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Geraniol prevents CCl_4-induced hepatotoxicity via suppression of hepatic oxidative stress, pro-inflammation and apoptosis in rats

Abdulmohsen I. Algefare [a], Manal Alfwuaires [a], Ademola C. Famurewa [b,c,*,1], Hany Elsawy [d], Azza Sedky [a,e,**]

[a] Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa 31982, Saudi Arabia
[b] Department of Medical Biochemistry, Faculty of Basic Medical Sciences, College of Medical Sciences, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo, Ebonyi State, Nigeria
[c] Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka State, India
[d] Department of Chemistry, Faculty of Science, Tanta University, Tanta 31527, Egypt
[e] Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt

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ABSTRACT

Carbon tetrachloride (CCl_4) is a classic chemical hepatotoxicant that triggers liver damage through hepatic exacerbation of oxidative stress. Geraniol (GRL) is a natural bioactive acyclic monoterpene with several pharmacological effects. We thus explored whether GRL could prevent CCl_4-triggered hepatic toxicity. Rats were divided and administered GRL (100 mg/kg) and/or CCl_4 (1 ml/kg of 1:1 v/v CCl_4: olive oil) in Control group, GRL group, CCl_4 group, GRL + CCl_4 groups 2 times per week for 4 consecutive weeks. CCl_4 caused significantly ($p < 0.05$) elevated serum activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TB), whereas the albumin (ALB) and total protein (TP) levels were significantly ($p < 0.05$) reduced relative to the control group. The liver activities of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) decreased significantly ($p < 0.05$), while malondialdehyde (MDA) level evidently elevated in comparison to the control group. The CCl_4 exposure caused significant increases in proinflammatory interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), apoptotic caspase-3 and caspase-9 levels, whereas the anti-inflammatory interleukin-4 (IL-4) and interleukin-10 (IL-10) were reduced in consistent with histopathological changes compared to the control. On the contrary, the GRL administration prevented the hepatic toxicity and lesions through restoration of liver status markers, antioxidant enzyme activities, MDA, cytokines and apoptosis in comparison to the CCl_4 group. Altogether, the findings reveal that GRL could abrogate CCl_4-provoked hepatic toxicity via inhibition of hepatic oxidative stress, inflammation and apoptosis in rats.

1. Introduction

Carbon tetrachloride is an industrial chemical and a classic hepatotoxicant. Carbon tetrachloride (CCl_4) is still found as a component of insecticides, fire extinguishers and grains exposed to fumigation [22]. Human exposure to CCl_4 is well recognised to cause liver fibrosis and injury to the liver organ; hence, it is an established experimental agent for development of hepatotoxicity model [29]. The target organ of CCl_4 metabolism is the liver, where it is metabolised by cytochrome P450-enzymes. The hepatic metabolism produces toxic oxidant metabolites like trichloromethyl peroxyl ($\bullet OOCCl_3$) and trichloromethyl ($\bullet CCl_3$) oxygen free radicals associated with hepatic oxidative degeneration and cell death. Several studies indicate that the oxygen free radicals orchestrate impairment in hepatic membrane organization and antioxidant homeostasis leading to activation of aberrant signalling that favours inflammatory cascades and apoptotic cues [25,3,6]. Specifically, trichloromethyl radical reacts with sulfhydryl (-SH) group of thiol proteins, glutathione and glutathione-based enzymes to overrun the hepatic

* Corresponding author at: Department of Medical Biochemistry, Faculty of Basic Medical Sciences, College of Medical Sciences, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo, Ebonyi State, Nigeria.
** Correspondence to: Department of Biological Sciences, College of Science, King Faisal University, PO Box 400 Al-Ahsa, 31982 Al-Ahsa, Saudi Arabia.
E-mail addresses: ademola.famurewa@funai.edu.ng (A.C. Famurewa), asadek@kfu.edu.sa (A. Sedky).
[1] ORCID: 0000-0002-8795-9964.

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antioxidant system as well as glutathione metabolism [6]. These intracellular alterations in the liver predominantly provoke pathophysiology progressing to hepatic injury, non-alcoholic fatty liver disease, fibrosis, cirrhosis, hepatitis, and even hepatocellular carcinoma depending on the dose and duration [33]. For example, studies have shown that CCl_4 induces hepatotoxicity and mitochondrial damage characterized by redox imbalance, inflammation and apoptosis with consistent histological necrosis and infiltration of inflammatory cells [25,34,7]. In recent studies, however, the role of CCl_4-induced reactive oxygen species (ROS), pro-inflammation and altered signalling cascades in hepatic fibrosis have been confirmed [22,24,8]. Therefore, antioxidants such as natural polyphenols with known anti-inflammatory potentials may exert inhibitory effects against CCl_4 hepatotoxicity.

