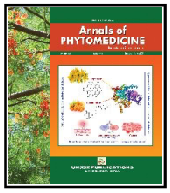
Annals of Phytomedicine 14(1): 672-684, 2025

DOI: http://dx.doi.org/10.54085/ap.2025.14.1.65

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

Hinokitiol, a monoterpenoid mitigates lypopolysachiride induced acute lung injury in mice *via* modulating TNF-α, COX-2, and Nrf-2/NF-κB pathway

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Article Info

Article history
Received 19 February 2025
Revised 30 March 2025
Accepted 31 March 2025
Published Online 30 June 2025

Keywords
Hinokitiol
Lung injury
Lipopolysaccharide
Cytokines
Antioxidant

Abstract

The therapeutic effect of hinokitiol mitigating lipopolysaccharide (LPS)-induced lung injury was investigated in mice. A total of 30, (6-week) Swiss albino male mice were obtained and acclimatised for one week. The mice were divided randomly into five groups with six animals each. Group I reserved as a normal control (NC) and was given normal saline orally, while Group II reserved as a disease control (DC) and was given LPS (10 µg/kg BW) by the oro-pharyngeal route on the 6th day of the trial, Groups IV hinokitiol low dose (HLD) and hinokitiol high dose (HHD) were kept as hinokitiol treatment @ 50 mg/kg BW and 100 mg/kg BW, (i.p. route), respectively, daily while Group III (HPS) was administered only hinokitiol (@ 100 mg/kg BW, (i.p. route) daily. On the 7th day, body weights were recorded, blood samples were taken for haematological analysis and mice were sacrificed to obtain bronchoalveolar lavage fluid for assessing inflammatory cells and lung weights. Antioxidant markers, cytokine profiles, histopathology, immunohistochemical assays and western blotting were all performed on the lung samples.

The present study revealed a substantial (p<0.05) alteration in relative and absolute lung weights, haematology (TEC, TLC, Hb and PCV) BALF (total cell count, neutrophils, macrophages and lymphocytes) inflammatory cytokines (IL-6; TNF-α; IL-1β; IL-22; IL-17; IFN-γ; TGF-β1 and IL-10) antioxidant profile (nitrite assay, TBARS, SOD, GSH and catalase), immunohistochemistry (Nrf-2; NF-κB; TNF-α and COX-2) and histopathology of mice treated with LPS (DC group) when compared to NC group and there was a significant improvement in treatment groups HLD and HHD. Groups HLD and HHD showed significant improvement in all the parameters in comparison to the DC group. The values of group HPS and NC group were comparable without any significant difference. Damaged alveoli with restricted lumen, emphysema, thickened intra-alveolar septa, pulmonary oedema and significant insinuation of inflammatory cells in the lung parenchyma when compared to Group I. In a dose-dependent manner, these alterations were reversed in the hinokitiol treatment groups HLD (@ 50 mg/kg BW) and HHD (@ 100 mg/kg BW). In group DC, immunohistochemical investigation revealed amplified expression of NF-κB, TNF-α and COX-2 but declined countenance of Nrf-2 which was considerably improved in treatment groups HLD and HHD.

Finally, hinokitiol was revealed to have powerful anti-inflammatory and antioxidant properties when used to treat LPS-induced ALI. The results showed significant improvement in the HHD (@ 100 mg/kg BW) group, which was evident in this study by reducing pro-inflammatory cytokines and restoring antioxidant enzymes, possibly hindering the initiation of the NF-κB signalling path and positively regulating the Nrf-2. As a result, hinokitiol can be employed as a pulmonary inflammation prevention strategy.

1. Introduction

Acute lung injury (ALI) and its additional unadorned type of acute respiratory distress syndrome (ARDS) leads to significant pain and transience in censoriously diseased patients (Johnson and Matthay, 2010). ALI is illustrated by rapid onset of bilateral pulmonary infiltrates of non-cardiogenic origin, respiratory failure due to alveolar collapse and hypoxemia (Guo *et al*., 2014). Further, there is a breach of the alveolar-endothelial barrier and resultant pulmonary oedema associated with proteinaceous alveolar exudates (Mei *et al*., 2007). Cytokines are vital regulators in the disease progression of LPS-induced lung injury, through interleukin (IL-6) and tumor necrosis factor-α (TNF-α) as pro-inflammatory cytokines upregulated majorly (Kumari *et al*., 2015). The consequences of ALI/ARDS are primarily subsidized by bilateral thoracic oedema, which results in the arrest of gas exchange, collapsed alveoli, and hypoxia (Am Lee *et al*., 2019).

Lipopolysaccharide (LPS) chemical entity existing in gram-negative bacteria cell walls (Kumari *et al*., 2015), is self-possessed with two components: the 'O'-antigen side chain and the core. The lipid A

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fraction is a polysaccharide core, it retains the toxicity of LPS (Szarka *et al*., 1997). Toll-like receptor 4 (TLR4) residing on alveolar macrophages and epithelial cells that spot LPS in lung infection, thus playing a majority of the innate immunity of the lungs (Luo *et al*., 2023) by activating various intracellular signalling cascades like NF-κB; MAPK; activator protein 1 (AP-1) (Hallstrand *et al*., 2014). Further, this acute inflammation leads to oxidative stress by rapid depletion of endogenic antioxidants such as SOD, catalase and glutathione peroxidase (GPx) (Freire *et al.,* 2023).

Hinokitiol is a natural monoterpenoid out-of-the-way from *Chamaecyparis taiwanensis*. It is also found in the heartwoods of trees in the family Cupressaceae. It is also known as β-thujaplicin. Hinokitiol has bactericidal, fungicidal, viricidal and anticancer activities (Potocka *et al*., 2023) and is extensively used as an antimicrobic mediator in hair tonics, toothpaste, make-ups and food. Also, hinokitiol hinders the production of pro-inflammatory cytokines like TNF-α by regulating the NF-κB through different mechanisms (Shih *et al*., 2014). According to existing literature, the therapeutic success rate for treating ALI/ARDS remains low, highlighting the need for further pharmacological research. Given hinokitiol's demonstrated anti-inflammatory properties, this study was designed to investigate its potential in mitigating LPS-induced lung injury in a mouse model by modulating inflammatory pathways to prevent ALI.

2. Materials and Methods

2.1 Animal model

An acute study was conducted on a total of 30 male Swiss albino mice, weighing around 22-30 g of the same age (8-12 weeks old;) procured from M/s Jeeva Life Sciences, Hyderabad. Animals persisted in polypropene cages with a provision of 12 h photoperiod cycle; at the animal house, at Dept. of Veterinary Pharmacology and Toxicology C. V. Sc Hyd. under the hygienic condition with an ambient temperature of 22-24°C. Mice were kept for one week for acclimatization ahead of the start of the experiment. All over the experiment, the mice were fed commercial standard and sterile feed in the form of pellets and provided with ad libitum reverse osmosis water. The experimental protocol was permitted by the Ethical Committee Endorsement No. 4/24/C.V.Sc, Hyd, IAEC-mice/ 12.06.2021. All the mice were uniformly divided into 5 sets consisting of 6 mice respectively designed for 7 days. All sets were subjected to different treatments as scheduled mentioned below.

The study involved five groups, each consisting of six mice, subjected to different treatment regimens over seven days. Group I (Normal Control, NC) received oral administration of normal saline daily. Group II (Disease Control, DC) was exposed to lipopolysaccharide (LPS) at 10 µg/kg *via* the oropharyngeal route to induce acute lung injury. Group III (Hinokitiol Per Se, HPS) was treated with hinokitiol alone (100 mg/kg, i.p route) for seven days. Group IV (Hinokitiol Low Dose, HLD) received pretreatment of hinokitiol (50 mg/kg, i.p.) for seven days, with LPS exposure on the sixth day. Group V (Hinokitiol High Dose, HHD) was administered LPS on day six, followed by hinokitiol (100 mg/kg, i.p.) for seven days. In Groups II, III, and IV, LPS (50 µg in 50 µl of normal saline) was introduced through the oropharyngeal route on the sixth day. The LPS-induced acute lung injury model was developed based on previous research, with ketamine (8-10 mg/kg, i.p.) and xylazine (40-50 mg/kg, i.p.) used to induce general anaesthesia. Body weights were recorded 12 h after LPS exposure, followed by blood and bronchoalveolar lavage (BAL) fluid sample collection. Lung tissues were separated and stored for further analysis on the seventh day. A schematic representation of the experimental design is shown in Figure 1.

