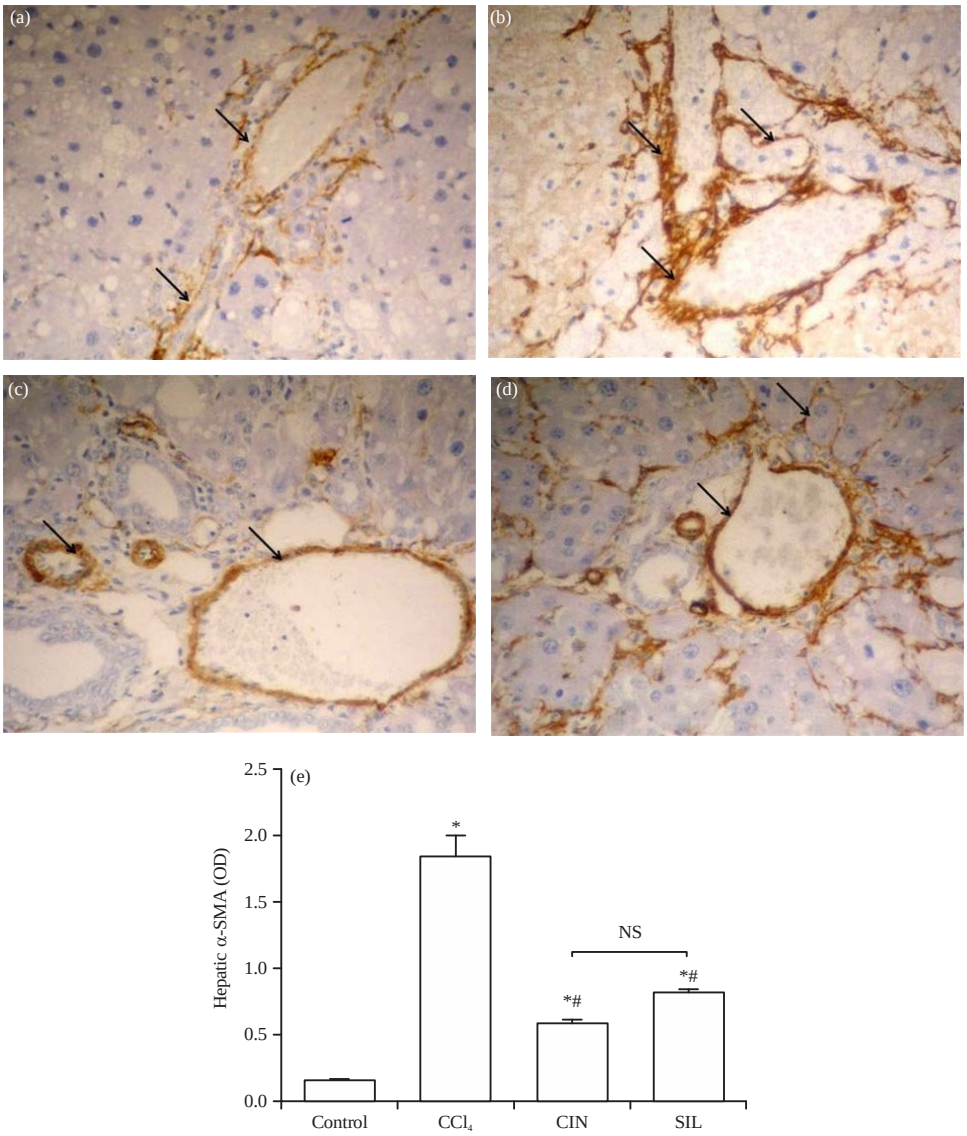

Fig. 4(a-e): Effect on hepatic α-SMA expression. Immunohistochemical staining of hepatic α-SMA (Streptavidin-biotin peroxidase stain with Mayer's hematoxylin counterstain x400) from (a) Control rats showing weak immunoreaction (arrows) around the blood vessels of portal area, (b) CCl_4-treated rats showing strong positive (arrows) immunoreaction predominantly between hepatocytes, the fibrous septa and portal area, (c) Cinnamaldehyde (CIN)-treated rats showing mild immunoreaction (arrows). This reaction is restricted to the portal area, (d) Silymarin (SIL)-treated rats showing moderate immunoreaction (arrows) and (e) Quantitative analysis for the Optical Density (OD) of positive staining. Data are expressed as Mean±SEM (n = 5), statistical analysis was performed using ordinary one-way ANOVA, followed by Tukey's post-test, *$p<0.05$ vs. control, $^{\#}p<0.05$ vs. CCl_4

DISCUSSION

In the current study, a significant hepatoprotective effect of CIN against acute liver injury induced by CCl_4 in rats was showed. In a comparable way to other studies in the literature, our CCl_4 model induced acute liver injury manifested as substantial increase in the serum activities of ALT, AST and LDH, which are considered as key markers for liver damage[34,35]. This injury was associated with impairment in the liver function revealed as a significant increase in serum level of total bilirubin and reduction in albumin, which are suggested as markers of hepatic function[36]. In addition, histological examination of liver tissues demonstrated many pathological changes escorting liver dysfunction including fatty

degeneration and increased percentage of damaged liver area on morphometric analysis (Fig. 1). Carbon tetrachloride-induced hepatotoxicity is attributed to the production of free radicals, via its metabolism by the hepatic monooxygenase (P450 2E1) system. These free radicals are implicated in the lipid peroxidation of hepatocyte membranes leading to the leakage of intracellular enzymes, steatosis and liver injury[8,37]. Lipid peroxidation and increased oxidative stress were demonstrated as increased level of hepatic MDA and decreased level of GSH, which is utilized in the detoxification process of free radicals. In addition, the ratio of GSH/GSSG was significantly decreased. The latter marker indicates the redox state of the GSH pool and is often used as a marker of cellular toxicity and oxidative stress[38].

