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Protocatechuic acid mitigates diazinon-induced lung injury in rats through modulation of oxidative stress, inflammatory, Keap-1/Nrf-2/HO-1 and ER stress-mediated apoptotic pathways

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ABSTRACT

Objective(s): Diazinon (DZN), a widely used organophosphate pesticide, induces pulmonary toxicity through oxidative stress, inflammation, endoplasmic reticulum (ER) stress, and apoptosis. This study investigated the potential protective effects of protocatechuic acid (PCA) against DZN-induced lung injury in rats.

Materials and Methods: Thirty-five adult rats were randomly assigned to five groups (n = 7): Control, DZN (20 mg/kg), PCA100 (100 mg/kg), DZN + PCA50, and DZN + PCA100. Lung tissues were evaluated histopathologically, and oxidative stress markers (GSH, SOD, CAT, and GPx) and inflammatory mediators (TNF-α, IL-1β, IL-6, NF-κB, COX-2, and iNOS) were measured by ELISA. The protein levels of Keap-1, Nrf2, and HO-1 were assessed via Western blotting. Expression of ER stress–related genes (XBP-1, eIF2, ATF4, CHOP) and apoptotic markers (Bax, Bcl-2, caspase-3, -6, -9) was analyzed by qRT-PCR.

Results: DZN exposure caused severe histopathological damage and significantly increased oxidative, inflammatory, ER stress, and apoptotic responses. PCA administration, particularly at 100 mg/kg, markedly improved lung morphology, normalized antioxidant enzyme levels, reduced cytokine production and NF-κB activation, and downregulated ER stress and apoptosis-related genes. PCA also enhanced Bcl-2 expression and activated the Nrf2/HO-1 signaling pathway.

Conclusion: PCA exerts dose-dependent protective effects against DZN-induced pulmonary toxicity by modulating oxidative stress, inflammation, ER stress, and apoptosis. These findings suggest that PCA may serve as a promising therapeutic candidate for mitigating pesticide-related lung injury.

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Introduction

Diatomaceous earth is a naturally occurring sedimentary material composed of the fossilized remains of diatoms, a type of hard-shelled algae (1). It is widely used in agriculture and pest control because of its effectiveness and environmental safety. However, the concurrent use of synthetic chemicals such as diazinon (DZN), an organophosphate insecticide, has raised significant toxicological concerns for both human health and the environment. Among its various adverse effects, pulmonary toxicity has emerged as a critical issue due to the essential role of the lungs in respiration and systemic homeostasis (2). This study, therefore, aims to elucidate the mechanisms underlying DZN-induced pulmonary toxicity and to explore the potential protective role of protocatechuic acid (PCA), a phenolic anti-oxidant compound.

DZN is commonly applied in agricultural settings to control crop and livestock pests (3). Despite its broad efficacy, DZN is classified as a toxic organophosphate compound, and its use is restricted in many countries. The toxicological profile of DZN includes neurotoxicity, hepatotoxicity, and pulmonary toxicity, with the latter being of particular concern due to the susceptibility of lung tissue to oxidative and inflammatory damage. The lungs not only mediate gas exchange but also contribute to immune regulation and detoxification processes. Consequently, understanding how DZN disrupts pulmonary function is crucial for developing strategies to mitigate its harmful impact.

Evidence indicates that DZN exposure triggers oxidative stress, inflammation, and apoptosis within lung tissue (4, 5). Oxidative stress results from an imbalance between reactive oxygen species (ROS) production and anti-oxidant defense

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mechanisms, leading to lipid peroxidation, protein oxidation, and DNA damage. Persistent ROS accumulation promotes the activation of inflammatory pathways, including NF-κB signaling, which further amplifies cytokine release and tissue injury. This cascade contributes to the development of chronic pulmonary inflammation and fibrosis.

Given these detrimental effects, identifying compounds capable of counteracting DZN-induced oxidative and inflammatory responses has become a priority in toxicological research. PCA, a naturally occurring phenolic compound found in many fruits and medicinal plants, possesses anti-oxidant, anti-inflammatory, and anti-apoptotic properties (6,7). By scavenging free radicals and activating protective cellular pathways such as the Nrf2/HO-1 axis, PCA may offer significant protection against DZN-induced oxidative and inflammatory lung injury.

This investigation provides experimental evidence for the protective potential of PCA against DZN-induced pulmonary toxicity in rats by evaluating oxidative stress markers, inflammatory mediators, ER stress, and apoptosis-related pathways. The findings are expected to contribute to a deeper understanding of the mechanistic basis of organophosphate-induced lung injury and to support the development of anti-oxidant-based therapeutic strategies.

Materials and Methods

Chemicals

PCAs with purity ≥97% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary and secondary antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). The following primary antibodies were employed: β-tubulin (sc-47778), Nrf-2 (sc-365949), and HO-1 (sc-390991). For detection, a goat anti-mouse IgG secondary antibody (sc-2005) was used. All antibodies were prepared and applied according to the manufacturer's instructions.

Ethical approval

Ethical approval was obtained from the Local Animal Experiments Ethics Committee of Ataturk University (31.01.2025-Decision No:15). All experiments were performed in accordance with the European Directive 2010/63/EU for animal experiments. In addition, all animal-related procedures used in this study were performed in accordance with ARRIVE guidelines.

Groups and experimental procedures

Thirty-five 8-week-old male Sprague Dawley rats (weighing 220–250 g) were procured from the Experimental Medicine Research and Application Center (ATADEM), Ataturk University, Erzurum, Turkey. The animals were kept under standard laboratory conditions and randomly allocated into five groups, each consisting of seven rats.

1- Control Group: Rats given saline solution orally for 28 days.

2- Protocatechuic Acid (PCA) Group: Rats given 100 mg/kg/body weight protocatechuic acid orally for 28 days.

3- Diazinon (DZN) Group: Rats given 20 mg/kg/body weight diazinon orally for 28 days.

4- Diazinon + Protocatechuic Acid-50 (DZN+PCA 50) Group: Rats given 50 mg/kg/body weight protocatechuic acid orally for 28 days, and 20 mg/kg/body weight diazinon administered 30 min later.

5-Diazinon + Protocatechuic Acid-100 (DZN+PCA 100) Group: Rats given 100 mg/kg/body weight protocatechuic acid orally for 28 days, and 20 mg/kg/body weight DZN administered 30 min later.

