



Original Article

Asian Pacific Journal of Tropical Biomedicine



apjtb.org

doi: 10.4103/apjtb.apjtb_413_24

Geraniol alleviates cyclophosphamide-induced cardiotoxicity in mice

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ABSTRACT

Objective: To explore the effect of geraniol on cyclophosphamide-induced cardiotoxicity.

Methods: Mice were divided into five groups: the control group, the cyclophosphamide group (200 mg/kg cyclophosphamide, *i.p.* on day 7), the group treated with geraniol 100 and 200 mg/kg from day 1 to day 14, along with a single dose of cyclophosphamide on day 7, and the geraniol alone group (200 mg/kg geraniol from day 1 to day 14). At the end of the study, animals were sacrificed, and blood and heart were collected and analyzed for biochemical, histopathological, and immunohistochemical changes.

Results: Treatment with 200 mg/kg geraniol significantly reduced the levels of cardiac injury markers, malondialdehyde, and inflammatory and apoptotic markers, while increasing antioxidant activities in mice with cyclophosphamide-induced cardiotoxicity. Moreover, it remarkably alleviated histopathological aberrations in cardiac tissue.

Conclusions: Geraniol attenuates cyclophosphamide-induced cardiotoxicity *via* antioxidant, anti-inflammatory, and antiapoptotic effects.

KEYWORDS: Geraniol; Cardiotoxicity; Natural product; Cyclophosphamide; Inflammation; Apoptosis

1. Introduction

Cancer is one of the major causes of mortality and morbidity worldwide. As per the published evidence, in 2022, more than 20 million confirmed cancer cases and more than 9.7 million cancer-related deaths were reported[1–3]. The global burden of cancer is projected to reach 28.4 million cases by 2040, representing a 47% increase compared to 2020. This rise is anticipated to be more pronounced in transitioning countries, where rates are expected to

increase by 64%-95%, in contrast to transition countries, which may see an increase of 32%-56%[4]. Moreover, it was predicted that by the end of 2050, cancer related cases may cross 35 million[3]. Additionally, the prevalence of subclinical left ventricular dysfunction among cancer patients is found in almost 42%. Various anticancer drugs have been developed worldwide and have significantly increased the life expectancy of patients, but at the same time, many such anticancer drugs have been shown to cause cardiovascular toxicity[5]. The extent of cardiotoxicity extensively and negatively affects patients' quality of life and clinical outcomes. Cardiotoxicity caused by such drugs also restricts their widespread clinical uses. Therefore, it is imperative to investigate the mechanism of cardiotoxicity exhibited by anticancer drugs so that more effective adjuvants can be developed.

Among various anticancer drugs, cyclophosphamide is one of the oldest and most extensively used drugs in different types of solid

Significance

Cyclophosphamide is a commonly used antineoplastic drug, but its use is often limited due to cardiotoxic manifestations. Geraniol shows a wide range of pharmacological activities and is used for various disease conditions. However, its cardioprotective potential is not explored yet. This study demonstrates that geraniol mitigates cyclophosphamide-induced cardiotoxicity by enhancing antioxidant, anti-inflammatory, and antiapoptotic activities. The exact mechanisms of its cardioprotective effect still need further investigation.

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How to cite this article: Karim S, Shaik RA. Geraniol alleviates cyclophosphamide-induced cardiotoxicity in mice. Asian Pac J Trop Biomed 2024; 14(10): 427-435.

Article history: Received 18 July 2024; Revision 19 August 2024; Accepted 15 October 2024; Available online 28 October 2024

tumors and hematological malignancies[2]. Apart from being a potent anticancer drug, it is also a potent immunosuppressant drug explicitly used in organ transplants and other immunological disorders[6]. Cyclophosphamide, a prodrug, undergoes hepatic metabolism and converts it into phosphoramidate mustard, acrolein, and another active metabolite. Phosphoramidate mustard is an anticancer moiety, binds with guanine residue at N-7 position and alters the DNA replication leading to the anticancer effect[2]. Acrolein is a lipophilic and reactive metabolite that exhibits marked oxidative stress, inflammation, and apoptosis. Based on clinical reports and case series, it was found that when cyclophosphamide was used at the clinical dose, 11%-43% of patients reported significant cardiotoxicity, and the symptoms of cardiotoxicity were seen within one week of its administration[2].

Considering the molecular mechanism of cyclophosphamide-induced cardiotoxicity, it was found that when cyclophosphamide is administered, a significant increase in the level of reactive oxygen species (ROS) and an alteration in the level of nuclear factor erythroid 2-related factor 2 (Nrf2) expression were found[7]. Additionally, it caused an increase in transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the level of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 β), and a reduction in the level of anti-inflammatory cytokines[7]. Apart from inflammatory cascade, it could augment the level and expression of proapoptotic proteins such as caspase-3[7]. Moreover, cardiotoxic manifestation due to cyclophosphamide administration is not only limited to oxidative stress, inflammation, and apoptosis; rather, a significant increase in the fibrotic markers has also been found[8]. Thus, the cumulative effect of cyclophosphamide-induced alterations in these signaling molecules and pathways leads to cardiotoxic manifestation that finally causes histopathological-ultrastructural aberrations, reduced left ventricular ejection fraction, cardiac hypertrophy, and heart failure[6].

Thus, it can be concluded that cyclophosphamide-caused cardiotoxicity is a serious concern and needs to be addressed. Currently, no adjuvant is approved to deal with this issue. Various studies have been conducted to establish the cardioprotective activity of drugs that can be used as an adjuvant to mitigate cyclophosphamide-induced cardiotoxicity[7]. Natural products have been extensively studied for cardioprotective potency[9].

