Evaluation of the activity of geraniol isolated from lemongrass (*Cymbopogon commutatus* Stapf.) on ochratoxin A-induced nephrotoxicity: role of the pPI3K/AKT-Nrf2 signaling pathway

Rebai Ben Ammar,[1,2*] Shouq Nafea Al Mutairi,[1*] Peramaiyan Rajendran,[1,3] Gamal M. Bekhet,[1] Maged Elsayed Mohamed,[4] Rashid Ismael Hag Ibrahim,[1] Hamad Abu Zahra,[1] Sarah Abdulaziz Alamer,[1] Mohammed Salem Moqbel,[5] Riadh Ksouri[2]

[1]Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa, Saudi Arabia; [2]Laboratory of Aromatic and Medicinal Plants, Center of Biotechnology of Borj-Cedria, Technopole of Borj-Cedria, Hammam-Lif, Tunisia; [3]COMManD, Department of Biochemistry, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences, Velappanchavadi, Chennai, Tamil Nadu, India; [4]Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia; [5]Department of Anatomy, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia

*Contributed equally.

Correspondence: Rebai Ben Ammar, College of Science, King Faisal University, Al-Ahsa 31982, Saudi Arabia.
Tel.: +966.135899429.
E-mail: rbenammar@kfu.edu.sa

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Abstract

Ochratoxin A (OTA) is a mycotoxin that causes immunotoxicity, teratogenicity, hepatotoxicity and nephrotoxicity in humans and animals. Numerous studies have suggested that oxidative stress may increase OTA's nephrotoxicity. Geraniol (GNL), a monoterpene found in many plant oils, is an antioxidant and free radical scavenger that helps repair multiple types of tissue damage. OTA-induced nephrotoxicity in mice was assessed using GNL as a protective natural compound. Swiss albino mice (six to eight weeks old, 25-30 g weight) were divided into four groups: control (normal saline), OTA (OTA 5 mg/kg body weight), GNL (GNL 40 mg/kg body weight), and GNL + OTA (GNL 40 mg/kg body weight + OTA 5 mg/kg body weight, 4 h later). Animals were treated for 42 days (2 times a week). Evaluations using body weight, kidney weight, spleen weight, Hematoxylin and Eosin (H&E) staining for tissue pathology, biochemical markers Alanine Transaminase (ALT), Aspartate Transaminase (AST), Creatinine (CREA), Blood Urea Nitrogen (BUN) and oxidative markers Malondialdehyde (MDA), Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx), as well as Western blot and DNA fragmentation assessments were performed. A significant decrease in body weight was observed after exposure to OTA, while a significant augmentation in spleen weight was noticed. As a result, tissue concentrations of SOD, CAT, and GPx were decreased, while serum concentrations of marker enzymes (ALT, AST, BUN, CREA and tissue MDA) were increased. In mice, GNL improved enzyme and antioxidant levels. OTA-induced renal injury was prevented by GNL based on histology examination by H&E. In the OTA group also upregulated cleaved caspase-3 and DNA fragmentation, while downregulated Phosphoinositide 3-kinase (pPI3K), Phosphorylated protein kinase (pAKT), Nuclear factor erythroid 2-related factor 2 (Nrf2), and B cell lymphoma-2 (Bcl2) protein expression were observed. GNL

increased the expression of pPI3K, pAKT, Nrf2, Bcl2, and decreased cleaved caspase-3. Based on these results, GNL protects nephrons via the pPI3K/AKT-Nrf2 signaling pathway. This study explained the molecular mechanism of OTA-induced renal injury and how GNL protects the kidneys.

Introduction

Mycotoxins are secondary metabolites produced by filamentous molds and fungi. Under humidity and temperature conditions, feeds and foods may accumulate mycotoxins, posing serious health risks.[1] The toxic secondary metabolite Ochratoxin A (OTA) is produced by strains of *Aspergillus* spp. and *Penicillium* spp. and is found in coffee, grains, legumes, dried fruits, juices, and meat.[2] OTA is toxic to humans and animals, mainly being able to affect the liver and kidney.[3] Mycotoxins like citrinin or deoxynivalenol cause oxidative stress in cells, which can lead to organelle damage or even cell death.[4] Animals exposed to OTA usually have the highest concentrations of OTA in their kidneys, then liver or muscles.[1] Skin, gastric mucosa, myocardium, bone marrow, adrenal medulla, and cortex also contain OTA.[1] OTA also affects antioxidant activity in the gut, kidney and embryo through oxidative stress.[4,5] Varieties of direct and indirect mechanisms trigger Reactive Oxygen Species (ROS) production and oxidative stress.[6] In the kidney, oxidative processes are severe, and ROS play an important role in the pathogenesis of various kidney disorders.[7] The Phosphoinositide 3-kinase/Phosphorylated protein kinase (PI3K/AKT) pathway is also involved in oxidative stress. PI3K/AKT affects Nuclear factor erythroid 2-related factor 2 (Nrf2) to detach from Kelch-like ECH-associated protein 1 (Keap1) and activates antioxidant enzymes.[7,10] Oxidative damage decreases PI3K/AKT signaling for cell survival. PI3K/AKT can be regulated to prevent ROS-induced renal apoptosis.[11] Antioxidants are natural compounds that protect kidneys from toxic chemicals-induced kidney damage.[12]

The natural monoterpene Geraniol (GNL) is found in lemongrass essential oil.[13] In addition to being hepatoprotective, it induces the regeneration of liver due to its high antioxidant effect, mitigates cisplatin-induced neurotoxicity, and decreases Trinitrobenzenesulfonic acid (TNBS)-induced colitis. In addition, GNL has antiangiogenic properties that make it useful for treating tumors.[14–16] This study explores the underlying action mechanisms of GNL extracted from the essential oil of lemongrass (*Cymbopogon commutatus*) on OTA-induced renal toxicity in mice. It was evaluated using organ morphology, Hematoxylin and Eosin (H&E) staining for tissue pathology, biochemical and oxidative markers.