The doses and administration methods of PCA and DZN were determined based on relevant literature (1, 2). Twenty-four hours after the final drug administration on day 28, the rats were euthanized under light sevoflurane anesthesia (Sevorane®; Queenborough, UK). Lung tissues were then collected; a portion was stored at −80 °C for biochemical analyses, while the remaining samples were fixed in 10% formaldehyde solution for histological evaluation.

ELISA analysis

Lung and testis tissues obtained from rats were snap-frozen in liquid nitrogen and pulverized into powder using a homogenizer (Tissue Lyser II, Qiagen, Netherlands). The powdered tissues were then diluted at a ratio of 1:20 with phosphate-buffered saline (PBS) and homogenized with stainless steel beads using the same homogenizer. The homogenates were centrifuged at 3000 RPM for 20 min, and the supernatants were collected for biochemical analyses. All analyses were performed using commercial ELISA kits (Brand: YL Biont). The following catalog numbers were used: Catalase (Cat: YLA0123RA), GPx-1 (YLA0120RA), GSH (YLA0121RA), MDA (YLA0029RA), SOD-1 (YLA1514RA), IL-6 (YLA0031RA), IL-1β (YLA0030RA), TNF-α (YLA0118RA), NF-κB p65 (YLA0513RA), iNOS (YLA0266RA), COX-2 (YLA0104RA), caspase-3 (YLA0017RA), Bcl-2 (YLA0086RA), and Bax (YLA0122RA). All procedures were carried out in accordance with the manufacturer's instructions.

Real-time PCR analysis

Total RNA was isolated from lung tissue using QIAzol lysis reagent (79306; Qiagen). Total RNA concentrations were determined using a NanoDrop (BioTek Epoch) device. In the next step, cDNAs were synthesized from total RNA using an RT2 First Strand Kit (330404; Qiagen). All procedures were performed according to the manufacturer's instructions. In the last step, the cDNAs obtained were reacted with RT2 SYBR® Green qPCR Mastermix (330500; Qiagen) and the primers whose sequences are given in Table 1 according to the manufacturer's instructions. After the reaction was completed, the genes were normalized to β-Actin using the $2^{-\Delta\Delta CT}$ method (3).

Western blot analysis

Lung tissues were homogenized in Radio-Immunoprecipitation lysis buffer and centrifuged at 16,000 × g for 20 min. Total protein concentrations were determined from the supernatant using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For Western blot analysis, 50 µg of protein from each sample was loaded into individual wells and subjected to SDS-PAGE. Following electrophoretic separation, proteins were transferred onto PVDF membranes. The membranes were blocked for 1.5 hours using 5% bovine serum albumin (BSA) prepared in PBS containing 0.1% Tween 20 (PBS-T), then washed and incubated overnight at 4 °C with primary antibodies. After another PBS-T wash, the membranes were incubated for 1.5 hours with an HRP-conjugated goat anti-mouse secondary antibody. Visualization was carried out using BioRad Clarity Max ECL substrate (Bio-Rad, Hercules, USA), and imaging was performed using

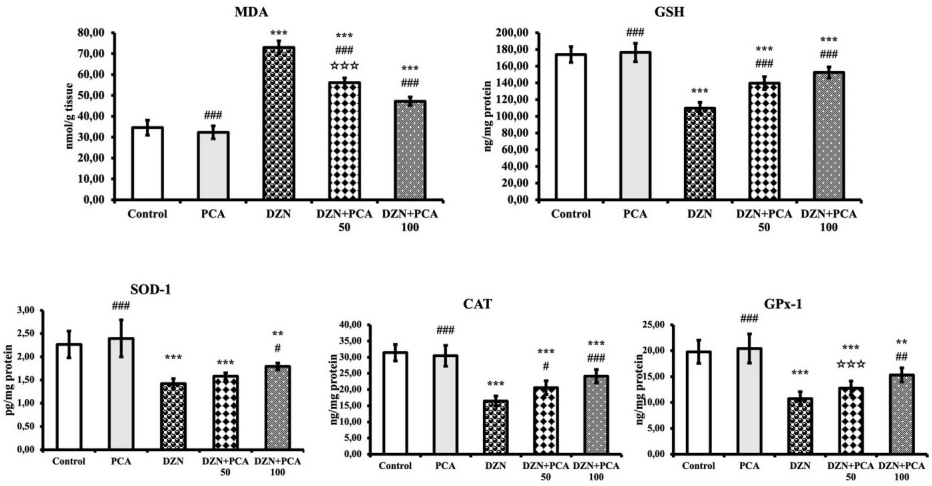

Figure 1. Effect of protocatechuic acid (PCA) on oxidative stress biomarkers in rat diazinon (DZN)-induced lung toxicity
Data are presented as mean ± SD (n = 7 rats per group). Levels of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined in lung homogenates to evaluate oxidative damage and antioxidant defense. DZN administration significantly increased MDA levels while decreasing GSH, SOD, CAT, and GPx activities compared to the control group, indicating marked oxidative stress. Treatment with PCA, particularly at 100 mg/kg, reversed these alterations, reducing MDA accumulation and restoring antioxidant enzyme activities toward control values. Statistical significance was analyzed using one-way ANOVA followed by Tukey's *post hoc* test (Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆*P*<0.01, ☆☆☆*P*<0.001).
DZN: diazinon; PCA: protocatechuic acid; MDA: malondialdehyde; GSH: glutathione; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase

the BioRad Gel Doc XR+ Imaging System. Densitometric analysis was conducted using ImageLab software (Bio-Rad, Hercules, USA), based on a protocol similar to that reported by Dogan et al. (4, 5).

Hematoxylin & eosin

Following sacrifice, lung tissue samples were rapidly removed and fixed in 10% neutral buffered formalin solution for 48 hours. After fixation, tissues underwent standard histological follow-up procedures. They were dehydrated for 1 hour each in a series of increasing concentrations of ethyl alcohol (70%, 80%, 90%, 95% and absolute alcohol) and then cleared using xylene (2 times, 1 hour each) to remove alcohol and facilitate paraffin infiltration. Tissues were kept in molten paraffin (56-58°C) 3 times (1 hour each) and then embedded in paraffin blocks. 5 µm thick sections were taken from the prepared paraffin blocks using a microtome (Leica RM2255, Germany). Sections were placed on adhesive (polylysine) slides and left to dry overnight at room temperature.