Several studies have demonstrated that natural phytoactive compounds exhibiting antioxidant potentials can combat the ROS-mediated hepatotoxicity caused by CCl_4 [6,33]. Geraniol is a natural bioactive acyclic monoterpene with the chemical formular $C_{10}H_{18}O$. GRL (3, 7-dimethylocta-trans-2, 6-dien-1-ol) is an important content of palmrosa, lemon, ginger, lavender, orange essential oils, rose, and about 20 % component of GRL oil content [18]. The antioxidant and several pharmacological effects of GRL have been reported [17,20,8], such as the enhancement of antioxidants. GRL enhances antioxidant enzyme activities in dopaminergic neurons against ROS injury and apoptotic cell death in Parkinson's disease [23]. GRL blocks lead-induced or 2-acetylaminofluorene-induced hepatotoxicity through attenuation of oxidative stress, inflammation and apoptosis in rats [13,19]. The report of El Azab et al., [8] was only found on the efficacy of GRL against CCl_4-induced hepatic oxidative stress and inflammation. Till date, it is unknown whether GRL could halt CCl_4-induced oxidative stress-mediated apoptosis. Therefore, the study was conceived to explore the possible protective action of GRL against CCl_4-induced hepatic damage via oxidative stress, inflammation and apoptosis in rats.

2. Materials and methods

2.1. Chemical and reagents

CCl_4 and GRL were purchased from Aladdin (China). Commercial kits were purchased from Jiancheng Co. (Nanjing, China).

2.2. Animals

Wistar rats (weight 200–240 g, eight weeks old,) were procured from the Animal House of Faculty of Science, King Faisal University, Saudi Arabia. The study protocols were in consistent with the research ethics at the King Faisal University (Reference number: KFU-REC-2022-DEC-ETHICS399). Animal cages with wire mesh that allows for good ventilation at 25 ± 2 °C, 50–60 % relative humidity and 12 h night/12 h day were used for this study. The rats were subjected to food and water without any restriction throughout the study period. Acclimatization was conducted by allowing the rats to feed on chow and water for one week.

2.3. Experimental design

At the end of acclimatization, the rats (male) were divided randomly into 4 groups (n = 6).

2.3.1. Group 1 (Control)

Rats received olive oil (0.5 ml/kg b.w) intraperitoneally (ip) on the 1st and 4th day of each week for 4 weeks.

2.3.2. Group 2 (GRL)

Rats received only GRL (100 mg/kg/day) orally (os) for 4 weeks.

2.3.3. Group 3 (CCl_4) group

Rats was administered only CCl_4 (1 ml/kg b.w of 1:1 v/v CCl_4: olive oil, ip) on the 1st and 4th day of each week for 4 weeks.

2.3.4. Group 4 (GRL+ CCl_4)

Rats was administered CCl_4 (1 ml/kg b.w of 1:1 v/v CCl_4: olive oil, ip) on the 1st and 4th day of each week for 4 weeks + GRL (100 mg/kg/day, os) for 4 weeks.

The doses of CCl_4 and GRL were chosen according to previous studies [14,21]. On the 29th day, rats were anesthetized, and blood samples collected into tubes for determination of liver function indices. The clotted blood was centrifuged at 2500*g* for 10 min at 4 °C. The serum obtained was stored at − 20 °C temperature. The rats were euthanized using 10 mg/kg xylazine and 100 mg/kg ketamine HCl for collection of liver samples for biochemical and histological analyses.

2.4. Biochemical analysis

2.4.1. Determination of hepatic markers

The serum samples were used for measuring the activities and levels of liver markers, AST, ALT, ALP, total protein (TP), albumin (ALB), and total bilirubin (TB) using standard kits and the kit instructions were followed for the assay steps.

2.4.2. Determination of oxidative stress markers

Liver sample (100 mg) was homogenized in PBS and centrifuged at 3000*g* for 20 min at 4 °C. The supernatant was separated into tubes. The liver activities of catalase (CAT, Cat. No. A007–1-1), superoxide dismutase (SOD, Cat. No. A001–1), and glutathione peroxidase (GPx, Cat. No. A005–1-1), as well as malondialdehyde (MDA, Cat. No. A003–1-2) level were estimated using commercial kits.

