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Garlic Volatile Oil Attenuates Lead Nitrate-induced Nephrotoxicity by Suppressing Apoptosis: Evidence from Immunohistochemical Analysis in Murine Models

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Abstract

Lead contamination remains a major global health concern due to its persistent nature and its ability to induce severe renal toxicity following prolonged exposure. The present study was undertaken to investigate the nephroprotective potential of garlic volatile oil (GVO) against lead nitrate $Pb(NO_3)_2$-induced nephrotoxicity in Swiss albino mice with particular emphasis on its anti-apoptotic and anti-genotoxic properties. For that, six experimental groups were established: control, $Pb(NO_3)_2$-intoxicated, low and high-doses of GVO-treated, silymarin-treated, and olive oil-treated. Lead accumulation in renal tissues was quantified using atomic absorption spectrometry (AAS), which confirmed significant lead deposition following $Pb(NO_3)_2$ exposure. Renal function was assessed by estimating serum urea and creatinine levels, while alterations in renal neurotransmitters (DA and AChE) were also evaluated. Molecular investigations were performed to assess the extent of renal injury and apoptotic markers. Immunohistochemical analysis of caspase-3 expression, together with the assessment of apoptosis-related proteins including caspase-8, caspase-3, Bax and Bcl-2, demonstrated that lead nitrate exposure markedly disrupted apoptotic signalling pathways in renal tissues. Oral administration of GVO markedly ameliorated lead-induced nephrotoxicity by reducing biochemical marker levels, restoring renal neurotransmitter status, normalizing apoptosis-related protein expression, reducing caspase-3 immunoreactivity, and improving renal architecture, thereby indicating effective suppression of apoptosis and enhanced tissue recovery.

Major Findings: The study highlights the therapeutic potential of GVO as a natural nephroprotective agent against lead-induced renal damage and provides experimental evidence supporting its anti-apoptotic and anti-genotoxic properties for managing heavy metal-induced nephrotoxicity.

Keywords: Caspases, Garlic Volatile Oil, Immunohistochemistry, Lead Nitrate, Nephrotoxicity

Abbreviations: AChE – Acetylcholinesterase; AAS – Atomic Absorption Spectrometry; DA – Dopamine; GVO – Garlic Volatile Oil; IHC – Immunohistochemistry; $Pb(No_3)_2$ – Lead (II) Nitrate; Pb – Lead

1. Introduction

Heavy metal toxicity significantly affects both animal and human health. Heavy metals are characterised by their high hydrophilic nature and rapid assimilation by biological systems, which facilitates their accumulation in living organisms. Among these metals, lead (Pb) exposure is particularly hazardous because of its widespread toxic effects[1]. Despite its well-established toxicity, it is still used in certain industries for a long time, including paints, plastics, cosmetics, mining, and battery recycling. Lead has a low melting point, is malleable, and resists corrosion. So, chronic exposure to lead, however, can cause major health difficulties, particularly in soft tissues like the kidneys and liver[2]. Since the kidneys are essential for eliminating toxins and preserving equilibrium, they are particularly vulnerable. Heavy metals and other toxicants tend to accumulate in these soft tissues of the

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body, where their burden can be quantified using various analytical techniques, including inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectroscopy (ICP-OES)/atomic emission spectroscopy (ICP-AES), X-ray fluorescence spectroscopy (XRF), and atomic absorption spectrometry (AAS)[3]. In the present study, the concentration of lead in the renal tissues of mice was determined using atomic absorption spectrometry (AAS). The findings confirmed that lead nitrate intoxication resulted in significant accumulation of lead in the renal tissues, thereby validating the successful establishment of lead-induced nephrotoxicity in the experimental animals[4]. Elevated renal biomarkers, including urea and creatinine, are the main indicators of lead-induced nephrotoxicity[5]. The delicate balance of neurotransmitters such as dopamine (DA) and acetylcholinesterase (AChE), which are essential for proper renal physiology, was also affected by the prolonged lead exposure. AChE is necessary for neurotransmitter signalling, while dopamine is crucial for controlling blood pressure and salt excretion in the kidneys. Moreover, chronic lead exposure usually intensifies renal failure by inhibiting these enzymes, which results in abnormal blood pressure and electrolyte imbalances that ultimately cause additional kidney damages[6]. One of the most detrimental effects of lead-associated toxicity within cells is the triggering of cell death by the mechanism of apoptosis, which indeed plays a vital role in the degradation of renal tissues. A number of chemical signals, especially those from the caspase family of proteases, are involved in the tightly controlled process of the apoptosis pathway[7]. Among these, caspase-3 is the most essential factor involved in the last stages of apoptosis as the executioner enzyme. When cell exposed to lead, oxidative stress and inflammation are elevated by the metal poisoning and thus induce caspase-3 activation, frequently through the caspase-8 pathway[8]. The up-regulation of the caspase cascade triggers the following apoptotic events, including DNA fragmentation, chromatin condensation, cell shrinkage, and eventual cell death[9]. Mechanistically, oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and the cellular antioxidant defence system, resulting in oxidative damage to lipids, proteins, and nucleic acids. Among these biomolecules, DNA is particularly susceptible to ROS-induced damage, which is primarily repaired through the base excision repair (BER) pathway. During this repair process, oxidised guanine residues are excised and released as 8-hydroxy-2′-deoxyguanosine (8-OHdG), a well-established and sensitive biomarker of oxidative DNA damage[10]. Elevated levels of 8-OHdG have been widely used to assess oxidative stress associated with environmental and occupational exposure to toxicants, including heavy metals. Therefore, the measurement of 8-OHdG provides a reliable and valuable approach for evaluating lead-induced oxidative DNA damage and the associated nephrotoxicity[1,11]. Additionally, the regulation of apoptosis requires the equilibrium among the pro-apoptotic and anti-apoptotic proteins, including Bax (Bcl-2-associated X protein) and Bcl-2 (B-cell lymphoma 2). Moreover, this equilibrium may be upset by lead exposure, which causes Bax expression to rise and Bcl-2 levels to decline, thereby forcing the cells toward apoptosis and worsening the renal damage[12]. Also, the present study uses Immunohistochemistry (IHC) for the further evaluation of apoptosis in renal tissues exposed to lead. IHC is a lab method that visualises the presence of proteins in tissue sections using certain antibodies[13,14].