Materials and Methods

Materials

Ochratoxin A (34037-2ML-R) was acquired from Sigma–Aldrich (St. Louis, MO, USA). The experiments were carried out using high-purity analytical grade chemicals obtained from British Drug Houses (Poole, Dorset, UK).

Isolation of geraniol

Wild lemongrass (*Cymbopogon commutatus* Stapf, Poaceae) was obtained from farms in Al-Asha, Eastern Province, Saudi Arabia (latitude: 25.3688, longitude: 49.6938, 25° 22′ 08″ North; 49° 41′38 ″ East, altitude 145 m above sea level), in April 2020. The whole plant was identified by a taxonomist at King Saud University.

GNL was previously isolated from lemongrass and identified by our research team as described by Younis *et al.*[17]

Animals treatment and experimental design

A total of twenty-four male albino mice, all weighing between 25 and 30 grams, were acquired from Charles River Laboratories located in Écully, France. The procedures were carried out in accordance with the relevant rules and in compliance with the norms of King Faisal University. Before commencing the experiments, all experimental procedures underwent thorough evaluation and received approval from the Research Ethics Committee (KFU-REC/2021-DEC-EA000283) King Faisal University. Mice were kept in a normal lab atmosphere at a temperature of 22±2°C with a 12/12 h light/dark cycle. Animals were randomly divided into 4 groups of 6 mice each as follows: Group 1 or Control (normal saline); group 2 treated with OTA alone (OTA 5 mg/kg body weight); group 3, mice were treated with OTA + GNL (40 mg/kg body weight, 4 h later) and group 4 (drug control treated with GNL 40 mg/kg body weight). They were treated for 6 weeks (2 times a week). Following the experiment, the animals were housed in cages with ventilation maintained at a flow rate equivalent to 15% of the cage volume to ensure adequate airflow. They were subsequently euthanized via cervical dislocation, and blood samples were collected from the retro-orbital plexus. The kidney and spleen were collected, measured, frozen to stop metabolic processes, and stored at a temperature of -80°C for subsequent examination. The renal tissue's relative weight was evaluated to assess the influence of GNL on alterations in kidney tissue mass generated by OTA.

Serum biochemical markers measurement

Serum Blood Urea Nitrogen (BUN) and Creatinine (CREA) levels were measured in the blood samples. Approximately 100 µL of blood was collected from the retro-orbital plexus. BUN and CREA were analyzed using assay kits from Sekisui Medical (Tokyo, Japan).

Biomarker evaluation

An assessment was conducted on the performance of biomarkers related to liver function. The levels of Aspartate Transaminase (AST) and Alanine Transaminase (ALT) were quantified using commercially available kits from RandoxTM Laboratories Limited (Crumlin, UK).

Biochemical analysis

The protein concentration of kidney tissue was measured according to the Bradford protocol.[18] Superoxide Dismutase (SOD) activity was evaluated by a previously reported method of Misra and Fridovich.[19] Glutathione Peroxidase (GPx) enzyme activitiy was assessed as previously reported.[20] In addition, the Glutathione (GSH) and Lipid Peroxidase (LPO) levels were measured as mmol MDA/mg tissue/60 min as per previously published methods.[21,22]

Western blot analysis

Western blot study was performed according to the methods described by Alzahrani *et al.*[23] We performed a Western blot analysis of pPI3K, pAKT, NRF2, Bcl2, cleaved caspase-3, and beta-actin as an internal loading control. The activation of pPI3K, pAKT, NRF2, Bcl2, and cleaved caspase-3 was measured using a

densiometric analysis, and the results were adjusted based on changes in beta actin levels.

The Bio-Rad protein assay (Hercules, California, USA) was utilized to determine the protein concentration and Bovine Serum Albumin (BSA) in all kidney tissue samples, serving as the reference standard. We loaded all wells with the same concentration (50 µg) of protein, separated the proteins using SDS-PAGE (8–15%), and then transferred them to nitrocellulose membranes. Alternatively, the membranes were blocked with 5% skimmed milk for 30 minutes at room temperature, followed by incubation with primary antibodies for a few hours and subsequent incubation with secondary antibodies. A 3600-00-C-Digit Blot Scanner (LICORBio, Lincoln, Nebraska, USA) was employed to visually observe the protein bands. The control band was standardized to a value of 1 using Image Studio Lite software, located in Lincoln, NE, USA. The experiment was replicated thrice.

Histopathology

The kidney tissues were preserved in 4% formalin, then encased in paraffin, sliced into sections that were 4 µm thick, and finally stained with H&E. The produced sections were observed and measured using an optical microscope (Leica D6000, Leica, Wetzlar, Germany), and the images were captured at a magnification of 200×.

Statistical analysis

The values are presented as the mean and Standard Error of the Mean (SEM). ANOVA was used to test for significance and Duncan's procedure was used for post hoc tests. Statistical analysis was done with SPSS 10.0; $p<0.05$ was considered statistically significant, $*p<0.05$ indicates significant differences between the OTA group and control group, and $\#p<0.05$ indicates significant differences between the OTA group and OTA + GNL group.

