Original Article

# The Inhibitory Effect of Geraniol on CCL4-induced Hepatorenal Toxicity in Pregnant Mice through the PI3K/AKT Signaling Pathway

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## Abstract

**Background:** Hepatotoxicity caused by $CCL_4$ is well known. Geraniol (GNL) has high antioxidant effect that can induces liver regeneration. However, the protective effect of GNL effect on $CCL_4$-induced hepatorenal toxicity in pregnant mice has not yet been studied.

**Objective:** To investigate whether GNL could protect against oxidative stress induced by $CCL_4$ via the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which is regulated by phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT), and has been found to have protective effects on renal and hepatic tissues.

**Materials and Methods:** Forty-eight female albino mice weighing 25–30 g were randomly allocated to 4 groups: Group I served as a control; Group II received a toxicity-inducing single dose of 15 µL of $CCL_4$ on the 4$^{th}$ day after mating; Group III received 40 mg/kg GNL + $CCL_4$ (with GNL from the 1$^{st}$ day of assimilation to delivery); and Group IV received GNL alone from the 1$^{st}$ day of assimilation to the end of the delivery period. GNL was evaluated for its protective effects on hepatotoxicity in $CCL_4$-treated pregnant mice. Litter size, weight, survival rate, and resorption were recorded. In addition, H & E staining was done for liver and kidney pathology as well as biochemical markers and oxidative markers malondialdehyde, superoxide dismutase, and catalase were analyzed.

**Results:** $CCL_4$ significantly reduced survival rate and increased resorption after exposure. Alanine transaminase and aspartate aminotransferase concentrations in the serum, tissue MDA, blood urea nitrogen, and creatinine were increased after $CCL_4$ exposure. GNL improved enzyme and antioxidant levels and prevented $CCL_4$-induced hepatic injury in mice. Caspase-3 cleavage was decreased by GNL, which increased PI3K, phosphorylated AKT, Nrf2, and B-cell lymphoma 2.

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**Submitted:** 11-May-2023 **Revised:** 21-Aug-2023 **Accepted:** 08-Oct-2023 **Published:** 15-Jan-2024

| Access this article online | |
|---|---|
| Quick Response Code: | Website: https://journals.lww.com/sjmm |
| | DOI: 10.4103/sjmms.sjmms_225_23 |



**How to cite this article:** Alzahrani SA, Bekhet GM, Ammar RB, Abdallah BM, Ali EM, Al-Ramadan SY, *et al*. The inhibitory effect of geraniol on CCL4-induced hepatorenal toxicity in pregnant mice through the PI3K/AKT signaling pathway. Saudi J Med Med Sci 2024;12:17-26.

**Conclusion:** GNL demonstrates a protective effect against CCl4-induced hepatorenal toxicity, mediated through the activation of the PI3K/AKT signaling pathway and the upregulation of Nrf2. These findings highlight the potential therapeutic implications of GNL in mitigating oxidative stress and inflammation in liver and kidney tissues.

**Keywords:** Carbon tetrachloride, geraniol, oxidative stress, phosphoinositide 3-kinases/protein kinase B, pregnant

## INTRODUCTION

Chemically induced toxicity refers to the harmful effects that occur when a living organism is exposed to chemicals that disrupt its normal physiological functions. These chemicals, often referred to as toxicants or toxins, can come from a variety of sources, including industrial processes, household products, and environmental pollutants. The harm caused by chemically induced toxicity can vary widely, depending on the type of chemical, the dose, the duration of exposure, and an individual's susceptibility. Overall, chemically induced toxicity underscores the importance of understanding and minimizing exposure to harmful chemicals, whether through proper regulation, safe handling practices, or the development of safer alternatives.

Humans and animals experience significant changes in blood parameters during pregnancy. The livers of pregnant rats have been reported to have lowered levels of cytochrome p450.[1] According to these findings, pregnant individuals probably experience different modes of chemically induced toxicity.[2] $CCL_4$ metabolizes into trichloromethyl free radicals, which cause liver damage.[3] Free radicals are now recognized for the key roles they play in a number of biological systems. Depending on oxygen tension, they are formed as an inevitable by-product of aerobic respiration and other cytoplasmic processes. Free radicals play key roles in intracellular signaling, homeostasis, and stress response at physiological concentrations. However, if the concentrations are high enough, lipids, proteins, and DNA can be damaged indiscriminately.[4] Apoptosis may result in severe cases when cell function is disrupted. Although there are significant species differences, many aspects of early mammalian development, from fertilization to organ differentiation, take place *in vivo* in low-oxygen environments. As early embryos are sometimes exposed to ambient oxygen concentrations or the products of maternal metabolic disorders, this may protect them from free radical damage.[5]

The $CCL_4$ toxicity mechanism has been studied since the 1950s, resulting in the widely accepted idea that $CCL_4$ hepatotoxicity is caused by cytochrome P450 in liver cells; cytochrome P450 catalyzes the reductive dehalogenation of $CCL_4$ metabolism and also triggers a cascade of secondary mechanisms; these mechanisms are responsible for the ultimate plasma membrane disruption and other effects that lead to cell death. After being metabolized by cytochrome P450, $CCL_4$ can produce free radicals and reactive oxygen species (ROS) that damage the liver.[6] These metabolites cause lipid peroxidation, which can lead to liver damage.[7] Many hepatoprotective drugs have been tested using this model. It has previously been reported that antioxidants can reduce the risk of liver disease by preventing oxidative damage.[8,9] $CCL_4$-induced liver damage can be prevented and treated by directly reducing ROS levels and inhibiting the oxidative chain reaction induced by $CCL_4$.