Liver inflammation is a common event in many acute and chronic liver diseases[39]. Toll-like receptors (TLRs), which are expressed on different hepatic structures, play an important role in the initiation and progression of liver inflammation. Theses receptors recognize PAMPs and DAMPs[40]. There is a growing body of evidences suggesting a link between oxidative stress and initiation of proinflammatory response, that is likely mediated through TLRs[41]. Eleven and thirteen functional TLRs have been identified in human and rodents, respectively[42]. Different studies demonstrated that TLR4, rather than TLR2 has a crucial impact in the pathogenesis of liver injury induced by ischemia/reperfusion[43,44]. TLR4 can interact with both MyD88 and TRIF, which are intracellular adaptor molecules to initiate its downstream signaling. It was interesting to evaluate the role of TLR4 in the pathogenesis of CCl_4-induced acute hepatotoxicity. The administration of CCl_4 significantly upregulated the expression of TLR4 and its adaptor proteins, MyD88 and TRIF (Fig. 2). Two separate TLR4-signaling pathways have been identified, which are known as MyD88-dependent and TRIF-dependent pathways. The MyD88 pathway involves the downstream activation of NF-κB and mitogen-activated protein kinases (MAPKs). On the other hand, TRIF pathway results in the activation of the transcription factors interferon regulatory factor (IRF)3, NF-κB and consequently in the induction of type I IFN and inflammatory cytokines. However, both pathways can result in the activation of a common downstream mediator, NF-κB[45,46]. Activated NF-κB is translocated to the nucleus where it acts as a transcription factor that promotes the expression of genes involved in inflammation, such as TNF-α, interleukin (IL)-1 and chemokines[46,47]. Indeed, IHC staining for the immunoexpression of NF-κB in liver tissues demonstrated a noticeable increase and nuclear translocation of activated NF-κB in CCl_4-treated rats. This was associated with a significant increase in the hepatic content of TNF-α (Fig. 3).

Furthermore, we detected by means of IHC staining a significant increase in the expression of α-SMA in liver tissues of CCl_4-treated rats. The elevation of α-SMA expression is considered as a marker for Hepatic Stellate Cells (HSCs) activation and fibrosis[48] that occurs mainly during chronic liver injury[49]. However, Dechene *et al.*[50] reported that acute liver injury can also be associated with activation of HSCs. It is likely that NF-κB induced by TLR4 stimulation is involved in HSCs activation[51]. These results suggest a pivotal role of oxidative stress, inflammation, mediated through TLRs signaling and the activated HSCs in acute liver injury induced by CCl_4.

Cinnamaldehyde (CIN) is a diterpene found in *Cinnamomum cassia*[52]. The CIN has a strong anti-oxidant activity both *in vivo* and *in vitro*[19]. Further, different studies demonstrated that CIN exerts anti-inflammatory effect in different experimental models including cardiac ischemia[53] and neuro-inflammation[54]. In addition, Youn *et al.*[55] showed that CIN has anti-inflammatory effect *in vitro* mediated through the suppression of TLR4. Therefore, the protective effect of CIN against acute liver injury induced by CCl_4 was evaluated. The administration of CIN ameliorated hepatic damage and improved liver function in rats treated with CCl_4, which was associated with a corresponding improvement in histopathological markers and reduction in the damaged area of the liver. These results are in harmony with Tung *et al.*[56] who reported that CIN exhibits a protective effect in another model of acute hepatitis induced by lipopolysaccharide/D-galactosamine in mice. The improvement in oxidative stress markers suggests that the hepatoprotective effect of CIN is mediated, in part through its antioxidant effect. In addition, it is likely that CIN was able to uncouple the crosstalk between oxidative stress and TLR4 signaling, because CIN was effective in suppressing the TLR4 expression and associated adaptor proteins. This is translated as a reduction in the release of downstream transcription factors and inflammatory cytokines responsible for the inflammatory response occurring during acute liver injury. The suppression of TLR4 signaling pathway has been attributed to the inhibition of receptor oligomerization[55].

The hepatoprotective effect exhibited by CIN is at least comparable to that of SIL. We observed a trend, albeit non-significant, for better improvement in different parameters (e.g., ALT, AST, LDH activities and GSH/GSSG ratio) in CIN-treated rats compared to SIL-treated rats. In particular, CIN resulted in significant reductions of the damaged liver area and NF-κB expression compared to SIL. The latter effect seems interesting because both agents suppressed TLR4 signaling pathway and reduced TNF-α level, which are implicated in NF-κB activation via the canonical pathway[57], in

a similar way. Therefore, we assume that CIN might have further suppressed NF-κB activation by inhibiting the alternative pathway. This non-canonical pathway can be triggered, independent of TNF-α, by B-cell activation factor (BAFFR), lymphotoxin β-receptor (LTβR), CD40 and receptor activator for NF-κB (RANK)[58,59]. However, this hypothesis is still to be further elucidated.

CONCLUSION

In conclusion, we provide evidence that CIN may be a therapeutic candidate for the protection of acute liver injury via suppression of oxidative stress and inflammation mediated by TLR4 signaling pathway. Considering its availability and safety, CIN can be a promising alternative to SIL in managing liver disorders. Further, hepatic patients or individuals at a high risk of liver injury can be advised to add CIN, as a flavoring agent into their diet.

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