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Supercritical Antisolvent Extraction of Grape (Vitis Vinifera) Leaves Bioactive Fractions and Their Effects on Ameliorating Pulmonary Damage Rat Model

[1]Huda E.Megahed, [1]Rasha H. Hussein*, [1]Huda M. Ismail Abo-Elfadl, [1]Bakinam A. Mohamed Abd-El Fattah, [1]Gehan M. Morsy

[1]Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

ABSTRACT

Pulmonary damage (Pd) refers to inflammation and damage to the lung parenchyma. Exposure to high doses of cypermethrin can cause respiratory paralysis and death due to its ability to induce free radical generation. Natural extracts, especially grape leaf extract, may protect against lung injury, but their protective effects against lung tissue fibrosis and necroptosis were not elucidated previously. The study aims to use the newly developed Supercritical antisolvent extraction method to extract the bioactive compounds in grape leaves and elucidate their potential to protect against pulmonary damage in the cypermethrin-intoxicated rat model. The animals were divided into five groups; two control groups and the others treated with grape leaves hydroalcoholic extract (GLHE), supercritical antisolvent extract (GLSAE), or dexamethasone (Dexa). The study involved the measurement of the specific marker of lung injury, surfactant-associated protein D, myeloperoxidase, some oxidative stress, inflammatory and fibrotic markers, and signaling molecules. It also involved histopathological examination of lung tissues. The results indicated that cypermethrin induced marked oxidative and inflammatory damage to lung tissues along with enhanced pulmonary fibrosis and necroptosis. However, treatment with either extract protected from lung tissue damage and fibrosis through regulation of Nrf2, NF-κB, RIP1 and MLKL genes expression along with PPAR-γ protein expression. Indicating an effective inhibition of lung tissue fibrosis and necroptosis. Obviously, GLSAE showed the most pronounced protective effects which are comparable to dexamethasone. In conclusion, supercritical antisolvent extraction might improve the efficacy of grape leaf bioactive compounds and provide an effective promising therapy against pulmonary damage.

KEYWORDS: Pulmonary damage, Cypermethrin, Supercritical antisolvent extract, hydroalcoholic extract, Grape leaves, Necroptosis, Lung tissue fibrosis.

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INTRODUCTION

Lung injury or pulmonary damage (Pd) refers to damage to lung tissue, which can lead to conditions like acute lung injury (ALI). This disorder is illustrated by inflammation and injury to lung parenchyma. It leads to impaired gas exchange and respiratory failure and often require intensive medical intervention (Park et al., 2020).

Pulmonary damage (Pd) can arise from a variety of causes of which viral infections, such as those caused by the COVID-19 virus, can induce a severe inflammatory response in the lungs, leading to acute respiratory distress syndrome (ARDS) in severe cases (Baboudjian et al., 2021). Inhalation of environmental toxicants, such as insecticides, chemical fumes, and smoke, can also damage delicate lung tissues, leading to immediate injury and chronic conditions like fibrosis over time (Park et al., 2020). Additionally, physical trauma to the chest, such as from blunt force injuries, can result in lung contusions, where the lung tissues are bruised, leading to inflammation, fluid buildup, and impaired oxygen exchange (Zhao et al., 2023). These causes, whether infectious, chemical, or traumatic, are central to the pathophysiology of lung injury, each requiring distinct therapeutic approaches.

Intricate interactions among inflammatory cells, cytokines, and mediators drive the pathophysiological mechanisms of pulmonary damage. Inflammatory responses play a critical role, particularly by increasing alveolar-capillary membrane permeability. Leading to the fluid leakage into the alveolar spaces, causing pulmonary edema and significantly impairing gas exchange, which can result in hypoxemia and respiratory failure (Zhao et al., 2023). Reactive oxygen species (ROS), produced during inflammation, further damage lung tissues by disrupting cell membranes, proteins, and DNA, exacerbating the injury (Liu et al., 2023). Together, these mechanisms create a feedback loop of inflammation and oxidative damage, worsening lung function and complicating recovery.

Because cypermethrin is used extensively as a broad-band insecticide, it is a common environmental toxic. Cypermethrin is a pyrethroid insecticide widely applied in agriculture and household pest control due to its effectiveness against a variety of pests. Its stability in the environment and low toxicity to mammals make it a popular choice for pest management (Soliman et al., 2021).However, despite its efficacy, cypermethrin poses potential toxicity and health risks to humans and animals through inhalation, ingestion, or dermal exposure. Specifically sustained inhalation of high doses can cause respiratory paralysis and death. Its capacity to cause free radical production explains its harmful consequences(Soliman et al., 2021).

Extensive efforts are being focused on phytomedicines comprising flavonoids, (vitamins, alkaloids, tannins, and terpenoids) as key therapeutic agents ascribed to their different biological activities (Majeed et al., 2021). Natural antioxidants are widely applied in medicine as they are able to minimize the hazards of numerous healthiness problems as pulmonary injury, cancers, and neurodegenerative disorders (Hrelia & Angeloni, 2020), possibly through their hydrogen-donating tendency and potential to quench free radicals thus preventing oxidative stress (Singh et al., 2023 and Ge et al., 2021).

Grape leaves (*Vitis vinifera*) are a popular culinary ingredient and contain a range of bioactive compounds that contribute to their health benefits. Rich in polyphenols, including catechins, quercetin, and tannins, grape leaves are known for their antioxidant properties, helping in reducing oxidative stress and inflammation (Harbeoui et al., 2019) and protect cells from oxidative damage linked to various chronic diseases (Di Pietro Fernandes et al., 2023). Grape leaves (*Vitis vinifera*) and their bioactive compounds may protect against lung injury. These leaves are rich in several polyphenolic compounds which exhibit strong antioxidant properties that can help mitigating lung injury (Arora et al., 2016)**.**

Plant extracts are often investigated and commercialized in liquid form which is characterized by reduced cost and high biodegradation rate. The extraction method and the used organic solvent paly important role on the activity of the final product of extract (Masud & Singh, 2013). The application of supercritical fluids (SCFs) is one of the innovative strategies for collecting plant extracts. Carbon dioxide (CO2) is the most used ingredient for high-pressure precipitation operations under supercritical circumstances. CO2 is environmentally favorable compound, non-flammable, non-toxic, and leaves no residue on processed specimens. Additionally, supercritical CO2-based micro-nization procedures enable the production of regular particles with small dimensions.

For that reason, our study aimed to extract the bioactive phenolic compounds of grape leaves using artificial ethanolic extraction and the supercritical antisolvent extraction techniques and to elucidate their role in ameliorating the pathophysiology of pulmonary damage induced by cypermethrin in rats. The study highlighted the role of grape leaf extracts against the oxidative, inflammatory, and fibrotic effects of cypermethrin in rats' lung tissue.