Lipid second messengers, which transmit signals, are generated by the phosphatidylinositol 3 kinase (PI3K) pathway. In particular, PI3K activates protein kinase B (AKT), a downstream signaling molecule involved in a wide variety of biological responses, including cell proliferation, apoptosis, and inflammation. The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is regulated by PI3K/AKT, according to previous studies.[10-12] In addition, Nrf2 controls the expression of many antioxidant genes. According to previous studies, the Nrf2 pathway plays a significant role in gestation.[13-15] This makes the PI3K/AKT/Nrf2 pathways potential primary targets for the successful treatment of gestation.

Lemongrass tea contains several bio-compounds, including those of its decoction, infusion, and essential oil extracts.[16] There are many biological and pharmacological benefits to lemongrass tea.[17-19] It is nontoxic and non-mutagenic; consequently, it is used as an alternative medicine in many developing countries.[20] The essential oil of lemongrass contains the natural monoterpene geraniol (GNL).[21] Due to its high antioxidant effect, it also induces liver regeneration, mitigates cisplatin-induced neurotoxicity, and decreases trinitrobenzene sulfonic acid-induced colitis. GNL also has antiangiogenic properties that make it good for treating tumors.[22-24] The protective effect of GNL on $CCL_4$-induced hepatorenal toxicity in pregnant mice

has not been studied. This study explores the underlying mechanisms of the action of GNL on $CCL_4$-induced hepatorenal toxicity in pregnant mice.

## MATERIALS AND METHODS

### Materials

GNL was purchased from Sigma–Aldrich (St. Louis, MO, USA). The experiments were conducted with pure analytical-grade chemicals purchased from British Drug Houses (Poole, Dorset, UK).

### Animal treatment and experimental design

Forty-eight female albino mice weighing 25–30 g each were used. The mice were maintained at a temperature of 22 ± 2°C with a 12/12 h light/dark cycle. All animal protocols were approved by the Committee of Ethics at the Deanship of Scientific Research, King Faisal University.

The animals were randomly divided into 4 groups of 12 female mice each. Group I comprised the control animals, which were treated with corn oil intraperitoneally (i.p.); the group II animals were treated with $CCL_4$ (15µl) i.p. on the 4$^{th}$ day after mating; the Group III animals were treated with i.p. GNL (40 mg/wt) thrice weekly from the 1$^{st}$ day of assimilation to end of the experiment, and they were also treated with $CCL_4$ on the 4$^{th}$ day after mating. The group IV animals were treated with i.p. GNL alone from the 1$^{st}$ day of assimilation to the end of the experiment by [Figure 1]. The mating of the animals utilized vaginal plug formation. After the experiment periods, the animals were put into cages (flow rate: 15% of cage volume), followed by cervical dislocation to complete the euthanizing process. Six animals were used for the examination of the litter size, ovary structure, and germinal epithelium. Blood samples were collected through the retro-orbital plexus. Kidney and liver tissues were collected, weighed, and frozen to stop any metabolic activity and stored at −80°C until further analysis.

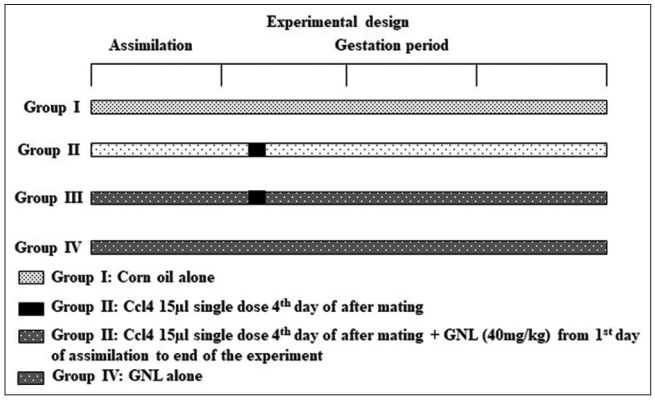


**Figure 1:** Animal experiment design

### Serum biochemical marker measurement

The blood urea nitrogen (BUN) and creatinine (Cr) levels were determined using blood samples. A small amount of blood was withdrawn from the retro-orbital plexus (about 0.5 mL). A Sekisui Medical (Tokyo, Japan) assay kit was used to measure the BUN and Cr levels.

### Biomarker evaluation

The serum activities of the hepatic function biomarkers were evaluated. The aspartate aminotransferase (AST) and Alanine transaminase (ALT) levels were measured using commercial kits (RandoxTM Laboratories Limited, Crumlin, UK).

### Biochemical analysis

The mouse liver and kidney samples were assayed for oxidative stress. The protein concentration was measured according to the Bradford protocol,[25] while the superoxide dismutase (SOD) activity was evaluated using a previously reported method.[26] We assessed the glutathione S-transferases (GST) and glutathione peroxidase (GPx) enzyme activities using previously reported methods.[27,28] In addition, the glutathione levels and malondialdehyde (MDA) were measured using previously published methods.[29,30]

### Western blot analysis

The Western blot study was performed according to the methods described by previous publication.[31] Membranes were blocked for 2 h with 5% nonfat milk, and then probed with primary antibodies. Dilution is shown in Supplementary Table 1. To visualize the protein bands, a 3600-00-C-Digit Blot Scanner was used. The control band was normalized to 1 with Image Studio Lite software (Lincoln, NE, USA). Each experiment was repeated three times.