Routine histopathological (H and E) evaluation of renal tissues has been comprehensively reported in our previous publication using the same experimental model. Therefore, the present study was specifically designed to extend those findings by investigating the apoptotic mechanisms through immunohistochemical analysis of caspase-3 [15]. This makes it possible to identify the patterns of protein expression in a particular cell type[12]. In this study, IHC enabled the precise detection of proteins like caspase-3, thereby providing valuable information regarding the location and level of apoptotic activity. IHC staining usually shows a significant increase in caspase-3 expression in lead-induced nephrotoxicity cases, especially in the renal tubules and glomeruli, indicating the amount of cellular damage happening in these areas[14]. Previous findings from our laboratory also demonstrated a strong interplay between oxidative stress, inflammation, and apoptosis induced by lead poisoning[11]. Pro-inflammatory cytokines enhance this effect by activating extrinsic apoptotic pathways through death receptors, further increasing caspase-8 and caspase-3 activation[15,16]. Lead exposure produces reactive oxygen species (ROS) which damage cellular structures and promote apoptosis. Renal function gradually declines as a result of the convergence of oxidative stress, inflammation, and apoptosis. Natural substances that have anti-inflammatory and antioxidant qualities have drawn interest recently because of their potential to mitigate heavy metal toxicity[17]. Among these,

the most effective bioactive compounds are the sulfur-containing ones, particularly allicin, which is found in fresh *Allium sativum* (garlic) bulbs[18]. This component is also present in garlic volatile oil (GVO), which is derived via hydrodistillation and makes GVO the most promising anti-toxic volatile fraction of the garlic. Additionally, GVO is considered a viable option for preventing lead-induced nephrotoxicity because of these bioactive constituents' potent anti-inflammatory, anti-apoptotic, and antioxidant characteristics[19,20]. In this line, some previous studies have reported that GVO provides substantial protection against lead-induced toxicity by attenuating oxidative stress and inflammatory responses[21]. In addition to its antioxidant and anti-inflammatory activities, GVO exhibits pronounced anti-apoptotic effects, as it is found to upregulate the anti-apoptotic Bcl-2 protein and downregulate the expression of pro-apoptotic mediators such as Bax, caspase-8, and caspase-3. This regulation of the equilibrium between pro-apoptotic and anti-apoptotic proteins of apoptotic signalling pathways is helpful in reducing the lead-induced cellular apoptosis, thereby protecting renal cells from damage and thus enhancing tissue integrity[12]. Moreover, many previous studies have demonstrated the protective effects of garlic and its extracts against heavy metal toxicity, including lead-induced hepatic and renal damage[22]. Additionally, garlic's sulfur-containing compounds have a chelating impact as they attach to lead ions and make their excretion easier from the body, which reduces the accumulation of lead in the vital organs and thus minimises the poisoning[23,24]. However, most of these investigations have focused solely on crude garlic extracts or general antioxidant properties[25]. Limited information is available regarding the nephroprotective potential of garlic volatile oil against lead nitrate-induced nephrotoxicity, particularly its role in suppressing apoptosis and modulating caspase-3 expression in renal tissues. Furthermore, immunohistochemical evidence explaining the anti-apoptotic mechanisms of garlic volatile oil remains scarce. Therefore, the present study was designed to investigate the ameliorative effects of garlic volatile oil on lead-induced kidney damage and apoptosis in Swiss albino mice. The novelty of the present study lies in evaluating the nephroprotective efficacy of garlic volatile oil against lead nitrate-induced renal toxicity through both biochemical and immunohistochemical assessment of caspase-3-mediated apoptosis. The hypothesis posits that lead poisoning induces renal toxicity by impairing cellular and molecular systems. Conversely, the volatile oil derived from herbal garlic is expected to demonstrate protective effects against lead toxicity, potentially serving as a therapeutic agent to mitigate the renal damage caused by heavy metal exposure. To the best of our knowledge, very few studies have comprehensively investigated the anti-apoptotic mechanism of garlic volatile oil in lead-induced nephrotoxicity using immunohistochemistry in Swiss albino mice[26-28]. Therefore, the purpose of this study is to investigate how GVO protects mice from renal damage caused by lead nitrate.

In particular, the study explores the possibility that GVO normalises neurotransmitter (DA and AChE) activity, restores renal indicators (urea and creatinine), may restore DNA damage by regulating 8-OHdG activity and decreases cell death by suppressing caspases (3 and 8) expression. Also, the results of this investigation will enhance comprehension of GVO's therapeutic possibilities in the management of lead-induced kidney damage and demonstrate its wider uses in reducing heavy metal toxicity.

2. Materials and Methods

2.1 Chemicals and Kits

All reagents used in this investigation were of laboratory quality and were acquired from corporations such as Sisco Research Laboratories Private Limited (SRL) - India, MERCK, RANBAXY, HIMEDIA, and SIGMA, as well as from purported research facilities.

2.1.1 General Scientific Diagnostic Kits

The various diagnostic kits employed for carrying out the experiments are displayed in Table 1.

2.2 Study Plant

The raw fresh garlic bulbs were used to extract the volatile oil after being chosen from the native lands of Banasthali Vidyapith, Rajasthan.

2.3 Garlic Volatile Oil Extraction

The volatile oil from fresh garlic bulbs was extracted using a procedure called Hydrodistillation, which is described completely below:

Before hydrodistillation, the fresh garlic bulbs were cleaned and scraped thoroughly. The bulbs were chopped,

Table 1. List of diagnostic kits purchased for the research study

S.No.	KIT	CATALOGUE NUMBER #	COMPANY
1.	8-OHdG (ELISA)	E-EL-0028	Elabscience
2.	Urea (Colorimetric)	43921	AutoZyme by Accurex Biomedical Pvt Ltd, Mumbai, India.
3.	Creatinine (Colorimetric)	38221	AutoZyme by Accurex Biomedical Pvt Ltd, Mumbai, India.
4.	Dopamine (ELISA)	E-EL-0046	Elabscience
5.	AChE (ELISA)	E-BC-K174-M	Elabscience
6.	Bcl-2 (ELISA)	E-EL-M0175	Elabscience
7.	BAX (ELISA)	E-EL-H0562	Elabscience
8.	Caspase3 (Colorimetric)	E-CK-A311	Elabscience
9.	Caspase 8 (Colorimetric)	E-CK-A312	Elabscience

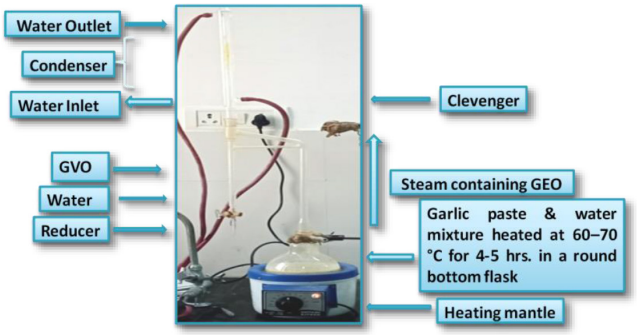


Figure 1. Visualisation of the hydrodistillation process using Clevenger apparatus; highlighting its precision in extracting volatile oil.

and distilled water was added to create a fine, uniform paste. This paste was then combined with boiling water in a flask with a flat bottom. To extract the volatile oil, a flask filled with raw garlic extract was linked to a Clevenger apparatus (ASGI). Additionally, until the last drop of volatile oil was extracted, the flask was heated to 60–70 °C for four to five hours. The condensed vapour that is produced as a result is separated using an oil-water separator[11,17]. Then, in a later study, fresh volatile oil was utilised (Figure 1).

2.4 Acquisition of Mice

The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were strictly adopted in every aspect of animal procedures. Male Swiss albino mice (*Mus musculus*), weighing between 20-30 g and aged 2-3 months, were selected as the study's subject animals. They were purchased from the Lala Lajpat Rai University of Veterinary and Animal Science, Hisar (Haryana).