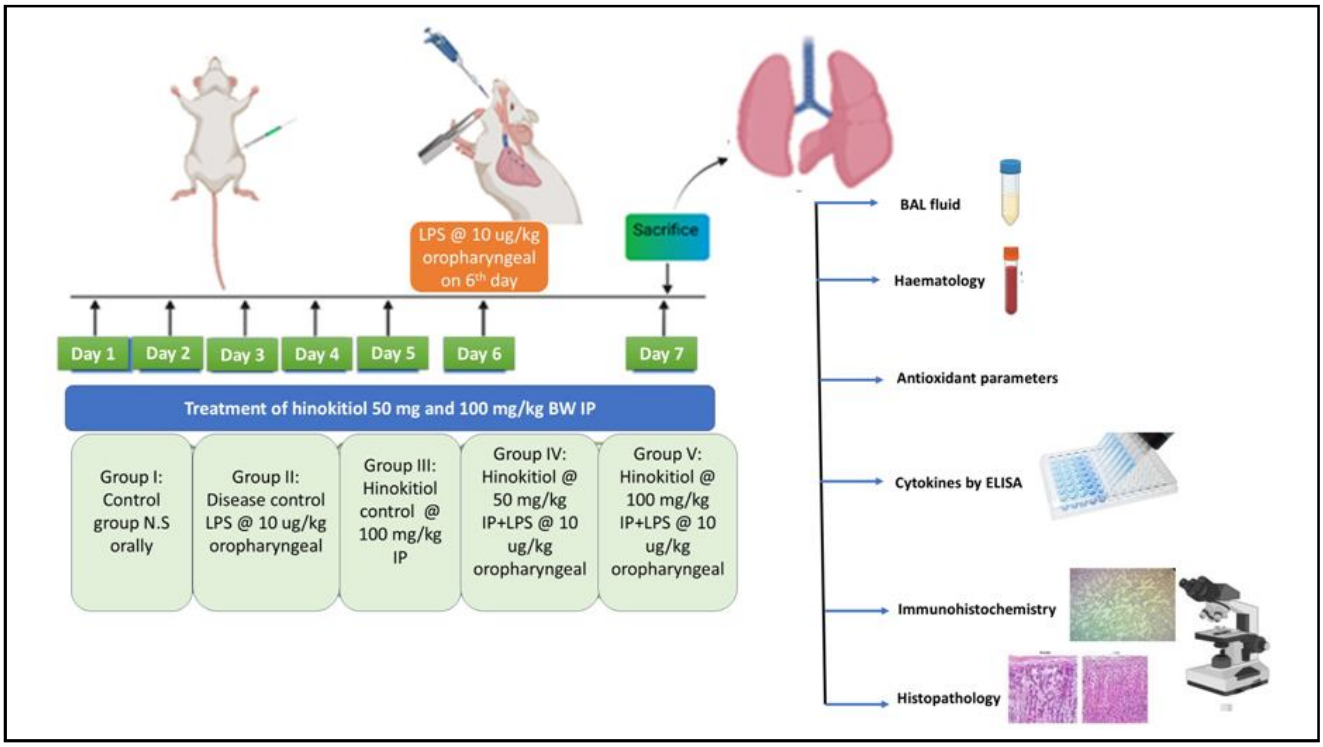


Figure 1: The experimental study design.

2.2 BAL fluid collection

From each experimental Group II representative mice, lungs were utilized for BALF collection and further processed in accordance with a study done by Van Hoecke *et al*. (2017). Take 0.5-1 ml of phosphate buffer solution (PBS) into a syringe and connect it to the catheter which was previously inserted into the trachea gently inject the PBS solution (pH 7.4) into the lung and aspirate the solution, repeat the procedure 3-5 times. The lavage fluid should be centrifuged for 5 min at 4000 g rpm at 4°C. Collected the supernatant for analysis of total leukocyte count and neutrophils count. The differential cells in BALF were counted with an automated cell counter. Lung tissues designated for oxidative stress analysis were homogenized in an ice-cold PBS buffer solution (pH 7.4). For ELISA assessments, the tissues were homogenized in a Tris-Triton buffer (pH 7.4) at 4°C.The leftover lung tissues were preserved in formalin for immunohistochemistry and histopathological examination.

2.3 Antioxidant profile

2.3.1 Thiobarbituric acid reactive substances estimation (TBARS)

In 500 µl of PBS, around tissue (100 mg) was homogenised.100 µl of supernatants of homogenized samples assorted in 100 µl of 8.1 per cent SDS; 100 µl of 20 per cent GAA; 750 µl of 0.8 per cent TBA; distilled water 950 µl. This mix is thermally heated for 60 min in a water bath at 100°C. Centrifuged for 10 min at 10,000 rpm after cooling. Then, 200 µl of supernatants were quantified at a wavelength of 532 nm and units of activity as nM/mg protein (Kumar and Reddy, 2012).

2.3.2 Estimation of glutathione (GSH)

Added 50 µl of homogenated lung tissue and 100 µl of GSH buffer to each well. Then each well was filled with Ellman's reagent and kept at room temperature for 10 min in the dark. The activity of the sample was measured using a 532 nm wavelength and units of activity of nM/mg protein (Chelpuri *et al*., 2022).

2.3.3 Estimation of super oxide dismutase (SOD)

Add 13 µl of PBS, followed by 6 µl of MTT reagent, 2 µl of homogenised samples and 15 µl of pyrogallol in each well. Incubate for 5 min and fill each well with 15 µl of DMSO. Quantified at 570 nm and Units of activity as IU/mg protein (Chen *et al.,* 2023).

2.3.4 Estimation of catalase activity

Add 50 µl of homogenate, 200 µl of 0.2M H_2O_2 and 250 µl of 0.01M PBS. Mixed them thoroughly with 1ml of dichromate acetic acid solution, and boil for 10 min in a water bath. The absorbance of green chromic acetate measured at 610 nm was compared to a blank after cooling and centrifuging for 5 min and values were expressed as IU/mg protein (Haritha *et al.,* 2013)

2.3.5 Estimation of nitrite

Took 100 µl of homogenized lung samples into a 96-well plate and added 100 µl Griess reagent in a dark place. Wait 15 min at room temperature and measure at a wavelength of 540 nm. Units of activity expressed as nM/mg protein Qamer and Bakar (2024).

2.4 Cytokine profile

The lung homogenate was analyzed for multiple cytokines, including proinflammatory markers (IL-1β, IL-6, IL-17, TNF-α, and IFN-γ) and anti-inflammatory markers (IL-10 and IL-22) were measured using a quantitative sandwich enzyme immunoassay method with the use of thermal fischer scientific ELISA kit, purchased from invitrogen.

2.5 Histopathological studies

Lung tissues were fixed in 10% NBF, rinsed overnight, dehydrated in graded alcohol, cleared with xylene, and embedded in paraffin at 55-56°C for histopathology. The paraffin blocks cut into 5-micron-thick slices were with microtome. The sliced pieces were placed on grease-free glass slides that had been pre-coated with Mayer's egg albumin and incubated for 12 h at 37°C. The stain Hematoxylin and Eosin were utilised to treat and stain the preserved tissues (Singh and Sulochana, 1996). The severity and extent of lung injuries were assessed using a 0-5 point scale, for separate categories indicating congestion, emphysema, damage of bronchioles and alveoli, and infiltration of inflammatory cells.

2.6 Immunohistochemical studies

The kit method was used to execute immunohistochemistry for quantitative measurement of NF-κB, TNF-α, COX-2, and Nrf-2 in lung tissues (Kumar *et al*., 2014).

2.7 Statistical analysis

The results of the trials are revealed as mean ± SE values. The arithmetical analysis was directed using Graph-Pad Prism (version 5.0) software, the test was applied one-way ANOVA for statistical difference followed by a Tukey's multiple comparison test and test results were checked for a different level of significance at $p<0.05$.

3. Results

3.1 The lung indices of lipopolysaccharide-treated mice are improved by hinokitiol

The hinokitiol effects were estimated in a lung-damage model caused by oropharyngeal LPS injection. When compared to NC, the mean body weight in DC was substantially lower ($p<0.01$). Furthermore, since the body mass index alterations in hinokitiol-administered mice were less significant than in the group DC, hinokitiol exhibited possible protection (Figure 2a). The effects were analytically not significant for HLD and HHD ($p<0.01$); despite the fact that they were dose-dependent. Average absolute lung weights (mg) in DC were highly substantially raised ($p<0.001$); when related to the NC. The results, however, were dose-dependent of HLD, and HHD was analytically substantial ($p<0.001$; Figure 2b). The pathognomic lesion is pulmonary oedema, which is characterized by an increased lung index (relative lung weights). The index of the lungs of DC was observed to be considerably more advanced ($p<0.01$) than that of the NC group, showing that LPS induction was successful (Figure 2c). In contrast, as compared to DC, the lung index of treatment groups was analytically decreased in HHD ($p<0.05$) and no significance with HLD.