Geraniol is among the extensively explored plant secondary metabolites that have been used since ancient times for various disease conditions[10]. To be more specific, it is an acyclic monoterpene obtained from the essential oils of lemongrass and rose[10]. It is also approved by the Food and Drug Administration to be used as a flavoring agent and for human consumption[10]. Considering the pharmacological attribute of geraniol, this natural bioactive possesses potent antioxidant, anti-inflammatory, antiapoptotic, antiarthritic, anti-hyperlipidemic, hepatoprotective, and anticancer potency[10]. In the current study, we aimed to explore

the protective effect of geraniol against cyclophosphamide-induced cardiotoxicity and elucidate its mechanisms of action.

2. Materials and methods

2.1. Chemicals and drugs

Cyclophosphamide was obtained from Baxter Pharmaceuticals to induce cardiotoxicity. Geraniol, as a test drug, was obtained from Sigma-Aldrich. ELISA kits for IL-1 β , IL-6, and TNF- α were procured from Abcam (Abcam, Cambridge, UK), ELISA kits for creatine kinase MB (CK-MB), and cardiac troponin T (cTnT), were obtained from MyBiosource (MyBiosource, San Diego, CA, USA.), and commercial calorimetric kits for lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate transaminase (AST). Reagents and chemicals were of analytical grade and utilized in the study.

2.2. Experimental animals

A total of 30 Swiss Albino mice were acquired from the animal house facility located at the Faculty of Pharmacy, King Abdulaziz University. The animals were housed under controlled environmental conditions, including a regulated room temperature (23-25 °C), relative humidity maintained between 50% and 55%, and a light/dark cycle of twelve hours each. The animals were provided standard diets and unrestricted access to water.

2.3. Experimental design and treatment paradigm

Experimental animals were randomly divided into five groups ($n=6$). Group I (normal control) was treated with normal saline from day one to day fourteen. Group II was treated with cyclophosphamide 200 mg/kg, *i.p.*, as a single dose on day seven. Group III and Group IV were treated with geraniol 100 and 200 mg/kg, *p.o.* from day one to day fourteen, respectively, along with a single *i.p.* dose of cyclophosphamide 200 mg/kg on day seven. Group V was treated with geraniol 200 mg/kg only from day one to day fourteen, as shown in Supplementary Figure 1[10,11].

2.4. Collection of blood and cardiac tissue

On day 15 *i.e.*, after 24 h of the last dosing, animals were weighed on the digital weigh-balance before sacrifice. A mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) was used to anesthetize the experimental animals. Cardiac puncture was used for the collection of blood. Once the blood was collected, it was centrifuged for 10 min at 3000 rpm at 4 °C for the collection of serum[12]. The obtained serum was stored at -80 °C for further estimation of biochemicals.

Carbon dioxide euthanasia method was used for the sacrifice of animals, and the heart was removed, washed with normal saline, dried using filter paper, and weighed. A part of cardiac tissue was dissected and kept in a 10% formalin solution for histopathological and immunohistochemical analysis. The rest of the tissue was stored for biochemical analysis[13].

2.5. Estimation of heart weight to body weight ratio

For the estimation of heart weight to body weight ratio, animals were weighed before being sacrificed and heart weight was also measured after sacrifice using digital weighing machine.

2.6. Antioxidant marker evaluation

To evaluate antioxidant and other biochemical markers, stored cardiac tissue was chopped, homogenized in 0.1 M buffer (phosphate buffer, pH 7.4), and centrifuged at 4 °C for 15 min to obtain supernatant. Malonaldehyde (MDA) level was determined using the method of Ohkawa *et al*[14]. Firstly, the supernatant was obtained from the tissue homogenate, and Tris-HCl was mixed with the supernatant and incubated for 2 h. The mixture was then centrifuged for 10 min at 1000 rpm and added with 10% trichloroacetic acid. The absorbance was recorded at 540 nm, and the values were expressed as nmol MDA/mg protein[14]. The antioxidant activity of superoxide dismutase (SOD) was performed according to the method of Marklund and Marklund[15]. Firstly, cytosolic supernatant was obtained, and pyrogallol and Tris-HCl were mixed. Absorbance was recorded using a spectrophotometer at 420 nm, and the antioxidant activity of SOD was expressed as U/mg protein[15]. Glutathione (GSH) level was evaluated as per the established method of Sedlak and Lindsay[16]. Absorbance was recorded using a spectrophotometer at 412 nm, and the level of GSH was expressed as μ mol of GSH/mg protein[1].

2.7. Evaluation of cardiac injury markers

Cardiac-specific and non-specific injury markers including CK-MB, LDH, cTnT, ALT, and AST were estimated in serum obtained from the blood of experimental animals. ELISA kits were used for the estimation of cTnT and CK-MB, whereas commercially available kits were used for the estimation of ALT, AST, and LDH according to the manufacturer's protocol[17].

2.8. Evaluation of cardiac inflammatory and apoptotic markers

IL-1 β , IL-6, TNF- α , and caspase-3 were estimated in the cardiac tissue using a commercially available ELISA kit. For this, a section

of cardiac tissue was homogenized in 0.1 M phosphate buffer and centrifuged at 4000 rpm at 4 °C to obtain supernatant. The obtained supernatant was transferred into the ELISA plates and proceeded according to the manufacturer's instructions. The absorbance was recorded at 450 nm[17].

2.9. Histopathological and immunohistochemical assessment

Stored tissue in 10% formalin was used for the histopathological and immunohistochemical analysis. Briefly, the apical sections of the cardiac tissue and sections from the liver and kidney were dissected and paraffinized blocks were prepared. Sections with a thickness of 4 μ m were cut using a microtome, floated in the water bath, immediately mounted on the slides, and dried for 1 h 20 min. The mounted slides were dehydrated and stained with hematoxylin and eosin. Similarly, for immunohistochemical analysis, 4 μ m thin sections were cut from the same section, dehydrated using alcohol, and treated with primary and secondary antibodies of NF- κ B (Bio SB), high sensitivity streptavidin-HRP conjugate, and DAB enhancer and slides were covered using cover slips[15]. The prepared slides were visualized under an inverted microscope (Motic).

