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Antioxidant Activity of the *Tithonia diversifolia*, *Moringa oleifera*, and *Curcuma longa* Extract Combination Against Oxidative Stress in a Lipopolysaccharide-Induced Acute Lung Injury in Mice

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Abstract. The induction of acute inflammation in the lung tissue is triggered by lipopolysaccharide (LPS)-mediated activation of the TLR4 receptor. The combination of *Tithonia diversifolia* (T), *Moringa oleifera* (M), and *Curcuma longa* (C) extracts holds potential for synergistic anti-inflammatory and antioxidant effects. This study aimed to evaluate the effect of the aqueous TMC extract combination on reducing malondialdehyde (MDA) levels and enhancing Superoxide Dismutase (SOD) and Nuclear Factor Erythroid-2 Related Factor 2 (NRF2) expression in LPS-induced acute lung injury (ALI) in BALB/c mice. Thirty-six BALB/c mice were divided into five groups: negative control, LPS positive control, and three treatment groups receiving varying doses of the combined extract (TMC-1: 500/300/200; TMC-2: 200/500/300; TMC-3: 300/200/500 mg/kg BW). Treatments were administered orally for 14 days, followed by intraperitoneal LPS induction (0.5 mg/kg BW). Extract combination (TMC) significantly mitigated LPS-induced oxidative stress in a mouse acute lung injury model. Post-LPS therapy reduced MDA levels dose-dependently (16.96–8.68% vs. LPS 36.52%), restored SOD activity near normal (5.74–13.84% vs. LPS 25.23%), and normalized NRF2 expression (9.58-11.42% vs. LPS 17.11%). The TMC combination significantly reduced MDA levels and normalized the NRF2–SOD pathway, with the strongest SOD restoration at TMC-2 (5.74%). These results confirm the role of TMC in strengthening the endogenous antioxidant system. This study provides scientific evidence for the development of the TMC extract combination as a potential phytopharmaceutical agent to mitigate oxidative stress and support tissue repair in inflammatory conditions such as ALI. Ultimately, this research supports UN SDG 3 (Targets 3.4 and 3.b) by developing an affordable respiratory protective agent, while simultaneously advancing SDG 15 through the sustainable pharmacological utilization of local plant biodiversity.

Keywords: acute lung injury; lipopolysaccharide; oxidative stress; TMC extract

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INTRODUCTION

Inflammation is the body's natural protective mechanism for eliminating pathogens, repairing damaged cells, and responding to allergens or irritants (Chen et al., 2018). However, this response becomes harmful if left uncontrolled; unresolved acute inflammation can develop into chronic inflammation, leading to extensive tissue damage. This pathological condition is a primary trigger for various severe systemic diseases, ranging from sepsis, asthma, and type 2 diabetes to neurodegenerative diseases such as Alzheimer's, cardiovascular disorders like atherosclerosis, and autoimmune diseases (Jogpal et al., 2022). Globally, chronic inflammatory

diseases are recognized as the most significant cause of death worldwide, with studies indicating that over 50% to over 60% of all deaths are linked to inflammation-related diseases (Furman et al., 2019). Based on 2018 Riskesdas data and subsequent analyses, Non-Communicable Diseases (NCDs) in Indonesia account for 73% of national deaths, with inflammation-related diseases being the most dominant cause (Arifin et al., 2022).

Systemic inflammatory mechanisms are often triggered by Lipopolysaccharide (LPS), an endotoxin from Gram-negative bacterial cell walls that activates monocytes and macrophages through interaction with Toll-like Receptor-4 (TLR4) (Gorman & Golovanov, 2022). This activation triggers an intracellular signaling cascade via the Mitogen-Activated Protein Kinase (MAPK) and Nuclear Factor-Kappa B (NF-κB) pathways, resulting in the overproduction of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and increased Nitric Oxide (NO) (Kim & Lee, 2025). This condition is exacerbated by an imbalance between Reactive Oxygen Species (ROS) production and endogenous antioxidant capacity, marked by a surge in malondialdehyde (MDA) levels and decreased Superoxide Dismutase (SOD) enzyme activity. In vital organs such as the lungs, LPS exposure increases vascular permeability and neutrophil infiltration, triggering Acute Lung Injury (ALI) or Acute Respiratory Distress Syndrome (ARDS) with a high mortality risk due to gas exchange failure (Liu et al., 2025). Whilst conventional treatments, such as corticosteroids, are available, their utilisation is frequently impeded by the potential for severe adverse effects when used over an extended period. This has prompted the World Health Organization (WHO) to advocate for the integration of traditional medicine, given that approximately 80% of the population in developing countries relies on herbal remedies due to factors such as accessibility, affordability, and a superior safety profile (Aware et al 2022). In this context, the combination of *Tithonia diversifolia*, *Moringa oleifera*, and *Curcuma longa* (TMC) extracts offers a synergistic therapeutic solution through complementary anti-inflammatory and antioxidant mechanisms (Abdel-Aziz et al., 2016). This combination or polypharmacological approach is expected to modulate inflammatory responses more effectively than single-component use while minimising toxicity.