2.4.3. Determination of inflammatory markers

The liver supernatant samples were used to estimate the liver levels of pro-inflammatory TNF-α (Cat No R019) and IL-6 (Cat No R016), and anti-inflammatory IL-4 (Cat No R013) and IL-10 (Cat No R017). Rat standard ELISA kits were used and the determinations were conducted according to the manufacturer's instructions.

2.4.4. Apoptotic caspases estimation

Hepatic caspase 9 (Cat No A069) and caspase 3 (Cat No A064) were quantitatively measured in line with the manufacturer's directions.

2.4.5. Histopathology

Hepatic tissues were fixed with 10 % buffered formalin followed by the routine processes of blocking, paraffin and sectioning (5 μm thick) with microtome, and then mounted on glass slides. The processed sections were stained with H&E solution. Any histopathological alterations were viewed and photographed with a light microscope (Nikon 80i, Japan). The histopathological changes were scored semi-quantitatively and mean values were determined: normal histostructure (0), mild (1), moderate (2), and severe alterations consistent with congested central vein and presence of inflammatory cells and vacuolation (3) [9].

2.4.6. Statistical analysis

Data were presented as mean ± standard error of mean (SEM) obtained from the 6 rats in each group. The Graph Pad Prism statistical program (version 8; GraphPad Software Inc., San Diego, CA, USA) was used to analyse data by one-way AVONA followed by Tukey's post-hoc test. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of GRL on liver function markers

Table 1 shows the effect of GRL on liver function markers in CCl_4-

Table 1
Effect of GRL on liver function indices in CCl_4-intoxicated rats.

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	TB (mg/dl)	ALB (g/dl)	TP (g/dl)
Control	35.5 ± 0.1	45.3 ± 0.2	25.9 ± 0.2	0.3 ± 0.1	4.30 ± 0.1	5.8 ± 0.11
GRL	36.2 ± 0.2	45.2 ± 0.2	28.4 ± 0.1	0.29 ± 0.1	4.30 ± 0.1	5.7 ± 0.14
CCl_4	50.3 ± 0.1*	153.3 ± 0.2*	55.0 ± 0.5*	0.66 ± 0.1*	2.11 ± 0.1*	3.1 ± 0.10*
GRL + CCl_4	40.2 ± 0.2#	84.2 ± 0.2#	30.1 ± 0.3#	0.38 ± 0.1#	3.20 ± 0.1#	4.1 ± 0.10#

Data are presented mean ± SEM (n = 6 rats/group). CCl_4: carbon tetrachloride; GRL: Geraniol; *P < 0.05: significant compared to the control group in the same column. #P < 0.05: significant compared to the CCl_4 group in the same column.

exposed rats. The CCl_4 exerted toxicity on the liver; CCl_4 significantly ($p < 0.05$) increased the serum activities of ALT, AST, ALP and level of TB in comparison to the control. Additionally, CCl_4 markedly reduced ($p < 0.05$) the levels of TP and ALB in comparison to the control. The administration of GRL prevented the toxic effect of CCl_4 demonstrated by significant reductions in serum activities of AST, ALP, ALT, and level of TB coupled with marked elevated levels of TP and ALB compared to the CCl_4 group.

3.2. Effect of GRL on oxidative stress markers

Fig. 1 presents the effect of GRL on oxidative stress indices in CCl_4-exposed rats. The CCl_4 significantly ($p < 0.05$) reduced the hepatic activities of CAT, SOD and GPx, while level of MDA was increased in comparison to the control. The GRL administration in GRL + CCl_4 group significantly ($p < 0.05$) increased the activities of CAT, SOD and GPx, while level of MDA decreased significantly compared to the CCl_4 group.

3.3. Effect of GRL on hepatic inflammatory and anti-inflammatory markers

Fig. 2 presents the effect of GRL on hepatic anti-inflammatory and pro-inflammatory marker levels in CCl_4-injected rats. The CCl_4 exposure induced significant increases ($p < 0.05$) in hepatic IL-6 and TNF-α levels compared to the control; CCl_4 triggered significant decreases in the anti-inflammatory IL-4 and IL-10 levels in the current study. GRL markedly ($p < 0.05$) inhibited the alterations in the hepatic TNF-α, IL-6, IL-4 and IL-10 levels compared to the CCl_4 group.