2.10. Statistical analysis

All the values were expressed as mean $\pm$ SEM ($n=6$). Statistical analysis was performed using GraphPad Prism version 5 by one-way ANOVA, followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

2.11. Ethical statement

The procurement of these mice and the experiment were approved by the Research Ethics Committee of the Faculty of Pharmacy, King Abdulaziz University, with approval reference number PH-1444-48 dated 02 May 2023.

3. Results

3.1. Effect of geraniol on the ratio of heart weight and body weight

Cyclophosphamide administration significantly increased the ratio of heart weight to body weight when compared to the control group ($P < 0.001$). Geraniol at 200 mg/kg effectively reduced the heart weight to body weight ratio ($P < 0.001$) when compared to the cyclophosphamide-treated group, whereas 100 mg/kg geraniol did not induce any significant effect ($P > 0.05$) (Figure 1).

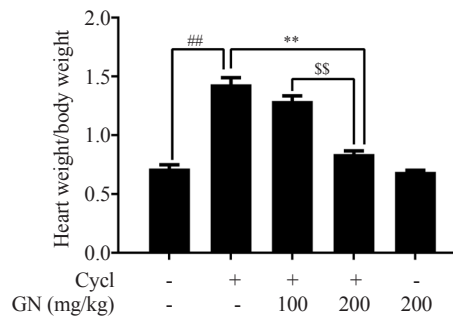


Figure 1. Effect of geraniol on cyclophosphamide-induced change in the ratio of heart weight and body weight. Cycl: cyclophosphamide; GN: geraniol. The values are expressed as mean±SEM ($n=6$) and analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. $^{##}P < 0.001$ compared with the control group. $^{**}P < 0.001$ compared with the cyclophosphamide-treated group. $^{SS}P < 0.001$ compared with the cyclophosphamide group treated with 100 mg/kg geraniol.

3.2. Effect of geraniol on hepatic biomarkers

Cyclophosphamide significantly increased the levels of hepatic biomarkers including ALT ($P < 0.001$), and AST ($P < 0.001$). Mice treated with 200 mg/kg geraniol showed obviously decreased levels

of ALT and AST ($P < 0.05$) compared with the cyclophosphamide-treated group. However, 100 mg/kg geraniol induced no significant effect on hepatic biomarkers ($P > 0.05$) (Figure 2).

3.3. Effect of geraniol on markers of oxidative stress

Cyclophosphamide significantly reduced SOD and GSH activities ($P < 0.001$), and enhanced MDA levels ($P < 0.001$). Treatment with 100 mg/kg geraniol significantly reduced the level of MDA ($P < 0.05$) with no notable effects on SOD and GSH ($P > 0.05$), while 200 mg/kg geraniol reversed cyclophosphamide-induced changes in antioxidant markers ($P < 0.001$) (Figure 3).

3.4. Effect of geraniol on cardiac injury markers

Cyclophosphamide administration significantly increased the levels of cardiac injury markers LDH ($P < 0.001$), CK-MB ($P < 0.001$), and cTnT ($P < 0.001$). Treatment with 100 mg/kg geraniol only significantly reduced LDH levels without noticeable effects on CK-MB and cTnT levels, while 200 mg/kg geraniol remarkably decreased the levels of cardiac injury markers ($P < 0.001$) (Figure 4).

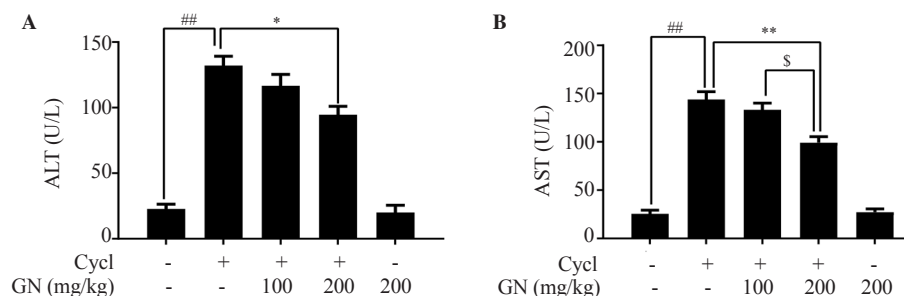


Figure 2. Effect of geraniol on cyclophosphamide-induced increases in ALT and AST levels. ALT: alanine transaminase; AST: aspartate transaminase. The values are expressed as mean±SEM ($n=6$) and analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. $^{##}P < 0.001$ compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.001$ compared with the cyclophosphamide-treated group. $^{S}P < 0.05$ compared with the cyclophosphamide group treated with 100 mg/kg geraniol.