MATERIALS AND METHODS

Chemicals

Cypermethrin ($C_{22}H_{19}Cl_2NO_3$, Molecular Weight**:** 416.30**)** purchased from Sigma-Aldrich (St. Louis, MO, USA) and ethanol was purchased from El Gomhouria Company (Egypt).

Preparing grape leaves hydroalcoholic extract (GLHE)

Fresh grape leaves were bought from the Agricultural Research Center, Giza, Egypt. Leaves were dried in the shade, powdered, then extracted by maceration with 70% (v/v) ethyl alcohol for 72 h at ambient temperature according to (Pari & Suresh, 2008). The extract was filtered, the solvent evaporated to dryness in a rotary evaporator. The residual extract was collected, kept in darkness at 4°C until using.

Supercritical antisolvent extraction of grape leaves (GLSAE)

Supercritical fluid extraction was applied to extract grape leaves according to the method described by (Valor et al., 2023). The supercritical solvent used is CO_2 (critical conditions = 40°C and 200 bar). The operating system used as the Supercritical CO_2, Green Extraction (Spe-edTM SFE-2/4, Applied separations,USDA1- USA). The sample was extracted by adjusting to the following conditions: Pressure at 150 bar, temperature at 40 C, flow rate 10 mL/min, static state and dynamic state for 60 min, and a total extraction time was more than 3 hours.

Animals

This study was approved by the Research Ethical Committee (REC) at Faculty of Science, Ain Shams University, Cairo, Egypt (Ethical approval). The adult male *Sprague Dawley* rats weighing 250 -300 gm and aged 3–4 months, were supplied from the animal Breeding House of the National Research Centre (NRC), Giza, Egypt. Rats were housed separately in stainless cages in air-conditioned rooms kept at 24°C with a 12h light/dark cycle. After the adapting period, animals were randomly grouped into five groups (n=6) as follows; **Group 1 (Healthy)**: animals were given 0.2% Tween 80 (5ml/kg/day) plus 1 ml normal saline orally (p.o.). **Group 2 (Control-Pd)**: Pulmonary damage was induced by oral administration of 50 µL cypermethrin pesticide (26.15mg/kg/day ($1/10^{th}$ LD50) dissolved in Tween 80) + 1 ml saline. **Group 3** (Pd+ GLHE): oral administration of cypermethrin + GLHE (100 mg/kg/day). **Group 4** (Pd+ GLSAE): oral administration of cypermethrin + GLSAE (65 µL/kg/day). **Group 5** (Pd+ DXA): orally administered cypermethrin + Dexamethasone (150 mg/kg in 1 ml saline solution). All cures were given by intragastric administration daily for 28 days.

At the end of the experiment, rats were fasted overnight, and anesthetized by 800 mg/kg of sodium pentobarbital (Zatroch et al., 2017) then sacrificed by decapitation. Blood samples were collected in nonheparinized, centrifugated for serum separation, and kept at -20°C for analysis. Lung tissues were separated, rinsed with sterile saline, weighed and used for histological studies, biochemical analysis, and measurement of gene expression.

Measurement of lung tissue myeloperoxidase

Myeloperoxidase in lung tissues was examined by quantitative sandwich enzyme immunoassay technique according to the kit's protocol (MyBioSource, U.S.A).

Determination of hydroxyproline in lung tissues, and serum activity of lactate dehydrogenase (LDH) enzyme
Lung tissue content of hydroxyproline was assessed by using enzyme linked immunoassay (ELISA) kit (MyBioSource). Lactate dehydrogenase (LDH) activity, was measured in serum samples using the LDH ELISA (MyBioSource).

Measurement of pulmonary surfactant-associated protein D (SP-D) in serum
Serum pulmonary surfactant-associated protein D was recently known as a specific marker of lung injury in rats, for that it is measured in our study using an ELISA kit (Cat No:E-EL-R0831, Elab science) .

Measurement of antioxidant and lipid peroxidation product markers
Determination of reduced glutathione (GSH) level in lung tissue was applied affording to the kit's protocol (CAT. No.: GR2511) , while serum total antioxidant capacity (TAC) was measured using (CAT.NO: MBS1600693, MyBioSource's, U.S.A) according to the manufacturer method. Lung tissue Malondialdehyde (MDA) was determined by a previously described colorimetric method (Draper & Hadley, 1990).

Measurement of gene expression of Nrf2, NF-κB, TGF-β1, RIP1 and MLKL
Measurement of mRNA levels of Nrf2, NF-κB, TGF-β1, RIP1, MLKL and the housekeeping gene GAPDH in lung tissues was done using real-time polymerase chain reaction (RT-qPCR) by using Applied Biosystems step one plus equipment. Total RNA was extracted from tissue lysate with Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP. USA) according to Kits directions. SuperScript IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) was utilized for reverse transcription of extracted RNA followed by PCR in one step. Primer sequences (Table 1) were constructed as explained earlier for Nrf2 (Li et al., 2016), NF-κB (Ismail Abo El-Fadl & Mohamed, 2022), TGF-β1, RIP1 and GAPDH as designed by primer plast.

Table (1): Forward and reverse sequences of the primers

Gene	Forward (5′-3′)	Reverse (5′-3′)
Nrf2	GAATAAAGTTGCCGCTCAGAA	AAGGTTTCCCATCCTCATCAC
NF-κB	TTCCCTGAAGTGGAGCTAGGA	CATGTCGAGGAAGACACTGGA
TGF-β1	GTGTGGAGCAACATGTGGAACTCTA	TTGGTTCAGCCACTGCCGTA
RIP1	TTACATGGAAAAGGCGTGATACA	AGGTCTGCGATCTTAATGTGGA
MLKL	AGGAGGCTAATGGGGAGATAGA	TGGCTTGCTGTTAGAAACCTG
GAPDH	TGGATTTGGACGCATTGGTC	TTTGCACTGGTACGTGTTGAT

Immunohistochemical analysis of PPAR-γ
Paraffin sections were framed on positively charged slides by using avidinbiotin- peroxidase complex (ABC) method. PPAR ɣ polyclonal antibody (ABclonal, CAT.NO. A11183). Sections were incubated with the mentioned antibodies, then reagents of ABC method were included (Vectastain ABC-HRP kit, Vector laboratories). Marker expression was identified with peroxidase and colored with diaminobenzidine (DAB, produced by Sigma) to find out antigen-antibody complex. IHC stained sections were scanned using Olympus microscope (BX-63). **Histological examination**
The microscopic examination of lung tissues was performed by fixing the lung tissues in neutral formalin. Tissue was sectioned inside paraffin-embedded blocks, followed by staining with Hematoxylin and Eosin (H&E). The tissue sections were then examined under light microscopy according to the previous method (Bancroft & Gamble, 2007)**.**

Statistical analysis
The SPSS program for Windows, (version 16) (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of data. Values were expressed as means ±SD. One Way Analysis of Variance (ANOVA) LSD test.