### Histopathology

After being embedded in paraffin, cut into 4-µm thick sections, and stained with H and E, the liver and kidney tissues were fixed in 10% formalin. An optical microscope (Leica D6000, Leica, Wetzlar, Germany) was used for the observation and measurements of the prepared sections, and images were acquired (200× magnification).

### Statistical analysis

Data are represented as means ± SEM and were analyzed by Prism 5.0 statistical program (GraphPad Software Inc., San Diego, CA, USA). Comparisons between experimental groups were performed using one-way ANOVA followed

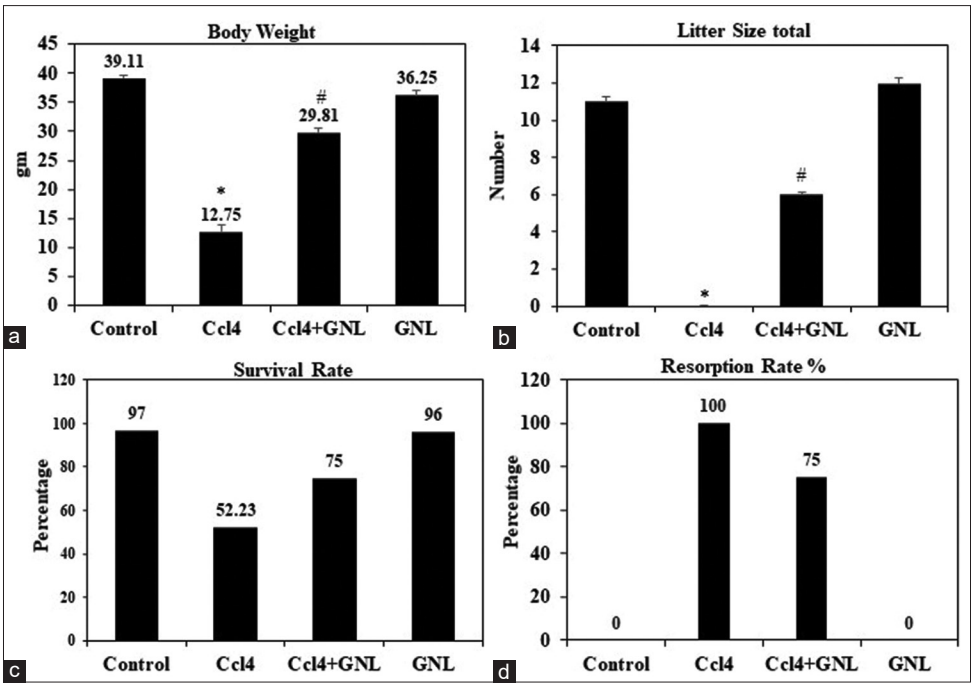


**Figure 2:** Effect of GNL on the body and organ index in mice: (a) Body weight; (b) Litter size total; (c) Survival rate; (d) Resorption rate. $^{*}P < 0.05$ indicates significant differences between the $CCL_4$ group and control group. $^{\#}P < 0.05$ indicates significant differences between the $CCL_4$ group and $CCL_4$ + GNL group

by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Effect of geraniol on body weight of pregnant mice

Figure 2a shows the effect of GNL on the $CCL_4$-induced body weight of the pregnant mice. In the second group, the $CCL_4$-induced body weights of the animals were significantly ($P < 0.05$) reduced in comparison to the body weights of the pregnant mice in the control group. The body weights of those mice that received GNL pretreatment were significantly elevated in comparison to the body weights of the mice treated with $CCL_4$ alone.

### Effect of geraniol on litter size

As seen in Figure 2b, $CCL_4$ exposure had a significant effect on litter size. The number of live-born pups dropped at the 15 μl/kg dose level; this was caused by the significant effects of $CCL_4$ on litter size ($P < 0.05$), such that $CCL_4$ administration led to no live-born pups (all the pups were resorbed). The number of live-born pups in the group treated with GNL and $CCL_4$ was significantly higher than that of the mice administered $CCL_4$ alone. Both the control mice and the mice treated with GNL alone had no noticeable differences.

### Survival rate effect of geraniol on carbon tetrachloride-induced pregnant mice

The effect of the GNL treatment on the survival rate was studied after the $CCL_4$ administration. In comparison with the $CCL_4$-treated groups, the GNL treatment significantly decreased mortality rates. Of the pregnant mice injected with $CCL_4$, 50% of them died [Figure 2c]. Compared to the control mice, GNL alone did not cause any significant differences.

### Geraniol effect on carbon tetrachloride-induced resorption rate

Figure 2d shows the resorption rates of the experimental and the control animals. In the mice treated with $CCL_4$, all the pups were resorbed. Compared to $CCL_4$ alone, the percentage resorbed in the animals pretreated with GNL was significantly lower.

### Geraniol effect on levels of blood urea nitrogen and creatinine in mice serum

As shown in Figure 3a and b, we found high levels of serum biochemical parameters, such as BUN and Cr, in CCL4-alone treated animals. However, the administration of GNL significantly ($P < 0.05$) inhibited these effects.