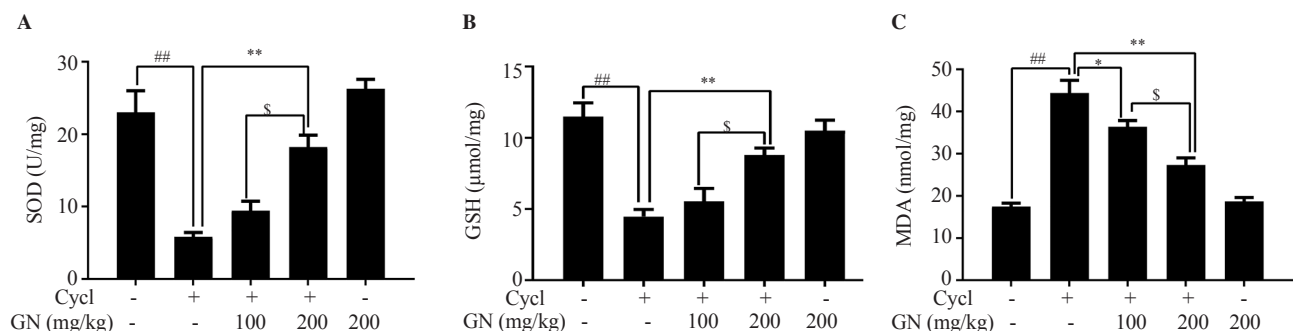


Figure 3. Effect of geraniol on cyclophosphamide-induced oxidative stress markers. SOD: superoxide dismutase; GSH: glutathione; MDA: malonaldehyde. The values are expressed as mean±SEM ($n=6$) and analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. $^{##}P < 0.001$ compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.001$ compared with the cyclophosphamide-treated group. $^{S}P < 0.05$ compared with the cyclophosphamide group treated with 100 mg/kg geraniol.

3.5. Effect of geraniol on cardiac inflammation and apoptosis

A significant rise in the levels of TNF- α , IL-6, IL-1 β , and caspase-3 ($P < 0.001$) was found in mice given 200 mg/kg cyclophosphamide. Treatment with 200 mg/kg geraniol decreased cyclophosphamide-induced TNF- α , IL-6, IL-1 β , and caspase-3 ($P < 0.05$) (Figure 5). In contrast, 100 mg/kg geraniol only diminished IL-6 level ($P < 0.05$) and had no remarkable effects on TNF- α , IL-1 β , and caspase-3 ($P > 0.05$).

3.6. Effect of geraniol on the expression level of NF- κ B

Cyclophosphamide administration significantly increased the level of NF- κ B in cardiac tissue when compared to the control ($P < 0.001$). Mice treated with 100 mg/kg geraniol showed no obvious effect on reducing the NF- κ B ($P > 0.05$), whereas treatment with 200 mg/kg geraniol significantly reduced NF- κ B level in cardiac tissue when compared to the cyclophosphamide-treated group ($P < 0.001$) (Figure 6).

3.7. Effect of geraniol on the histopathology of cardiac, liver and renal tissue

As seen in Figure 7, the normal group and the group treated

with 200 mg/kg geraniol alone exhibited histomorphological characteristics of cardiac tissue (black arrow). The cyclophosphamide-administered group showed marked pyknosis (red arrow), fibrotic changes (yellow arrow), congestion (white arrow), vacuolation (green arrow), and myofibrillar disintegration (blue arrow). Treatment with 100 mg/kg geraniol showed no marked reversal of histopathological damage induced by cyclophosphamide. In contrast, 200 mg/kg geraniol effectively and significantly reversed the histological damage towards normal, where minimal pyknosis (red arrow), vacuolation (green arrow), and myofibrillar disintegration were seen (blue arrow).

Histopathological analysis of hepatic tissue was also done. The normal control group and the geraniol *per se* treated group showed normal histomorphological characteristics of hepatic tissue. The cyclophosphamide-treated group demonstrated marked pyknosis (red arrow), fibrotic changes (yellow arrow), central vein congestion (blue arrow), significantly damaged hepatocytes (white arrow), and vacuolation (orange arrow). The group treated with 100 mg/kg geraniol showed no marked improvement in histopathological damage induced by cyclophosphamide, whereas 200 mg/kg geraniol notably reversed histological damage, as evidenced by minimal pyknosis (red arrow), central vein congestion (blue arrow), and vacuolation (orange arrow). No evidence of damaged hepatocytes or fibrotic changes was seen (Supplementary Figure 2).

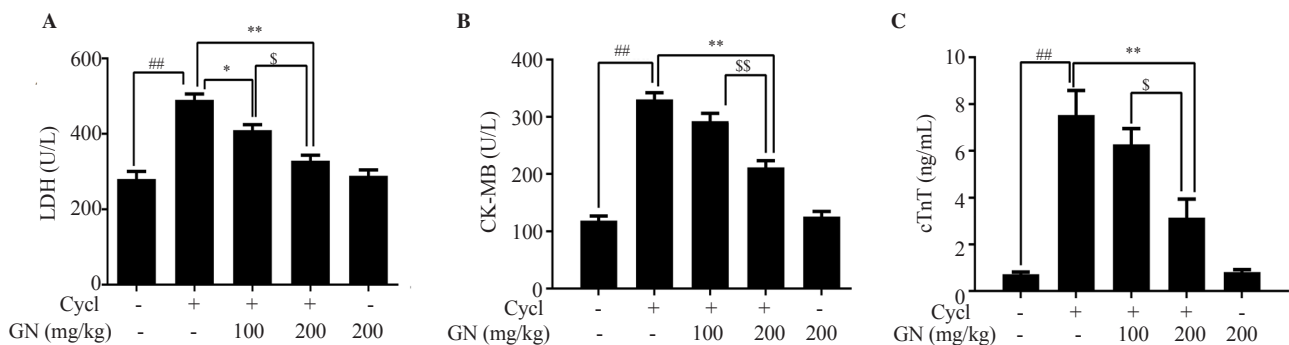


Figure 4. Effect of geraniol on cyclophosphamide-induced increases in cardiac injury markers. LDH: lactate dehydrogenase; CK-MB: creatine kinase-MB; cTnT: cardiac troponin T. The values are expressed as mean $\pm$ SEM ($n=6$) and analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. ## $P < 0.001$ compared with the control group. * $P < 0.05$, ** $P < 0.001$ compared with the cyclophosphamide-treated group; \$ $P < 0.05$, \$\$ $P < 0.001$ compared with the cyclophosphamide group treated with 100 mg/kg geraniol.