Results

Effect of GNL on spleen and kidney morphological changes

The design of animal experiments is shown in Figure 1a. After six weeks of OTA administration hyperemia, swelling, and cirrhosis

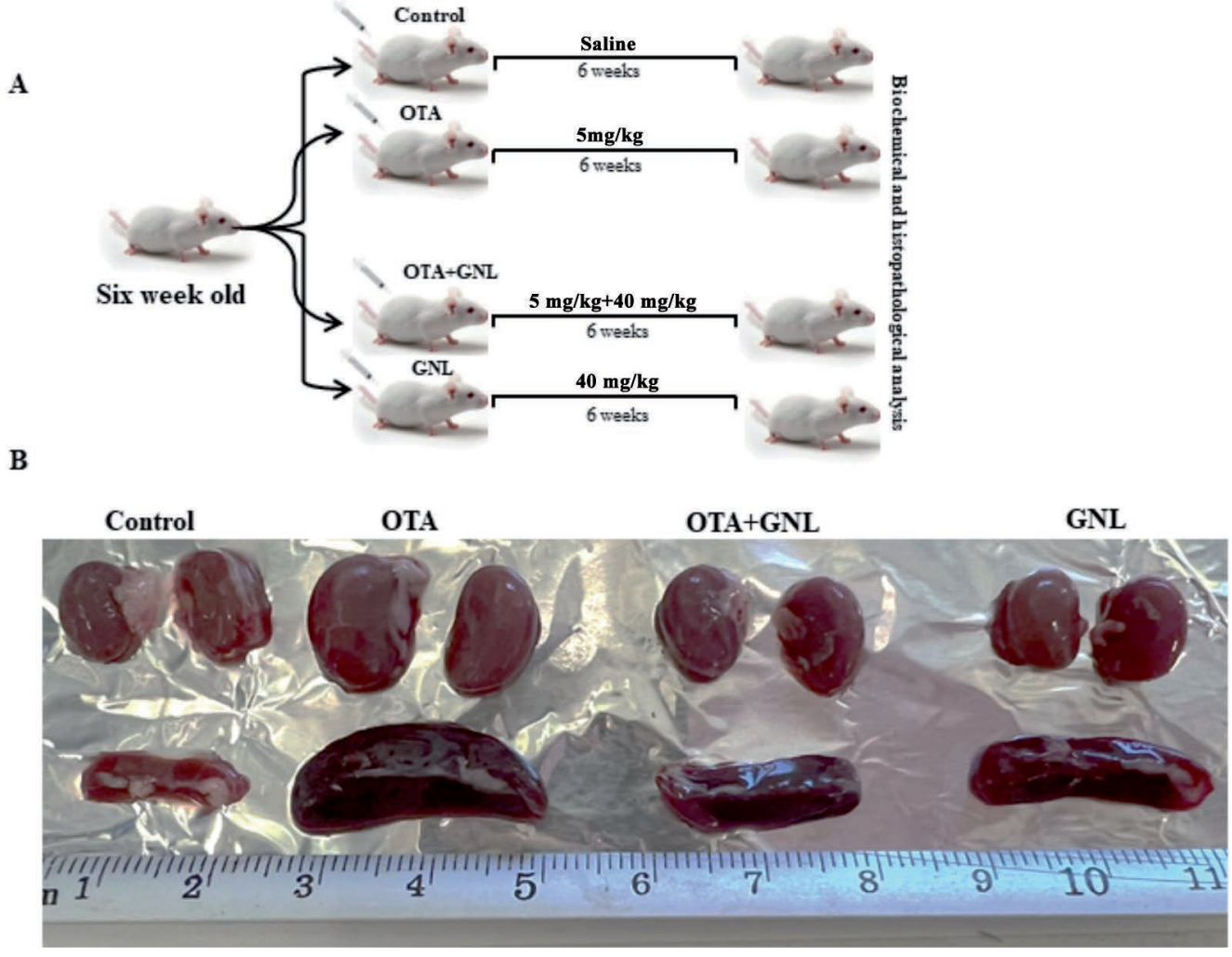

Figure 1. Changes in spleen and kidney morphology caused by GNL: A) Experimental design for animals, B) Morphology of kidney and spleen.

were seen in the kidney and spleen of OTA-treated mice. While mice pre-treated with 50 mg/kg GNL displayed normal morphology in both kidney and spleen tissues, similar to the normal control group (Figure 1B).

Effect of GNL on the body and organ index in mouse

During treatment, body weight was measured every two days. The kidney and spleen were weighed after the experiment. OTA-treated mice had significantly lower body and increased organ weights than control-treated mice (Figure 2A, B and C). After OTA administration, the body weight of the mice decreased but was restored to baseline levels following GNL treatment (Figure 2A). OTA-treated animals exhibited significantly higher spleen and kidney weights compared to controls, while the OTA + GNL-treated group showed significantly reduced organ weights (Figure 2B and C). These results suggest that GNL mitigated the adverse effects of OTA on organ weight.