### Effect of geraniol on alanine transaminase and aspartate aminotransferase

GNL was assessed for its renal-protective effects through the assessment of serum biochemical parameters, such

as ALT and AST. Figure 3c and d show how GNL affects the serum biochemical parameters induced by $CCL_4$. Compared with the control group, the serum ALT and AST levels in the $CCL_4$-treated mice were increased significantly ($P < 0.05$). The animals administered GNL, however, did not have the increased serum ALT and AST levels indicated in the $CCL_4$-treated mice ($P < 0.05$). According to the results, GNL almost completely protected against $CCL_4$-induced renal and hepatic toxicity.

## Effects of geraniol on antioxidant enzymes in liver and kidney tissues

We measured the levels of the antioxidant enzymes SOD, catalase (CAT), and MDA in the liver and kidney tissues to understand how GNL functions. The $CCL_4$ treatment

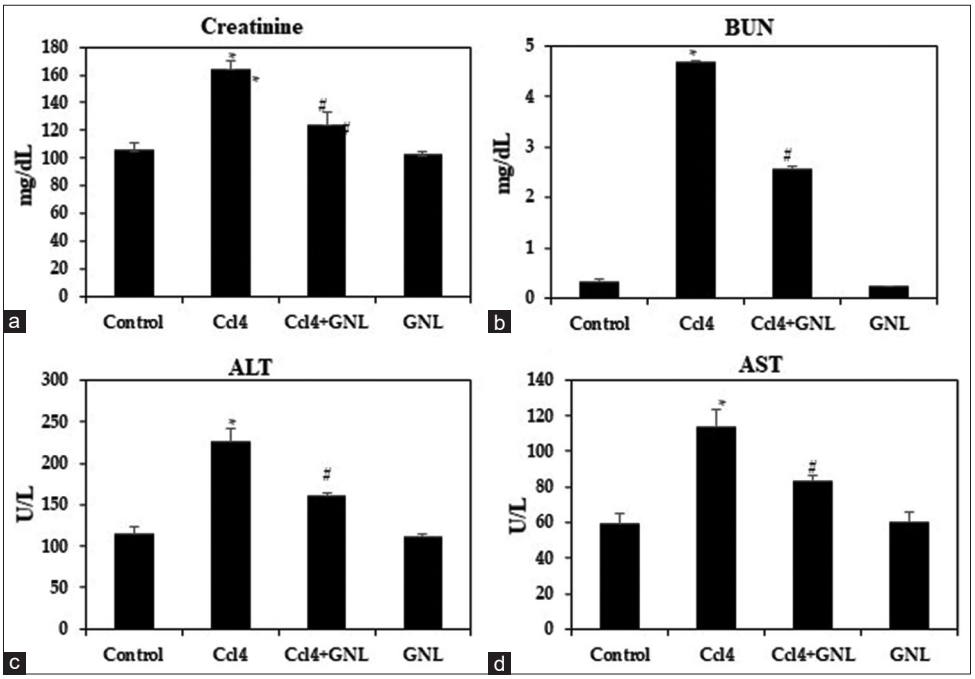


**Figure 3:** Effect of GNL on blood markers ALT, AST, BUN, and Cr in $CCL_4$-induced animals: (a) Creatinine (mg/dL); (b) Serum BUN (mg/dL); (c) ALT (U/L); (d) AST (U/L). Three repetitions were performed for each experiment. The data are represented as mean ± SEM ($n = 3$). *indicates $P < 0.05$ vs. control group; # indicates $P < 0.05$ vs. $CCL_4$ treatment alone

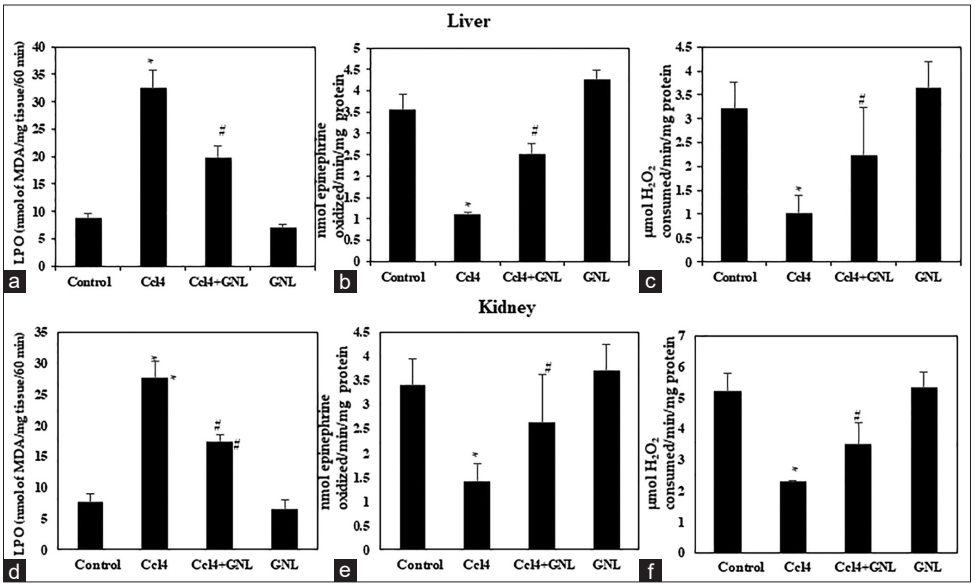


**Figure 4:** GNL reduces $CCL_4$-induced renal oxidative stress and liver and renal toxicity in mice. (a and d) Liver and renal LPO levels (μmoL/mg protein). (b and e) SOD activities (nmol epinephrine oxidized/min/mg protein). (c and f) Liver and renal CAT activities (μmol $H_2O_2$ consumed/min/mg protein). Data are represented as mean ± SEM ($n = 3$). * indicates $P < 0.05$ vs. control group; # indicates $P < 0.05$ vs. $CCL_4$ treatment alone