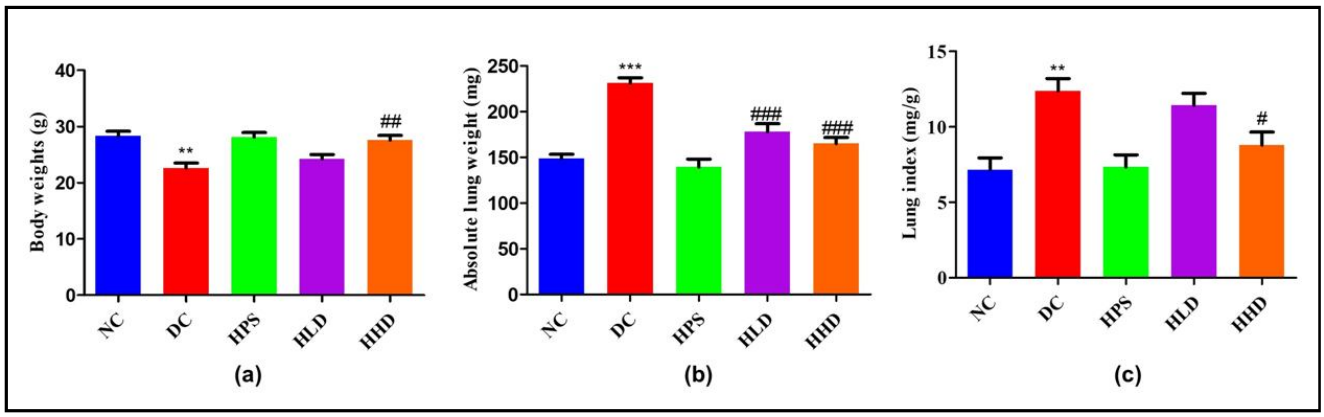


Figure 2: Hinokitiol's effect on lung index parameters (a) Body weights (b) Absolute lung weight, and (c) Lung index.

3.2 The LPS-induced changes in BALF parameters are normalised by hinokitiol administration

Cytological characteristics in BALF were influenced significantly after oropharyngeal by LPS treatment. Total cell, lymphocyte, neutrophil and macrophage ($p<0.001$) counts were considerably greater in DC mice than NC mice. In the assessment of DC, hinokitiol delivery led to substantial decreases in total cell ($p<0.01$in HLD and HHD), lymphocyte ($p<0.001$ in HHD; no significance with HLD), neutrophil ($p<0.01$ in HLD; $p<0.001$ in HHD), macrophage ($p<0.05$ at HLD; $p<0.001$ in HHD) counts (Figures 3a-d). These findings suggest that hinokitiol inhibits LPS-induced lung damage by directing macrophages, neutrophils and lymphocytes. Surprisingly, BALF characteristics *per se* were identical to those in the NC group, implying that this prospective chemical is safe.

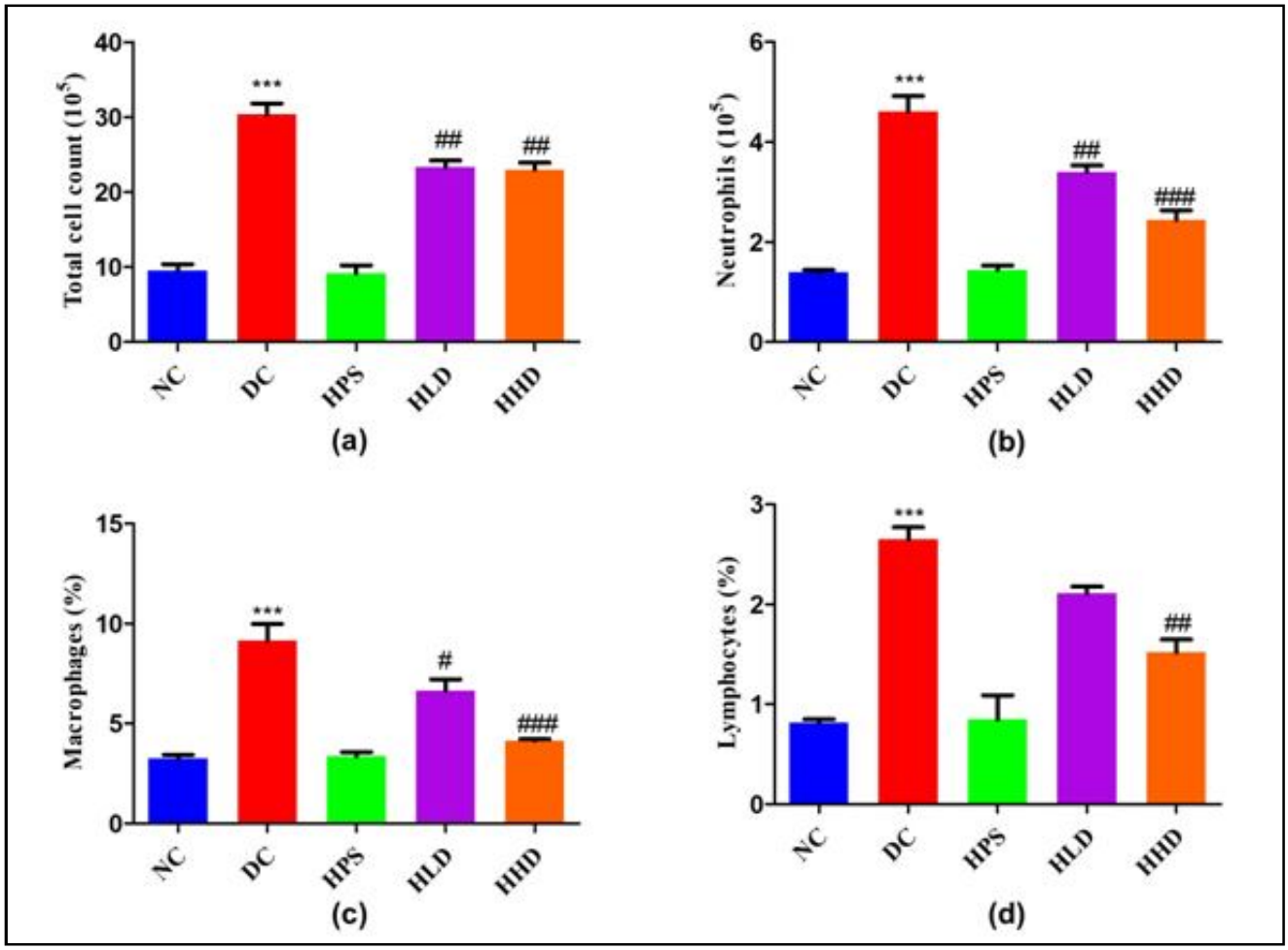


Figure 3: Hinokitiol's effect on BALF parameters (a) TCC, (b) Neutrophils, (c) Macrophages, and (d) Lymphocytes.

3.3 The LPS-induced changes in blood parameters are normalised by hinokitiol administration

The TEC (10^6/ml) and Hb (g/dl) in DC ($p<0.05$); TLC (10^3/cu.mm) and hematocrit values (%) in DC were considerably ($p<0.001$) less than the NC. In contrast to the DC, hinokitiol therapy resulted in a substantial no significance in TEC and TLC (HLD; HHD), (Figures 4a and 4b). The Hb and Hematocrit values in hinokitiol treatment groups were not significant in HLD and $p<0.05$ in HHD (Figures 4c and 4d).