Effect of GNL on ALT and AST enzymes in mice serum

OTA-induced kidney damage can trigger inflammatory pathways that may also affect the liver, leading to elevated ALT and AST levels as secondary markers of systemic damage. The protective effects of GNL were evaluated by measuring serum biochemical parameters, ALT and AST. Figures 3A and B illustrate the impact of GNL on serum biochemical markers as affected by OTA. Compared to the control group, mice treated with OTA showed a significant increase in serum levels of ALT and AST (p

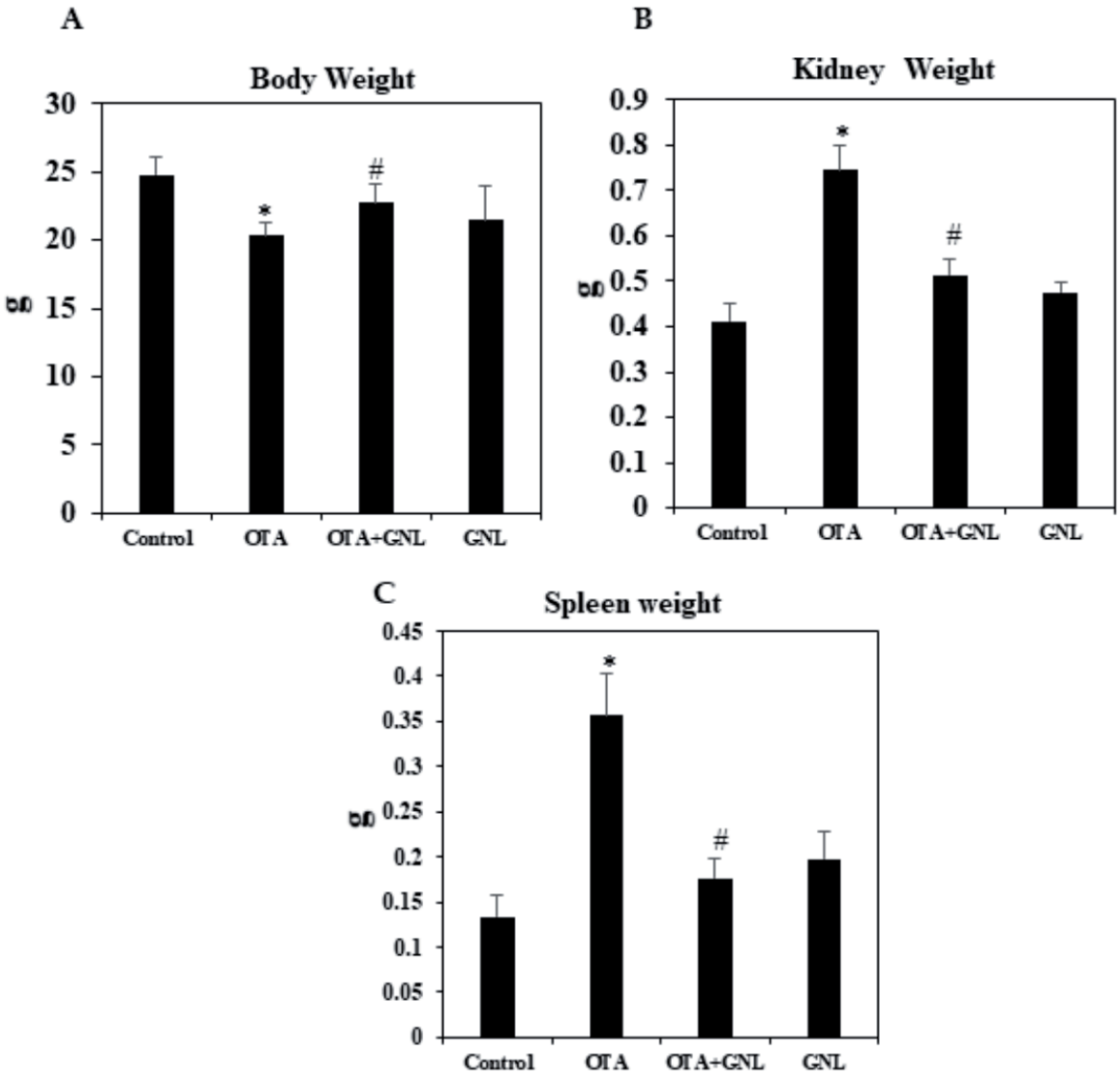

Figure 2. Effect of GNL on the body and organ index in mouse. (A) Body weight. (B) Kidney weight. (C) Spleen weight. Statistical significance of the differences between the treatment groups and the control group. Values are expressed as mean±SEM (n=3). p <0.05 was considered statistically significant. *p <0.05 indicates significant differences between the OTA group and control group. #p <0.05 indicates significant differences between the OTA group and OTA + GNL group only.

= 0.034). However, GNL-treated mice did not exhibit the elevated serum ALT and AST levels seen in the OTA group ($^{\#}p$ = 0.021). These results suggest that GNL nearly entirely protected the mice from OTA-induced renal toxicity.

GNL effect on levels of CREA and BUN in mice serum

As shown in Figures 3C and D, we found high levels of CREA and BUN. Oral administration of GNL with OTA significantly (p = 0.017) inhibited these parameters.

GNL reduces renal oxidative stress in OTA-treated mice

To understand the mechanism of GNL, the levels of antioxidant enzymes SOD, GSH-PX, CAT, and MDA in kidney tissue were assessed. OTA treatment significantly increased MDA production and decreased levels of SOD, CAT, and GPx in the kidneys (p = 0.22) (Figure 3E-H). OTA treatment increased MDA levels by 2.8-fold compared to the control group. However, administering GNL at 40 mg/kg prior to OTA exposure effectively inhibited this increase, bringing MDA levels close to those observed in the control group (p = 0.031) (Figure 3E). Figures 3F-H show that SOD, CAT, and GPx activities were nearly restored to normal levels (p = 0.024) after GNL administering. These findings indicate that GNL protects the kidneys from OTA-induced damage by mitigating oxidative stress.