markedly increased MDA production and reduced the SOD and CAT levels in the liver [Figure 4a-c] and kidney tissues [Figure 4d-f]. $CCL_4$ significantly increased the MDA level compared with that in the control group, but the 40 mg/kg GNL administered before the $CCL_4$ treatment almost completely inhibited the $CCL_4$-induced elevation ($P < 0.05$) [Figure 4a and d]. According to Figure 4b, c, e and f, the SOD and CAT activity were almost normal ($P < 0.05$). By preventing oxidative injury, GNL protects the liver and kidney from $CCL_4$-associated damage.

## Effect of geraniol on liver pathology

Figure 5 provides a photomicrograph showing a section of untreated mouse liver (control group), which shows the central vein surrounded by cords of polygonal hepatocytes with granular cytoplasm and central, rounded, vesicular nuclei. Blood sinusoids are lined by flat endothelial cells(s) and 3 branches of the portal space portal vein, bile duct, and hepatic artery. The histopathological examination of liver samples in the control group showed normal liver morphology and no inflammatory cell infiltration or necrosis. In the photomicrograph showing a section of mouse liver treated with $CCL_4$ (Group II), it was found that the central vein was dilated and congested. In addition, necrosis of hepatocytes with hemorrhage was within the congested central vein, portal vein, and hepatic sinusoids. The necrosis was observed predominantly around both the centrilobular and the portal space regions; the necrotic hepatocytes barely have discerniblenuclei, vacuolization, and cells. There is an acute infiltration of inflammatory cells around the portal area, which is seen near the congested central vein; extensive foamy change in Kupffer cells is also seen. $CCL_4$ is commonly used to induce acute type A liver injury and hepatitis with granulomata (H and E ×400). In the photomicrograph showing a section of mouse liver treated with $CCL_4$ and GNL (Group III), centrilobular necrosis, cell swelling, and vacuolar degeneration were found (red arrows) along with hemorrhage within the dilated portal vein. The low level of inflammation around the bile duct was also noted (black arrow). The portal area branches of the portal vein, hepatic artery, and bile duct were seen at the portal tract. Therefore, GNL did improve liver histopathology (H and E ×400). In the photomicrograph showing a section of mouse liver treated with GNL alone (Group VI), it was found that the architecture of liver was normal with the central vein surrounded by cords of polygonal hepatocytes with granular cytoplasm and central, rounded, vesicular nuclei; blood sinusoids(s) and 3 branches of the portal space portal vein, bile duct, and hepatic artery were seen at the portal tract.

## Effect of geraniol on kidney pathology

Figure 6 is the photomicrograph of control mouse kidney showing normal architecture renal corpuscles with their glomeruli surrounded by proximal and distal renal tubules and a blood vessel. In the photomicrographs of a mouse kidney treated with $CCL_4$, many of the renal tubules show apoptotic renal tubules and necrotic renal tubules (arrows) and the associated mild focal tubular vacuolization of the tubular cell cytoplasm compared with the control kidney. Interstitial hemorrhage and intra-tubular hemorrhage are shown. The photomicrographs of a mouse kidney treated

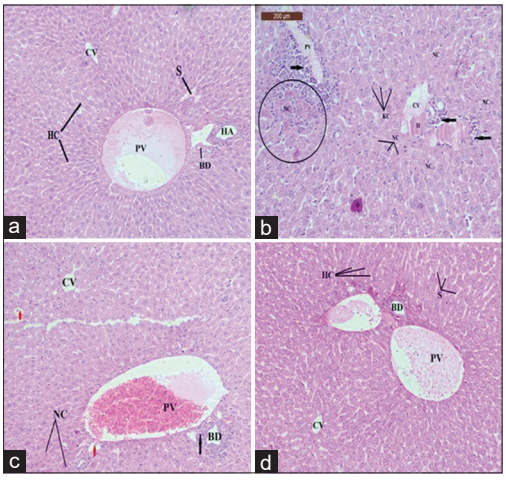


**Figure 5:** Photomicrography showing a sections liver tissues stained by haemotoxyloin and eosin (H and E ×200). (a) Control animal. (b) $CCL_4$ (Group II) (c). $CCL_4$ and GNL (Group III). (d) GNL (Group VI). Central vein (CV), Vesicular nuclei (HC), Portal vein (PV), Bile ductile (BD) and Hepatic artery (HA). Central vein (CV), Necrosis (NC), hemorrhage (H), Kupffer cells (K), Centrilobular necrosis (NC), hemorrhage (H). Vesicular nuclei (HC), blood sinusoids (S)