3.4. Effect of GRL on hepatic apoptosis caspases

Fig. 3 presents the effect of GRL on hepatic apoptosis marker levels in CCl_4-injected rats. The CCl_4 exposure induced significant increases ($p < 0.05$) in the hepatic levels of caspase-3 and caspase-9 compared to the control. GRL significantly ($p < 0.05$) exerted reduction in the levels

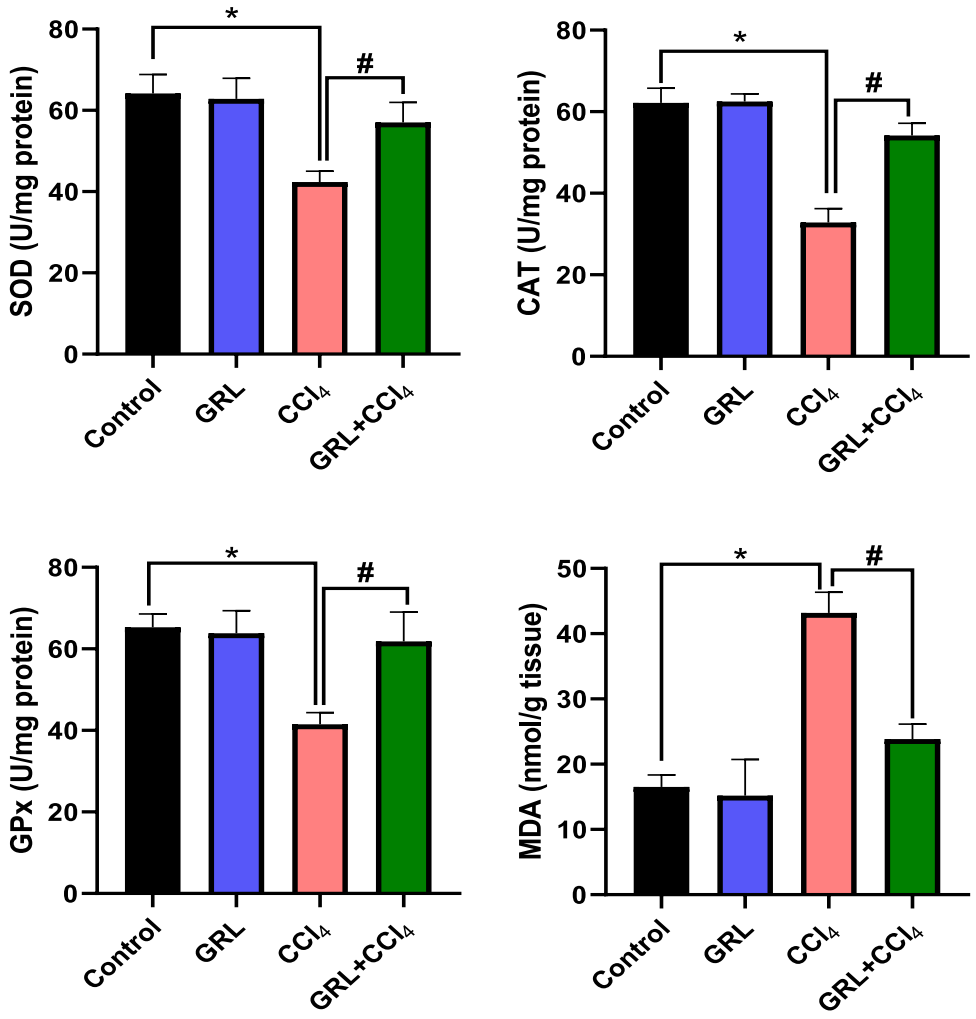

Fig. 1. Effect of GRL on hepatic activities of oxidative stress markers in rats. GRL: Geraniol; CCl_4: Carbon tetrachloride; data are shown as mean ± SEM (6 rats/group). *p < 0.05: significant in comparison to the control group. #p < 0.05: significant in comparison to the CCl_4 group.