RESULTS

The effects of grape leaf extracts on rat body weight and relative lung weight
Oral administration of cypermethrin caused a significant decrease in animals' body weight accompanied by a substantial elevation in the relative weight of lung tissue in the control-Pd rats related to healthy rats. However, treating Pd-rats with GLHE and GLSAE significantly improved body weight and relative lung weight compared to the control-Pd rats. Results in the Pd+GLSAE were not significantly comparable to those of Pd-rats treated with dexamethasone.

Table 2: body weight and relative lung weight in experimental groups

	Body weight (g)	Relative lung weight
Healthy	284.6± 12.87	0.56± 0.036
Control-Pd	214.3± 3.66 #	0.82± 0.034 #
Pd+ GLHE	231± 3.74 *	0.74± 0.034 *
Pd+ GLSAE	266.6± 11.25 *	0.63± 0.022 *

Pd+ Dexa	276.6± 7.39 *	0.61± 0.068 *

Means are significantly different at (P≤0.05). # means are significant compared to healthy group. * values are significant compared to the control-Pd group.

Modulation of myeloperoxidase level in lung tissues

From the results of the study, the levels of myeloperoxidase were significantly elevated after oral administration of cypermethrin in rats (figure 1). However, their levels were markedly reduced after the treatment of Pd-rats with grape leaf extracts. The results of GLSAE were more pronounced to be comparable to those of dexamethasone. Indicting their efficiency over the traditional hydroalcoholic extract against pulmonary damage.

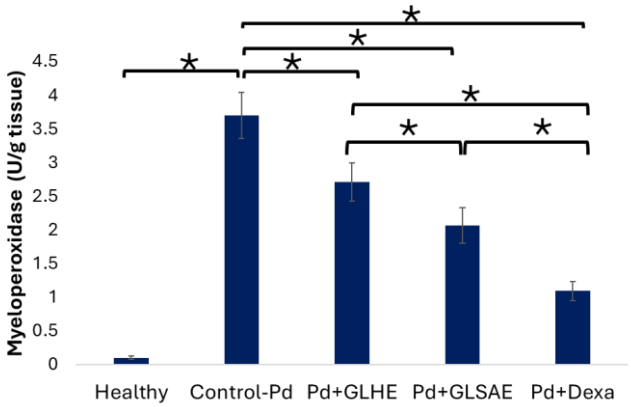

Figure (1): Mean values of lung tissue myeloperoxidase in the examined groups. * Means are significantly different at (P≤0.05).

Antioxidative effects of grape leaf extracts against pulmonary damage

Regarding the antioxidant effects of grape leaf extracts, we determined the gene expression of Nrf2, tissue level of MDA and GSH, and serum TAC (figure 2). The results indicated that cypermethrin induced a marked oxidative stress status in pulmonary tissues and appeared to significantly reduce Nrf2 gene expression, lung tissue level of GSH, and serum TAC along with elevation in lung tissue MDA compared to healthy rats. However, the lipid peroxidation product MDA and the antioxidant markers; Nrf2, GSH and TAC were significantly improved in the lung tissue of rats administrated grape leaf extracts orally. Moreover, the values were highly regulated by GLSAE compared with GLHE.

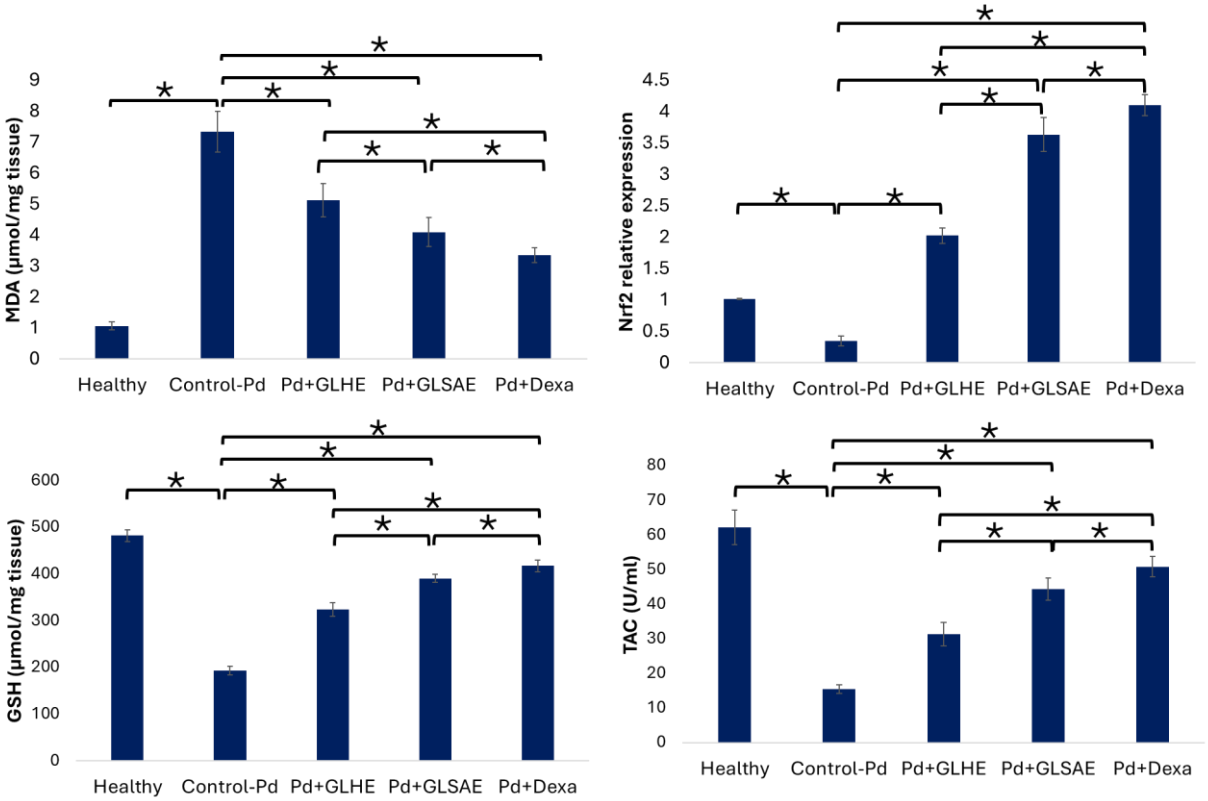

Figure (2): levels of lung tissue MDA, Nrf2 gene expression, tissue GSH content and serum TAC in the examined groups. * Means are significantly different at (P≤0.05).