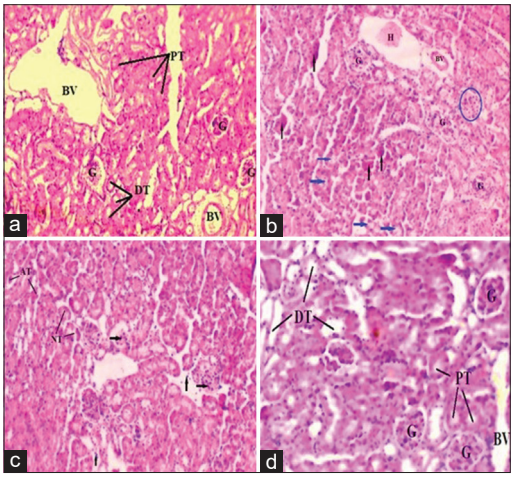


**Figure 6:** Photomicrography of kidney tissues stained by haemotoxyloin and eosin (H and E ×200). (a) Group I: Control group, (b) Group II: $CCL_4$, (c). Group III, $CCL_4$ and GNL. (d) GNL alone. glomeruli (G) proximal and distal renal tubules (PT and DT respectively) and blood vessel (BV). Apopototic renal tubules (AT) and necrotic renal tubules (NT) hemorrhage and intra-tubular hemorrhage (H)

with $CCL_4$ and GNL show focal interstitial inflammation with diffuse and marked inflammatory infiltrate (black arrows); in addition, some apoptotic and necrotic renal tubules were seen. The photomicrograph of mouse liver treated with GNL alone shows normal architecture of renal corpuscles with their glomeruli and proximal and distal renal tubules; blood vessels are also shown.

### Effects of geraniol on the activation of phosphoinositide 3-kinases/protein kinase B in carbon tetrachloride-induced mice

The activation of phosphatidylinositol 3 kinase (pPI3K)/pAKT in the liver was examined to determine how GNL inhibits $CCL_4$-induced liver toxicity. Studies have shown that PI3K/AKT signaling facilitates an essential cell survival signal in liver tissues.[32-34] Our study investigated whether exogenous GNL protects against the liver toxicity caused by $CCL_4$ by activating the PI3K/AKT pathway. In comparison with the control animals, $CCL_4$ treatment decreased PI3K/AKT phosphorylation in the liver. However, as shown in Figure 7, GNL activated pPI3K/pAKT phosphorylation.

### Effects of geraniol on the activation of nuclear factor-erythroid-2-related factor 2 in carbon tetrachloride-induced mice

The Nrf2 protein prevents genome instability through its role as a sensor of oxidative or electrophilic stress. As well as adjusting the expression of antioxidant proteins, it protects cells from the damage caused by oxidative stress.[9,31] Therefore, we hypothesized that GNL could activate antioxidant genes in $CCL_4$-induced liver oxidative stress. The expression levels of the Nrf2 signaling proteins were assessed. As shown in Figure 8, $CCL_4$ stimulation increases the total Nrf2 levels in liver tissue pretreated with GNL. In these studies, Nrf2 signaling was implicated in the protective effects of GNL against $CCL_4$-induced liver toxicity.

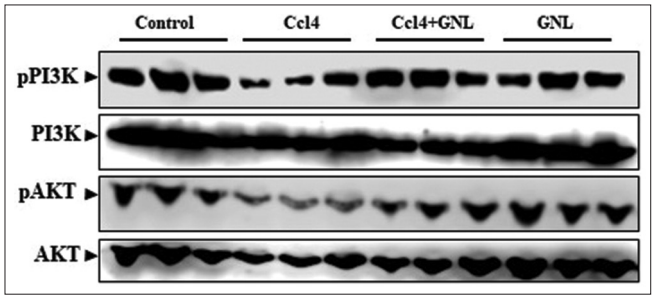


**Figure 7:** GNL induces the activation of pPI3K/pAKT signaling pathways. Western blot analysis showed the activation of pPI3K/pAKT signaling pathways. Data are represented as mean ± SEM ($n$ = 3). * indicates $P < 0.05$ vs. control group; # indicates $P < 0.05$ vs. $CCL_4$ treatment alone

### Effects of geraniol on B-cell lymphoma 2 expression

Our study examined the effect of GNL on B-cell lymphoma 2 (Bcl-2) expression, another major antiapoptotic regulator. As shown in Figure 8, we first determined the protein level expression of Bcl-2 in $CCL_4$-treated mice and found that the Bcl-2 expression was reduced. In contrast, the GNL treatment prevented apoptosis by upregulating Bcl-2, which is essential for survival.

### Effect on geraniol on cleaved caspase-3

A classical apoptosis marker was measured to determine whether GNL reduced $CCL_4$-induced apoptosis. Cleaved caspase-3 is considered to be the most significant apoptosis effector. As shown in Figure 8, GNL treatment significantly inhibited caspase-3 activity in $CCL_4$-induced liver tissues in comparison to the respective untreated controls ($CCL_4$ alone).