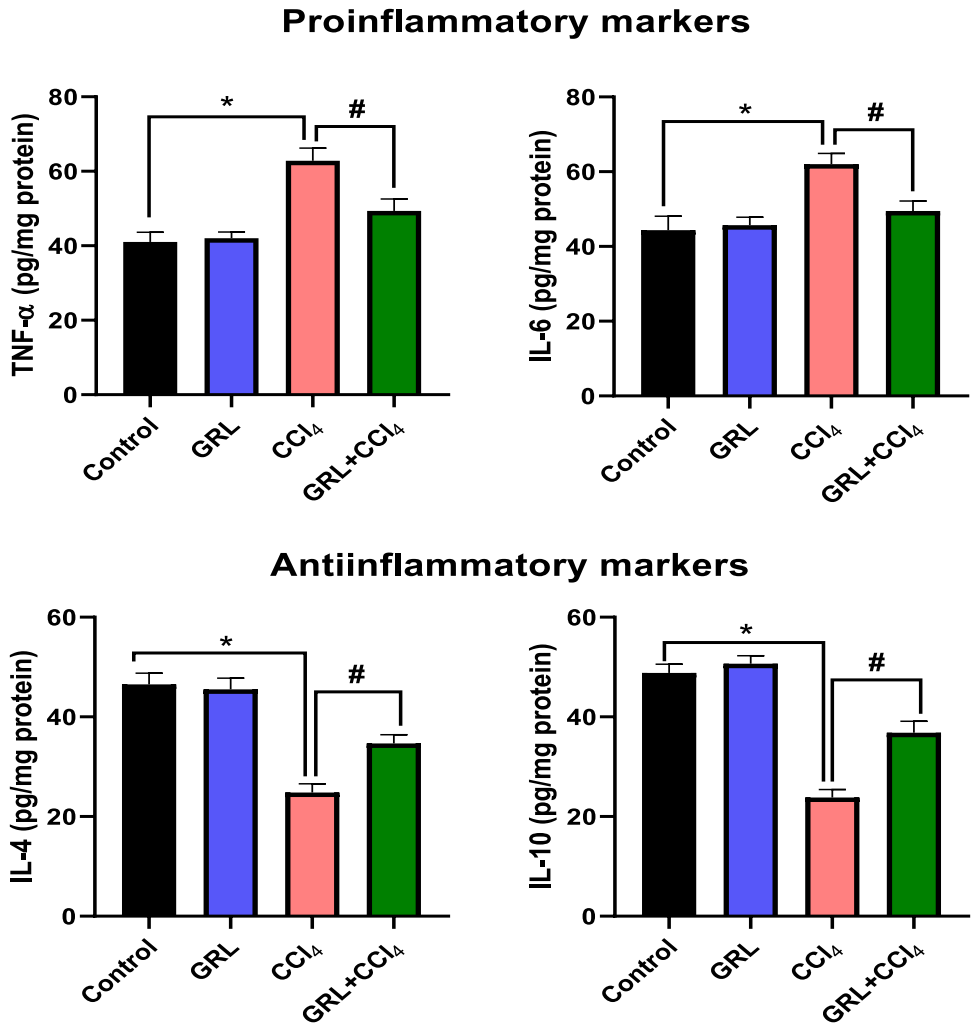

Fig. 2. Effect of GRL on liver cytokine levels in CCl4-injected rats. NRL: Geraniol; CCl_4: Carbon tetrachloride; data are shown as mean ± SEM (n = 6). *p < 0.05: significant in comparison to the control group. #p < 0.05: significant in comparison to the CCl_4 group.

of caspase-3 and caspase-9 compared to the CCl_4 group.

3.5. *Effect of GRL on the liver histology*

Fig. 4 depicts the results of the histopathological analysis of the effect of GRL and CCl_4 on the liver of rats. The control and the GRL group liver samples revealed normal ultrastructure with evident normal hepatocyte, blood sinusoid, and central vein (a and b). In the CCl_4 group, histopathological abrasions like congested central vein and inflammatory cell infiltration; the hepatocytes appeared degenerated with vacuolated cytoplasm (c–e). However, in the GRL + CCl_4 group, we observed amelioration regarding the degeneration, congestion and infiltration of inflammatory cells (f).

4. Discussion

CCl_4 is a potential carcinogen and an established hepatotoxicant; nevertheless, it remains an industrial solvent, and humans are exposed to it at workplaces [6]. Given the critical biochemical role of the liver and its susceptibility to serious health problems worldwide, its protection from the attacks of exogenous toxicants is very important. Herein, our findings demonstrate that GRL could protect the liver from the oxidative and degenerative effects of CCl_4 via inhibition of oxidative inflammation and hepatic apoptosis.