Grape leave extracts reduced Hydroxyproline in lung tissue and LDH release

Hydroxyproline as a marker of collagen deposition in lung tissue was significantly upregulated in rats administrated with cypermethrin compared with the normal control rats. Also, a significant elevation in serum level of LDH was recorded as an indicator of cytotoxicity in the damaged lungs of rats. On the other hand, treatment with GLHE, GLSAE, and dexamethasone significantly improved hydroxyproline levels and cytotoxicity. Obviously, the best improvement was found in rats treated with GLSAE (figure 3).

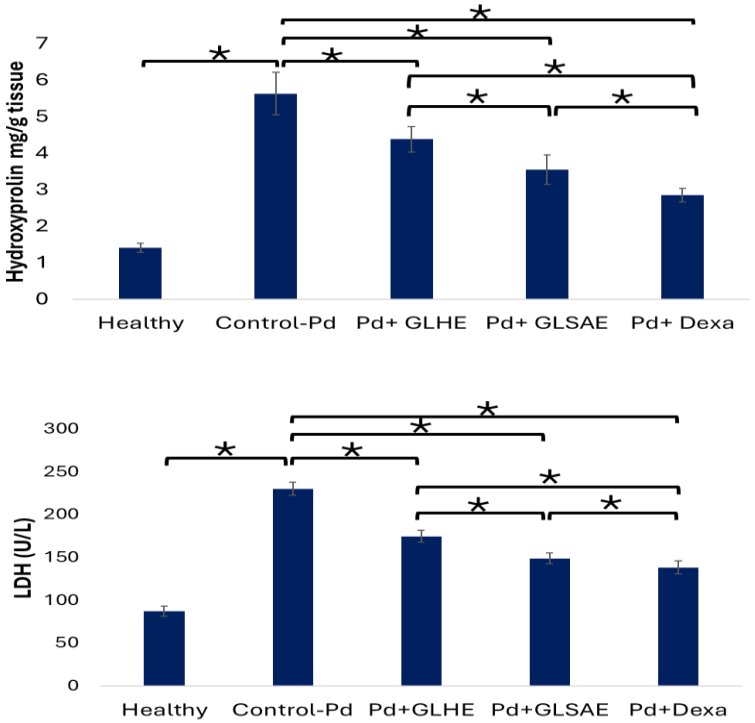

Figure (3): lung tissue levels of hydroxyproline and serum LDH in the examined groups. * Means are significantly different at (P≤0.05).

Grape leave extracts reduced the gene expression of inflammatory markers in lung tissue inflammation

The animals administered oral doses of cypermethrin exhibited an increased expression of NF-κB and TGF-β1 in their lungs (figure 4). NF-κB is an inflammatory transcription factor affecting other pro-inflammatory cytokines of as TGF-β1, which is also related to lung tissue fibrosis. This indicates a massive proinflammatory and profibrotic condition induced by cypermethrin in lung tissues. However, grape leaf extracts and Dexa treatment could effectively inhibit the proinflammatory effects of cypermethrin in lung tissue.

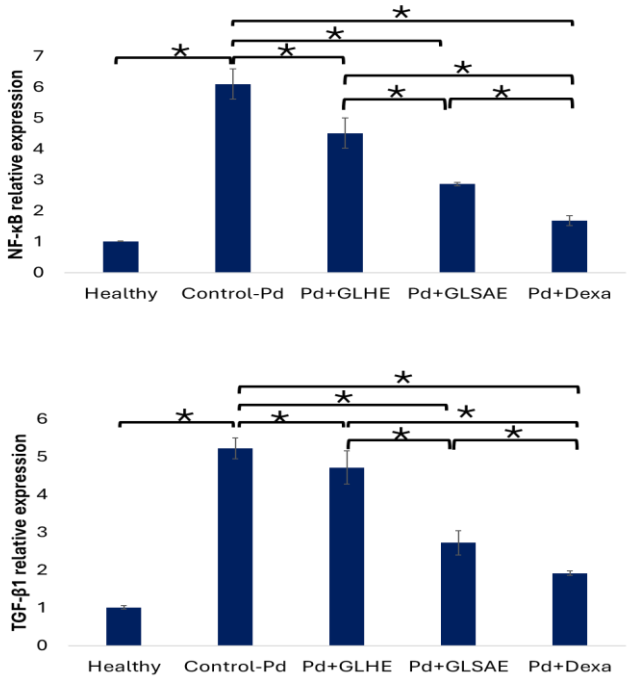

Figure (4): the gene expression level of pro-inflammatory markers in lung tissue of all groups. * Means are significantly different at (P≤0.05).

The relative expression of RIP1 and MLKL was reduced by grape leaf extracts

The relative gene expression levels of RIP1 and MLKL were significantly elevated in lungs after cypermethrin administration by rats. Meanwhile, grape leaf extracts and Dexa caused a significant reduction in RIP1 and MLKL expression levels as compared to control Pd-rats. The more pronounced reduction was found in the animals treated with GLSAE which is comparable to that of Dexa (figure 5).

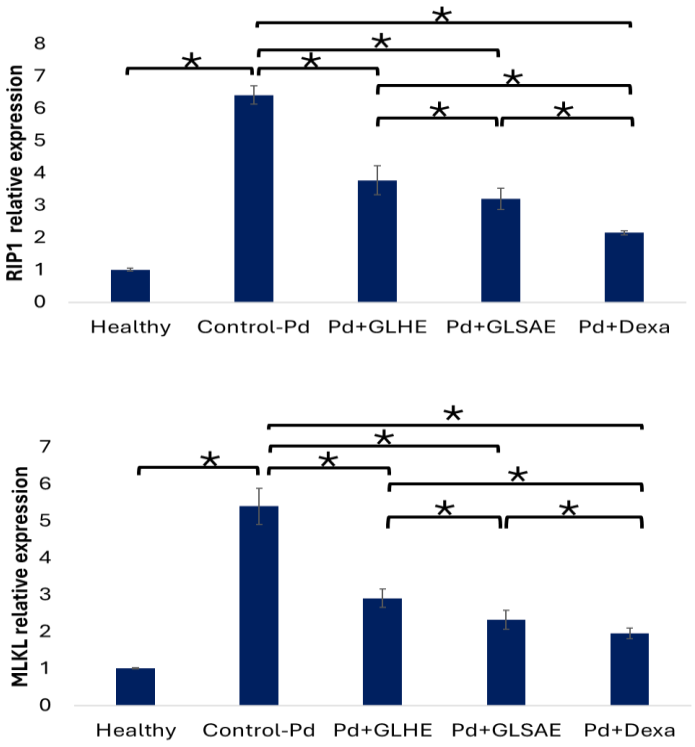

Figure (5): The gene expression level of RIP1 and MLKL . * Means are significantly different at (P≤0.05).