2.5 Animal Experiment Authorisation

Prior to the allocation of the animal groups for further experimental design, the Institutional Animal Ethics Committee at Banasthali Vidyapith gave its approval in terms of ethics with the approval number BV/IAEC/January/2020/10. Each experiment was conducted in accordance with the generally acknowledged practices.

2.6 Preservation of Animals

The animals were housed in an enclosure with a pathogen-free environment, well-maintained air conditioning, a temperature of 23 to 25 °C, a humidity of 50 ± 15%, and a sufficient pallet to consume food and water. Approximately one week before the studies, the mice were acclimated for a certain period of time.

2.7 Experimental Setup

A total of 36 male Swiss albino mice were used for the present study and randomly assigned to 6 groups (n = 6) (Figure 2). Further, Group I served as the untreated control and received double-distilled water only. Group II animals were administered lead nitrate (50 mg/kg) for 30 days. This group was further subdivided into five subgroups (IIa-IIe). In addition to lead nitrate, the mice in subgroups IIb, IIc, IId, and IIe were also given low and high doses of garlic volatile oil, a standard dose of Silymarin and olive oil, respectively, starting from the 12th day to completion of the study, while subgroup IIa

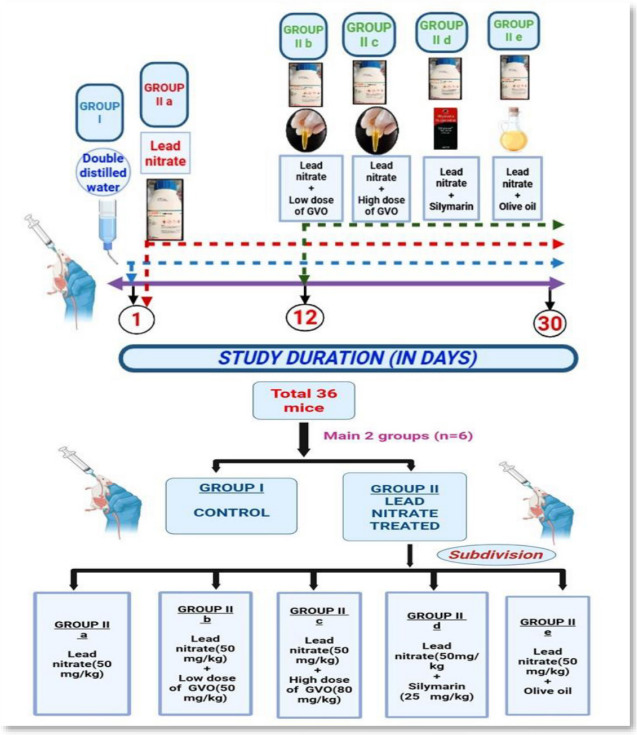


Figure 2. Schematic representation depicting the duration of the study and the grouping arrangement.

received only lead nitrate. The dose of lead nitrate was prepared in double-distilled water, whereas GVO doses were prepared using olive oil as a vehicle. All treatments were administered orally via gavage throughout the whole experimental period. The selection of the doses was based on the earlier findings from our laboratory[11,15,29,30].

2.8 Collection of Samples

Mice were given an overnight fast to reset their metabolism after the treatment was finished. The subjects were euthanised by cervical dislocation on the 30th day of the experiment, and the blood was drawn and centrifuged (5000 rpm for 10 minutes at 4°C). After this, the yellowish fluid (serum) was carefully pipetted out and kept at -20°C for the examination of the nephro-biomarkers (urea and creatinine). Along with that, after the dissection, the mice kidneys were also taken out and immediately kept in pre-chilled 0.9% saline. Extra tissues were removed, and the renal tissues were washed 2-3 times with saline until the complete removal of residual blood was achieved. Then, with a digital balance, the moist weight of each kidney sample was precisely determined. After weighing, tissues were then homogenised (10% w/v) in 0.1 M sodium phosphate buffer (pH = 7.4) using a homogeniser (YS1-471 York Tissue Homogeniser)[11]. Next, the mixture was spun for 10 to 15 minutes at 4°C at 10,000 rpm. Subsequently, the supernatant was extracted in order to analyse the parameters of oxidative stress, renal neurotransmitters, inflammation, and programmed cell death in the mice renal tissues.

2.9 Estimation of Lead (Pb) Content

In this study, the analysis of renal tissues for the estimation of lead (Pb) concentration was carried out in a systematic manner. Initially, 0.2 g of renal tissue was subjected to digestion using a Microwave Digestion System with a mixture of 3:1 wet acid ultrapure concentrated nitric acid and perchloric acid. Then, the tissue samples were further heated for roughly 18 hours at 80°C in a water bath shaker, followed by cooling them at room temperature. After cooling, 6 mL of ion-free water was poured into each sample and vigorously shaken for 6 hours. The Whatman filter paper was utilised to purify the resultant mixture, and a final step involved adding 10 mL of double-distilled water to the filtered solutions. These prepared samples were subsequently stored at 4°C to maintain stability. To determine the concentrations of renal Pb content, a Hydride Vapour Generation System (Model MHS-10, Perkin–Elmer) equipped with a Flame Atomic Absorption Spectrometer (ICE 3000 series AA spectrophotometer) was employed. The concentrations of Pb were reported as µg/g dry weight tissue. This comprehensive process ensured accurate assessment of Pb concentrations in the renal tissue samples[22,31-33].

2.10 Renal Biomarkers

The amounts of urea and creatinine in blood were assessed using Autozyme diagnostic kits.

2.11 Estimation of Renal Neurotransmitters

Renal Acetylcholinesterase (AChE) enzyme activity and Dopamine were determined using competitive ELISA kits.

2.12 Cell Death Biological Markers

The levels of apoptosis regulator proteins, including Caspase-8, Caspase-3, Bax and Bcl-2, in the lead nitrate-treated renal tissues were determined. Keeping the manufacturer's instructions in mind, these protein levels were evaluated with the readily accessible ELISA and colourimetric testing kits.