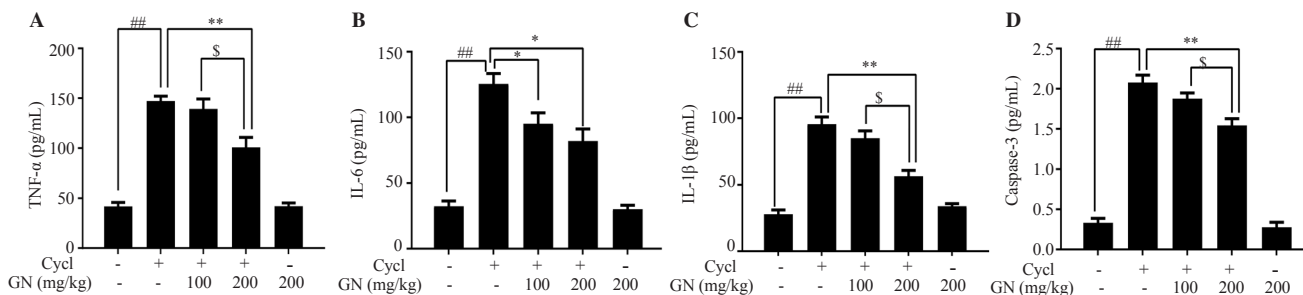


Figure 5. Effect of geraniol on the markers of cardiac inflammation and apoptosis in cyclophosphamide-administered mice. The values are expressed as mean $\pm$ SEM ($n=6$) and analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. ## $P < 0.001$ compared with the control group. * $P < 0.05$, ** $P < 0.001$ compared with the cyclophosphamide-treated group; \$ $P < 0.05$ compared with the cyclophosphamide group treated with 100 mg/kg geraniol.

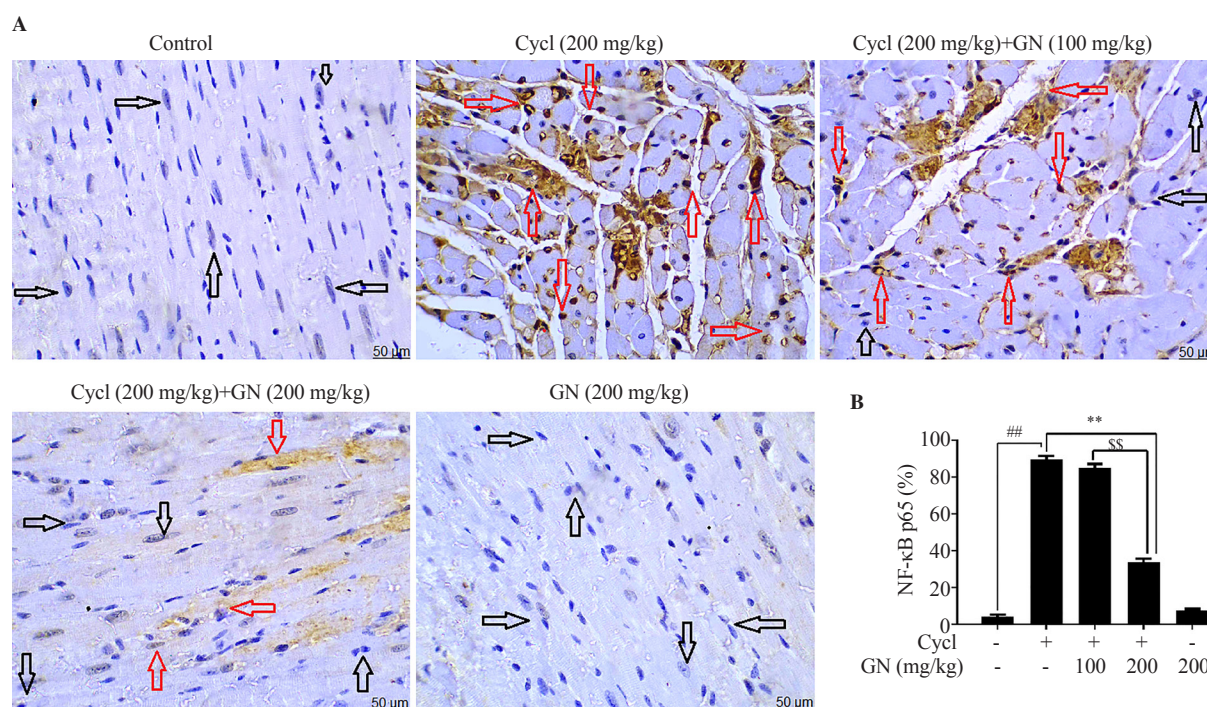


Figure 6. (A) Immunohistochemical analysis using NF-κB antibodies (scale bar: 50 μm, magnification: ×400). The normal control group and the group treated with 200 mg/kg geraniol alone show minimal or almost negative expression of NF-κB (black arrow). The cyclophosphamide-administered group shows a marked increase in NF-κB level (red arrow). There is no marked reduction in NF-κB level in the group treated with cyclophosphamide and 100 mg/kg geraniol. In contrast, 200 mg/kg geraniol significantly reduces cyclophosphamide-induced increase in NF-κB level (the black arrow represents negative whereas the red arrow represents positive NF-κB expression). (B) Semiquantitative analysis of NF-κB level. The values are expressed as mean ± SEM (n=3) and analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. ^{##}*P* < 0.001 compared with the control group. ^{**}*P* < 0.001 compared with the cyclophosphamide-treated group. ^{ss}*P* < 0.001 compared with the cyclophosphamide group treated with 100 mg/kg geraniol.