Effect of GNL on kidney tissue histopathologic changes in OTA-treated mice

As indicated in Figure 4, when compared with the normal control, OTA-treated mice exhibited damage to kidney tissue structure. H&E-stained kidney sections from mice in the control group showed normal histological structure of Glomerulus (G) Proximal convoluted Tubules (PT), and Distal convoluted Tubules (DT). Histopathological section of OTA-treated mice kidney showed marked disorganization of renal tubules with Necrotic Changes (NT), marked Apoptotic Tubules (AT) and sloughing of Tubular epithelial cells (T), Vacuolation of renal tubules (V), diffuse hydropic tubular degeneration (red arrows) and multifocal chronic interstitial nephritis, infiltration of lymphocytes and mononuclear cell interstitium (black arrows), condensation of cellular casts in the lumen (*). The OTA+GNL-treated groups showed substantial protection, with near-normal tissue morphology. The GNL-alone groups exhibited normal kidney histology with no adverse effects.

GNL induces the activation pPI3K/pAKT signaling pathways

The activation of pPI3K/pAKT in the kidney was analyzed to understand how GNL counteracts OTA-induced renal toxicity. Compared to the control group, OTA treatment reduced the phosphorylation of PI3K and AKT in the kidney. However, as illustrated in Figure 5A and B, GNL increased the phosphorylation of pPI3K and pAKT.

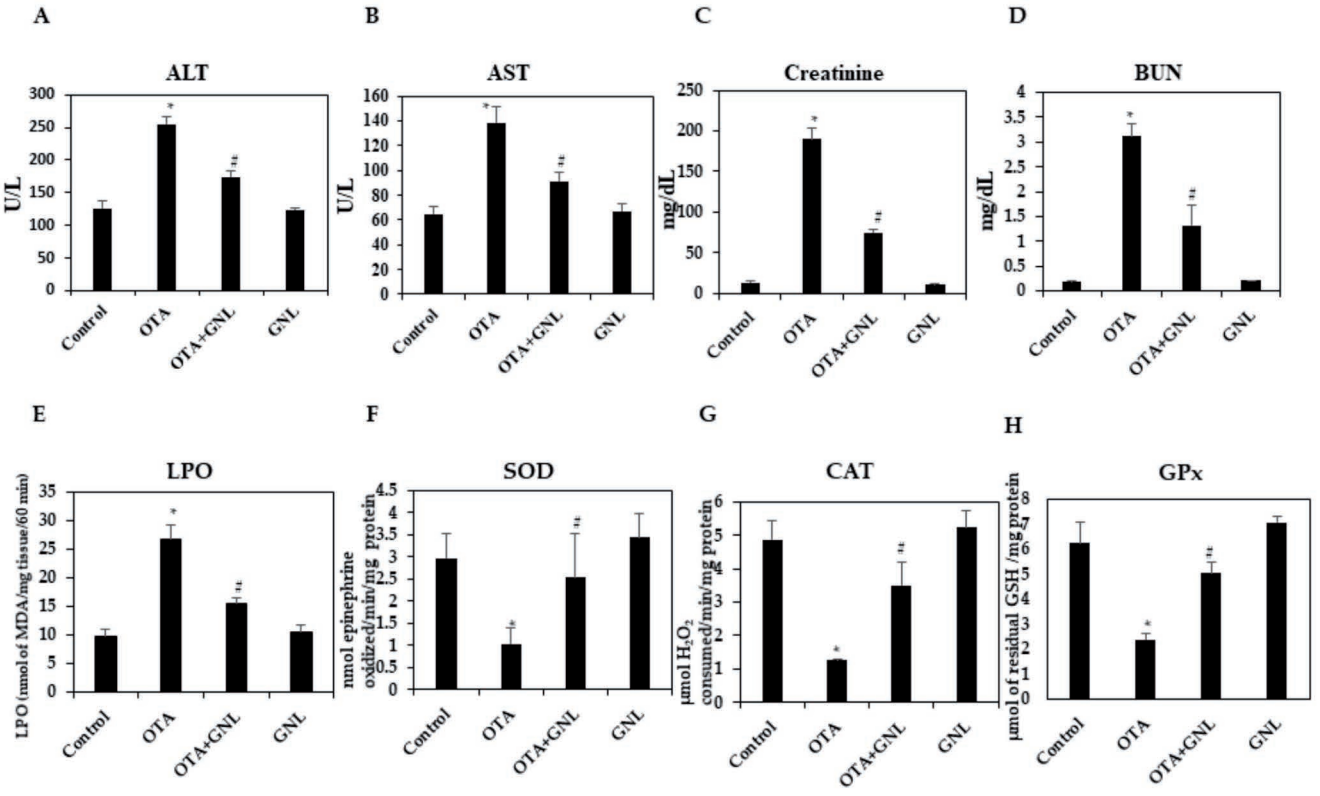

Figure 3. Geraniol effect on alanine (ALT) and aspartate (AST) transaminase, serum markers enzymes and oxidative stress markers enzymes. (A) ALT, (B) AST, (C) Creatinine, (D) Serum BUN. (E) Renal LPO levels. (F) SOD activities. (G) Renal CAT activities. (H) Renal GPx activities. Values are expressed as mean±SEM (n=3). *p <0.05 indicates significant differences between the OTA group and control group. #p <0.05 indicates significant differences between the OTA group and OTA + GNL group.

GNL induces the activation of Nrf2 signaling pathways

As shown in Figures 5C and D, OTA exposure increased total Nrf2 levels in renal tissue pretreated with GNL. In this study, Nrf2 signaling was found to play a role in the protective effects of GNL against OTA-induced renal toxicity.

Effects of GNL on Bcl2 expression

This study investigated the impact of GNL on the expression of Bcl-2, a crucial regulator that prevents cell death. Figures 5C and D demonstrate our initial measurement of Bcl-2 protein expression levels in mice treated with OTA, revealing a decrease in Bcl-2 expression. Nevertheless, the GNL therapy effectively mitigated this effect by increasing the expression of Bcl-2, hence inhibiting programmed cell death and enhancing cell viability.