Staining Procedures: A standard Hematoxylin-Eosin (H&E) staining protocol was applied to examine general tissue morphology and cellular details. Briefly, deparaffinized and rehydrated sections were stained with hematoxylin for 10 min and eosin for 1 minute. At the end of the staining procedure, sections were dehydrated, cleared with xylene, and covered with a synthetic covering agent (Entellan).

All histopathological evaluations were performed by a researcher blinded to the groups using a light microscope (Olympus BX43, Japan). From each subject's lung section, 10 randomly selected different fields were examined at 40X objective magnification. Semi-quantitative scoring criteria were used to assess histopathological changes in rat lung tissue. The degree of lung damage was evaluated according to a semi-quantitative scoring system previously described (10.1165/rcmb.2009-0210ST) and modified for our study, and the evaluation parameters (leukocyte infiltration, alveolar wall thickening, alveolar damage, alveolar hemorrhage, airway degeneration, and vascular degeneration criteria). The scores given for each parameter were graded as 0=none, very little; 1=mild; 2=moderate, and 3=severe, and were summed to obtain a total histopathological damage score. According to the total score obtained, the lesions were divided into three grades: Grade 1 (mild damage: 1-6 points), Grade 2 (moderate damage: 7-12 points), and Grade 3 (severe damage: 13-18 points).

Statistical analysis

The data obtained at the end of the study were analyzed using IBM SPSS software. Data were presented as mean ± standard deviation. Tukey *post hoc* tests and one-way analysis of variance (ANOVA) were applied for multiple comparisons. Statistical significance was determined at $P<0.05$, $P<0.01$, and $P<0.001$ levels.

Results

PCA alleviates the oxidative stress triggered by diazinon in lung tissue

In rats exposed to DZN, marked suppression of testicular anti-oxidant enzyme activities, including SOD, CAT, and GPx, was observed compared with the control group. Furthermore, a notable reduction in GSH levels was detected, indicating depleted anti-oxidant reserves. This impairment in anti-oxidant defense was accompanied by a significant elevation in MDA levels, reflecting increased lipid peroxidation. Conversely, PCA administration mitigated the DZN-induced decline in anti-oxidant enzyme activities in a dose-dependent manner and effectively restored GSH concentrations. These findings are summarized in Figure 1.

PCA interrupts the inflammatory pathway triggered by diazinon in lung tissue

As shown in Figures 2 and 3, ELISA analysis revealed that levels of key inflammatory markers NF-κB, COX-2, iNOS, TNF-α, IL-6, and IL-1β were significantly elevated in testicular tissues of DZN-treated rats compared with the control group. Co-treatment with PCA, especially at a dose of 100 mg/kg, led to a marked reduction in TNF-α and

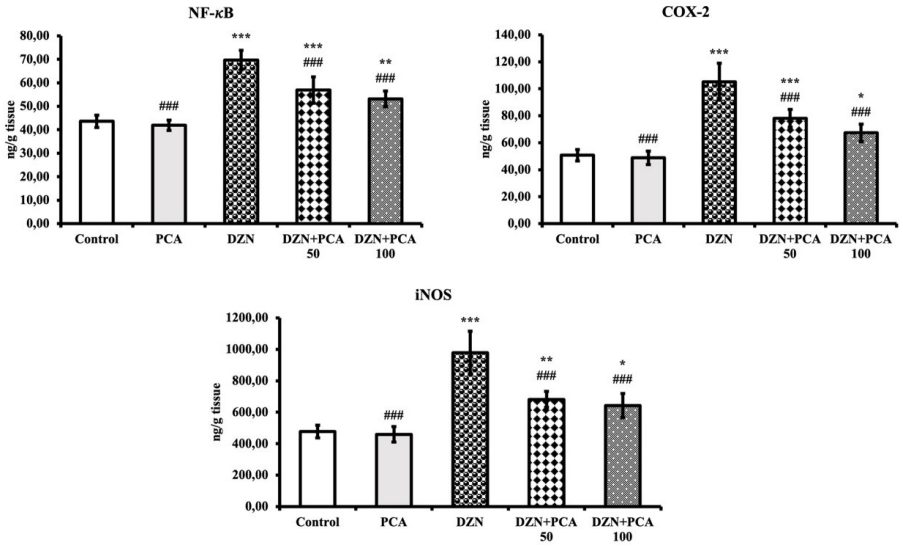

Figure 2. Effects of diazinon (DZN) and protocatechuic acid (PCA) on NF-κB, COX-2, and iNOS levels in rat lung tissue
The concentrations of nuclear factor kappa B (NF-κB), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) were quantified using ELISA. Data are expressed as mean ± SD (n = 7 rats per group). DZN exposure markedly increased NF-κB, COX-2, and iNOS levels compared with the control group, indicating activation of inflammatory pathways. Treatment with PCA, particularly at 100 mg/kg, significantly reduced these inflammatory mediators, demonstrating its anti-inflammatory potential. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test (Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆*P*<0.01, ☆☆☆*P*<0.001). DZN: diazinon; PCA: protocatechuic acid; NF-κB: nuclear factor kappa B; COX-2: cyclooxygenase-2; iNOS: inducible nitric oxide synthase