While the hepatoprotective and cardioprotective properties of these plants have been demonstrated, research on the synergy of the three in addressing systemic inflammation remains limited, particularly with regard to the optimal dosage and the molecular interaction mechanisms. The potential anti-inflammatory activity of the combination *of Tithonia diversifolia*, *Moringa oleifera*, and *Curcuma longa* extracts has been supported by several previous studies. Research by Solfaine et al. (2021) and (Abd-Elnaby et al., 2022) demonstrates that each extract can significantly inhibit NO production, with inhibitory activity exceeding 70% at therapeutic concentrations. *In vitro* and *in silico* approaches have been utilised to identify bioactive compounds, including vaidilin C, quercetin, and curcumin, which have been shown to possess anti-inflammatory properties. The existence of a positive correlation between *in vitro* results and in silico predictions serves to reinforce the validity of this combination's therapeutic potential. Nevertheless, there is a paucity of *in vivo* evidence on the combined effect on modulating key oxidative stress markers (MDA, SOD, and NRF2) in an LPS-induced ALI model.

Based on the existing scientific evidence, this study is designed to investigate the effect of the combined aqueous TMC extract on oxidative stress marker parameters, specifically the reduction of MDA and the increase in SOD activity and NRF2 expression. This research is important as it aims to provide empirical data on a safe, accessible, and potentially more effective multi-target therapy derived from local medicinal plants to counteract the damaging cascade of LPS-induced inflammation and oxidative stress. For science, this study will contribute to the pharmacological understanding of herbal synergism, specifically within the NRF2 antioxidant pathway. For society, it offers the prospect of developing an affordable, evidence-based phytopharmaceutical formulation that could serve as an adjunct or alternative therapy for managing inflammatory conditions, thereby reducing dependency on synthetic drugs with significant side effects

METHODS

Experimental Design

This research employed a Completely Randomized Design (CRD). Mice were allocated into six groups (Table 1). Each treatment group consisted of six replicates. The total number of mice used was 36.

Table 1. Treatment groups for LPS and TMC combination administration

Group	LPS (mg/kg BW)	*Tithonia diversifolia* (mg/kg BW)	*Moringa oleifera* (mg/kg BW)	*Curcuma longa* (mg/kg BW)
Healthy/normal (K0)	-	-	-	-
LPS (K-)	0.5	-	-	-
TMC 3 (K+)	-	300	200	500
TMC 1 + LPS (P1)	0.5	500	300	200
TMC 2 + LPS (P2)	0.5	200	500	300
TMC 3 + LPS (P3)	0.5	300	200	500

Note:
LPS = Lipopolysaccharide, TMC = *Tithonia diversifolia-Moringa oleifera-Curcuma longa*, BW = Body Weight

Preparation of *Tithonia diversifolia-Moringa oleifera-Curcuma longa* (TMC) Extract

Powdered extracts of *Tithonia diversifolia* (Batch No. 240429.PTN.F.MLG.357.121), *Moringa oleifera* (Batch No. 240130.KLR.F.KJY.001), and *Curcuma longa* (Batch No. 240614.KNT.L.MLG.596.227), collectively referred to as TMC, were procured from Materia Medica Batu, Malang, East Java, Indonesia. To prepare the aqueous extract, 100 g of each dried botanical was weighed and infused in 1 L of boiling water for 10 minutes. The mixture was then cooled, filtered through cheesecloth to obtain the liquid TMC extract, and stored in a freezer at -80°C for 24 hours. The frozen extract was subsequently lyophilized using a freeze-dryer to obtain a dry powder. This dry TMC extract was reconstituted in distilled water prior to oral administration to the mice (modified from Adharini et al., 2020).