2.13 Immunohistochemistry of Caspase-3

To assess caspase-3 expression in kidney tissues of mice using immunohistochemistry, the kidneys were primarily fixed in 10% neutral buffered formalin for 1-2 days to preserve the tissue morphology. Afterwards, the mice renal tissues were embedded in paraffin wax and then microtomed at 4- 5 µm by a Leica Microtome. These sections were then transferred onto poly-L-lysine-coated microscope slides to ensure optimal tissue adherence throughout the staining process. The slides were then dried at 60°C for 1-2 hours. The staining procedure began by deparaffinising the tissue sections with two successive 5-minute intervals in xylene. Thereafter, rehydration was performed using graded ethanol (100%, 95%, 70%, and 50%) before being rinsed in distilled water. Heat-induced epitope retrieval (HIR) was executed by dipping the mounted slides in citrate buffer (DIVA, pH 6.8) and heating them in a microwave for 10-20 minutes[34,35]. This retrieval step was critical to unmask the caspase-3 epitopes and enhance antibody binding efficiency. After the antigen retrieval, the slides were cooled at standard temperature for about 20 minutes and then rinsed off in phosphate-buffered saline (PBS). To inhibit endogenous peroxidase activity and minimise background staining, Peroxidase Blocking Solution was applied to the tissue sections for 10 minutes. The slides were then washed in PBS. Non-specific interactions were reduced by treating the sections with 5% bovine serum albumin (BSA) or normal goat serum for 30 minutes. Without washing off the blocking solution, the slides were allowed to incubate overnight at 4°C with anti-caspase-3 antibodies diluted 1:50 (1 mg/ml). The following day, the microtomed sections were washed three times in PBS to get rid of any unbound 1° antibody. Subsequently, the sections were incubated with a 2° HRP-conjugated anti-mouse IgG antibody for about 1 hour at standard temperature. After this step, three washes in PBS were performed, and a DAB (3, 3′-diaminobenzidine) substrate solution was applied to visualise caspase-3 expression. The reaction resulted in a brown precipitate indicating positive caspase-3 immunoreactivity in the cytoplasm, particularly within the renal tubules and glomeruli. Care was taken to monitor the staining under a light microscope to prevent overdevelopment. The reaction was terminated by washing the slides in deionised water. To enhance cellular morphology, the slides were secondarily stained with hematoxylin for about 1-2 minutes to stain the nuclei blue. After rinsing in tap water, these slides were subjected to dehydration through graded ethanol solutions (70%, 95%, and 100%) and then cleared in xylene. The sections were then mounted using Permount or an aqueous-based mounting medium and covered with coverslips. At last, slides were examined under a light microscope, and images were then taken. Furthermore, to quantitatively assess caspase-3 expression, five random fields from each section were evaluated using ImageJ software (version 1.46a, NIH, Bethesda, MD, USA), allowing for precise measurement of the percentage area of positive staining[36].

2.14 DNA Damage Assessment

A commercial kit from Elabscience was used to quantify the quantities of 8-hydroxy-2′-deoxyguanosine (8-OHdG) across the distinct experimental groups of renal tissues in order to determine the extent of DNA disruption.

2.15 Statistical Analysis

To determine whether the means of various groups were significant, the data were statistically examined. For each group (n = 6), the values are displayed as the mean ± SEM. Using the Statistical Package for the Social Sciences (SPSS) software, version 20.0, we performed a statistical analysis to compare the untreated and treated groups using a one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test. To identify statistically significant variations between the means under comparison, a significance level of $p<0.05$ was applied. Prism software, version 4.02, created by GraphPad Software in San Diego, CA, USA, was used for all computations.

3. Results

3.1 Lead Content Assessment

In the lead nitrate subjected experimental set, the Pb concentration was substantially higher (8.09 µg/g), $p<0.05$, relative to the untreated one (I) (1.68 µg/g) whereas, in the GVO low and high dose (II b; II c) reduction of Pb concentration was noticed ($p<0.05$) (4.44 µg/g and 4.04 µg/g respectively), as compare to the toxicant group (II a). Similarly, in the Silymarin and olive-treated groups (II d and II e), reduced levels of Pb content were seen, $p<0.05$ (2.61 µg/g and 4.57 µg/g) as compared to the mice treated lead nitrate-treated group (II a); results are represented in Figure 3. From this, it could be concluded that garlic

volatile oil has potential for dissipating lead nitrate from the renal tissue; therefore, it may facilitate reduction of tissue lead burden from the biological systems.

3.2 Creatinine and Urea Levels

The study assessed the damage caused by lead nitrate exposure and the subsequent recovery facilitated by GVO in kidneys. The evaluation was conducted using crucial renal toxicity markers, namely urea and creatinine. Initially, the lead nitrate-intoxicated groups exhibited a significant increase, up to 32.12 mg/dL for urea and 42.24 mg/dL for creatinine, compared to the control groups with levels of 19.33 and 24.91 mg/dL, respectively. Conversely, mice given a low dosage of GVO demonstrated a significant reduction in urea levels (27.74 mg/dL) and a non-significant reduction in creatinine levels (35.20 mg/dL) relative to the lead nitrate-intoxicated group (32.12 and 42.24 mg/dL). On the other hand, the higher dosage of GVO showed a significant decrease in both urea (22.54 mg/dL) and creatinine (27.49 mg/dL) levels compared to the lead nitrate-intoxicated group (32.12 and 42.24 mg/dL). For the dosage of Silymarin, both urea and creatinine levels showed a significant reduction by 25.22 mg/dL and 22.60 mg/dL, respectively, relative to the lead nitrate category (32.12 and 42.24 mg/dL). Finally, in the olive oil group, urea levels significantly declined to 26.42 mg/dL, while creatinine levels showed a non-significant reduction to 35.9 mg/dL compared to the lead nitrate-intoxicated group (32.12 and 42.24 mg/dL) depicted in Figure 4.

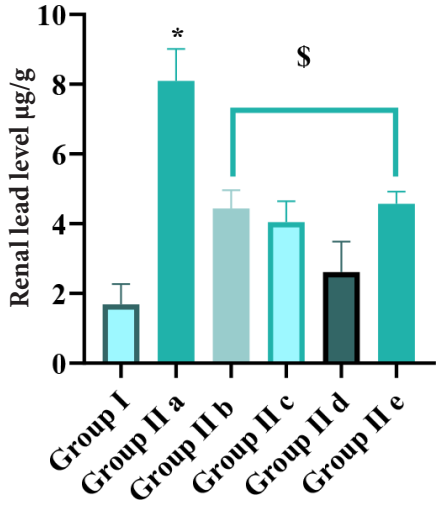


Figure 3. The histogram displays the variations in the study groups for renal lead content. The mean ± SEM (n = 6) values are shown, and they are statistically significant at $p<0.05$. In comparison to group C (I), the LN (II a) is shown by * $p<0.05$. In contrast to (II b), (II c), (II d), and (II e) categories, $ $p<0.05$ shows the LN (II a).

3.3 Renal Neurotransmitters

3.3.1 Dopamine (DA) Level

The dopamine level was estimated in the treated group, as dopamine plays a crucial role in kidney salt-water regulation. In the present study, the group subjected to lead intoxication demonstrated a notable reduction in dopamine levels ($p<0.05$) (6.154 pg/mL) in the renal tissues

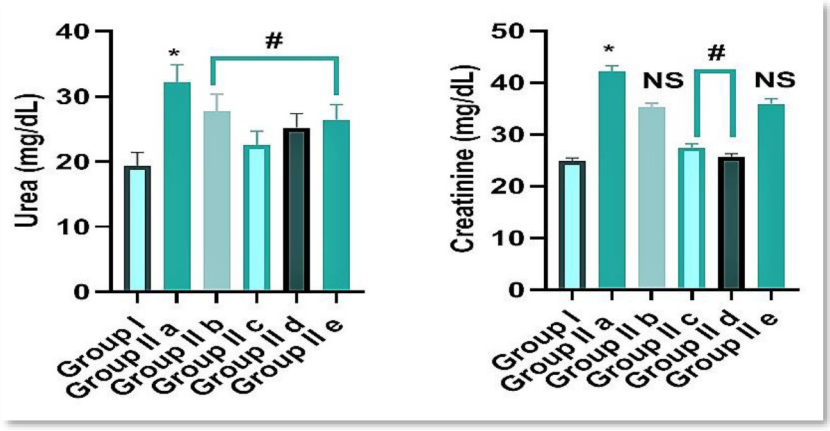


Figure 4. The study groups' variations in kidney toxicity parameters are displayed in the given histogram. The values, which are statistically significant at p<0.05, are displayed as mean ± SEM (n = 6). The LN (II a) is shown by $p<0.05$ * in comparison to the group under C (I). In contrast to (II b), (II c), (II d), and (II e) categories, # $p<0.05$ shows (II a). NS stands for Non-significant.