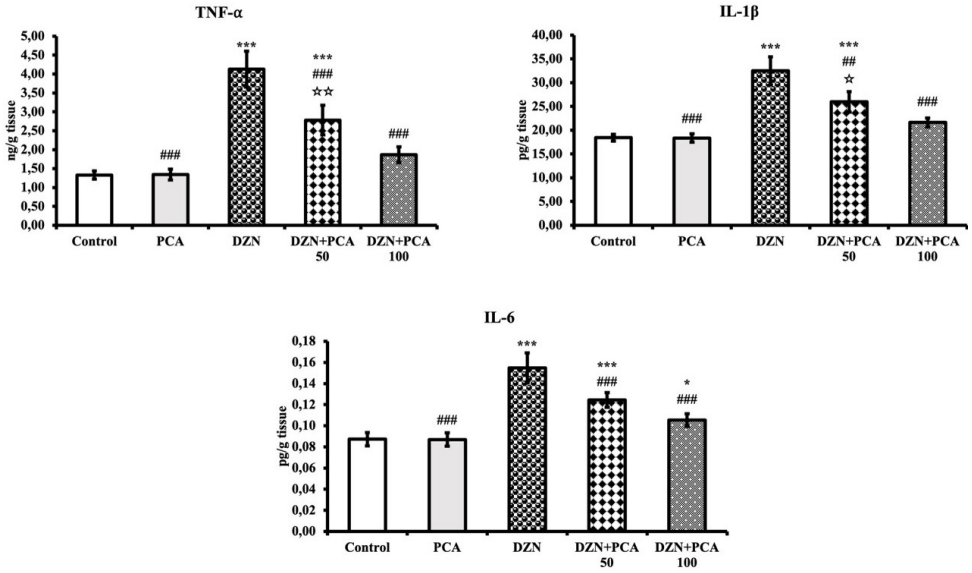

Figure 3. Effects of diazinon (DZN) and protocatechuic acid (PCA) on proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in lung tissue of rats
The concentrations of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) were determined in lung homogenates using ELISA. Data are presented as mean ± SD (n = 7 rats per group). DZN exposure significantly increased TNF-α, IL-1β, and IL-6 levels compared to the control group, indicating a strong inflammatory response. PCA administration, particularly at 100 mg/kg, markedly reduced cytokine production, demonstrating its anti-inflammatory and lung-protective effects. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. (Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆*P*<0.01, ☆☆☆*P*<0.001). DZN: diazinon; PCA: protocatechuic acid; TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1 beta; IL-6: interleukin-6

COX-2 protein levels in a dose-dependent manner (*P*< .01). Both doses of PCA significantly suppressed NF-κB and IL-1β levels; however, no significant difference was noted between the two doses. Interestingly, iNOS levels decreased following 50 mg/kg PCA treatment, while the 100 mg/kg dose did not result in a significant difference compared to the DZN-only group. These findings indicate that PCA modulates DZN-induced inflammatory responses at the protein level in a dose-specific manner.

PCA reduces the apoptotic effect of diazinon in lung tissue

As summarized in Figure 4, ELISA results showed that DZN exposure significantly increased the protein levels of pro-apoptotic markers Bax and caspase-3, while reducing the level of the anti-apoptotic protein Bcl-2 in testicular tissue compared to the control group. PCA treatment reversed these changes by elevating Bcl-2 levels and suppressing Bax (*P*< .01) and caspase-3 (*P*< .001) in a dose-dependent manner, with the 100 mg/kg dose showing

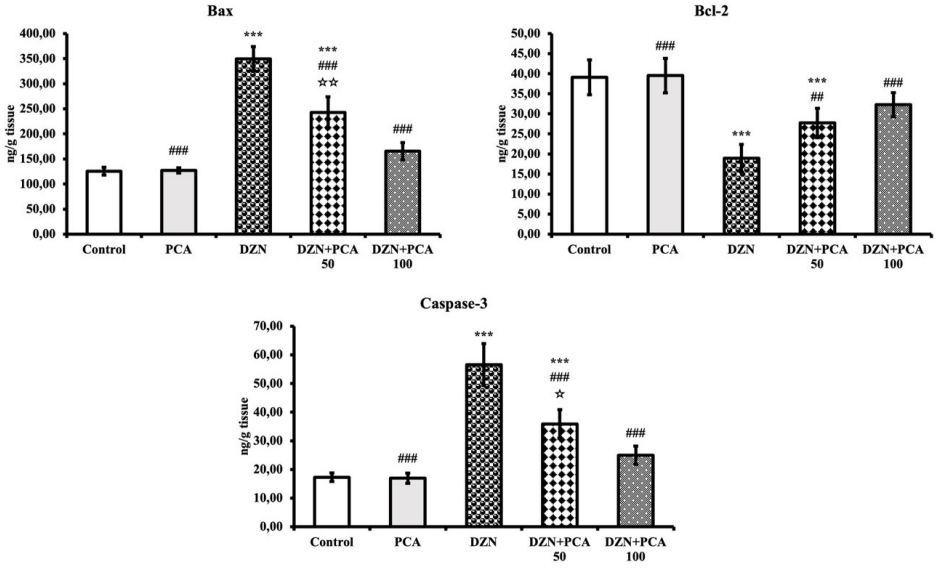

Figure 4. Effects of diazinon (DZN) and protocatechuic acid (PCA) on apoptosis-related proteins (Bax, Bcl-2, and Caspase-3) in rat lung tissue
Protein levels of Bax, Bcl-2, and Caspase-3 were quantified using ELISA. Data are expressed as mean ± SD (n = 7 rats per group). Exposure to DZN significantly increased pro-apoptotic proteins Bax and Caspase-3, while decreasing the anti-apoptotic protein Bcl-2, compared with the control group, indicating activation of apoptosis in lung tissue. PCA treatment, particularly at 100 mg/kg, effectively reversed these effects, downregulating Bax and Caspase-3 while up-regulating Bcl-2, demonstrating its anti-apoptotic and cytoprotective properties. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆*P*<0.01, ☆☆☆*P*<0.001). DZN: diazinon; PCA: protocatechuic acid; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; Caspase-3: cysteine-aspartic acid protease-3

a more pronounced effect. Additionally, qRT-PCR analysis revealed that DZN significantly up-regulated the mRNA expression of caspase-3, caspase-6, and caspase-9. PCA administration mitigated this apoptotic gene activation, and the 100 mg/kg dose led to greater suppression of caspase-3 and caspase-9 expressions compared to the 50 mg/kg group. These findings indicate that PCA confers dose-related anti-apoptotic effects against DZN-induced testicular damage at both the protein and gene expression levels. As summarized in Figure 5.

PCA reduces the ER stress effect of diazinon in lung tissue

As shown in Figure 6, qRT-PCR results revealed that DZN administration significantly elevated the mRNA expression levels of endoplasmic reticulum (ER) stress-related genes XBP-1, eIF2, ATF4, and CHOP in lung tissue compared to the control group, indicating a pronounced ER stress response. Co-treatment with PCA attenuated this effect in a dose-dependent manner. Notably, the 100 mg/kg PCA dose led to a more substantial downregulation of ATF4 and CHOP transcripts ($P < .01$), while reductions in