Effect of GNL on cleaved caspase-3 and DNA fragmentation

Figure 5E demonstrates that GNL therapy effectively suppressed caspase-3 activity in OTA-induced renal tissues, as compared to the untreated control (OTA alone). Conversely, DNA laddering (or internucleosomal DNA cleavage) is a sign of late-stage apoptosis. Renal tissues exposed to GNL treatment were analyzed by agarose gel electrophoresis for DNA laddering to see if apoptosis occurred. GNL inhibits apoptotic DNA fragmentation similar to the control. Overall, these results indicate that GNL inhibits apoptosis in OTA-induced renal tissue death (Figure 5F and G).

Discussion

Food safety and contamination of animal feed have become critical concerns due to the evident toxicity of OTA. Furthermore, there is a pressing need to explore alternative strategies to mitigate OTA toxicity. Although the harmful effects of OTA are well-documented, the molecular mechanisms involved in its detoxification remain poorly understood and require further investigation. Recently, GNL's antioxidant properties have prompted researchers to study its biological effects.[24–27] The present study examined how GNL protects kidneys from OTA-induced damage. As mentoned above, animals are mainly affected by OTA in the kidney and liver. According to Li *et al.*, 6-week-old mice treated with OTA at 5 mg/kg for 6 weeks had a slight decrease in body weight compared to control

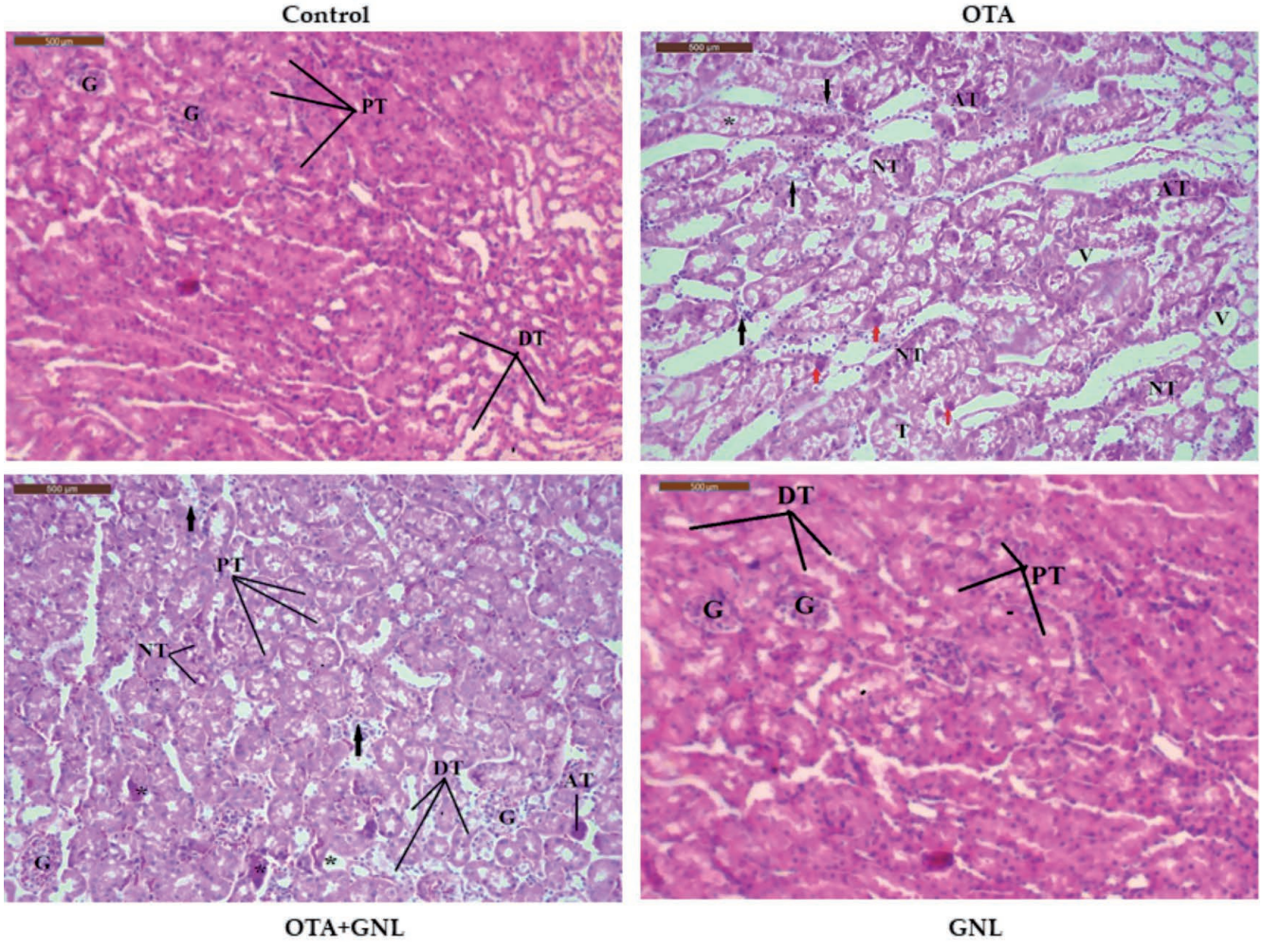

Figure 4. Effect of GNL on OTA-induced tissue histopathologic changes in mice kidney. Histological staining (H&E) of kidney tissue (200× magnification), scale bar: 50 µm.

mice. In addition, they found that the urine output of the OTA-exposed group was higher than that of the normal control group.[28] We noticed depression and weight loss after OTA treatment in the established animal model. Kidney damage is often detected by blood biomarkers. Elevated BUN and CREA levels in serum indicate impaired renal function.[29–31] In our study, the mice lost weight, and their CREA and BUN levels increased significantly after OTA exposure. Staining with H&E confirmed this deduction. OTA primarily affects renal tubular reabsorption function, not glomerular filtration function.[6] According to our previous study, OTA causes toxic effects via oxidative stress.[3] It is worth noting, however, that treatment with both GNL and OTA is effective at restoring the levels of the above-mentioned biomarkers (CREA and BUN) to levels similar to those of the control animals. In this study, it was found that GNL was able to effectively alleviate the damage caused to the renal cells by exposure to OTA.