In the current study, two times injection of CCl_4 per week for 4 consecutive weeks was used following the study of Hussein and Khan Ref. [14]. We thus found considerable liver damage revealed by significant elevations in the serum activities of liver enzymes, AST, ALT, ALP, and TB levels in comparison to the control group. Furthermore, the markers of hepatic protein synthetic capacity, ALB and TP, were markedly reduced in comparison to the control (Table 1). It therefore corroborates the earlier reports that CCl_4 is an hepatotoxicant [1,22,3, 8]. The liver is involved in the catabolism of drugs and xenobiotics. These enzymes, ALT and AST are preponderant in the hepatocytes and are used to monitor the effect of drugs and xenobiotics on the liver health status. The damage to the hepatocytes by the CCl_4 caused the outflow of the enzymes into the serum [11], whereas the increased activity of ALP is an indicator of hepatic-cholestatic damage [12]. Liver synthesises serum proteins to main transport and blood osmotic balance. The reduced levels of TP and ALB confirmed the reduced synthetic capacity of the liver occasioned by the CCl_4. Therefore, our results on the distortions of hepatic functional indices created by the CCl_4 injection are in agreement with the previous report [16,6,7]. In contrast, our concomitant administration of GRL to rats inhibited the CCl_4-induced hepatic damage in this study. We found that the activities of AST, ALT, ALP, as well as levels of TB, ALB and TP were significantly restored in the serum compared to the CCl_4-injected rats (Table 1). This demonstrates that GRL prevented the hepatic damage and also preserved the hepatic integrity such that the enzyme leakage into the blood circulation was significantly limited. Literature reveals the hepatoprotective effect of

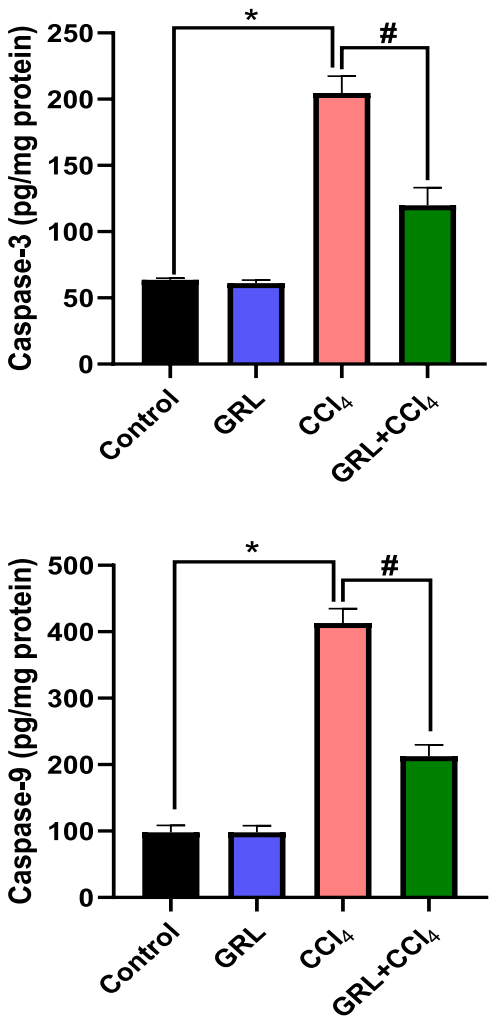

Fig. 3. Effect of GRL on liver caspase levels in CCl_4-injected rats. GRL: Geraniol; CCl_4: Carbon tetrachloride; data are shown as mean ± SEM (n = 6). *Significant in comparison to the control ($p < 0.05$); #significant in comparison to the CCl_4 group ($p < 0.05$).

GRL in animal studies [13,19]. Its beneficial effect has also been associated with its antioxidant properties [22,30].