Experimental Animals

The study used 36 male Balb/c mice (*Mus musculus*), obtained from Kemuning, Karanganyar, Central Java. All experimental protocols were reviewed and approved by the Health Research Ethics Committee with Ethical Clearance Number: 45-KEP-FKHUB-2025 (Appendix 1). The mice, aged 6-7 weeks with an average body weight of 20-25 grams, were confirmed healthy based on active movement, bright eyes, normal limbs, dense, white, and smooth fur, and normal gait. Before treatment, the mice were acclimatized to the new environment for one week. They were housed in plastic cages with wire lids, divided into treatment groups. Bedding was changed every 2-3 days, and the animals were provided with adequate food and water daily. The housing room was maintained at 18-26°C with a relative humidity of 40-70% (Agustina, 2015; Mutiarahmi et al., 2021).

LPS Induction and Oral TMC Treatment

The 36 mice were divided into six treatment groups (Table 1). The TMC extract was administered orally for 14 consecutive days. On the 15th day, ALI was induced using purified LPS (*Escherichia coli* O111:B4, phenol-extracted, Sigma-Aldrich, USA, Product No. L2630) at a dose of 0.5 mg/kg BW dissolved in 0.1 mL of phosphate-buffered saline (PBS). LPS was administered via intraperitoneal injection twice within a 5-day interval, specifically on days 1 and 4 after the completion of the two-week gavage period (modified from Wang et al., 2021).

Organ Isolation

On the 33rd day after treatment, mice from each group were euthanized by cervical dislocation, followed by dissection for organ isolation. The liver and spleen were isolated for flow cytometry analysis, while the lungs were preserved for histopathological examination. The isolation of the spleen and liver for flow cytometry followed a modified method from Lestari et al. (2022). Isolated organs were rinsed with PBS, homogenized in a Petri dish containing 2 mL PBS using a syringe plunger, and the resulting cell suspension was transferred to a propylene tube. PBS (10 mL) was added, and the homogenate was centrifuged at 2500 rpm, 10°C for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of sterile PBS (Rifa'i, 2018). The lungs were weighed, stored in neutral buffered formalin, and subsequently processed into Hematoxylin and Eosin (H&E)-stained slides for histopathological analysis.

Antibody Staining and Flow Cytometry Analysis

The obtained cell suspensions from the liver and spleen were divided into 1.5 mL microtubes (50 µL each) containing 400 µL PBS. The

homogenate was centrifuged at 2500 rpm, 10°C for 5 minutes. After discarding the supernatant, the splenocyte pellet was stained with 50 µL of extracellular antibodies. The mixture was vortexed and incubated for 20 minutes at 4°C in the dark. Cells were then fixed using a CytoFix buffer (BioLegend, USA) for 20 minutes. Following fixation, cells were washed with a perm/wash buffer (BioLegend, USA) for 5 minutes and centrifuged again. The pellet was stained with intracellular antibodies, vortexed, and incubated for 20 minutes at 4°C in the dark. Finally, cells were resuspended in 500 µL PBS, transferred to flow cytometry tubes, and analyzed using a BD Bioscience FACS Calibur™ flow cytometer. Relative quantification of each parameter was performed using BD CellQuest™ software (Hermanto et al., 2020). The following antibody panels were used for flow cytometry analysis: MDA (FITC Anti-Malondialdehyde antibody, abcam ab27615), Nrf2 (PE-Cy7 conjugated Rabbit anti-rat Nrf2 Polyclonal Antibody, Bioss bs-1074R-PE-Cy7), and SOD (Cy5.5 conjugated Rabbit anti-rat SOD1 Polyclonal antibody, Bioss bs-10216R-Cy5.5).

Data Analysis

Flow cytometry results were analyzed using BD CellQuest Pro™ software. Data were tabulated in Microsoft Excel, presented as mean ± standard deviation (SD) from three replications. Percentage data falling within the ranges of 0-30% or 70-100% were arcsine-transformed. Statistical analysis was performed using SPSS version 26. One-way analysis of variance (ANOVA) was conducted, followed by Tukey's HSD post-hoc test, with statistical significance set at $p < 0.05$ (Adharini et al., 2020).

RESULTS AND DISCUSSION

The liver is a crucial organ in metabolism and detoxification, making it highly susceptible to oxidative stress induced by exogenous compounds such as Lipopolysaccharide (LPS) (Ferro et al., 2020). Oxidative stress occurs when there is an imbalance between the production of ROS and the body's endogenous antioxidant capacity (Schieber & Chandel, 2014).

Histological Evaluation of Mice Lung Tissue

Intraperitoneal induction of LPS triggers significant histopathological changes in the lung organs of mice (Figure 1). This damage includes massive neutrophil infiltration into the interstitial and alveolar spaces, as well as alveolar septal thickening. The normal group shows no alterations in lung tissue due to the absence of inflammatory response initiation.