OTA was found to have a significant effect on serum enzyme levels such as ALT and AST, with OTA causing a marked increase in their levels. There is a possibility that some of these effects are attributed to kidney damage caused by OTA. OTA-induced kidney damage can trigger inflammatory pathways that may also affect the liver, leading to elevated ALT and AST levels as secondary markers of systemic damage. In this regard ALT and AST are biomarkers of liver damage, and ALT is considered a gold standard.[32] Dysregulated permeability of the renal membrane and/or disturbed biosynthesis of these enzymes may cause higher levels of circulating enzymes.[33] Increased serum levels of these enzymes can cause pathologic changes in the kidney, which can lower total protein and albumin synthesis. As reported in our previous study, GNL is known to have significant antioxidant properties.[27] In the present study, it was found that AST and ALT elevated levels could be significantly reduced by GNL. The results of this study are in accordance with the

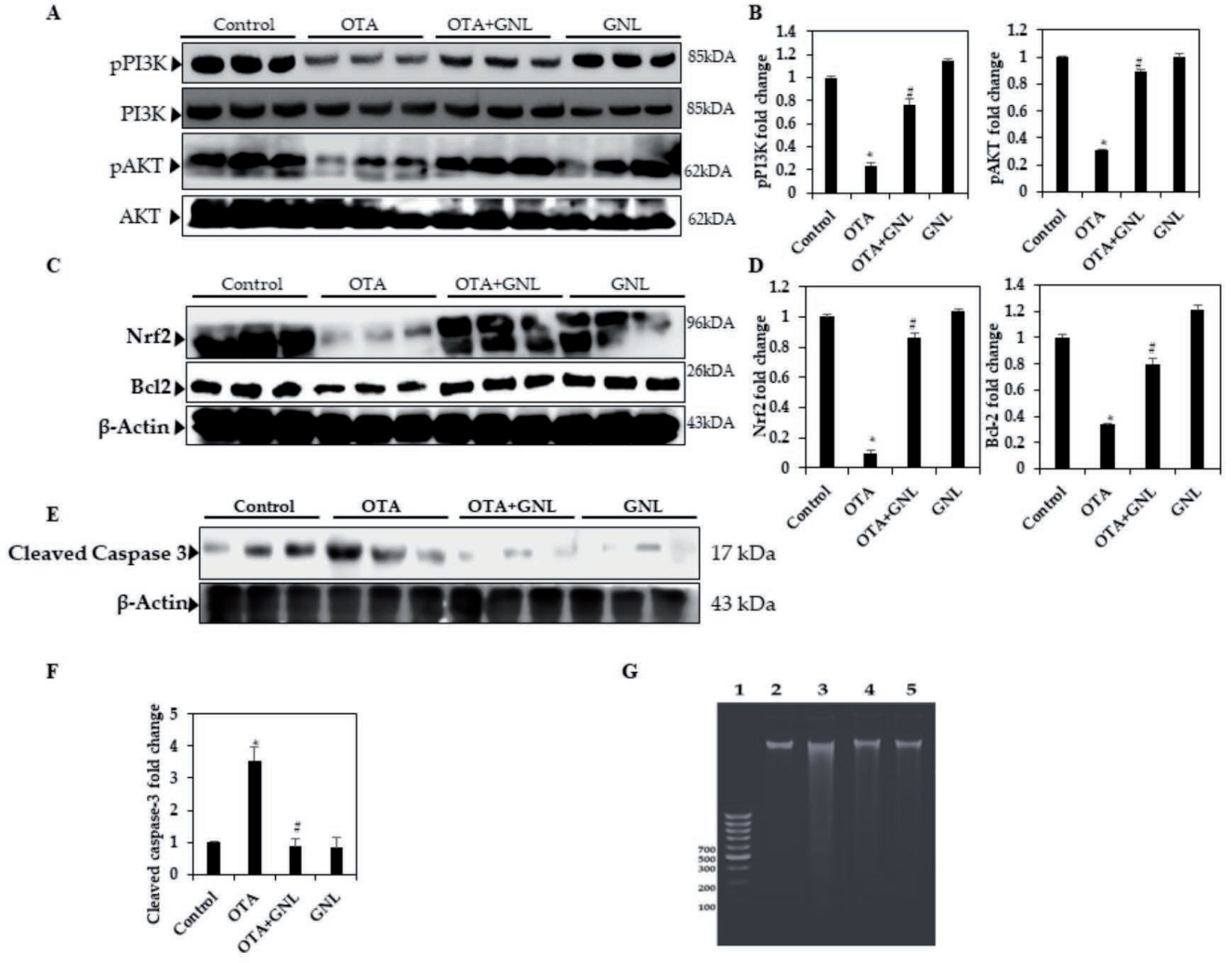

Figure 5. GNL induces the activation pPI3K/pAKT, Bcl2 and NRf2 signaling pathways. A) Western blot analysis of the activation of pPI3K/pAKT signaling pathways; B) Quantitation of both pPI3K/PI3K and pAKT/AKT, as obtained by densiometric analysis and normalized by fold changes; C) Activation of Nrf2 and Bcl2 protein expression; D) Quantitation of Nrf2 and Bcl2, as obtained by densiometric analysis and normalized by fold changes; E) DNA fragmentation analyzed by agarose gel electrophoresis. Lane: 1) DNA ladder, 2) Control, 3) OTA alone, 4) OTA with GNL, 5) GNL alone; F) cleaved caspase-3 protein expression; G) Quantitation of cleaved caspase-3, as obtained by densiometric analysis and normalized by fold changes. Values are expressed as mean±SEM (n=3). *p <0.05 indicates significant differences between the OTA group and control group; #p <0.05 indicates significant differences between the OTA group and OTA + GNL group.

fact that GNL has antioxidant properties in preventing the damage induced by various toxins to organs.[34] MDA is a marker for lipid peroxidation and is thought to be the most representative end product of lipid peroxidation.[35,36] A significant increase in MDA levels was observed in the OTA-treated group, suggesting that the body overproduces ROS and undergoes lipid peroxidation reactions in response to OTA. OTA-induced MDA levels were reduced by GNL pretreatment in mouse kidneys. It may be associated with the reduction in lipid peroxidation, through which GNL protects against kidney damage. The *in vivo* antioxidant systems include SOD, CAT and GPx. As a result of OTA exposure, SOD, CAT, and GPx activities were reduced. The mice were significantly less damaged by OTA-induced kidney damage after GNL treatment, where SOD, CAT, and GPx activities were significantly improved. The antioxidant effect of GNL seems to protect kidneys from damage caused by OTA exposure. GNL's distinctive molecular structure enables it to effectively neutralize singlet oxygen and scavenge free radicals. This action significantly reduces oxidative stress, making GNL a potent antioxidant capable of protecting cells and tissues from damage caused by ROS.[15,37,38] The survival pathways involving pPI3K and pAKT may also play a role in mediating renal cell survival.[26] PI3K and AKT were found to be significantly inhibited in phosphorylation when exposed to OTA, based on the results of this study. In renal tissue treated with GNL, pPI3K, and pAKT levels were elevated and restored.