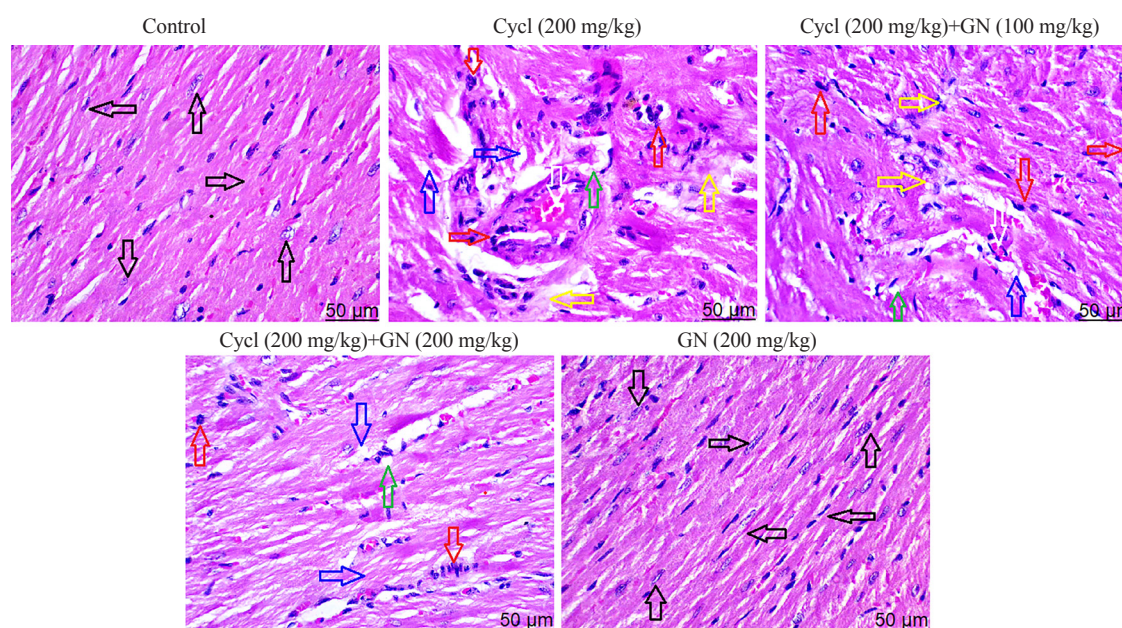


Figure 7. Histopathological analysis of cardiac tissue using hematoxylin and eosin stain (scale bar: 50 μm, magnification: ×400). The control group and the group treated with 200 mg/kg of geraniol alone show normal histomorphological features in cardiac tissue (black arrow). In contrast, the group treated with cyclophosphamide displays significant pyknosis (red arrow), fibrotic alterations (yellow arrow), congestion (white arrow), vacuolation (green arrow), and disintegration of myofibrils (blue arrow). The group receiving cyclophosphamide and 100 mg/kg of geraniol does not exhibit a notable improvement in the histopathological damage caused by cyclophosphamide. However, treatment with 200 mg/kg of geraniol significantly improves histological damage, showing only minimal pyknosis (red arrow), vacuolation (green arrow), and myofibrillar disintegration (blue arrow).

We also performed the histopathology of renal tissue. It was found that the normal control group and the geraniol *per se* treated group showed normal histomorphological characteristics of renal tissue. The cyclophosphamide-treated group exhibited marked pyknosis (red arrow), fibrotic changes (yellow arrow), damaged proximal convoluted tubule and distal convoluted tubule (light green arrow), damaged Bowman's capsule (brown arrow), and significantly damaged mesangium and podocytes (blue arrow). Treatment with 100 mg/kg geraniol showed no marked reversal of histopathological damage induced by cyclophosphamide, whereas 200 mg/kg geraniol effectively and significantly alleviated histological damage (Supplementary Figure 3).

4. Discussion

It is well established that the prevalence as well as incidence of various types of cancers have increased considerably. As a result, many anticancer drugs have also been developed to treat and increase patients' life expectancy and quality of life[5]. Among conventional and targeted anticancer drugs, cyclophosphamide is explicitly used in various solid tumors and hematological malignancies[16]. Despite being a potent drug, cardiotoxicity caused by this drug is a limiting factor for its widespread use. Cyclophosphamide-induced cardiotoxicity has been well documented in the preclinical and clinical findings[17]. Thus, the clinical management of cyclophosphamide-induced cardiotoxicity remains a major challenge for clinicians. In the present study, we explored the protective effect of geraniol against cyclophosphamide-induced cardiotoxic manifestations. The primary reason for selecting our test drug is its multifactorial mechanism of action, such as potent antioxidant, antiapoptotic, and anti-inflammatory[10]. Moreover, geraniol has also been previously explored for cardioprotective potential in isoproterenol-induced cardiotoxicity and against neonatal ventricular cardiomyocytes and oxidative stress[18]. Cyclophosphamide caused considerable oxidative stress, inflammation, apoptosis, and histopathological alterations. Treatment with geraniol could ameliorate these changes, demonstrating its cardioprotective effect. Similar findings have also been reported previously[19–21].

Change in body weight or organ or their ratio is always considered a reliable gravimetric marker of diseased conditions, including cardiotoxic manifestations. In the present study, we found that cyclophosphamide administration increased heart weight and reduced body weight. Increased heart weight signifies the hypertrophy of auricles and ventricles, resulting from imbalanced apoptosis or necrosis leading to increased cardiomyocyte density and impaired contraction-relaxation mechanism. However, geraniol reduced the ratio of heart weight to body weight, especially at 200 mg/kg. In clinical practice, cardiac-specific injury markers such as CK-MB, cTnT, LDH or non-specific injury markers such as ALT and AST are used to investigate cardiac injury[22]. These cardiac-specific

markers are usually found in the serum post-cardiotoxicity because, upon cardiac injury or under stress conditions, cardiomyocytes get damaged, and permeability increases, leading to their leakage into the system circulation[20,22]. Similarly, non-specific markers are also released in the systemic circulation during cardiac injury. In the present study, administering a single dose of cyclophosphamide caused increased serum levels of CK-MB, cTnT, LDH, ALT, and AST. Similar findings were reported in previous studies[20,22]. Geraniol treatment decreased cyclophosphamide-caused alterations in these markers.