Redox homeostasis modulates the physiological production of ROS for benefits in immune responses and balance by antioxidant mechanism [2]. However, exogenous agents are known to cause impaired redox balance provoking oxidative stress-mediated damage [10]. We explored the effect of CCl_4 on the hepatic antioxidant status. CCl_4 impaired the antioxidant mechanism in the liver of rats in this study revealed by significant diminution in the hepatic activities of CAT, SOD and GPx, and the consequent elevation in MDA levels (Fig. 1). Our finding herein corroborates the literature reports that the oxidant metabolites produced upon the hepatic metabolism of CCl_4 do overwhelm hepatic antioxidant milieu [3,32,33,8]. Robust body of evidence shows that covalent binding of CCl_4 metabolites, trichloromethyl free radicals, to cell proteins and antioxidant apparatus is the first step in a chain reaction that eventually leads to membrane lipid peroxidation, liver lesions and oxidative stress [29]. The cooperative effect of these conditions might contribute to the depressed SOD, CAT, and GPx activities and elevated MDA levels shown in this study [7,25]. Evidently, the histological results showed liver lesions such as congested central vein and degenerated cells that could be linked with the oxidative milieu [29]. The antioxidant efficacy of GRL is reported in the literature [21,23]. However, the sulfhydryl groups in glutathione, GPx and other thiol proteins can react with trichloromethyl to form an oxidative complex, and by extension, exerts deleterious effects on cellular antioxidant apparatus such as SOD and CAT, GPx, and reduced glutathione [6]. In this study, GRL exhibited antioxidant effect via improving the SOD, GPx and CAT activities against CCl_4 herein. These enzyme expressions were significantly elevated in the GRL + CCl_4 group compared to the CCl_4 group. Consequently, the level of MDA was also decimated significantly relative to the CCl_4 group. In oxidative stress-mediated diseases/disorder such as diabetes, diabetic cardiomyopathy, osteoarthritis, neuropathy and drug-induced toxicity, GRL has been underlined as a potent antioxidant agent for improvement of redox cycle balance [17,27,5,8]. Mechanistically, the GRL antioxidant action has been linked to its intracellular potential to activate the redox-sensitive pathway of nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) that promotes gene expression of antioxidant enzymes such as CAT, SOD and GPx among others via nuclear transcription [30]. Therefore, the GRL action in our study is in conformity with the earlier findings and could be attributed to the underlying activation/expression of antioxidant genes which were manifested via the increased activities of CAT, SOD and GPx.

Following the existing reports, CCl_4 oxidative stress causes activation of inflammatory and apoptotic cascades. CCl_4 action stimulates nuclear factor-kappa B (NF-κB), a nuclear transcriptional factor implicated in the expression of inflammatory cytokines and caspase-dependent apoptosis in liver damage [1,26,31,34]. To this end, we evaluated the effect of CCl_4 on pro-inflammatory (IL-6 and TNF-α), anti-inflammatory (IL-4 and IL-10) cytokines and caspase markers of apoptosis. It was observed that the levels of TNF-α and IL-6 significantly increased, whereas IL-4 and IL-10 levels significantly reduced in CCl_4-injected rats relative to the control rats (Fig. 2). This result shows that CCl_4 provoked pro-inflammation and reduces anti-inflammation [25,28]. NF-κB is translocated to the nucleus during cellular oxidative stress in the hepatic tissue [4], and its nuclear effect in terms of cytokine gene expression has been reported during CCl_4 hepatotoxicity [1,25]. In addition, we found histological infiltration of inflammatory cells (Fig. 4). It is therefore conceivable, in consonance with the previous findings, that CCl_4 could induce pro-inflammation in the liver [26,29,31]. Moreover, the cross-talk of inflammation and apoptosis is widely reported in published papers. For example, in a model of liver mitochondrial damage orchestrated by CCl_4, the network of oxidative stress and hepatic inflammation was found to trigger apoptosis [28,34]. Our findings herein also agree with the earlier papers in that the expression of caspase 3 and caspase-9 were significantly elevated in the liver of CCl_4-injected rats (Fig. 3). In contrast, the treatment with GRL evidently reversed the aberrant levels of the cytokines as well as the caspases. The hepatic IL-6 and TNF-α levels reduced while IL-4 and IL-10 levels increased significantly in GRL + CCl_4 group compared to the CCl_4 group in this study. We hereby confirm the anti-inflammatory effect of GRL in CCl_4-mediated inflammatory liver damage. On the other hand, the caspases were also reduced significantly as a result of the antiapoptotic effect of GRL. The anti-inflammatory and antiapoptotic effects of GRL may be associated with the mechanistic inhibitory effect of GRL on the inflammatory factors, activators and mitochondrial integrity as suggested by the literature [15,17,21,30].

In conclusion, the present study has demonstrated the hepatotoxicity of CCl_4 in consistent with the literature. Importantly, the present work unravels that GRL could exert hepatoprotective effect against CCl_4-triggered hepatic toxicity through mitigation of oxidative stress, inflammation and apoptosis. The effect could be adduced to the antioxidant, anti-inflammatory and antiapoptotic activities of GRL in this study.