of Swiss albino mice, as opposed to the control group (9.327 pg/mL). Furthermore, treatment with GVO at varying doses (50 mg/kg and 80 mg/kg) reinstated DA levels (7.95 and 8.83 pg/mL) in comparison to the lead-impaired group (6.154 pg/mL), displaying statistical significance (P<0.05). The reference drug, Silymarin, significantly increased DA levels up to 9.208 pg/mL relative to the lead nitrate group (6.154 pg/mL) (p<0.05). Moreover, DA activities showed a significant elevation in the vehicle group (7.795 pg/mL) relative to the lead nitrate-intoxicated group (6.154 pg/mL) (p<0.05), as depicted in Figure 5.

3.3.2 Acetylcholinesterase (AChE) Level

The AChE level was also estimated in the treated groups. In the present study, the group subjected to lead intoxication demonstrated a notable reduction in AChE levels (p<0.05) (1.34 U/mg protein) in the renal tissues of Swiss albino mice, as opposed to the control group (1.77 U/mg protein). Furthermore, treatment with GVO at varying doses (50 mg/kg and 80 mg/kg) reinstated AChE levels (1.37 and 1.90 U/mg protein) in comparison to the lead-impaired group (1.34 U/mg protein), displaying statistical significance (p<0.05). The reference drug, Silymarin, significantly increased AChE levels to 1.58 U/mg protein compared to the lead nitrate group (1.34 U/mg protein) (p<0.05). Moreover, AChE activities exhibited a significant reduction in the vehicle group (0.97 U/mg protein) relative to the lead nitrate-intoxicated group (1.34 U/mg protein) (p<0.05), as depicted in Figure 5.

3.4 Mitigating Lead Nitrate-induced Apoptosis with GVO

Our data revealed that the renal tissues may undergo apoptosis after contact with lead nitrate, as shown by a significant elevation in pro-apoptotic mediators like caspase-8, caspase-3, and Bax, along with that there is reduction in the anti-apoptotic mediator Bcl-2. Through keeping all this in mind, our findings reveal that the caspase-8, caspase-3 and Bax levels get significantly enhanced in the toxicant category (II a) in contrast to the untreated one (I) (p<0.05), besides this in case of GVO dosages (II b and II c), silymarin (II d) and olive oil (II e) treated groups the extent of caspase-8, and Bax get reduced as correlate to the toxicant treated (II a) significantly, and only in case of caspase-3 olive oil treated-group (II e) reduces non-significantly relative to the toxicant-treated group (II a) (Figure 6). Further to that, Bcl-2 level get reduced in the toxicant-treated renal tissues (II a), when compared to the untreated one (p<0.05). Moreover, in the case of GVO-treated dosages (II b and II c) and silymarin, Bcl-2 level get restored and increased (p<0.05) when compared to the toxicant-treated category (II a), whereas in the case of the olive oil-treated group (II e), Bcl-2 level got increased (p>0.05) as compared to the toxicant-treated category (Figure 6). Thus, Pb deposition and the activation of apoptosis in renal tissues were shown to be connected, as demonstrated by a statistically significant positive correlation coefficient. The amount of caspase-8,

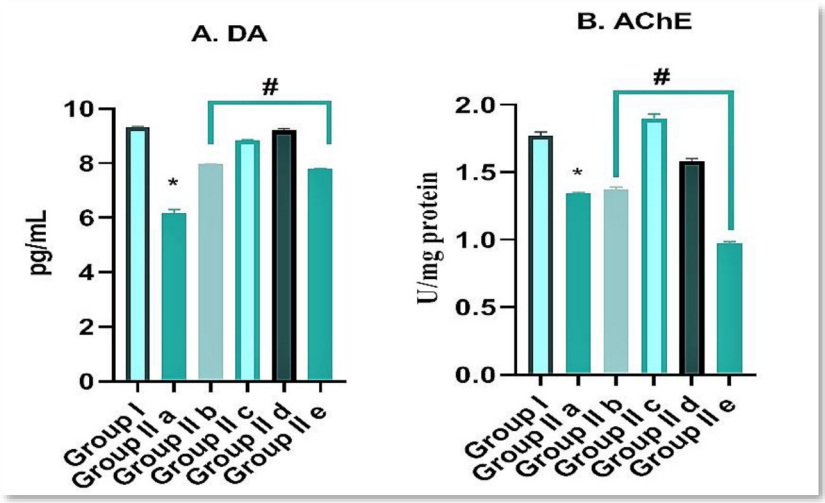


Figure 5. The histogram displays the post-treatment variations in dopamine and acetylcholinesterase levels. The mean ± SEM (n = 6) values were displayed, and they are statistically significant at p<0.05. In comparison to the group under C (I), the LN (II a) is shown by *p<0.05. The LN (II a) is shown in comparison to (II b), (II c), (II d), and (II e) categories with #p<0.05.

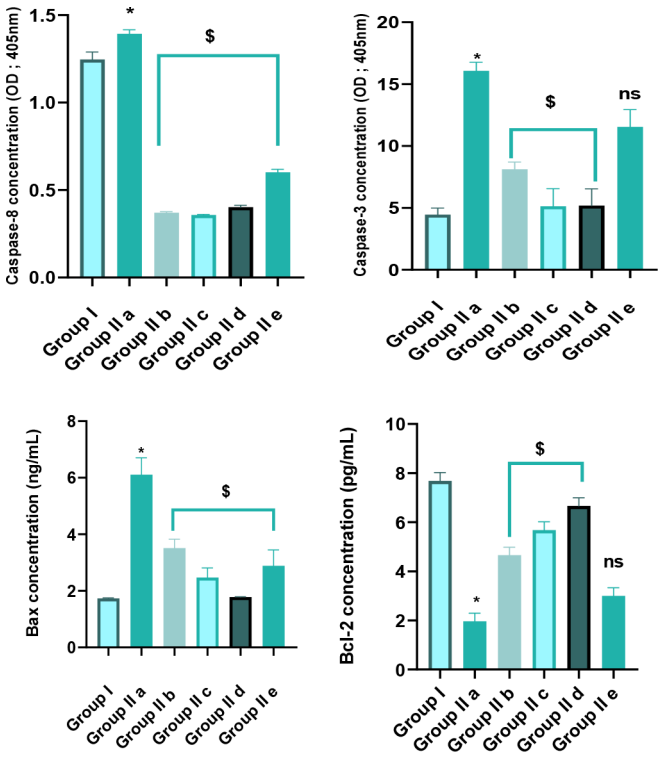


Figure 6. The pharmacological response of GVO on the extent of apoptotic parameters in the mice renal tissue is depicted in the histograms. Data are displayed as Mean ± SEM at $p<0.05$. Here, $^{*}p<0.05$ indicates the comparison between the toxicant-treated group (II a) and the untreated group (I); on the other hand, $^{\$}p<0.05$ depicts the correlation between the toxicant-treated group (II a) and the other groups (II b), (II c), (II d) and (II e) respectively. Additionally, ns stands for non-significant.

caspase-3 and Bax was significantly reduced, and the amount of Bcl-2 was increased when GVO dosages were supplied following lead nitrate. Hence, based on the study results, it is concluded that GVO may improve cellular survival and reduce apoptosis.