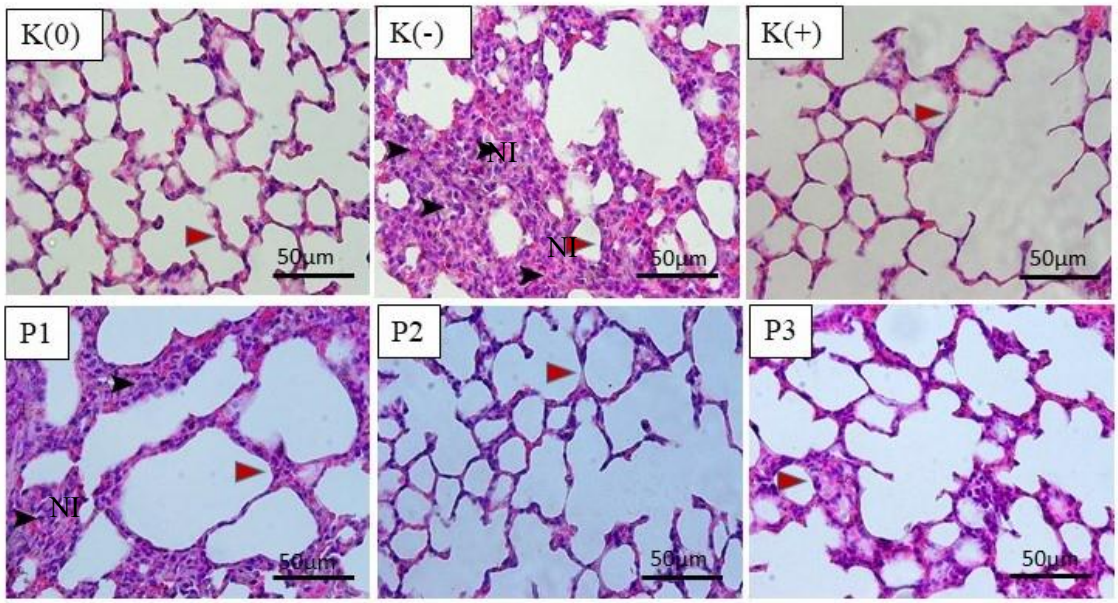

Figure 1. The effect of TMC combination extract on histological changes in the lungs of mice. The comparison showed among untreated [K(0)], LPS-induced [K(-)], LPS + TMC1 [P1], LPS + TMC2 [P2], and LPS + TMC3 [P3]. Lungs were stained using HE for identification of tissue injury characteristics. Images were observed at higher magnification (400×). Neutrophil infiltration (black arrow) and septal thickening (red arrow).

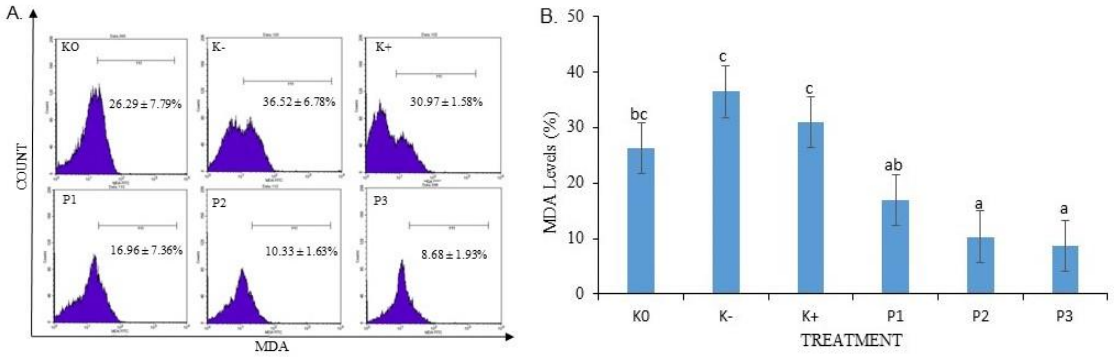

Figure 2. Administration of TMC in LPS-induced mice reduces MDA expression. The comparison showed among untreated [K(0)], LPS-induced [K(-)], LPS + TMC1 [P1], LPS + TMC2 [P2], and LPS + TMC3 [P3]. (A) Flow cytometry results, and (B) Results of the one-way ANOVA statistical test presented as mean ± standard deviation. Different letters indicate significant differences at $p \leq 0.05$ using the Tukey's HSD post-hoc test.