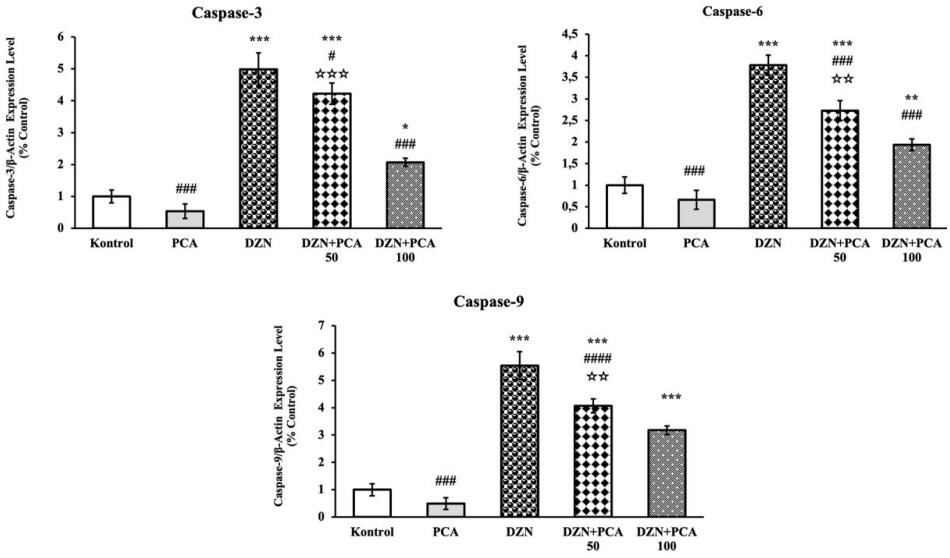

Figure 5. Effects of diazinon (DZN) and protocatechuic acid (PCA) on Caspase-3, Caspase-6, and Caspase-9 mRNA transcription levels in rat lung tissue
mRNA expression levels of Caspase-3, Caspase-6, and Caspase-9 were quantified using quantitative real-time PCR (qRT-PCR). Data are presented as mean ± SD (n = 7 rats per group). DZN exposure significantly up-regulated Caspase-3, Caspase-6, and Caspase-9 gene expression, indicating activation of intrinsic apoptotic pathways in lung tissue. PCA co-administration, especially at 100 mg/kg, markedly suppressed the overexpression of these caspases, suggesting that PCA exerts anti-apoptotic protection by downregulating apoptotic signaling at the transcriptional level. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test (Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆*P*<0.01, ☆☆☆*P*<0.001). DZN: diazinon; PCA: protocatechuic acid; qRT-PCR: quantitative real-time polymerase chain reaction; Caspase-3/6/9: cysteine-aspartic acid proteases involved in apoptosis

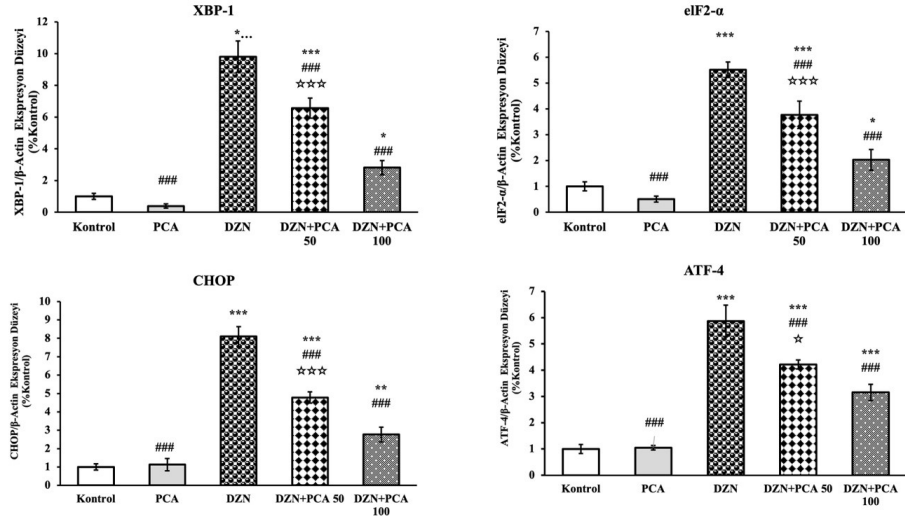

Figure 6. Effects of diazinon (DZN) and protocatechuic acid (PCA) on ER stress-related gene expression (XBP-1, eIF2-α, CHOP, and ATF-6) in rat lung tissue

mRNA transcription levels of X-box binding protein-1 (XBP-1), eukaryotic initiation factor 2-alpha (eIF2-α), CCAAT/enhancer-binding protein homologous protein (CHOP), and activating transcription factor-6 (ATF-6) were determined using quantitative real-time PCR (qRT-PCR). Data are presented as mean ± SD (n = 7 rats per group). DZN exposure significantly up-regulated XBP-1, eIF2-α, CHOP, and ATF-6 gene expression, indicating activation of endoplasmic reticulum (ER) stress-mediated signaling pathways. PCA co-treatment, particularly at 100 mg/kg, markedly downregulated these stress markers, demonstrating its potential to attenuate ER stress and associated apoptotic responses in lung tissue. Statistical analysis was conducted using one-way ANOVA followed by Tukey's *post hoc* test (Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆*P*<0.01, ☆☆☆*P*<0.001). DZN: diazinon; PCA: protocatechuic acid; ER: endoplasmic reticulum; XBP-1: X-box binding protein-1; eIF2-α: eukaryotic initiation factor 2-alpha; CHOP: C/EBP homologous protein; ATF-6: activating transcription factor-6; qRT-PCR: quantitative real-time polymerase chain reaction

XBP-1 and eIF2-α expressions were also significant ($P < .05$). These findings suggest that PCA effectively alleviates DZN-induced ER stress in lung tissue by modulating key components of the unfolded protein response.

PCA reduces the Keap-1, Nrf-2 and HO-1 effect of diazinon in lung tissue

As illustrated in Figure 7, Western blot analysis showed that DZN exposure led to a significant downregulation of NRF2 and HO-1 protein levels in lung tissue, accompanied by an up-regulation of KEAP-1, compared to the control group. These changes indicate a disruption of the cellular anti-oxidant defense mechanism mediated by the KEAP-1/NRF2/HO-1 pathway. Treatment with PCA effectively reversed these alterations in a dose-dependent manner. In particular, the 100 mg/kg PCA group exhibited a marked increase in NRF2 and HO-1 protein expressions ($P < .01$), along with a significant reduction in KEAP-1 levels ($P < .05$), relative to the DZN group. These findings suggest that PCA restores redox homeostasis in the lungs by activating