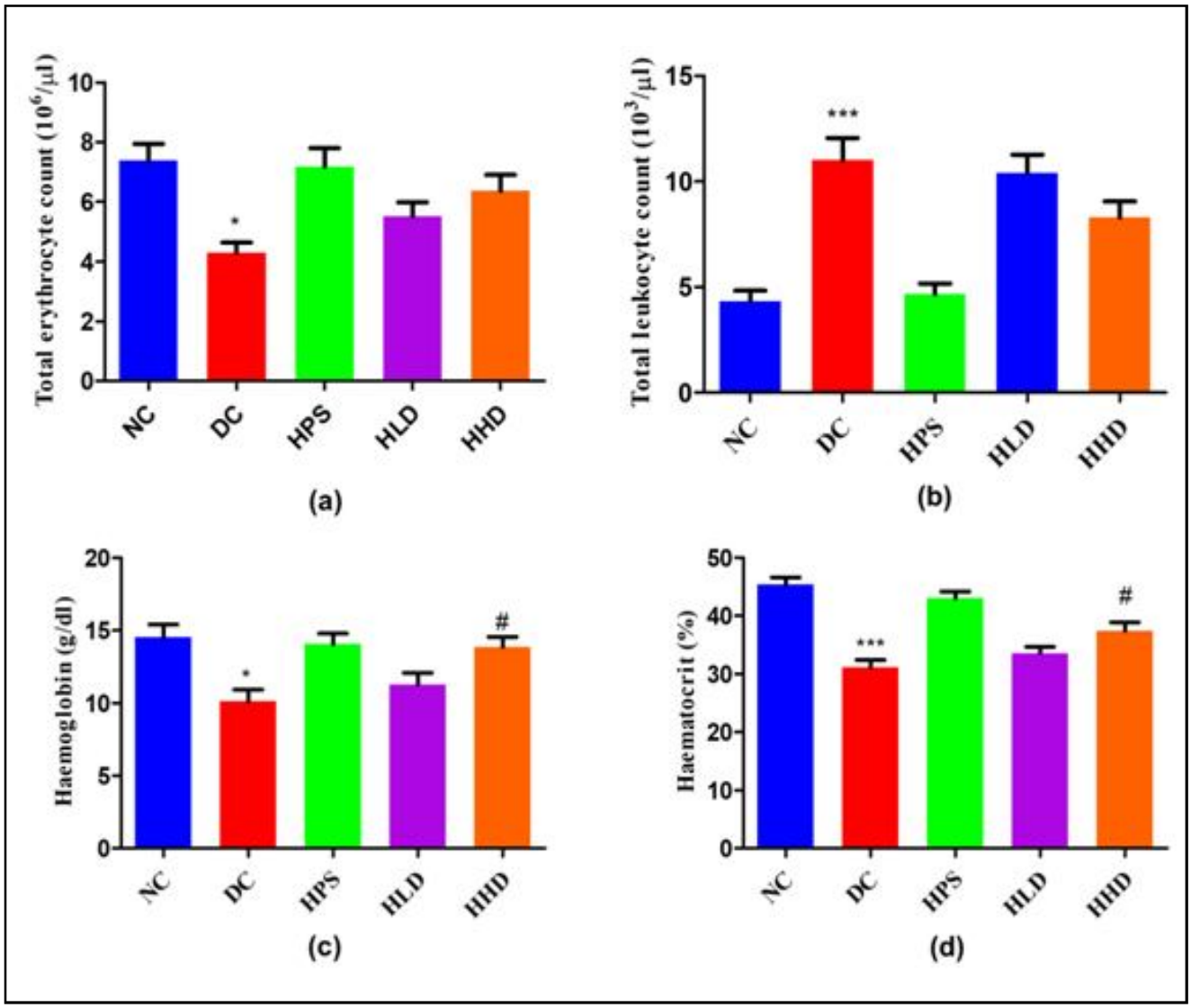


Figure 4: Hinokitiol's effect on haematology (a) TEC, (b) TLC, (c) Haemoglobin, and (d) Haematocrit.

3.4 Hinokitiol attenuated oxidative stress and nitrosative stress

The concentration of MDA in the group DC was substantially greater ($p<0.001$) than NC, as illustrated in Figure 5a. In contrast to the group-DC, hinokitiol action caused a substantial reduction ($p<0.001$ at HLD and HHD) of MDA concentration. SOD activity in DC was highly significantly lesser ($p<0.001$) than in the NC, while in HLD and HHD increased with substantial ($p<0.05$); highly substantial ($p<0.001$) levels than DC. Furthermore, in group DC, GSH concentrations were substantially lesser ($p<0.001$) than NC (Figure 5b). In comparison to the DC group, hinokitiol management led to a substantial rise ($p<0.001$ HLD and HHD) in GSH concentrations (Figure 5c). The DC group had considerably lower catalase concentrations ($p<0.001$) than the NC. In the assessment of the DC, hinokitiol action resulted in a substantial surge ($p<0.001$ at HHD) in catalase concentration, although the decline at HLD was significant ($p<0.01$) (Figure 5d). Further, the tissue nitrite concentrations in DC revealed a substantial ($p<0.001$) rise to the DC. Whereas, HLD, HHD have shown a significant decrease than DC and these values reflected progressive decline in a dose concentration method ($p<0.01$ and $p<0.001$) (Figure 5e). Per se group levels of these biological markers were normal, suggesting that they were safe. The antioxidant and anti-nitrosative stress possessions of hinokitiol may be responsible for the observed protection, according to these findings.

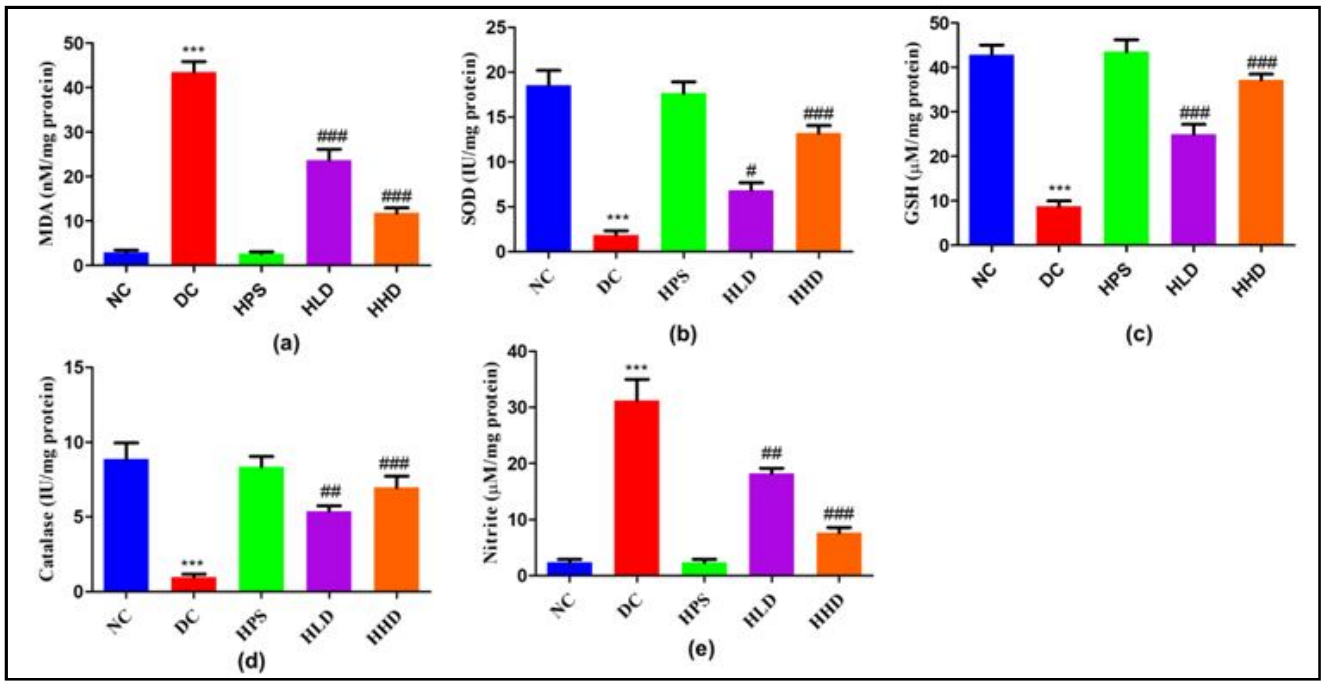
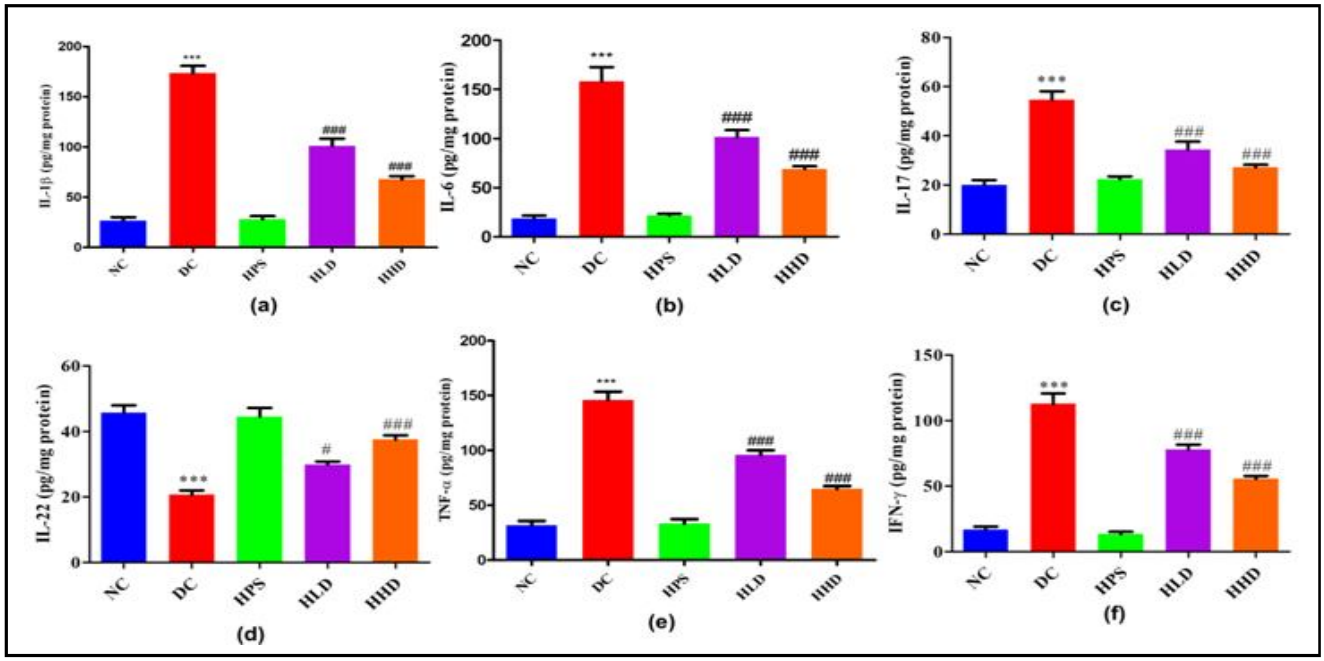