3.5 DNA Damage Level

As shown in Figure 7, the lead nitrate exposure group (II a) significantly elevated the 8-OHdG levels relative to the untreated one (I), indicating greater DNA destruction in the renal cells. However, treatment with GVO dosages, as well as with silymarin and olive oil, demonstrated a protective effect by mitigating DNA damage and reducing the elevated 8-OHdG levels caused by lead nitrate. Among these, the higher dose of GVO and silymarin showed the most prominent results, highlighting their effectiveness in minimising DNA damage.

3.6 Impact of GVO Expression of Caspase-3

To explore the mechanism behind the anti-apoptotic effects of garlic volatile oil (GVO), the expression of apoptosis-

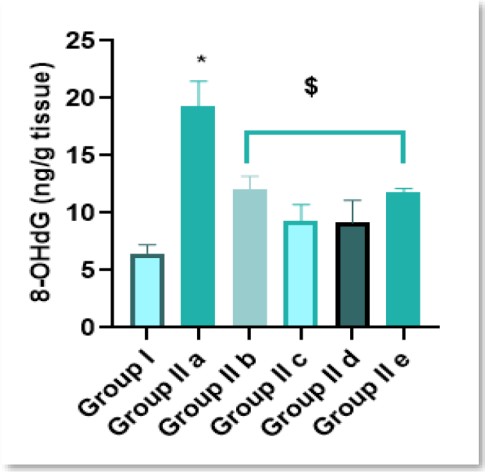


Figure 7. The pharmacological impacts of GVO on the extent of 8-OHdG levels in the mice renal tissue are depicted in the given histograms. Data are displayed as Mean ± SEM (n = 6) at $p<0.05$. Here, $^{*}p<0.05$ indicates the comparison between the toxicant-treated group (II a) and the untreated one (I); on the other hand, $^{\$}p<0.05$ depicts the comparison of groups including the toxicant-treated (II a) with the other groups like (II b), (II c), (II d) and (II e), respectively.

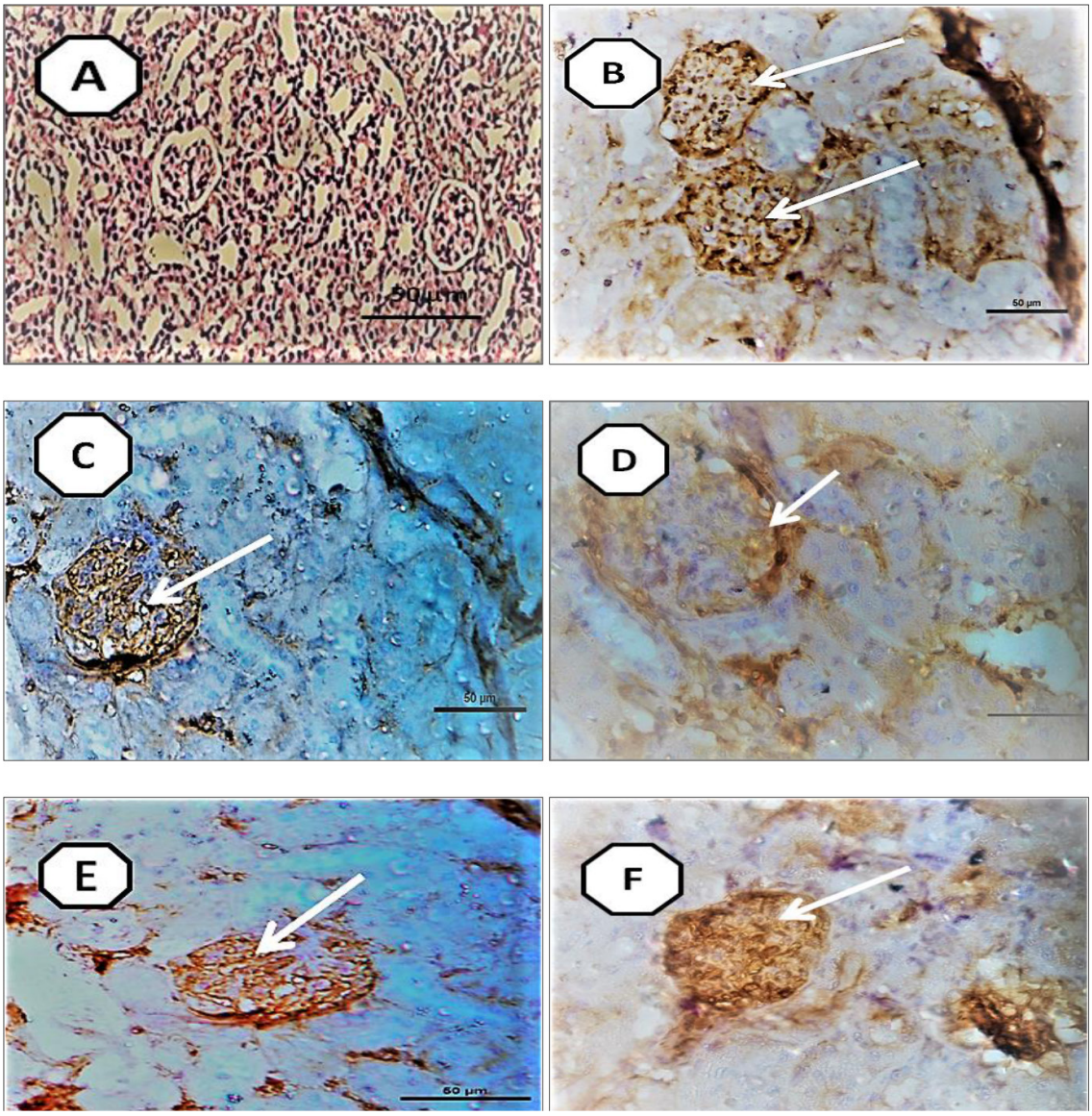


Figure 8. IHC micrographs of immunostained renal sections for caspase-3 expression, indicated by brown staining (white arrows). In the control group **[A],** caspase-3 expression is absent or minimal. Intense caspase-3 expression is evident in the toxicant-treated group **[B],** indicating significant apoptosis. The lead nitrate + low-dose GVO group **[C]** shows reduced caspase-3 expression, while the lead nitrate + high-dosage of GEO group **[D]** shows a further decrease in expression. Both the lead nitrate + silymarin group **[E]** and the lead nitrate + olive oil group **[F]** display weak caspase-3 staining. [Anti-caspase-3 immunostaining, X400] Scale bars: 50 µm.

related genes, particularly caspase-3, a key effector in both extrinsic and intrinsic apoptosis pathways, was examined using the immunohistochemistry technique (IHC). The findings showed that caspase-3 was primarily localised in the cytoplasm of renal cells, with significantly higher expression in the toxicant-treated group (II a) compared to the untreated category of mice. This increase in caspase-3 expression indicates heightened apoptotic activity in the lead-exposed category of mice (Figure 8), suggesting that lead nitrate induces renal apoptosis by upregulating caspase-3 expression. However, co-administration of GVO effectively reduced caspase-3 expression in the kidneys, pointing to its protective and anti-apoptotic role. Caspase-3-positive cells were visualised as brown

precipitates within the cytoplasm, especially in renal tubules and glomeruli, while areas without caspase-3 expression remained unstained, except for the blue nuclear hematoxylin counterstain. These results suggest that GVO dosages modulate caspase-3 expression and thus contribute to its anti-apoptotic effects.