Effect of grape leaf extracts on SP-D levels in serum

Serum level of SP-D was markedly elevated in the injured lungs, which was obvious in the animals administered with cypermethrin orally (figure 6). This could reflect the damaging effect of cypermethrin on pulmonary tissues. Meanwhile, the values were significantly reduced by GLHE, GLSAE, and Dexa in Pd-rats. Compared with the traditional hydroalcoholic extract, the most pronounced protective effects are seen in the animals treated with Dexa and GLSAE.

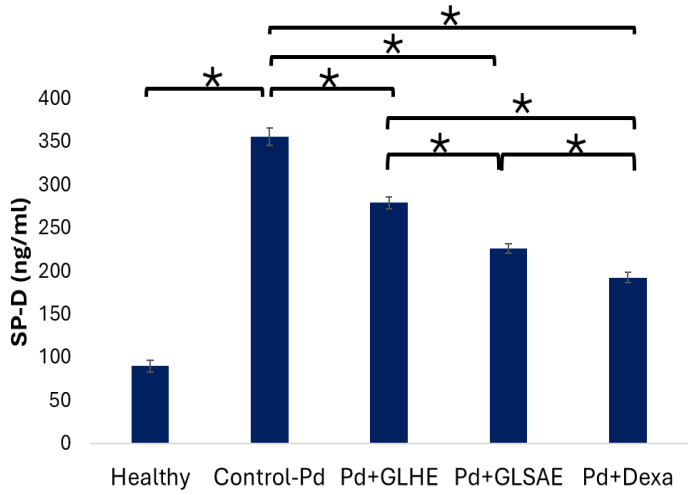

Figure (6): Serum levels of SP-D in all groups. * Means are significantly different at (P≤0.05).

Immunohistochemical analysis of PPAR-γ

The immunohistochemical expression of PPAR-γ is found to be severely upregulated in the lung tissue sections of rats administered with cypermethrin orally indicating marked inflammatory consultation. Their effect is significant compared to that in the healthy control group. However, it was moderately downregulated after treatment of animals with GLSAE in Pd-rats, which is non-significantly comparable to that of Dexa treatment in group 5. An effect is not observed after treatment with the traditional ethanolic extract of grape leaves in group 3.

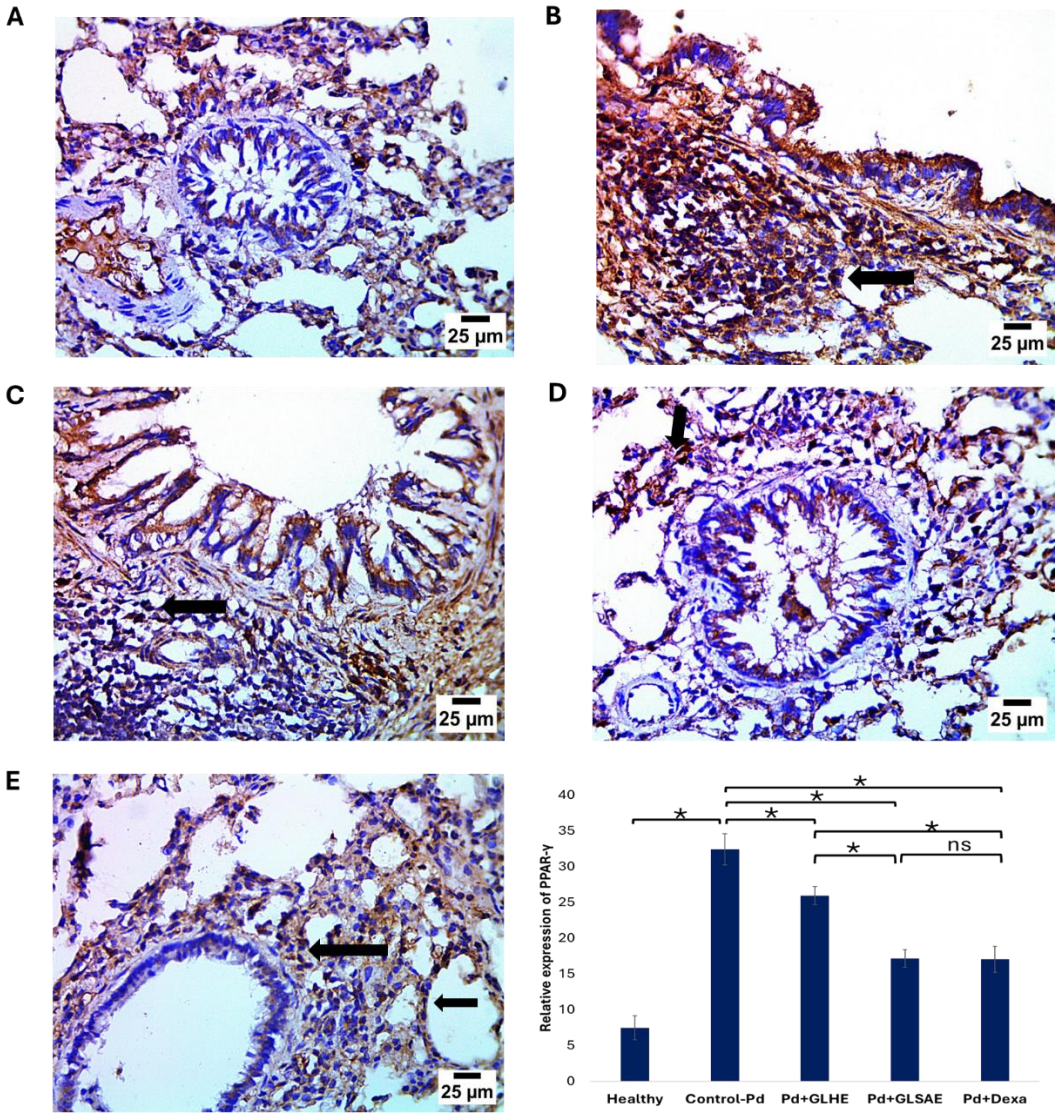

Figure (7): Immunohistochemical photomicrograph for PPAR-γ in lung tissue sections (IHC-Peroxidase-DAB) showing A) mild expression for PPAR-ɣ in peribronchiolar tissue (arrow) from group 1. B) very sever expression for PPAR-ɣ in peribronchiolar tissue (arrow)in group 2. C) sever expression for PPAR-ɣ in peribronchiolar tissue (arrow) in group 3. D) moderate expression for PPAR-ɣ in peribronchiolar tissue (arrow) in group 4. E) moderate expression for PPAR-ɣ in peribronchiolar tissue and alveolar tissue (arrow) in group 5. *means are significantly different at (P≤0.05), ns: means are not significantly different at (P≤0.05).