Figure 5: Hinokitiol's effect on LPS-induced oxidative-nitrite stress (a) MDA, (b) SOD, (c) GSH, (d) Catalase, and (e) Nitric oxide.

3.5 Hinokitiol reduced inflammatory signalling in LPS treated mice

In LPS-treated mice, hinokitiol decreased inflammatory signals. It spurred us to look into the involvement of inflammatory signals. Using an ELISA kit, we measured the levels of proinflammatory cytokines in pulmonary tissues. These cytokines were found to be elevated in group-DC animals. In relation to NC, LPS led to a substantially greater level with $p<0.001$ in IL-1β; IL-6; TNF-α; IFN-γ; IL-17 and IL-22 were significantly raised. Hinokitiol was used in our pharmaceutical intervention. IL-1β with $p<0.001$ in (HLD and HHD), IL-6 with $p<0.001$ in (HLD and HHD), IL-17 with $p<0.001$ in (HLD and HHD), IL-22 with $p<0.05$ in HLD and $p<0.001$ in HHD), TNF-α with $p<0.001$in (HLD and HHD), IFN-γ with $p<0.001$ in (HLD and HHD) and TGF-β1 with $p<0.001$ in (HLD and HHD) as markers of inflammation in comparison to the DC group. IL-10, an anti-inflammatory cytokine, was considerably ($p<0.001$) lower in DC than in NC, despite the fact that HLD had no effect and HHD had an effect on DC with ($p<0.001$) (Figures 6 a-h). We saw no abnormal countenance of cytokines in the group *per se*, indicating that this novel phytoconstituent is safe. Our findings clearly show that hinokitiol has anti-inflammatory properties.



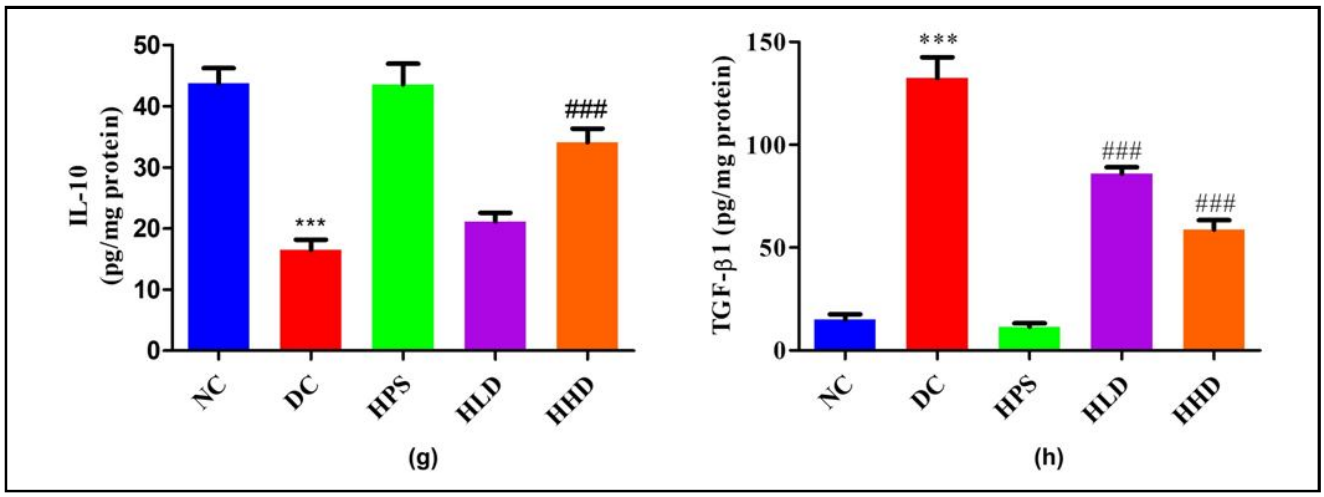


Figure 6: Hinokitiol's effect on pro-inflammatory cytokines (a) IL-1α, (b) IL-6, (c) IL-17, (d) IL-22, (e) TNF-α, (f) IFN-γ, (g) IL-10, and (h) TGF-β1.

3.6 Morphology of lung and histopathological analysis

We noticed aberrant lung morphology in the DC group, as well as severe congestion and oedema when related to animals from the NC group. The lung morphology of hinokitiol-treated mice, on the other hand, was less impacted by LPS. Hinokitiol did not induce any aberrant responses when administered alone to *per se* group, showing that the treatment is safe (Figure 7A). Histopathological study of lung tissue sections in NC revealed the presence of numerous alveoli with normal histological architecture, intact bronchioles surrounded by epithelium, normal alveolar septa, and normal appearance of venules (Figure 7B, a). DC's lung architecture was significantly altered, with severe congestion and emphysema, a collapsed alveolar wall, an accretion of edematous fluid in the alveolar lumen, a confined bronchiole, an alveolar lumen with flake-off epithelium, peri-bronchiole oedema, mild hyperplasia, and infiltration of inflammatory cells (Figure 7B,b). HLD had moderate lung architecture abnormalities, moderate alveolar and bronchial lumen, moderate thickness of alveolar septa, emphysema, and inflammatory cell infiltration (Figure 7B,d). Alveolar epithelial cell rebuilding, typical bronchial epithelium, normal venule morphology, mild congestion, emphysema and inflammatory cell infiltration were all seen in HHD lung slices (Figure 7B,e). The histoarchitecture of *per se* lung pieces was identical to that of NC lung sections, which exhibited a normal architecture (Figure 7B,c).