4. Discussion

Heavy metal exposure remains a major global health concern owing to their extensive application in diverse manufacturing and anthropogenic processes. Among these toxic elements, lead (Pb) in particular is recognised as one of the most toxic elements that exerts its harmful effects by disrupting essential cellular and molecular activities[15,37,38]. The renal system is one of the most essential organ systems, primarily responsible for the filtration of blood and the detoxification of harmful substances, and is particularly susceptible to damage caused by heavy metal ions. One of the more specific impacts of the toxic metal lead on renal tissue is the induction of inflammation and cell death[39,40]. The renal tissues of the lead nitrate-intoxicated group exhibited significantly higher levels of Pb than those of the untreated control group, as demonstrated by the atomic absorption spectrometry (AAS) results presented in Figure 3. This is in line with past studies which revealed that the soft tissues serve as the principal reservoirs for lead accumulation[15,41]. The study revealed a significant reduction in renal lead concentration within the group treated with garlic volatile oil. These findings aligned with previous research conducted in our laboratory, where garlic aqueous extract (GAE) was utilised, which is known for its high concentration of flavonoids and phenolic compounds, particularly allicin. Garlic aqueous extract was used in previous studies to show a comparable decrease in lead-nitrate levels in liver tissue, indicating that GVO may have a chelating effect and is able to reduce lead toxicity[42,43].

Moreover, it is noteworthy that the phenol, flavonoid and other organic sulfur compounds found in GVO have been acknowledged for their strong potency to chelate out Pb^{2+} metal ions[16]. More significantly, it has been shown that these organosulfur components of garlic volatile oil can bind to metal ions, reducing their negative impact on biological systems by facilitating the excretion of divalent metal cations such as lead from the body and preventing their absorption in renal tissue[44]. Therefore, organosulfur compounds possess the ability to act as chelating agents, lowering lead traffic in renal tissues[45]. The co-administration of garlic oil decreases the accumulation of lead in tissues and consequently reverses metal-induced biochemical alterations. This indicates the complexation of lead with the free "lone pair" of electrons on the sulfur atom of various organosulfides present in garlic volatile oil and the subsequent excretion of such complexes[46]. Hence, the study highlights the therapeutic potential of garlic volatile oil in reducing the harmful effects of lead exposure due to its numerous health benefits.

In addition to this, these bioactive compounds also have health-supportive benefits like antioxidant, anti-inflammatory, anti-apoptotic, and genoprotective effects. Furthermore, our study finding too highlights the remedial benefits of GVO in neutralising the toxic consequences exerted by lead toxicity, owing to its multifaceted biological activities. This is important because earlier findings have also shown a strong relation between heavy metal toxicity and initiation of processes including oxidative stress, inflammation, and apoptosis in the affected tissues[11,47]. As they are natural and demonstrate minimal physiological adverse effects on biological systems, herbal interventions are the primary choice among various natural remedies for neutralising the noxious effects caused by these heavy metals. In that context, for the current study, garlic-derived volatile oil was exclusively chosen due to its well-documented remedial benefits for curing renal toxicity, which is indirectly linked with the various stress-induced cellular alterations as supported by previous research[15,48,49].

Over time, lead heavy metal builds up in the kidneys and can have several detrimental effects. Oxidative stress, inflammation, DNA damage, cell death and direct damage to the renal tubular cells are the main ways through which lead can cause nephrotoxicity[50]. The kidney's capacity to manage electrolyte balance, remove waste from the blood, and preserve fluid homeostasis may be compromised by this toxic metal exposure[51]. Along with this, urea and creatinine concentrations in serum (waste products of protein and muscle metabolism) are also elevated, which is a clear sign of impaired renal function.

The kidneys are unable to keep these waste products within their usual levels when renal toxicity sets in. Based on muscle mass, serum creatinine is created at a steady rate and completely removed from the circulation via proximal tubular secretion and glomerular filtration. These markers are frequently employed to assess kidney

injury since they are thought to be the most sensitive and specific indicators of renal failure in preclinical studies. Serum creatinine and urea levels are normally low in the blood, but they can increase in cases of kidney injury or chronic infection. Creatine phosphate, a high-energy substance found in muscles, is the precursor of serum creatinine[52]. Creatinine tubular reabsorption can be efficiently evaluated by precise diagnostic techniques and assays. Lead deposition in the kidneys can cause damage to nephrons and the renal parenchyma, which worsens the glomerular filtration rate (GFR) and raises levels of urea and creatinine. Lead-induced nephrotoxicity can seriously reduce the kidney's capacity to filter blood, as measured by GFR[53]. Hence, reduced GFR is a sign of the kidneys' decreased ability to excrete waste and excess fluid, which can result in an accumulation of toxins and a decline in renal function[54]. According to research by Rana *et al.*, (2020), lead interacts with cell junctions to change the morphology of epithelial cells, which induces oxidative stress in the kidney proximal tubules and enhances membrane permeability, which permits urea and creatinine to re-enter the bloodstream[55]. Moreover, elevations in serum creatinine and urea levels can result from exposure to lead-nitrate, which can cause necrosis and gradual damage to the glomerulus and tubules[35,56]. Urine is the main way that urea filtered by the glomerulus is excreted; hence, measuring the levels of creatinine and urea in renal tissue is a superior way to evaluate renal abnormalities. Reduced kidney function is directly associated with nephrotoxicity, as seen by an increased urea level after lead nitrate treatment[57]. Our findings are also in line with these earlier investigations, as lead nitrate-exposed mice showed substantial elevations in serum urea and creatinine, which may indicate renal injury. On the other hand, groups that received GVO dosages experienced a decrease in these markers, suggesting that the volatile oil may have the ability to lower blood waste products and lessen kidney toxicity (Figure 4).

Lead toxicity can affect kidney function by inhibiting AChE activity and altering dopamine levels, leading to oxidative stress, inflammation, and kidney damage[58]. Dopamine (DA) is an essential component of the kidney's physiology, controlling the excretion of water and salt. The kidney possesses a well-developed intra-renal dopaminergic system, and growing evidence suggests that disturbances in this dopamine signalling pathway may be involved in causing hypertension, which can eventually lead to chronic kidney disease in certain human populations[59]. Dopamine also directly inhibits renal sodium reabsorption and suppresses the renin-angiotensin system (RAS) at multiple levels, thereby helping to regulate and maintain normal blood pressure[60]. Moreover, it is possible to conclude from past scientific and experimental evidence that, at a certain dose, dopamine could widen the kidney arteries, which then increases blood flow, urine production, and helps in the removal of potassium and salt[61]. Previous investigations have also proven that activating dopaminergic pathways within the kidneys can mitigate the effects of metal toxicity[62].