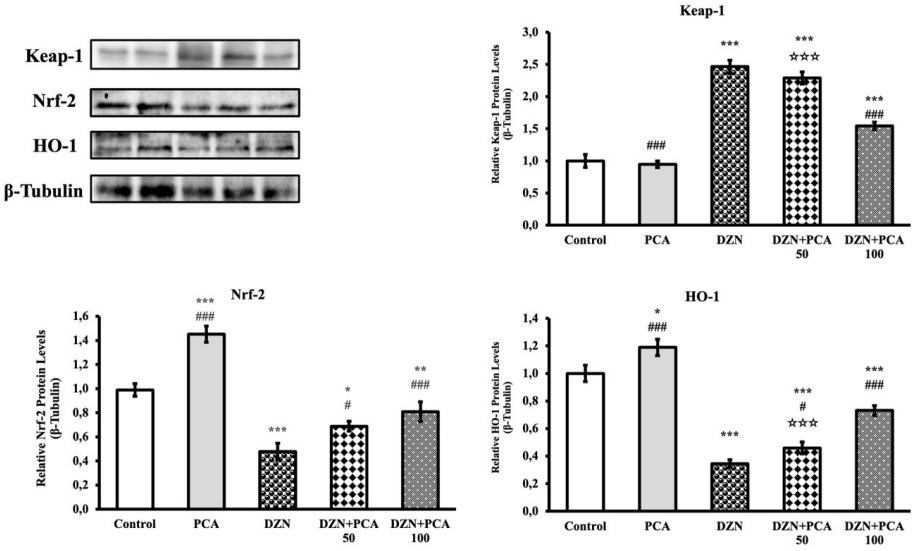

Figure 7. Effects of diazinon (DZN) and protocatechuic acid (PCA) on Keap-1, Nrf-2, and HO-1 protein levels in rat lung tissue

Protein expressions of Kelch-like ECH-associated protein 1 (Keap-1), nuclear factor erythroid 2-related factor 2 (Nrf-2), and heme oxygenase-1 (HO-1) were determined using Western blot analysis. Data are presented as mean ± SD (n = 7 rats per group). DZN exposure significantly increased Keap-1 and decreased Nrf-2 and HO-1 protein levels, indicating suppression of the antioxidant defense system. PCA co-administration, particularly at 100 mg/kg, restored Nrf-2 and HO-1 expression while reducing Keap-1, demonstrating activation of the Nrf-2/HO-1 signaling pathway and enhancement of cellular antioxidant capacity. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test ((Control vs others: **P*<0.05, ***P*<0.01, ****P*<0.001; DZN vs others: #*P*<0.05, ##*P*<0.01, ###*P*<0.001; DZN + PCA 50 vs DZN + PCA 100: ☆*P*<0.05, ☆☆P<0.01, ☆☆☆ *P*<0.001). DZN: diazinon; PCA: protocatechuic acid; Keap-1: Kelch-like ECH-associated protein 1; Nrf-2: nuclear factor erythroid 2–related factor 2; HO-1: heme oxygenase-1

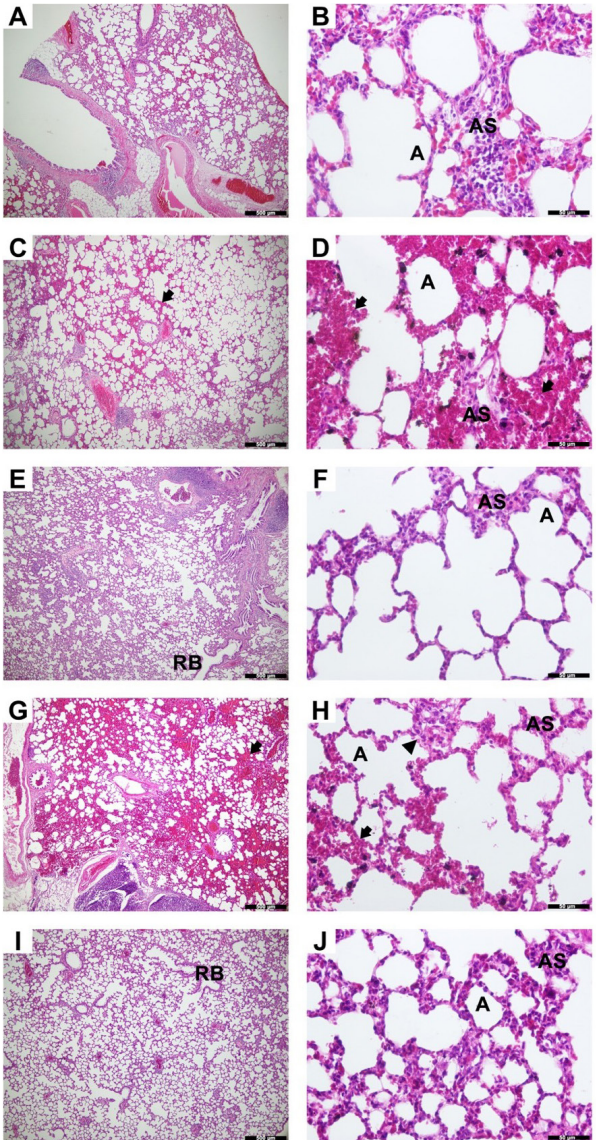

Figure 8. Representative histological micrographs of rat lung sections from the experimental groups stained with hematoxylin and eosin (H&E)
Panels (A, C, E, G, I) display low-magnification (4×) overviews of the lung tissue architecture, while panels (B, D, F, H, J) show high-magnification (40×) cellular details. (A-B) The control group exhibits normal lung histology. (C-D) In the group administered only diazinon (DZN), severe alveolar damage, prominent thickening of alveolar walls (arrowheads), and widespread hemorrhage (arrow) are observed. (E-F) The protocatechuic acid (PCA)-only group shows a preserved morphology similar to the control group. (G-H) Moderate alveolar damage is evident in the group treated with DZN plus 50 mg/kg PCA (DZN+PCA50). (I-J) In the group treated with DZN plus 100 mg/kg PCA (DZN+PCA100), the damage is significantly ameliorated, with only mild histopathological alterations remaining. A: Alveolus; AS: Alveolar Septum; RB: Respiratory bronchiole. Scale bars: 500 µm (A, C, E, G, I); 50 µm (B, D, F, H, J).

the NRF2 pathway and enhancing anti-oxidant defense mechanisms suppressed by DZN toxicity.

Histopathology results

The structural alterations induced by DZN exposure in lung tissue and the potential protective effects of PCA were evaluated through light microscopic examination of sections stained with Hematoxylin and Eosin (H&E) (Figure 8A–8J). Semi-quantitative scoring results are summarized in Table 2.