## DISCUSSION

The liver plays a crucial role in pregnancy due to its vital functions in maintaining maternal health, supporting fetal development, and ensuring a successful pregnancy outcome. The liver is responsible for metabolizing and detoxifying various substances that enter the body, including drugs, hormones, and waste products. During pregnancy, the metabolic demands on the body increase, and the liver plays a vital role in processing these additional substances to prevent their accumulation, which could be harmful to both the mother and the developing fetus.[35] The liver is involved in regulating hormone levels in the body, including sex hormones, insulin, and thyroid hormones. Hormonal changes are significant during pregnancy, and the liver helps maintain hormonal balance to support maternal adaptations to pregnancy and fetal growth. The liver stores glycogen, a stored form of glucose, and releases glucose into the bloodstream when needed to maintain stable blood sugar levels. During pregnancy, the body's demand for glucose increases, especially in the later stages, to provide energy for both the mother and

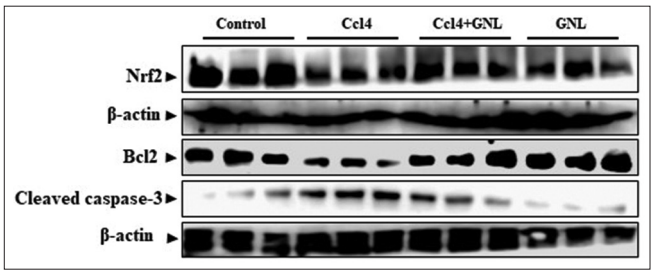


**Figure 8:** GNL-induced activation of the Nrf2 signaling pathways. Western blot analysis showed the activation of Nrf2, Bcl-2, and caspase-3 protein expression. Data are represented as mean ± SEM ($n$ = 3). An *indicates $P < 0.05$ vs. control group; # indicates $P < 0.05$ vs. $CCL_4$ treatment alone

the growing fetus. The liver helps regulate blood sugar levels to meet these demands. The immune system and hormones change during pregnancy, making it a unique physiological state.[36] Despite being rare during pregnancy, liver disorders can be deadly, causing both morbidity and mortality.[37] While researchers are aware of the harmful effects of $CCL_4$, their understanding of the molecular mechanisms underlying detoxification has not been fully clarified. In the present study, we examined how GNL protects the liver and kidneys from $CCL_4$-induced damage. Animals are mainly affected by $CCL_4$ in the liver and kidneys.

As a result of lipid peroxidation, oxidative lipid damage markers are created. MDA represents the progression of lipid peroxidation, while thiobarbituric acid reactive substance represent the final product. Acute liver damage is marked by the biochemical markers ALT and AST. ALT is mainly localized in the cytoplasm, while AST is mainly found in the mitochondria.[38] We found that $CCL_4$ exposure increased the pro-oxidative marker MDA and the liver damage markers (ALT and AST), and it reduced the antioxidant defense capacity (SOD and CAT); these findings are similar to those of other studies. As a result of damage to the cell and subcellular membranes, $CCL_4$ increases ALT and AST activity. According to the $CCL_4$ studies, the antioxidant defense capacities decrease because the toxic and reactive metabolites of $CCL_4$ consume them, but also because the enzymes become nonfunctional due to their modification by $CCL_4$.[39-41]

When GNL was pretreated before $CCL_4$ exposure, the acute liver injury markers and the pro-oxidative and proinflammatory markers were significantly reduced, as was the antioxidant enzyme consumption, compared with the toxic effects of $CCL_4$ in group III. A histopathological study also found that pretreatment with GNL significantly reduced centrilobular necrosis and the macrovesicular and microvesicular hepatocytes, as well as inflammatory infiltrate in animals exposed to the toxic effects of $CCL_4$. There is some evidence that GNL has hepatoprotective effects due to its ability to inhibit oxidative radicals and neutralize cytotoxic agents.[42,43] The induction of antioxidants by GNL can explain the reduced consumption of the antioxidant defense capacities and the significantly lower hepatotoxicity. We found that GNL induced antioxidants in a manner similar to the results of a study examining GNL's protective effect in chronic hepatotoxic models. This shows that GNL has a hepatoprotective effect beyond suppressing oxidative stress.

The survival pathways associated with p-PI3K and p-AKT can also mediate the liver survival pathway. Based on the results of our study, PI3K and AKT were found to be significantly inhibited in phosphorylation when exposed to $CCL_4$. In liver tissue treated with GNL, the p-PI3K and p-AKT levels were elevated and restored, which is consistent with previous findings.[43]

There are a number of antioxidant genes that are regulated by the transcription factor Nrf2, and there are those that are induced by this transcription factor. A very important aspect to keep in mind is that they are ubiquitously expressed in the human liver system. By suppressing oxidative stress, Nrf2 and its downstream targets play a crucial role in the maintenance of liver tissue homeostasis, thereby contributing to liver health. In addition, they are also involved in the onset and progression of liver failure.[44,45] Through its interaction with antioxidant responsive element, Nrf2 controls the transcriptional activation of antioxidant genes. GNL's antioxidant properties have been validated in several studies, but its underlying mechanism remains unclear. GNL inhibited the $CCL_4$ toxicity in mouse liver by activating the Nrf2 pathway. There is a low level of expression of Nrf2 during physiological conditions; however, in cases of oxidative stress caused by factors such as poisonings, hyperglycemia, hypoxia, and volatility, the expression of Nrf2 can be significantly enhanced.[46] This is consistent with the classical pattern of Nrf2 activation.[47] Furthermore, we found that the total level of Nrf2 was significantly decreased after $CCL_4$ treatment, whereas the total level of Nrf2 was increased after pretreatment with GNL, and the levels of Nrf2 were significantly decreased after $CCL_4$ treatment. This finding is consistent with the previous research.[9] We found that GNL promotes Nrf2 transfer from the cytoplasm to the nucleus, resulting in the increased expression of its downstream targets, which improves redox balance, protects mouse liver cells, and makes the body better at producing antioxidants.