Histopathological analysis of lung tissue

Microscopic investigation of lung tissue sections (figure 8) of the normal control rats showed a normal histologic formation of lung tissue including bronchioles and alveoli. Meanwhile, the animals administered cypermethrin exhibited severe multifocal to coalescing interstitial pneumonia that was characterized by numerous aggregations of peribronchial and perivascular inflammatory cells. Some examined sections revealed alveolar hemorrhages. Moderate improvement was determined in Pd+ GLHE rats' group that is characterized by moderate interstitial pneumonia with mild reduction of inflammatory cell infiltration compared to Pd-rats. Higher improvement was observed in Pd rats administered GLSAE, which exhibited apparently normal lung tissue in several examined sections with mild perivascular inflammatory cell infiltration and mild interstitial pneumonia in fewer sections. Similar results were also observed in Pd rats treated with Dexamethasone showing normal lung tissue structure in several sections with mild interstitial pneumonia in other sections.

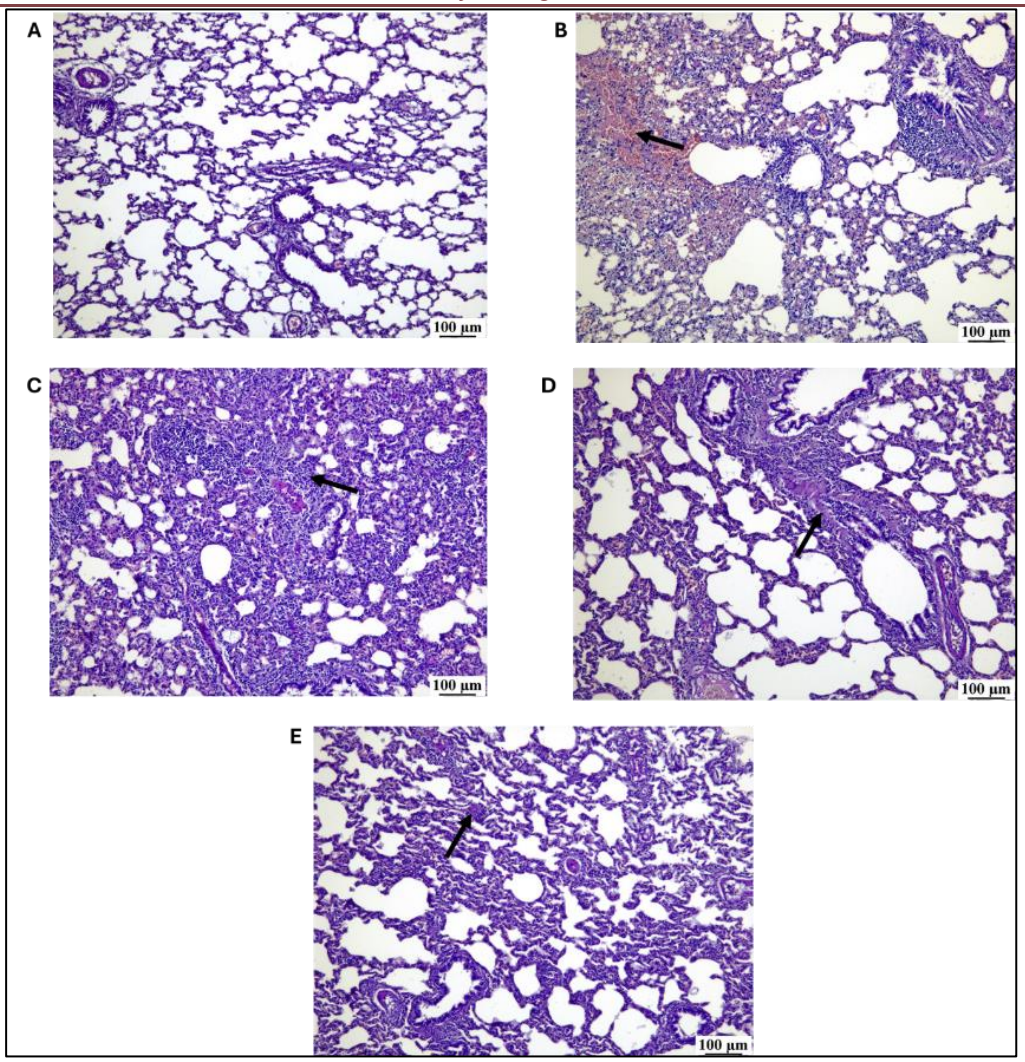

Figure (8): Photomicrograph of lung tissue sections (H&E) showing A) normal structure of bronchiole from group 1. B) numerous peri-bronchial inflammatory cells infiltration with pulmonary hemorrhages (arrow) in group 2. C) moderate interstitial pneumonia (arrow) in group 3. D) mild perivascular and peri-bronchial inflammatory cells infiltration (arrow) in group 4. E) mild interstitial pneumonia group 5.

DISCUSSION

Global toxicity of insecticides is becoming life-threatening, with an increased risk of compromised lung function. Moreover, the respiratory system is particularly vulnerable to the harmful effects of pesticides, as they can cause irritation, allergic reactions, and even long-term pulmonary conditions. Recent studies have highlighted the adverse effects of cypermethrin on lung health. Exposure to cypermethrin has been linked to oxidative stress and inflammation in lung tissues, contributing to respiratory distress and lung injury (Sule et al., 2022). A correlation has been demonstrated between the utilization of therapeutic plants and the mitigation of harmful consequences caused by environmental pollutants, like pesticides (Majeed et al., 2021).

Many works have proven the optimistic impact of plant polyphenols on pharmacy and biochemical aspects. But apply them, polyphenols need to be extracted from natural substrates first, then analyzed and characterized. For obtaining ,maximum yield of polyphenols and avoiding the loss of functional characteristics, a suitable effective extraction method should be applied (Tang et al., 2018).

In this study, to extract grape leaves polyphenols, two clean extraction technologies such as hydroalcoholic extraction and the Supercritical antisolvent extraction SAE, were used and examined the effect of each extraction on cypermethrin-induced Pulmonary damage.

Hydroalcoholic extraction is a technique in plant polyphenols extracted by maceration with 70% (v/v) ethyl alcohol. While Supercritical antisolvent extraction SAE method utilizes supercritical fluids, typically carbon dioxide CO2, as the primary solvent to extract desired compounds while employing a non-solvent (antisolvent) to precipitate the extracted compounds. This approach has gained attention due to its environmentally friendly nature and ability to selectively extract compounds without the use of toxic organic solvents (Valor et al., 2023). Accordingly, this innovative technique could be used to extract bioactive compounds from various plant materials, including grape (*Vitis vinifera*) leaves.