Comparative histopathological analyses revealed that lung sections from the Normal (N) and PCA100 groups (receiving 100 mg/kg PCA alone) exhibited essentially normal histological architecture, with mean total scores of 6 and 2, respectively, both corresponding to Damage Grade 1. In these groups, the alveolar septa were thin and intact, and the alveolar spaces were open and transparent. Key pathological findings such as leukocyte infiltration, alveolar wall thickening, alveolar damage, hemorrhage, airway and vascular degeneration were either absent (e.g., leukocyte infiltration and hemorrhage scores: 0 in PCA100) or observed only at mild levels (all parameters scored 1 in the N group; remaining parameters in PCA100 scored between 0–1) (Figure 8B–8F). These findings suggest that PCA at 100 mg/kg does not cause notable adverse effects in healthy lung tissue. In contrast, DZN administration in the DZN group resulted in severe and widespread histopathological alterations in lung tissue, with a mean total score of 11, corresponding to Damage Grade 2. The primary findings included moderate leukocyte infiltration, alveolar wall thickening, alveolar damage, and airway and vascular degeneration (generally scored 2), along with mild alveolar hemorrhage (score 1) (Figure 8D). These changes indicate that DZN induces a pronounced inflammatory response and structural deterioration in the lungs. In the DZN+PCA50 group, which received 50 mg/kg PCA alongside DZN, some improvements were observed in comparison to the Sham group; however, no significant reduction in the total damage score (mean: 11; Damage Grade 2) was noted due to a marked increase in alveolar hemorrhage. While leukocyte infiltration and alveolar wall thickening remained moderate (score 2), alveolar damage and airway degeneration were alleviated (score 1). Vascular degeneration persisted at a moderate level (score 2), and notably, severe alveolar hemorrhage was recorded (score 3) (Figure 8G–8H). This suggests that 50 mg/kg PCA was insufficient to prevent DZN-induced alveolar hemorrhage.

The most prominent protective effect was observed in the DZN+PCA100 group, which received 100 mg/kg PCA with DZN. In this group, all histopathological parameters showed marked improvement compared to the Sham group.

Table 2. Comparison of mean histopathological scores in lung tissue according to groups

Parameters	Control (n=6)	DZN (n=6)	PCA100 (n=6)	DZN+PCA50 (n=6)	DZN+PCA100 (n=6)
Leukocyte infiltration	1	2	1	2	0
Alveolar wall thickening	1	2	0	2	1
Alveolar damage	1	2	0	1	1
Alveolar hemorrhage	1	1	0	3	0
Airway degeneration	1	2	1	1	1
Vascular degeneration	1	2	0	2	1
Total score	6	11	2	11	4
Damage level	Level 1	Level 2	Level 1	Level 2	Level 1

The mean total histopathological damage score decreased to 4 (Damage Grade 1), approaching values observed in the Normal group (Figure 8I–8J). Specifically, leukocyte infiltration and alveolar hemorrhage were absent (score 0), while alveolar wall thickening, alveolar damage, airway, and vascular degeneration were mild (score 1). These findings demonstrate that PCA mitigates DZN-induced lung injury in a dose-dependent manner, with the 100 mg/kg dose exerting a more robust and comprehensive protective effect than the 50 mg/kg dose. Notably, 100 mg/kg PCA substantially prevented DZN-induced inflammation and structural damage, thereby restoring lung histology to near-normal levels.

Discussion

The study of organophosphate compounds, particularly DZN, has garnered significant attention due to their widespread use in agriculture and the consequent implications for human health and environmental safety (6). DZN is known for its potent insecticidal properties; however, its neurotoxic effects and potential toxicity to various organ systems, including the lungs, raise critical concerns that necessitate further investigation (7, 8). This discussion delves into the implications of DZN's pulmonary toxicity and the protective role of protocatechuic acid, integrating current literature and highlighting the importance of understanding these interactions for public health and regulatory policies.

DZN exerts its toxic effects primarily by inhibiting acetylcholinesterase, an enzyme critical for the breakdown of acetylcholine in the nervous system. This inhibition leads to an accumulation of acetylcholine, resulting in overstimulation of cholinergic receptors, which can manifest in various symptoms, including respiratory distress (9). The lungs, being essential for gas exchange, are particularly vulnerable to toxic insults (10). Emerging evidence suggests that DZN exposure can lead to pulmonary inflammation, oxidative stress, and even apoptosis of lung cells, contributing to a spectrum of respiratory ailments (8, 11).

Research has demonstrated that exposure to DZN can result in significant histopathological changes in lung tissue. These changes may include edema, infiltration of inflammatory cells, and alterations in lung architecture, which collectively compromise respiratory function (11, 12). The mechanisms underlying DZN's pulmonary toxicity warrant further exploration, particularly in the context of vulnerable populations, such as agricultural workers and those living in proximity to treated fields.

Protocatechuic acid, a naturally occurring phenolic compound found in various fruits and vegetables, has garnered interest for its potential protective effects against oxidative stress and inflammation (13, 14). Recent studies suggest that PCA exhibits anti-oxidant properties, scavenging free radicals and enhancing the activity of endogenous anti-oxidant enzymes (13, 15). The protective role of PCA against DZN-induced lung toxicity is particularly noteworthy, as it may mitigate the oxidative damage and inflammatory response associated with exposure to this organophosphate.

The mechanism by which PCA exerts its protective effects may involve several pathways. Firstly, PCA can modulate signaling pathways related to oxidative stress, such as the Nrf2 and HO-1 pathways, which are crucial for the cellular response to oxidative stress. Activation of Nrf2 leads to the transcription of various cytoprotective genes, thereby enhancing the cell's ability to cope with toxic insults (16). Secondly, PCA's anti-inflammatory properties may inhibit the release of pro-inflammatory cytokines and chemokines, thus reducing the inflammatory response in the lungs following DZN exposure (15).

In addition to its anti-oxidant and anti-inflammatory properties, PCA may also promote cellular repair mechanisms. The ability of PCA to enhance the proliferation and differentiation of epithelial cells could play a crucial role in lung tissue recovery following injury (17). This is particularly relevant in the context of DZN exposure, where lung tissue integrity is compromised.