Antiapoptotic proteins such as Bcl-2 protect the liver cells from apoptosis and oxidative stress.[48,49] Caspase-3 executes the apoptotic program and is mainly responsible for the cleavage of poly (ADP-ribose) polymerase when cell death occurs. This cleavage is produced by caspase-3 and capase-7, which are activated during apoptosis. The $CCL_4$-induced downregulation of Bcl-2 and the elevation of cleaved caspase-3 were upregulated and suppressed by GNL treatment in liver tissue, respectively. However, it is important to note that GNL treatment prevented the caspase cascade from being activated.

The kidney biomarkers in mice exposed to $CCL_4$ confirmed the histopathological lesions observed in this study. The renal tissue showed numerous apoptotic and necrotic renal

tubules, along with focal mild tubular vacuolization of the tubular cell cytoplasm. These findings were compared with those of the control kidney, which exhibited interstitial hemorrhage and intratubular hemorrhage. Focal mild acute tubular necrosis and apoptosis in the adjacent tubules, focal interstitial chronic inflammation with diffuse and marked inflammatory infiltrate and perivascular neutrophils, and inside the glomerulus also some shrinkage glomeruli were seen. These changes could be due to $CCL_4$ cause injury to the various membrane components of the cell. In addition, histological studies on the kidney of $CCL_4$-treated mice were similar with other studies.[50-53]

## CONCLUSION

This study demonstrated that oxidative stress contributes to the mechanism of $CCL_4$-induced liver and renal toxicity and that exposure to $CCL_4$ triggers both adverse effects and significant changes in the liver and renal functions in pregnant mice. Through the release of PI3K/AKT signaling, GNL treatment contributes to the restoration of liver and renal function through the activation of Nrf2 by PI3K/AKT. The antioxidant activity of GNL could possibly be useful in protecting human and animal health by decreasing oxidative stress. It may also reduce the biosynthesis of mycotoxins in food sources by inhibiting oxidative stress. Despite the fact that, to the best of our knowledge, no evidence has demonstrated that GNL can be safely used in patients with $CCL_4$-induced oxidative stress in liver and kidney diseases, it is increasingly evident that GNL has an antioxidant effect by upregulating the Nrf2 signaling pathway both *in vitro* and *in vitro*. Consequently, GNL might become a candidate for clinical applications in the treatment of liver and renal diseases. However, further clinical trials are necessary to determine its efficacy.

### Ethical considerations

The study was conducted according to the guidelines of King Faisal University and the "Executive Regulations for Research Ethics on Living Creatures (Second Edition)," published by the National Bioethics Committee, Saudi Arabia. All animal care and experimental procedures were approved by the Animal Research Ethics Committee at King Faisal University, Al-Ahsa, Saudi Arabia, and approved by its Institutional Review Board (Ref no.: KFU-REC-2022-DEC-EA000576, date: April 21, 2022).

### Peer review

This article was peer-reviewed by two independent and anonymous reviewers.

### Data availability statement

Data are contained within the article or Supplementary Material.

### Author contributions

Conceptualization: G.M.B., P.R.; Methodology, S.A.A, P.R.; Software, R.B.A; validation, B.M.A, E.A., and D.A.; formal analysis, S.Y.R.; investigation, S.A.A., P.R and D.A.; resources, R.B.A.; data curation, P.R.; writing – Original draft preparation, S.A.A.; writing – Review and editing, B.M.A, E.A., and P.R.; visualization, G.M.B.; supervision, G.M.B.; Funding Acquisition, G.M.B.

All authors have read and agreed to the published version of the manuscript.

### Financial support and sponsorship

This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia, Student Researcher Track (GRANT2, 893).

### Conflicts of interest

There are no conflicts of interest.

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**Supplementary Table 1: Antibodies used in the study**

| Antibody | Molecular weight | Company | Dilution |
|---|---|---|---|
| pPI3K | 85 | PA5-104853 Thermo Fisher Scientific, Inc. (Waltham, MA, USA) | 1.2000 |
| PI3K | 85 | #PA5-29220 Thermo Fisher Scientific, Inc. (Waltham, MA, USA) | 1:2000 |
| pAKT | 60 | Cat #44-602G Thermo Fisher Scientific, Inc. (Waltham, MA, USA) | 1:5000 |
| AKt | 60 | Cat #44-609G Thermo Fisher Scientific, Inc. (Waltham, MA, USA) | 1:2000 |
| Nrf2 | 62 | (AB-M-018) MOLEQULE-ON (New Lynn, Auckland, New Zealand) | 1:5000 |
| Bcl-2 | 28 | CAT#15071 Cell signaling (Danvers, MA, USA) | 1:1000 |
| Cleaved Caspase-3 | 17 | CAT#9661 Cell signaling (Danvers, MA, USA) | 1: 1000 |
| secondary antibodies goat anti-rabbit | - | (AB-M-010) MOLEQULE-ON (New Lynn, Auckland, New Zealand) | 1:10,000 |
| secondary goat anti-mouse | - | (AB-M-009) MOLEQULE-ON (New Lynn, Auckland, New Zealand) | 1:10,000 |
| β-actin | 43 | (AB-M-003) MOLEQULE-ON (New Lynn, Auckland, New Zealand) | 1:10,000 |