CRediT authorship contribution statement

Azza Sedky: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Hany Elsawy:** Formal analysis, Funding acquisition, Investigation, Writing – review & editing. **Ademola C. Famurewa:** Conceptualization, Formal

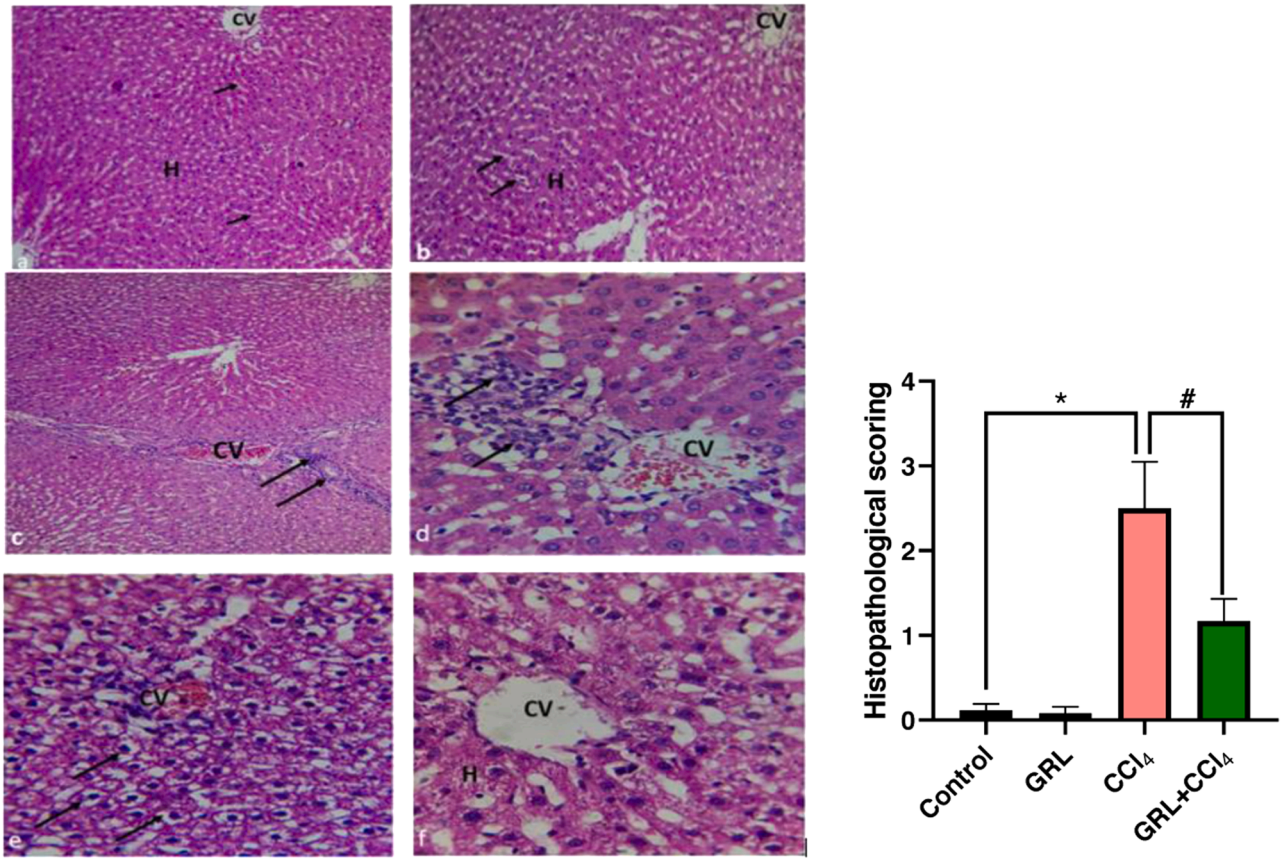

Fig. 4. Photomicrographs of liver tissue (H&E). a) Liver tissue of the control group showed normal architecture, hepatocyte (H) with normal structure, sinusoid (arrows) and central vein (CV) (X100); b) GRL-treated group showed normal hepatocytes (H) between blood sinusoid (arrows) and central vein (CV) (X100); c-e) CCl_4-injected group showing c) absence of hepatic architecture with congested central vein (CV) and inflammatory cell infiltration (arrows) (X100);d) congested central vein (CV) and inflammatory cell infiltration (arrows) (X400); e) congested central vein (CV) and hepatocytes with vacuolated cytoplasm (arrows) (X400); f) GRL + CCl_4 group showing improvement of structure of hepatocytes (H) and central vein (CV) (X400).

analysis, Writing – original draft, Writing – review & editing. **Manal A. Alfwuaires:** Conceptualization, Formal analysis, Writing – review & editing. **Abdulmohsen I. Algefare:** Conceptualization, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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