However, the results obtained revealed that the extractive yield was maximum in Supercritical antisolvent extraction than by cold

percolation method Hydroalcoholic extraction. The advantages of SAE over conventional extraction methods include higher extraction efficiency, reduced extraction times, and improved purity of the extracted compounds. Studies have shown that SAE can yield higher concentrations of polyphenolic compounds compared to traditional methods such as maceration or Soxhlet extraction (De Aguiar et al., 2022). Additionally, the adjustable parameters in SAE, such as temperature, pressure, and the ratio of solvent to antisolvent, allow for optimized extraction conditions tailored to specific target compounds. Overall, the application of supercritical antisolvent extraction in extracting bioactive compounds from grape leaves offers a promising method for enhancing the nutritional and therapeutic value of this abundant agricultural byproduct (Franco & De Marco, 2021).

Results represented a reduction in animals' body weight accompanied by a substantial elevation in the relative weight of lung tissue in rat groups administered cypermethrin as compared to control rat group. Which were similar to results obtained by (Shuklan et al., 2023) stated that administering cypermethrin at a dose of 20 mg/kg/day produces body weight loss, due to its effect on the gastrointestinal tract, which reduces appetite and the absorption of nutrients from the gut, or it may be the result of cypermethrin's direct toxicity. Additionally, (Hughes et al., 2016) hypothesized that the decrease in body weight might be the result of gastrointestinal tract malabsorption of nutrients, while (Taha et al., 2021) reported that the decrease in body weight might be caused by pesticides' cytotoxic effect on somatic cells or by affect on central nervous system, which controls food intake. On the other hand, the increase of lung relative weight following cypermethrin administration may be due to the deposition of collagen and appearance of histopathological lesions, such as thickening of the alveolar walls, occurrence of edematous fluid and blood congestion causing pulmonary edema, as previously described by (Soliman et al., 2021). While treating the intoxicated rats with GLE markedly reversed and improved the animal's body weight and relative weight of lung tissues. An effect might be due to their high phenolic and antioxidant content (Di Pietro Fernandes et al., 2023) which markedly attenuated the oxidative damage of lung tissue induced by cypermethrin and protected against inflammatory and fibrotic lesions of lung tissue.

Cypermethrin induced a marked oxidative stress status in pulmonary tissues as the reduction in Nrf-2, GSH and TAC and elevation in MDA compared to healthy rats. The precise chemical mechanism via which the environmental toxin cypermethrin causes lung damage is unknown, however it could be mediated by the release of reactive oxygen species (ROS), which causes lung damage and a loss of basement membrane integrity (Arafa et al., 2015).

Oxidative stress could result while cypermethrin is broken down by the cytochrome P450 microsomal system. Because cypermethrin is lipophilic, it can readily penetrate the lipid bilayer and compromise the integrity of cells (Taha et al., 2021). A previous study by (Ghazouani et al., 2020) reported that the thiol group of cysteine residues and polyunsaturated fatty acids of biological membranes may be attacked by reactive oxygen species during the metabolism of cypermethrin, resulting in cell damage.

Results indicated that GLSAE could normalize all oxidative stress markers, which have been closely linked to pulmonary oxidative stress as compared with GLHE. It is known that grape leaves contain different types of phenolic compounds such as tannins, flavonoids, procyanidins, and anthocyanins, Flavanols, flavonols, and resveratrol are secondary metabolites produced by plants that have important biological activity (Nassiri-Asl & Hosseinzadeh, 2009).

Research findings indicate that grape leaf extract has antioxidant action because of its high polyphenolic content, which inhibits the production of free radicals and oxidation (Chernousova et al., 2022). Using the extracts of Grape leaves counteracted the enzymatic alterations induced by H_2O_2, showing the role of polyphenol in scavenging reactive oxygen scavenger (Nassiri-Asl & Hosseinzadeh, 2009).

Another study by (Yang et al., 2019) showed the abundance of polyphenols in GLE casing its potent antioxidant effects. While (Di Pietro Fernandes et al., 2023) stated that GLE extract exhibits an antioxidant capacity to decrease peroxyl radicals.Parallel outcomes were got by (Murshed et al., 2023) which indicated that GLE ameliorated the changes in GST and MDA levels in infected mice. Similarly, other research indicated that the bioactive compounds in grape leaves may help ameliorate lung injury through several mechanisms: their antioxidant properties can neutralize reactive oxygen species (ROS) generated during lung injury, reducing oxidative stress and promoting recovery (Di Pietro Fernandes et al., 2023).

In the same manner, (D. D. S. Lacerda et al., 2014) suggested that chronic treatment with an extract of grape leaves may represent an adjuvant therapy for the treatment and/or prevention of diabetic complications because of its antioxidant effects. The caffeic acid content found in grape seed leaves is reliable on reducing oxidative stress and recovering the antioxidant defense mechanism (Ehtiati et al., 2023). Moreover, quercetin has the ability to scavenge superoxide radicals, hydroxyl radicals, and lipid peroxyl radicals through its antioxidant activities (Abdou & Abd Elkader, 2022).

While (Joo Sul et al., 2024) stated that GLE are rich in proanthocyanidins which exhibit positive effects due to their ability to modulate cell signaling pathways, thus providing significantly greater protection against oxidative stress . Additionally, the aqueous extract of grape leaves also presents an in vivo antioxidant effect, increasing the GHS levels in the heart of streptozotocin-induced diabetic rats (D. S. Lacerda et al., 2016).

The study results revealed that MPO levels were significantly elevated after oral administration of cypermethrin in rats. These findings demonstrated that MPO plays a role in the inflammatory process of lung illnesses and that tissue inflammation and neutrophil infiltration are strongly connected with elevated MPO levels in the lungs. Meanwhile, measuring MPO activity is a well-established method for estimating inflammation (Davies & Hawkins, 2020).

Similar results were obtained by (Alak et al., 2019) which revealed that, Cypermethrin dramatically raised tissue MPO levels. This elevated level was counteracted by both extracts of grape leaves. (Salvador et al., 2019) showed that GLE is rich in resveratrol and quercetin which appears to prevent tissue neutrophil infiltration, hence providing protective effects and suppress MPO activity.

Additionally, rats administered oral doses of cypermethrin exhibited an increased expression of NF-κB and TGF-β1 in their lungs. In animal models, inhalation of cypermethrin resulted in increased levels of pro-inflammatory cytokines, indicating an inflammatory response that can exacerbate lung injury (Sule et al., 2022).