Oxidative stress has always been considered a significant contributor to the cardiotoxic events caused by various toxic agents, including anticancer drugs such as doxorubicin and cyclophosphamide[20]. In physiological conditions, antioxidants counteract the produced ROS or reactive nitrogen species. In the cardiotoxic state, the enzymatic activity of SOD or catalase and the level of GSH reduced considerably, whereas the level of TBARS increased significantly[23]. Additionally, upon cardiotoxic injury, the lipidic component of cardiomyocytes gets oxidized, resulting in an increased level of TBARS that further aggravates the inflammation and apoptosis, thus leading to the worsening of cardiac injury[23]. In the present study, cyclophosphamide caused a significant increase in oxidative stress. This finding is consistent with that found in previous studies[21,23–26]. However, geraniol treatment reversed cyclophosphamide-induced changes in antioxidant and MDA levels. Crespo *et al.* also found the antioxidant effect of geraniol at the dose of 5–200 μ M against oxidative stress in neonatal ventricular cardiomyocytes[18]. In a study by Kshirsagar *et al.*, geraniol conferred protection against high fat-induced endothelial dysfunction *via* antioxidant activity[27].

In addition, cardiac inflammation caused by cyclophosphamide is another major contributor to cardiotoxicity[28]. Accumulating evidence has shown that cyclophosphamide administration increases the expression of NF- κ B along with the level of proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) and reduces the level of IL-10[27,29,30]. NF- κ B is a transcription factor and one of the extensively explored mediators of cardiac inflammation in various preclinical models[1]. In normal physiological conditions, NF- κ B is sequestered in the cytoplasm in association with I κ B. When a toxic cardiac stimulus, such as oxidative stress or damage to cardiac injury, occurs, I κ B becomes phosphorylated, and as a result, NF- κ B translocates into the nucleus[31–33]. In the nucleus, NF- κ B regulates the expression of proinflammatory cytokines and causes cardiac inflammation.

Additionally, the importance of NF- κ B can be validated by the ongoing Phase III clinical trial for NF- κ B inhibitors to evaluate its cardioprotective potency[33]. In the present study, cyclophosphamide administration increased the levels of NF- κ B, IL-1 β , IL-6, and TNF- α , which is consistent with other previously published reports[21,24–26]. Treatment with geraniol notably diminished their levels. Wu *et al.* revealed that geraniol exerted an anti-inflammatory

effect *via* modulation of MAPK/Akt/NF- κ B/PI3K pathways[34]. In contrast, Soubh *et al.* showed its anti-inflammatory effect against 2,4,6-trinitrobenzene sulphonic acid-induced colitis *via* modulation of p38 MAPK/NF- κ B/glycogen synthase kinase-3 pathways[35].

Apoptosis is critically regulated by the balanced activity of proapoptotic proteins such as Bax, caspase-3, and antiapoptotic protein Bcl-2[7,30]. Bcl-2 is associated with the mitochondrial membrane and abrogates cytochrome C's release, leading to an antiapoptotic effect[36]. Bax and caspase-3 become activated in response to cardiotoxic stimuli. In this study, cyclophosphamide triggered apoptosis, which was alleviated by geraniol. The results are in line with those found in previous studies[37].

Moreover, pyknosis, myofibrillar disintegration, and loss of myofibrils were found in the cardiomyocytes of cyclophosphamide-treated group. Treatment with geraniol alleviated the aforementioned structural damage. Since the cardio-renal-hepatic system is closely related, we also performed histopathological analyses of the kidney and liver. We found that cyclophosphamide-induced marked damage to renal and hepatic tissue. Treatment with 100 mg/kg geraniol showed no marked improvement. However, 200 mg/kg geraniol effectively mitigated cyclophosphamide-caused damage to renal and hepatic tissue. Notably, the extent of protection was more pronounced in liver and cardiac tissue as compared to renal tissue (Supplementary Figures 2-3). However, more detailed studies are needed to confirm the protective effects of geraniol. Additionally, the gene expression of other inflammatory and apoptotic markers should also be studied to substantiate the findings.

In conclusion, geraniol exerts protective effects against cyclophosphamide-induced cardiotoxicity by reducing apoptosis, inflammation, and oxidative stress. However, more detailed cellular and molecular studies are needed to affirm its efficacy.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

The Deanship of Scientific Research at King Abdulaziz University, Jeddah, Saudi Arabia has funded this project under grant no. RG-21-166-43.

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

Conception or design of the work was done by SK. Investigation and data collection were done by RAS. SK and RAS contributed to interpretation. RAS was involved in writing original draft and preparation. Critical revision of the article and final approval of the version to be published were done by SK.