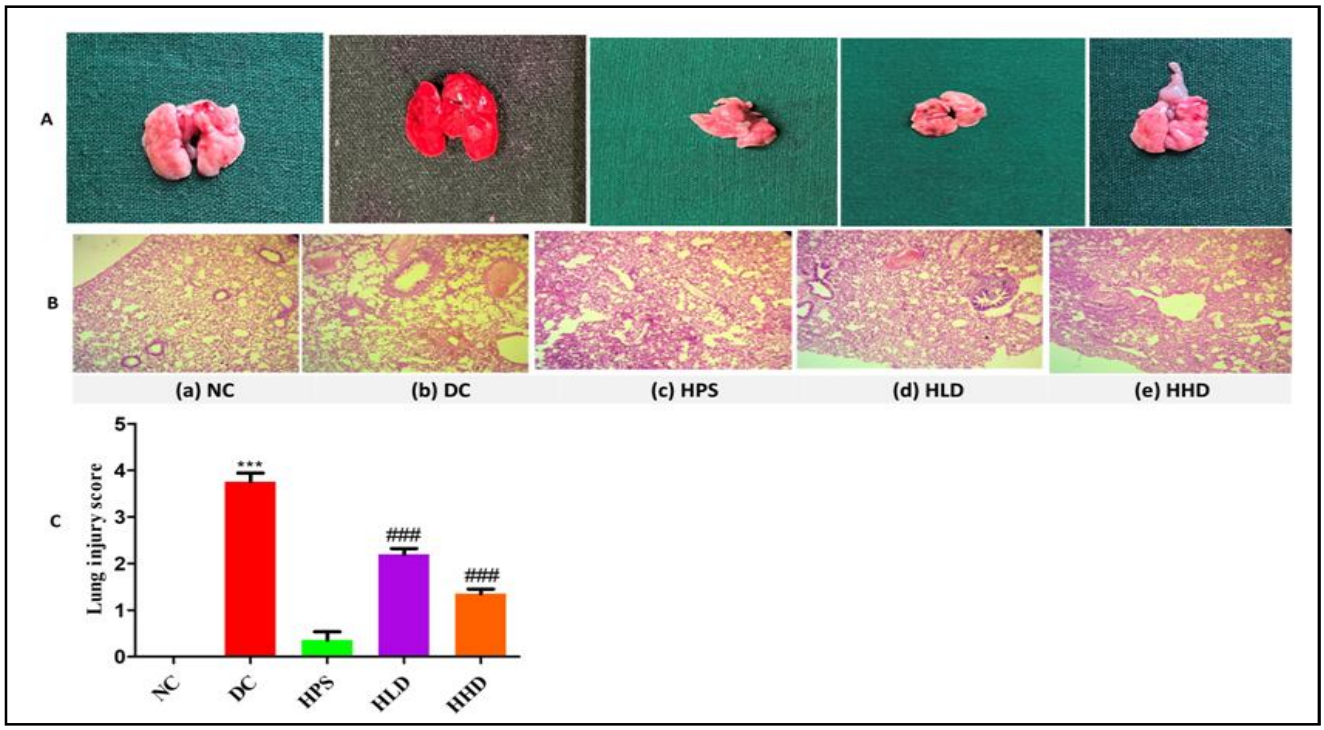


Figure 7: A. Lung morphology, B. Histopathology examination, and C. Lung injury score, (a) NC, (b) DC, (c) HPS, (d) HLD, and (e) HHD.

3.6.1 Lung injury score

To assign a lung injury score to different treatment groups, we observed the quantity of inflammatory cells infiltrating the bronchial and alveolar lumen, accumulation of edematous fluid in and around the bronchial and alveolar lumen, the thickness of inter-alveolar septa and haemorrhages in different lobes, and emphysema. A 0-5 point scale was used to rate the severity and degree of lung damage. When compared to NC, DC showed highly significant (p<0.001) lung injury, while HLD and HHD showed mild and moderate lung injury. Furthermore, the *per se* group's histological characteristics and score were similar to the NC group, demonstrating that our intervention had no harmful consequences (Figure 7C).

3.7 Immunohistochemistry profile

Immunohistochemistry of lung sections of DC revealed intense positive immunoreactivity for the COX-2 protein, TNF-α protein and NF-κB protein, *i.e.*, high intensity of brown color compared to section from NC. *Per se* which showed mild immunostaining, while sections from HLD and HHD showed moderate to mild immunostaining of COX-2 expression protein; TNF-α protein; NF-κB protein. In contrast, Nrf-2 revealed a significant reduction in immunostaining for the Nrf-2 in DC when compared to NC. The treatment groups HLD, HHD, and HPS showed intense positive immunoreactivity for the Nrf-2 protein. The expression of Nrf-2 in lung tissue suggested a positively regulated antioxidant profile. The immune reactivity of these markers are shown in Figure 8.

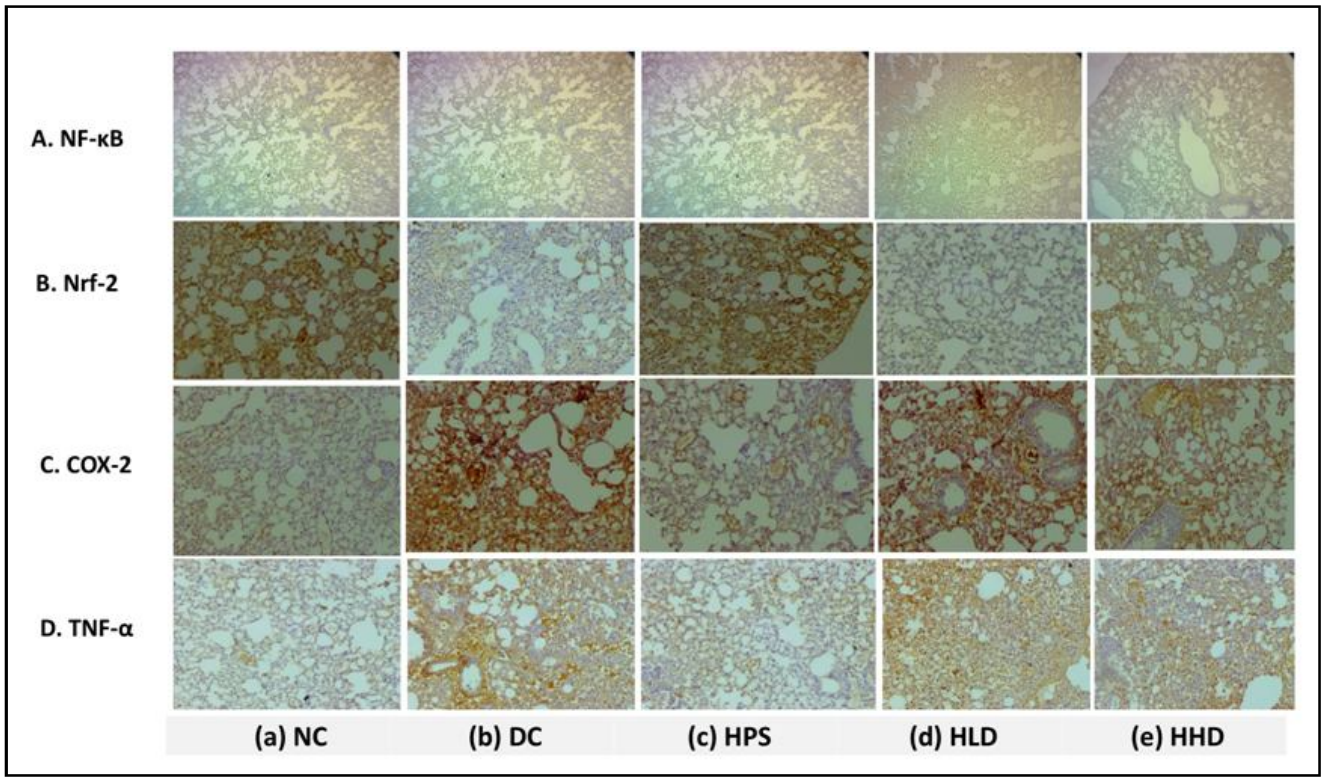


Figure 8: Hinokitiol's effect on immune histochemistry of A. NF-κB, B. Nrf-2, C. COX-2, D. TNF-α, (a) NC, (b) DC, (c) HPS, (d) HLD, and (e) HHD.