A noticed anti-inflammatory effect following treatment with both grape seeds extracts was thought to be due to the rich content of proanthocyanidin. A study by (Valcheva-Kuzmanova et al., 2014) reported the anti-inflammatory role of proanthocyanidin in inhibiting amiodarone-induced lung toxicity .Furthermore, (Sul et al., 2022) showed that using GLE in treating bleomycin - induced lung fibrosis mice model, reduced pulmonary fibrosis and alveolar damage. However, (Kaur et al., 2022) revealed that an aqueous extract from grape leaves inhibited TGF-β1 level by altering the NF-κB pathway; thus, lowering gastric inflammation. This anti-inflammatory effect is mediated by quercetin glycosides content of grape leaves extract.

Moreover, (Peng et al., 2020) revealed that piceatannol, one analog of resveratrol, isolated from grapes leaves , was showed to acquire anti-inflammatory, anti-oxidative, anti-proliferative activities. Additionally, flavonoids like quercetin and kaempferol present in GLE were able to reduce inflammation by inhibiting the production of pro-inflammatory cytokines and modulating inflammatory pathways, thereby protecting against lung injury (Murshed et al., 2023). Tannins found in grape leaves also possess antioxidant and anti-inflammatory properties, contributing to lung protection by stabilizing cell membranes and reducing the severity of lung damage. While not extensively studied in grape leaves specifically, saponins are known for their immune modulation and anti-inflammatory effects, which may also benefit lung health (Arora et al., 2016). All these compounds can mitigate the inflammatory response associated with lung injury by inhibiting pro-inflammatory cytokines and modulating immune responses (Murshed et al., 2023). A significant increase was noticed in Hydroxyproline showing an increase of collagen after cypermethrin treatment compared with the normal control rats. The increase of collagen deposition over time leads to fibrosis (Zhu et al., 2024). Some studies also suggest that bioactive compounds in grape seed extract, can enhance lung function and reduce fibrosis, thus improving overall pulmonary health after injury (Di Pietro Fernandes et al., 2023).

The relative gene expression levels of RIP1 and MLKL were significantly elevated in lungs after cypermethrin administration by rats. RIP1 kinase is a critical regulator of inflammation and responsible on maintaining immune responses and ideal tissue homeostasis (Park et al., 2020). While, MLKL was identified as the final effector of necroptosis completion (Martens et al., 2021). Necroptosis can induce programmed cell death (PCD). Receptor-interacting protein kinase 1 (RIP1), RIP3, and mixed-lineage kinase domain-like (MLKL) protein can regulate necroptosis and play important roles in Pd. Tumor necrosis factor-alpha (TNF-α) could be apart of necroptosis induction (Fulda, 2016). Those effects were markedly reversed by grape extracts in our study, showing inhibitory effects on necroptosis. This might be related to the inhibitory effects of GLEs on NF-κB expression with its consequent suppression of TNF-α /RIP1/ MLKL pathway. This effect was not elucidated previously; however, their high content of phenolic compounds might be the causative effector. Of which the study of (Hu et al., 2021) reported that Resveratrol (RES) which is extracted from grape skin could protect myocardial cells from hypoxia/reoxygenation (H/R)-caused injury due to its inhibitory effects on TNF-α /RIP1/ MLKL pathway.

The study results showed a marked elevation in SP-D serum level in the injured lungs, which was evident in the animals administered with cypermethrin orally. The hydrophilic lung surfactant protein D (SP-D) performs essential functions in immune defense system and in the homeostasis of surfactant phospholipids. There are several works representing the connection of SP-D and chronic obstructive pulmonary disease (Walther et al., 2019). A previous work by (Elmore et al., 2023) showed that SP-D regulation is influenced by the proinflammatory cytokines, the pulmonary intravascular is the main cause of the SP-D elevation in the circulation during lung injury. On the other hand, GLE markedly decreased SP-D levels in intoxicated rats. From our results, the antioxidant and anti-inflammatory effects of the extracts might reflect on the reduction of SP-D expression and inhibition of pulmonary damage.

Pulmonary fibrosis is characterized by variations in fibroblast phenotypes causing excessive extracellular matrix accumulation (Milam et al., 2008). Our study represents a very sever expression patterns of the PPAR-γ immunohistochemistry in peribronchiolar tissue in rats treated with cypermethrin. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone receptor superfamily that activated by numerous xenobiotics, thus causing alterations in metabolic and inflammatory responses (Zhang et al., 2019). Additionally, PPAR-γ agonists inhibit the ability of transforming growth factor-β1 (TGF-β) to induce myofibroblast differentiation and collagen secretion. These remarks show the important role of PPAR-γ in regulating fibroblast/myofibroblast activation in fibrotic lung diseases (Milam et al., 2008) and (Zhang et al., 2019). Furthermore, treatment with both types of extract were found to be beneficial in ameliorating the degree expression for PPAR-ɣ as observed from the immunohistochemical results of the current work. This agreed with (Ghrir et al., 2024) who have documented the anti-xenobiotic detoxication role of GSE against chlorpyrifos-induced liver toxicity. Obviously from our results, the inhibitory effects of grape extracts on TGF-β and collagen deposition in lung tissues might be mediated by the inhibition of PPAR-ɣ expression.

Histopathological assessment revealed that cypermethrin exhibited severe multifocal to coalescing interstitial pneumonia that characterized by numerous aggregations of peri-bronchial and perivascular inflammatory cells. The results examined proposed that cypermethrin emitted irritant influence on the pulmonary tissue, due to the proliferation of pulmonary interstitial. Meanwhile,

lung tissues became more compact due to cells clumping and nuclei condensation in following administration of cypermethrin. The findings are in agreement with those of (Yanagihara et al., 2023) stating that inhalation of pyrethroids causes alveolitis, and pulmonary edema as it induce oxidative stress and collagen deposition. An improvement was noticed following the treatment of both grape seed extracts. Similar results obtained by (Galaly et al., 2018) showing the role of supplementing GLE (150 mg/kg) in amelioration of lung fibrotic damage and lung histology. Similarly, (Joo Sul et al., 2024) showed that GLE improved lung collagen deposition and fibrosis pathology induced by bleomycin in rats.

CONCLUSION

Research findings indicate that both grape leaf extracts mitigate pulmonary toxicity in rats resulting from cypermethrin treatment. Overall, while cypermethrin remains a widely used insecticide, its potential effects on lung health warrant careful consideration and regulation to moderate associated risks. In our study GLEs showed a comparative modulatory effect on protecting them from pulmonary damage. Their effects might be related to their antioxidant, anti-inflammatory, antifibrotic, and anti-necroptotic effects. Obviously, their effects on necroptotic markers were not previously elucidated. Comparing results showed that GLSAE was mostly effective in protecting from the pulmonary damage associated with cypermethrin. This might indicate that GLEs provide an effective promising therapeutic approach against pulmonary damage.

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