Several antioxidant genes are regulated by the transcription factor Nrf2 and are activated by this transcription factor. It is crucial to note that they are widely present in the human's renal apparatus.[39,40] Nrf2 and its downstream targets have a vital function in preserving the balance of renal tissue by reducing oxidative stress, which ultimately promotes kidney health. Moreover, they play a role in both the initiation and advancement of renal failure.[41,42] GNL suppressed the harmful effects of OTA on mice kidneys via stimulating the Nrf2 pathway. Nrf2 expression is typically low under normal physiological conditions. However, when oxidative stress is induced by factors such as poisoning, hyperglycemia, hypoxia, or volatility, Nrf2 expression can increase significantly. This is consistent with the established model of Nrf2 activation, where its expression is elevated in response to stress.[43,44] Additionally, it was found that the overall amount of Nrf2 was significantly reduced following OTA treatment, but the overall amount of Nrf2 was enhanced following pretreatment with GNL. This discovery aligns with prior investigations. Moreover, it was noticed that GNL facilitates the translocation of Nrf2 from the cytoplasm to the nucleus, leading to enhanced expression of its downstream targets. This, in turn, enhances redox balance, safeguards mice kidney cells, and enhances overall well-being by utilizing antioxidants.[45]

Bcl-2 and similar proteins have the ability to shield renal cells against apoptosis and oxidative damage.[46,47] Caspase-3 carries out the process of programmed cell death, known as apoptosis, during cell death. The formation of this cleavage is initiated by caspases-3 and -7, which become active during the process of apoptosis. The administration of OTA resulted in the decrease of Bcl2 and increase of cleaved caspase-3 in kidney tissue. However, these effects were reversed by the treatment with GNL. It is crucial to emphasize that GNL administration effectively inhibited the activation of the caspase cascade. Our investigation revealed a correlation between renal tissues exhibiting high levels of ROS and a reduced antioxidants enzymes activity. GNL demonstrates renoprotective properties by inhibiting apoptosis in renal cells. This finding is consistent with previous studies.[47] Based on the findings, GNL provides protection to renal cells. While GNL shows promise as a potential treatment for avoiding nephrotoxicity caused by OTA, further research is needed to understand its pharmacological effects in a xenograft model that is clinically relevant. This study primarily focused on short-term exposure to OTA and GNL. The long-term effects of GNL on OTA-induced nephrotoxicity remain unclear, and future studies should examine chronic exposure to evaluate the potential for sustained protective effects.

Conclusions

This work provides evidence that oxidative stress is a crucial factor in the renal toxicity caused by OTA. Furthermore, exposure to OTA results in detrimental consequences and substantial alterations in kidney function. The GNL administration facilitates the restoration of renal function by stimulating the activation of Nrf2 through the PI3K/AKT signaling pathway. GNL possesses antioxidant properties that can safeguard the well-being of mice by diminishing oxidative stress. GNL has been demonstrated to be safe for mice with kidney disorders caused by OTA-induced oxidative stress. Furthermore, there is growing evidence suggesting that GNL exhibits antioxidant properties by enhancing Nrf2 signaling *in vivo*. The exact protective mechanism of GNL is yet to be elucidated; further study remains required to pinpoint the details of its action. Therefore, GNL has promise for future application in the treatment of renal disorders, however, additional laboratory studies and clinical trials are necessary to ascertain its efficacy.

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