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Geraniol alleviates cyclophosphamide-induced cardiotoxicity in mice

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Running title: Cardioprotective effect of geraniol

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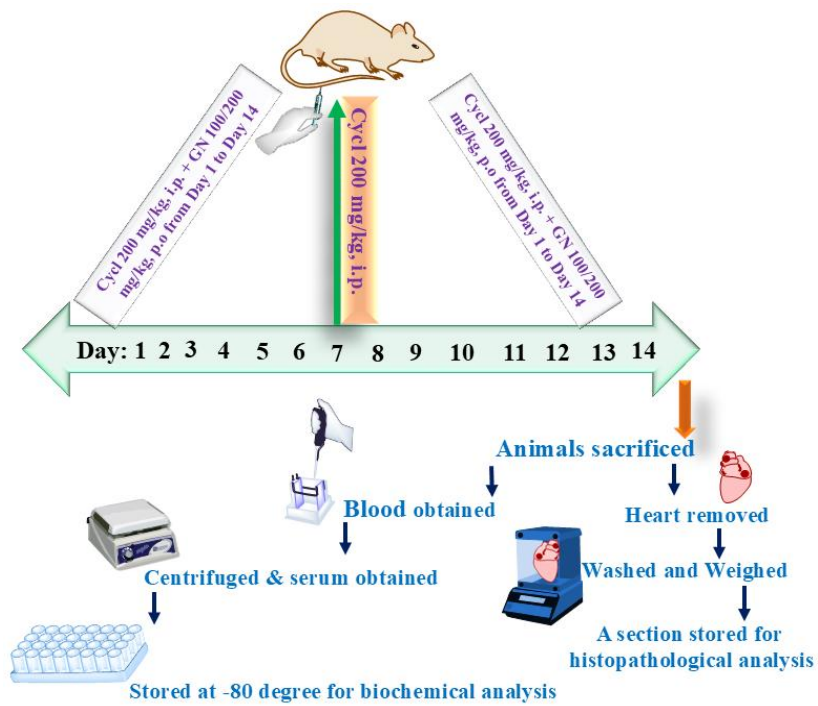
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Supplementary Figure 1. Treatment paradigm and dosing schedule. Cycl: cyclophosphamide;
GN: geraniol.

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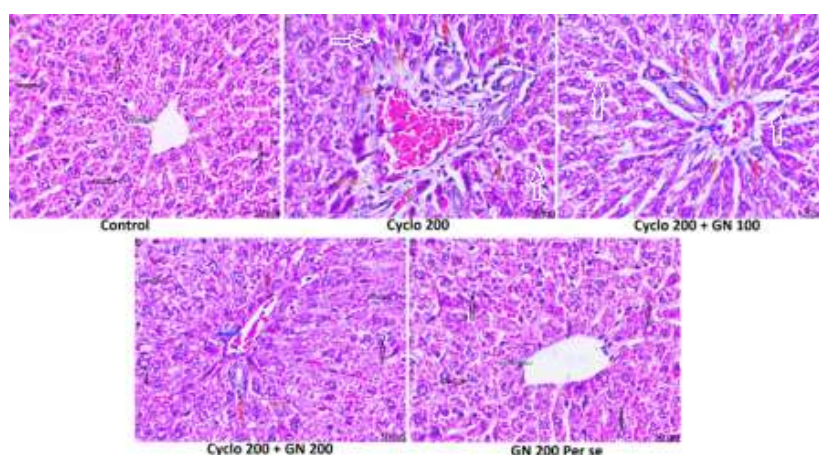
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Supplementary Figure 2. Histopathological analysis of hepatic tissue using hematoxylin and eosin stain (scale bar: 50 μ m; magnification: $\times$ 400). The normal and geraniol *per se* treated group show normal histomorphological characteristics of hepatic tissue. The black arrow represents trabecularly arranged normal polygonal hepatocytes with no pyknosis, apoptotic changes, or disintegration. The green arrow represents a normal central vein with no evidence of congestion. The cyclophosphamide-treated group shows marked pyknosis (red arrow), fibrotic changes (yellow arrow), central vein congestion (blue arrow), significantly damaged hepatocytes (white arrow), and vacuolation (orange arrow). The group treated with 100 mg/kg geraniol shows no marked reversal of histopathological damage induced by cyclophosphamide, whereas 200 mg/kg geraniol effectively and significantly reverses histological damage, where minimal pyknosis (red arrow), central vein congestion (blue arrow), vacuolation (orange arrow) and no evidence of damaged hepatocytes or fibrotic changes are seen.

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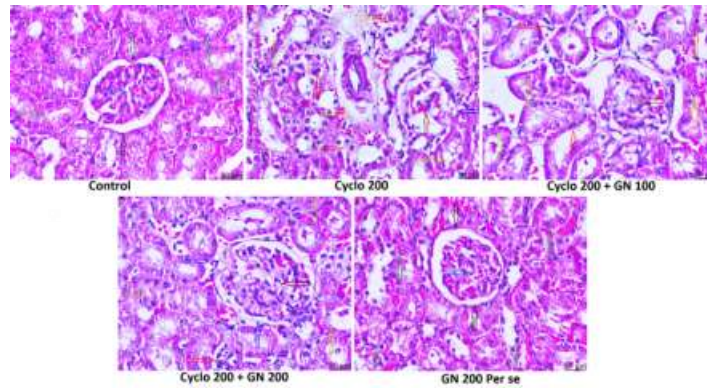
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Supplementary Figure 3. Histopathological analysis of renal tissue using hematoxylin and eosin stain (scale bar: 50 μ m; magnification: $\times 400$). The normal and geraniol *per se* treated group show normal histomorphological characteristics of renal tissue. The green arrow represents normal proximal convoluted tubule (PCT) and distal convoluted tubule (DCT), the black arrow represents normal Bowman's capsule, and the brown arrow represents normal mesangium along with intact podocytes. The cyclophosphamide-treated group shows marked pyknosis (red arrow), fibrotic changes (yellow arrow), damaged PCT and DCT (light green arrow), damaged Bowman's capsule blue (brown arrow), and significantly damaged mesangium and podocytes (blue arrow). Treatment with 100 mg/kg geraniol shows no marked reversal of histopathological damage induced by cyclophosphamide, whereas 200 mg/kg geraniol effectively and significantly alleviates histological damage, where minimal pyknosis (red arrow), minimally damaged PCT and DCT (light green arrow), Bowman's capsule blue (brown arrow) and mildly damaged mesangium and podocytes (blue arrow) are seen.