Oxidative stress is a well-established contributor to pulmonary tissue injury (18, 19), particularly following exposure to environmental toxicants such as organophosphates (11). Among these, DZN has been shown to generate excessive ROS, thereby overwhelming the lung's endogenous anti-oxidant defense system. Enzymes such as SOD, CAT, and GPx are crucial in mitigating oxidative insults by detoxifying ROS, while reduced GSH plays a central role in maintaining redox balance and scavenging free radicals. Previous studies have demonstrated that DZN significantly reduces the activities of SOD, CAT, and GPx and depletes GSH content in lung tissue, leading to enhanced lipid peroxidation, as reflected by elevated MDA levels (11, 20). Consistent with these findings, our results showed that DZN exposure caused a marked decline in anti-oxidant enzyme activities and GSH levels, alongside a significant increase in MDA, indicating severe oxidative stress in the lungs.

Importantly, PCA administration was found to mitigate these adverse effects in a dose-dependent manner. PCA restored SOD, CAT, and GPx activities and replenished GSH stores, thereby significantly reducing MDA accumulation. The anti-oxidant effects of PCA may be attributed to its phenolic hydroxyl groups, which can directly scavenge ROS and enhance the activity or expression of anti-oxidant enzymes. Similar protective outcomes have been reported in other models of oxidative lung injury, where PCA exerted its effect through modulation of the Keap1/Nrf2/HO-1 signaling pathway (21). Overall, these results suggest that PCA can effectively counteract DZN-induced oxidative pulmonary damage by reinforcing both enzymatic and non-enzymatic anti-oxidant defenses.

Inflammation plays a pivotal role in the pathogenesis of toxicant-induced organ damage, and the lung is particularly susceptible due to its constant exposure to environmental insults (10). In this study, DZN administration significantly elevated the protein levels of proinflammatory mediators such as NF-κB, COX-2, iNOS, TNF-α, IL-6, and IL-1β in lung tissue, consistent with previous reports highlighting DZN's ability to trigger robust inflammatory responses (8). The activation of NF-κB, a master regulator of inflammation, likely contributed to the up-regulation of downstream cytokines and enzymes, including IL-1β, TNF-α, and COX-2. Notably, PCA co-administration mitigated this proinflammatory response in a dose-dependent manner. PCA at 100 mg/kg effectively reduced TNF-α and COX-2 levels, suggesting potent inhibition of the NF-κB signaling axis (22). Both PCA doses significantly suppressed NF-κB and IL-1β, indicating that even lower concentrations of PCA can disrupt the early stages of the inflammatory cascade.

Apoptosis is a central mechanism of cellular injury

following exposure to toxic environmental agents, including organophosphates such as DZN (23). In the present study, DZN exposure markedly up-regulated pro-apoptotic proteins Bax and caspase-3 while downregulating the anti-apoptotic marker Bcl-2 in lung tissue, reflecting a shift toward programmed cell death. These results are consistent with earlier reports demonstrating that DZN activates mitochondrial apoptotic pathways through oxidative stress and inflammatory signaling (24, 25). The increased expression of caspase-3, -6, and -9 observed at the mRNA level further supports the activation of the intrinsic apoptotic cascade. Importantly, co-treatment with PCA significantly attenuated these apoptotic responses. PCA restored Bcl-2 levels while reducing Bax and caspase-3 expression in a dose-dependent fashion, with 100 mg/kg exhibiting stronger effects. This protective influence was also evident at the gene expression level, as PCA suppressed DZN-induced overexpression of caspase-3 and caspase-9, key executioners in apoptosis. These findings suggest that PCA's anti-oxidant and anti-inflammatory properties may stabilize mitochondrial integrity and inhibit caspase activation, thereby reducing apoptotic progression. Similar anti-apoptotic effects of PCA have been documented in various models of tissue injury, where PCA inhibits ROS-mediated signaling and preserves cellular viability (Kang *et al.*, 2018; Kim *et al.*, 2015). Taken together, our data highlight the efficacy of PCA in modulating apoptosis-related pathways and mitigating DZN-induced lung cell death at both transcriptional and translational levels.

Endoplasmic reticulum stress plays a crucial role in toxicant-induced lung injury by disrupting protein folding homeostasis and activating pro-apoptotic signaling cascades (26). In the present study, DZN administration significantly up-regulated the expression of major ER stress marker XBP-1, eIF2, ATF4, and CHOP in lung tissue, indicating the activation of the unfolded protein response (UPR). These findings align with previous reports demonstrating that organophosphate compounds, including DZN, provoke ER stress by generating excessive reactive oxygen species and impairing calcium homeostasis (27, 28). Notably, CHOP and ATF4 are key mediators of ER stress-induced apoptosis, and their overexpression is associated with progressive lung tissue injury. PCA co-treatment was found to significantly alleviate this ER stress response in a dose-dependent manner. The 100 mg/kg dose exerted a more pronounced effect, particularly in suppressing ATF4 and CHOP mRNA levels, while XBP-1 and eIF2 expression also decreased significantly. These results suggest that PCA may interfere with the PERK-eIF2α-ATF4-CHOP axis and suppress apoptotic signaling initiated by prolonged ER stress. Similar protective effects of PCA against ER stress have been reported in models of oxidative tissue damage, where PCA improved protein folding capacity and reduced CHOP-mediated apoptosis (29). Collectively, our findings indicate that PCA mitigates DZN-induced ER stress by modulating UPR components and may prevent downstream apoptotic events in lung tissue.

Conclusion

The investigation of DZN's pulmonary toxicity and the protective role of protocatechuic acid underscores the complexity of interactions between environmental pollutants and human health. The findings of this study contribute to the growing body of literature emphasizing the need for comprehensive risk assessments of organophosphate exposure and the importance of dietary interventions in promoting health. As we advance our understanding of these interactions, it is imperative to adopt a holistic approach that encompasses regulatory, educational, and therapeutic strategies to safeguard public health in an increasingly toxic world.

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Ethics Approval

All experimental procedures involving animals were conducted in accordance with ethical standards and were approved by the Atatürk University Animal Experiments Local Ethics Committee (ATADEM) under decision no. 15 dated 31.01.2025.

Data Availability

Data will be made available upon request.

Authors' Contributions

T D, C G, O A, MHB, E AS, and ICC contributed to conceptualization, investigation, and manuscript preparation. MB H, I C, and C G supervised. T D, C G, and I C handled statistical analysis and reviewed the final results. MB H, M A, and I B reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript. The authors declare that all data were generated in-house and that no paper mills were used.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Declaration

During the preparation of this manuscript, the authors used ChatGPT, a generative AI tool, to assist with grammar improvement and language refinement. The authors have thoroughly reviewed and edited the content after using AI and accept full responsibility for the integrity and accuracy of the final manuscript.

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