For instance, the divalent cation lead (Pb^{2+}) competes with calcium (Ca^{2+}), affecting the activity of enzymes such as nitric oxide synthase (NOS). This competition reduces nitric oxide (NO) production, leading to disruptions in neurotransmitter synthesis. Consequently, the reduced availability of neurotransmitters disrupts the intricate molecular circuits that regulate bodily functions, ultimately contributing to aggressive behaviour[63]. In this current study, lead nitrate poisoning caused a drop in dopamine levels (Figure 5), indicating disturbances in blood pressure and electrolyte balance, which ultimately lead to kidney damage and cell death[64]. Recent research has also shown that both rats and mice experienced a decrease in dopamine when exposed to lead[65]. More specifically, the impacts of dopamine on the kidneys and various aspects of renal function are thoroughly supported by previous research[60,66]. Additionally, this study observed lower levels of AChE in the kidneys of mice subjected to lead nitrate (Figure 5), suggesting renal cell damage. AChE deficiency may be caused by changes in lipids and membrane proteins specifically involving polyunsaturated fatty acids and protein carbonyl content.

Oxidative stress, which can lead to acute symptoms such as nausea, dizziness, difficulty breathing, and even death, is associated with AChE inhibition. In contrast, the animal groups administered GVO dosages depicted elevated levels of these neurotransmitters (Figure 5), suggesting a protective effect of GVO in contrast to lead nitrate toxicity in the mice renal tissues[15,67].

Lead toxicity at the cellular level significantly triggers apoptosis, primarily through caspase-dependent pathways, contributing to the degradation of renal tissues. When the body is exposed to lead, it creates oxidative stress that forms reactive oxygen species (ROS), which harm DNA and disrupt mitochondrial function. This triggers apoptotic cascades, including both intrinsic and extrinsic pathways. The intrinsic pathway is marked

by increased pro-apoptotic proteins like Bax, which disrupt the mitochondrial membrane, ultimately release cytochrome c, and subsequently activate caspase-9 and finally the executioner caspase-3 [68]. At the same time, death receptor signalling activates the extrinsic pathway and inflammatory molecules such as TNF-α, which activate caspase-8 and further enhance caspase-3 activity, leading to more severe kidney tissue damage. Lead also enters renal cells through divalent metal transporters (DMT1), mistaking lead for essential metals like calcium or iron. Once inside the cell, lead disrupts intracellular calcium homeostasis, increasing ROS production and further damaging proteins, lipids, and DNA, resulting in mitochondrial dysfunction and cytochrome c release. This triggers the intrinsic apoptotic pathway, while lead-induced oxidative stress activates pro-inflammatory cytokines that stimulate the extrinsic pathway via death receptors, leading to additional caspase-3 activation and apoptosis[69]. GVO modulates these apoptotic processes by balancing among the apoptosis-regulating proteins. GVO upregulates Bcl-2, which is a cell-death-suppressing protein, while downregulating the Cell-death-inducing proteins like Bax, thus reducing the cytochrome c release from mitochondria. This prevents further triggering of caspase-9 and caspase-3, ultimately reducing apoptosis in the tissue (Figure 6).

Along with the above findings, previous research has made it clear that DNA is the most sensitive macromolecule which is susceptible to oxidative stress within the cell[70]. For this reason, 8-OHdG is a robust marker for measuring the oxidative DNA lesions induced by reactive species[71,72]. It is mainly detectable in urine, blood, and soft tissues. Moreover, elevated concentrations of 8-OHdG are correlated with various diseases like cancer, diabetes, cardiovascular, and neurodegenerative disorders[73]. According to the recent findings of this study, lead nitrate exposure elevated the production of 8-OHdG by inducing oxidative damage in DNA, thus indicating more DNA damage. In contrast, treatment with GVO alleviated the lead nitrate-induced oxidative DNA damage through its antioxidant properties and thereby reducing 8-OHdG levels to near those observed in the control group (Figure 7). Consistently, earlier studies have also shown that GVO significantly lowers 8-OHdG levels in a dose-dependent fashion, confirming its antioxidant and protective effects.

Immunohistochemical (IHC) analysis confirmed this, with photomicrographs showing a marked reduction in caspase-3 expression in GVO-treated groups, especially at higher doses relative to the lead-nitrate-treated group (Figure 8). GVO's remedial properties offer a multifaceted defence against lead-induced nephrotoxicity, making it a most promising therapeutic agent[74]. Earlier investigations by Nasr *et al*., and Han *et al*., support these findings, showing that lead accelerates pro-apoptotic protein migration to mitochondria, inhibiting anti-apoptotic proteins and disrupting cellular balance[75-77].

GVO effectively counteracts this by modulating apoptotic indicators, enhancing Bcl-2 expression, and reducing caspase-3, caspase-8, and Bax levels[77]. These sulfur compounds in GVO exhibit strong anti-apoptotic properties, protecting renal tissue from lead-induced damage and preserving kidney functions. Additionally, similar protective effects have been observed with silymarin and olive oil, highlighting the therapeutic potential of natural compounds in mitigating heavy metal toxicity.

5. Conclusion

The findings of the present study conclusively demonstrate that chronic lead nitrate exposure induces marked nephrotoxicity through the disruption of renal function, impairment of neurotransmitter homeostasis, induction of oxidative stress, activation of inflammatory and apoptotic pathways, and extensive structural damage to renal tissue. Lead-induced oxidative stress was identified as a central mechanism driving renal injury by promoting lipid peroxidation, suppressing endogenous antioxidant defences, triggering inflammatory mediators, and upregulating caspase-3-mediated apoptosis, ultimately resulting in significant renal dysfunction.

Treatment with garlic volatile oil effectively mitigated these pathological alterations by restoring renal biochemical markers, improving antioxidant status, normalising neurotransmitter levels, reducing inflammatory responses, attenuating caspase-3 expression, and preserving renal architecture. Furthermore, the reduction in renal lead accumulation following GVO administration suggests that its nephroprotective efficacy may also involve metal-chelating activity in addition to its potent antioxidant, anti-inflammatory, and anti-apoptotic properties.

Overall, this study provides compelling experimental evidence that GVO exerts significant nephroprotective effects against lead nitrate-induced renal toxicity. These

findings support the potential of GVO as a promising natural therapeutic candidate for preventing or alleviating heavy metal-induced nephrotoxicity. Future investigations focusing on the precise molecular signalling pathways, pharmacokinetic characteristics, and long-term efficacy of GVO are warranted to facilitate its translation into clinical applications.

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Author Contributions

The entire experimental work was conceptualised by S.S. and V.S. The manuscript's principal author, S.S., contributed the majority of writing activities, but support was also provided by V.S. A Literature search was done by S.S. Figures were developed by S.S. The study design was framed by V.S. The experimentation and data analysis were carried out by S.S. The statistics and data interpretation were done by S.S. The final manuscript was prepared by S.S. and V.S. The authors have read and approved the final manuscript.

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