4. Discussion

ALI is a serious clinical consequence with collapse of the alveolar-capillary membrane, with high morbidity rates and death (Renushe *et al*., 2022). Epithelial integrity breakdown, neutrophil build-up, pulmonary oedema, severe hypoxemia, and acute pulmonary inflammatory responses are trademarks of the development of ALI. Microbial infection, sepsis, trauma and ischemia all produce epithelial integrity breakdown, neutrophil buildup, pulmonary oedema, severe hypoxemia, and acute pulmonary inflammatory responses, involved in the progression of ALI (Puneet *et al*., 2005). The most often utilised clinically related severe lung damage model in laboratory animals for reviewing the pathophysiologic trail for the advance of ALI is the administration of lipopolysaccharide (LPS). Acute LPS exposure activates the innate immune system, causing an influx of inflammatory cells, cytokine release, and increased lung capillary permeability, all of which contribute to pulmonary oedema progression (Matute-Bello *et al*., 2008). The aetiology of apoptotic alterations in the lung tissue of ALI patients is thought to be due to a delay and abnormal production of neutrophil granulocytes, as well as irregularities in the release of cytokines. Infiltrated neutrophils, which are important in the pathophysiology of neutrophil-mediated ALI damage and for oxidant/antioxidant disproportion by free radicle (Grommes and Soehnlein, 2011). Furthermore, pro-inflammatory cytokines have been found to be released by macrophages and neutrophils. In the later stages of ALI, these may be significant intermediaries at the beginning of the inflammatory reaction, modulating fibroblast activity and stress caused by oxidation (Pinheiro *et al*., 2015; Blondonnet *et al*., 2016). For a higher therapeutic success rate in the treatment of ALI/ARDS, a variety of pharmacological treatments are necessary (Xiao *et al*., 2021). In this study used hinokitiol as a pre-treatment to reduce the negative effects of LPS and to assess the clinical efficacy of hinokitiol against LPS-induced lung damage. The antioxidant and cytokine profiles, as well as body

weights, absolute lung weights, lung index, haematological parameters, bronchoalveolar lavage fluid (BALF) analysis, and antioxidant and cytokine profiles, were evaluated. At the conclusion of the experiment, lung samples were obtained for histological and IHC evaluation.

The body weights of LPS-treated mice (@ 10 µg/kg BW) were set up to be significantly lower (p<0.01) than those of NC mice given normal saline (NS) orally in the current experimental investigation. Increased pro-inflammatory cytokines and oxidative stress may have contributed to weight loss by causing fast catabolism and tiredness. These results are agreed with reports of previous investigations (Karkale *et al*., 2018; Am Lee *et al*., 2020). Body weight growth in mice given low doses of hinokitiol (HLD) and high doses of hinokitiol (HHD) is dose-dependent and similar to the mean body weights of mice in the NC group. This positive effect could be attributed to the anti-inflammatory and antioxidant characteristics of hinokitiol.

Lung oedema is a pathognomonic lesion of LPS-induced ALI, characterised by significantly elevated absolute and relative lung weights, which may be owed by amplified oxidative stress, inflammatory cytokines, capillary damage and favouring the leakage of protein and other cellular compartments into the lung interstitium (Xinqiang *et al*., 2020). In the present study, the LPS treated group mice had a substantial, increase in absolute and relative lung weights when related to the NC mice. The large increase in absolute and relative lung weights may be caused by a strong inflammatory response (expression of numerous cytokines storm), and these findings are consistent with those of Karkale *et al*. (2018), Am Lee *et al*. (2019) and Xinqiang *et al*. (2020). Absolute organ weights were notably reduced (p<0.001) in the groups treated with hinokitiol to HLD and HHD. The antioxidant system may be restored with reduced oxidative damage caused by free radicals and hinokitiol's anti-inflammatory activity, as evidenced by a significant (p<0.05) reduction in relative organ weight or lung index in HLD and HHD. During lung damage, neutrophils are the most frequent inflammatory cells, and they are known to show a perilous role in the course of most instances of ALI (Abraham, 2003). Through the production of iNOS, active alveolar macrophages and neutrophils release a lot of nitric oxide (NO), which damages lung tissue and secretory cells. TNF-α, IL-1β and IL-6, as well as other pro-inflammatory mediators, are known to be released by monocytes and macrophages, and these cytokines are known to initiate, amplify, and prolong lung injury (Goodman *et al*., 2003). In LPS-treated rats, augmented accumulations of total cells, neutrophils, macrophages, and lymphocytes were found in BALF, which are linked to the production of pulmonary oedema during inflammation. The total cell count, neutrophils, macrophages, and lymphocytes were all considerably (p<0.001) higher in the LPS group in our study. The treated mice outperformed the untreated control mice. The administration of LPS may overstimulate the host immune response, allowing cytokines from activated neutrophils to overwhelm the area, resulting in increased inflammatory cell infiltration. These results are concurrent with reports of Huang *et al*. (2019) research. When related to the LPS treated group, the hinokitiol treatment groups HLD and HHD showed a substantial (p<0.001) decrease in total cells, neutrophils, macrophages, and lymphocytes. Hinokitiol's anti-inflammatory and antioxidative capabilities may contribute to its ameliorative impact, implying that hinokitiol has a protective effect in lowering the increased pulmonary capillary permeability caused by LPS. There is currently no research literature on the influence of hinokitiol on BALF analysis, and the findings of this study may aid future scientific research.

The purpose of this research was also to see if hinokitiol could protect against haematological changes caused by LPS. When compared to NC, the LPS treated group had significantly lower TEC, Hb, and hematocrit levels, as well as a significantly higher TLC value. The decreased TEC might be explained by increased lipoperoxidation in the LPS-treated group, which was reflected in a significant rise in MDA concentration in the current study. Increased MDA may make RBCs vulnerable, causing them to be damaged and leading to anaemia. A decline in hematocrit per cent could be due to a faster rate of RBC breakdown. Inhaling LPS resulted in rapid and long-lasting neutrophilic airway inflammation, which resulted in an increase in neutrophils and other leucocyte cells. The chance of oxidative damage-induced cellular lysis increases as an outcome of the chemotactic effect of multiple chemokines and cytokines released by stimulated macrophages near the site of injury, resulting in decreased TEC, Hb and PCV. These findings are in line with Ayushi *et al*. (2024) observations. During the trial period, the treatment groups HLD, HHD showed considerable improvement in hematological parameters in a dose-dependent manner. Hinokitiol's hemoprotective effects may be attributable to its increased free radical scavenging and antioxidant activities.

The antioxidant profile (GSH, SOD, CAT, MDA and Nitrate levels) in LPS-induced ALI was also investigated. Glutathione (GSH) is a universal antioxidant that enhances the defence against reactive oxygen species (ROS) by scavenging free radicals and so protects against external harmful damage (Anilkumar *et al.,* 2013). It is done by immediately donating a hydrogen atom and neutralising free radicals ($OH^{\bullet}$). GSH is abundant in the epithelial lining of the lungs and protects against a range of inhaled oxidants (Namratha *et al.,* 2021). Nitrite is a nitrosative stress marker that plays a vital part in the pathophysiology of acute cases, where nitrate levels are useful for indirectly measuring NO levels, which are amplified by the overactivation of an important measure for nitrosative stress in lung tissue by inducible nitric oxide synthase (Anchi *et al*., 2018). By catalysing the dismutation of superoxide radicals to form intermediate hydrogen peroxide, superoxide dismutase (SOD) inhibits lipid peroxidation and has a protective function in tissue necrosis. Malonaldehyde (MDA) is a lipid peroxidation by-products that has been used as a reaction rate barometers in studies of lipid peroxidation.Catalase (CAT) is a crucial enzyme in the cellular antioxidant protection system because it catalyses the conversion of H_2O_2 to water and oxygen, both of which are significant antioxidants (Gebicka and Krych-Madej, 2019). When comparing the LPS treated group mice to the NC group mice, there was a substantial drop in SOD, GSH and CAT, as well as an increase in nitrite and MDA in lung tissue. The pathogenesis and aggravation of lung disease are caused by airway inflammation, and oxidative stress is a causal agent in a variety of pathological circumstances, including the aetiology and aggravation of pulmonary disease. A high quantity of various ROS is created in critical circumstances such as sepsis or ALI, resulting in oxidative stress, which plays a vital role in sepsis, and free radicals are created (Crimi *et al*., 2006). ROS affects the cell membrane's lipid layer, causing unsaturated fatty acids in cellular membranes to peroxide, releasing cytotoxic compounds like MDA and compromising cellular integrity. ROS inactivates antioxidant enzymes like SOD, GSH and CAT (Jyothi Kumari and Anoop Kumar, 2024). Oxidants activate Nrf-2 transcription factors in lung tissue, which increase antioxidant response elements (AREs)-mediated production of antioxidant enzymes and cytoprotective proteins and preserve cellular homeostasis. The NF-κB and MAPK pathways are also activated by oxidants. (Lin *et al*., 2017). The current findings

were consistent with those of Rungsung *et al*. (2018) and Zhao and Du (2020), who discovered that inducing ALI and lung tissue damage increases MDA while decreasing GSH, SOD and CAT levels. LPS treatment causes a decrease in CAT, SOD and an increase in MDA and nitrite, which promotes oxidative damage in lung tissue due to excessive free radical formation, according to Gould *et al*. (2011). These studies back up what we have found in our current study. SOD, GSH and CAT expression, as well as nitrite and MDA levels, were considerably higher in the hinokitiol treatment groups. The findings of Xu *et al*. (2017), who revealed the protective role of hinokitiol in human corneal epithelial cells, back up these findings. In a hepatogenesis study conducted by Fathima *et al*. (2018), hinokitiol was discovered as an antioxidant mechanism against diethylnitrosamine.

The current study also estimated the levels of cytokine expression. Cytokines such as IL-1, IL-6, and TNF-α were considerably greater in the lung tissue of mice in the LPS disease group compared to mice in the normal control group. LPS is renowned for inducing severe lung injury by activating cytokines such as IL-6, TNF-α and IL-1β (Ding *et al*., 2019; Li *et al.*, 2019). LPS induces a flurry of inflammatory reactions in the early stages of lung impairment (Lee *et al*., 2016). Pro-inflammatory cytokines and their regulatory mechanisms have been established to be very effective in decreasing LPS-induced lung inflammation (Huang *et al*., 2019; Ding *et al*., 2019). Inflammatory cytokines have been found to be intricated in the pathophysiology of LPS-induced acute lung damage (Ran *et al*., 2014; Li *et al*., 2019). LPS increases signal transduction, subsequent in the initiation of the NF-κB transcription factor family and macrophages produce a large number of immunoregulatory chemicals, including pro-inflammatory compounds such as TNF-α, IL-1β and IL-6 (Amsa *et al.,* 2024). The innate immune system produces IL-6, which is one of the initial cytokines unconfined during the acute phase of ALI (Liu and Chen, 2016). IL-6 has a number of immunoregulatory actions that can aid the immune system's performance (Zhe *et al*., 2017). IL-1β is a pro-inflammatory cytokine generated mostly by macrophages that are complicated in inflammatory and allergy responses (Basak *et al*., 2005) and that often works in tandem with TNF-α. The inflammatory cytokine IL-1β occupies a key position at the commencement of the cytokine overflow (Goodman *et al*., 2003). TNF-α may trigger an inflammatory response and harm vascular endothelial cells. Enzyme leakage occurs when TNF-α interacts with a TNF-α receptor in lung tissue (Marsh and Wewers, 1996). In the mouse model of LPS-induced ALI, suppression of TNF-α and IL-1β effectively turns down pulmonary damage as per the findings of Mei *et al*. (2007) and Chen *et al*. (2008).

In this study, the concentrations of pro-inflammatory cytokines were evocatively greater ($p<0.001$) in the LPS-induced acute lung injury group and were shown to be more severe than normal control mice. These results support the findings of Bhardwaj *et al*. (2020) and Shi *et al*. (2020). After pre-treatment with hinokitiol, the countenance of IL-1β, IL-6, and TNF-α was dramatically decreased ($p<0.001$) in different dosage groups of mice, showing that NF-κB activation, translocation, and transcription of pro-inflammatory cytokines were blocked. These findings in hemorrhagic shock-induced liver damage were corroborated by Lu *et al*. (2019). Hinokitiol was also shown to prevent periodontic bone loss in mice with ligature-induced experimental periodontitis (Hiyoshi *et al*., 2020). IL-10 is a potent anti-inflammatory cytokine that inhibits macrophage and T-cell activation while also lowering the creation of inflammatory cytokines, making it important in the prevention of inflammatory and autoimmune diseases. IL-10 deficiency or abnormal expression might increase the inflammatory response to a microbial challenge, indicating the severity of lung injury. When related to the NC, the LPS treated group's IL-10 expression was considerably ($p<0.001$) lower. According to Wu *et al*. (2009), IL-10-mediated preservation of the injured lung was most likely due to a reduction in neutrophil activation and a decrease in macrophage inflammatory protein-2 production (MIP-2). When related to the LPS-treated group, the hinokitiol high dose set had significantly ($p<0.001$) progressive stages of IL-10.

Immunohistochemistry was used in this investigation the expression of inflammatory markers in LPS-induced ALI. TNF-α is a TLR-4 ligand that activates the NF-κB intracellular signalling pathway, resulting in the secretion of pro-inflammatory cytokines and COX-2, which enhances prostaglandin release and causes inflammation (Shishodia, 2013). In ALI/ARDS, NF-κB is a key transcript factor implicated in the development of inflammation. LPS phosphorylates IKB in the cytoplasm, allowing it to enter the nucleus, where NF-κB connects to certain promoter gene sequences, resulting in brown immunohistochemistry expression (Li *et al*., 2019). Crosstalk between Nrf-2 and the transcript factor NF-κB, which is the key modulator of inflammatory responses and many aspects of innate and adaptive immune activities, has been linked to Nrf-2's anti-inflammatory effect. The LPS-exposed group II animals had significantly higher levels of Nrf-2 protein expression than the NC group. The cell's innate antioxidant enzyme system keeps Nrf-2 inactive within the cytosol. Nrf-2 is activated and translocated to the nucleus when the body is exposed to oxidative stress, where it regulates the genes involved in the antioxidant enzyme system (Ali *et al*., 2020). When related to the NC group, the appearance of NF-κB, TNF-α and COX-2 in the DC group revealed a considerably higher intensity of countenance by revealing a brown colour. While sections from hinokitiol pre-treated groups HLD and HHD revealed moderate to mild immunostaining expression of NF-κB, TNF-α and COX-2. The expression of Nrf-2 revealed a marked reduction in immunostaining in DC in contrast to NC and treatment groups HLD and HHD showed intense positive immunoreactivity for Nrf-2 protein. The expression of Nrf-2 in lung tissue suggested a positively regulated antioxidant profile.

The gross pathology of the lungs in group II revealed considerable oedema and congestion, which was supported by the findings of Karkale *et al*. (2018). While the lungs of group IV and V revealed a dose-dependent reduction in the degree of congestion and oedema. Hinokitiol treatment minimised gross changes by reducing inflammatory responses and preventing neutrophil and macrophage migration. The aforementioned findings were validated by a histopathological examination of lung tissue. Acute lung inflammation with significant inflammatory cell infiltration, thickening of intra-alveolar septa, ruptured alveoli and bronchioles, constricted bronchial lumen with epithelial desquamation, congestion, and emphysema were observed in the LPS-treated group, indicating serious lung injury (based on lung injury score 5). Inflammatory cell infiltration was reduced, pulmonary oedema was cleared, and ruptured alveolar structures were restored in hinokitiol treatment groups, implying that it has a powerful anti-inflammatory effect. In a gradient dependent manner, HLD and HHD dramatically amended the lung damage score when compared to DC.

In hinokitiol *per se* group, mice did not vary with normal control groups in all the parameters thus suggesting the safety of the compound. Further pretreatment with hinokitiol Hinokitiol, particularly at a high dose (100 mg/kg BW), effectively mitigated LPS-induced lung injury by suppressing NF-κB - driven inflammation, enhancing Nrf2-mediated antioxidant defense, reducing COX-2 expression, and restoring lung architecture.

5. Conclusion

We conclude that LPS causes ALI by insulting the alveolar epithelial-endothelial barrier, owing to pulmonary oedema and impairment of normal lung physiological function, by upregulating the NF-κB pathway (higher levels of expression of pro-inflammatory cytokines) and downregulating Nrf-2 (significantly altering oxidative stress parameters) and upregulating the NF-κB pathway (increasing the countenance of pro-inflammatory cytokines). Because of its considerable anti-inflammatory effect via downstream control of the NF-κB pathway and renewal of antioxidant capacity *via* upstream regulation of the Nrf-2 pathway, mice treated with hinokitiol at a high dose (100 mg/kg BW) were able to reduce LPS-induced ALI. Hence, hinokitiol could have promising therapeutic value for the prophylactic management of ALI.

Acknowledgements

We sincerely appreciate the PV Narsimha Rao Telangana Veterinary University Rajendranagar, Hyderabad, for its valuable support and keen involvement in this research.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Nagarjuna Gandham, Anilkumar Banothu, Kalakumar Bharani, Ravikumar Yadala, Amit Khurana, Ambica Gadige, Durga Veera Hanuman Donga and Akshatha Renushe (2025). Hinokitiol, a monoterpenoid, mitigates lypopolysachiride induced acute lung injury in mice *via* modulating TNF-α, COX-2, and Nrf-2/NF-κB pathway. Ann. Phytomed., 14(1):672-684. http://dx.doi.org/10.54085/ap.2025.14.1.65.**