Administration of the TMC extract under normal conditions induces no pathological changes, as this group maintains a tissue architecture closely resembling the normal control. This indicates that the TMC extract combination is safe for consumption and does not trigger inflammation under normal physiological conditions. The protective and preventive effects of the TMC extract combination become clearly evident in the P1, P2, and P3 groups, all of which exhibit a significant reduction in alveolar septal thickening and minimal to absent neutrophil infiltration compared to the LPS group. Histologically, the normal lung displays a well-organized alveolar structure with thin alveolar septa (1–2 μm) composed of squamous type I pneumocytes and cuboidal type II pneumocytes (Zhao et al., 2023). Intraperitoneal (IP) administration of LPS induces pathognomonic changes of acute lung injury (ALI) within 6–12 hours post-induction, characterized by an increase in alveolar septal thickness up to 5–8 μm due to interstitial edema and massive neutrophil infiltration (reaching 40–60% of total inflammatory cells) (Jiao dkk., 2024; Wang dkk., 2021).

This study demonstrates that the TMC extract combination is capable of regulating neutrophil migration and infiltration into the lung tissue. The P2 group (LPS + TMC 2) demonstrates the most optimal recovery rate among all LPS-induced groups. The alveolar architecture in P2 appears the most well-organized and closely approaches normal physiological conditions, characterized by minimal septal thickening and a highly significant reduction in inflammatory cell count. The P2 formulation contains the highest dose of *Moringa oleifera* extract, which is rich in antioxidant compounds such as quercetin and kaempferol. This study demonstrates that the TMC extract combination effectively regulates neutrophil infiltration into lung tissue by modulating ROS levels. Pathophysiologically, LPS-induced migrating neutrophils release massive amounts of ROS via the respiratory burst phenomenon. This excessive ROS accumulation acts as an aggressive free radical cascade that drives cell membrane lipid peroxidation, leading to vascular leakage and alveolar septal thickening.

The anti-inflammatory potential of the TMC extract combination as a preventive agent significantly alleviates ROS, driven synergistically by the bioactive constituents of the three plants. Flavonoid compounds such as quercetin and kaempferol from *Moringa oleifera*, alongside the active components of *Tithonia diversifolia*, function as exogenous radical scavengers that directly neutralize ROS, thereby preventing cellular oxidative stress (Jaja-Chimedza et al., 2017). The suppression of ROS levels subsequently inhibits their role as second messengers required to activate the pro-inflammatory NF-κB, MAPK, and NLRP3 pathways (further supported by curcumin from *Curcuma longa*). Consequently, cellular homeostasis is maintained without triggering secondary inflammation, as structurally evidenced by the preservation of thin alveolar septa and alveolar spaces cleared of inflammatory cells (Wang et al., 2021; Yang et al., 2024).

Expression Levels of MDA in Liver Cells

MDA levels (Figure 2b) in the LPS group showed no significant difference from the normal and TMC groups, with MDA levels of 26.29% in the normal group, 30.97% in the TMC group, and 36.52% in the LPS group, indicating that TMC pretreatment prevents MDA increase due to LPS.

In contrast, MDA levels in the LPS group versus the P1, P2, and P3 groups showed significant differences, with MDA levels of 16.96%, 10.33%, and 8.68%, respectively (Figure 2a). These results demonstrate that TMC extract provides strong antioxidant effects against oxidative stress in addressing LPS-induced lung inflammation.

MDA is a primary marker of lipid peroxidation, and it is widely used as an indicator of oxidative damage in liver tissue (Yekti et al., 2018). Elevated MDA levels are consistently observed in various liver injury models induced by LPS, CCl_4, paraquat, or hepatotoxic drugs (Zhou et al., 2023). The analysis of MDA levels revealed significant differences among the treatment groups. LPS induction (K-) increased MDA levels to 36.52%, substantially higher than the healthy control group (K0) at 26,29% but statistically not significant. Administration of TMC extract (K+) resulted in an MDA level of 30,97%, lower than the LPS group but still higher than the healthy control, despite being statistically not significant compared with both groups. This finding suggests that, under physiological conditions, TMC extract exerts a moderate antioxidant effect, capable of suppressing free radical formation without disrupting cellular redox balance. Conversely, the combination treatment groups that received TMC extract prior to LPS (P1–P3) exhibited a significant reduction in MDA levels in comparison to the LPS group. LPS is known to elevate oxidative stress and lipid peroxidation across various tissues (Martins et al., 2022). This increase reflects oxidative stress characterized by intensified lipid peroxidation due to the accumulation of reactive oxygen species (ROS) (Yekti et al., 2018). LPS activates pro-inflammatory pathways via TLR4–NFκB, leading to increased ROS production and oxidative damage to cell membranes (Mahdavi-Roshan et al., 2022).

These results demonstrate that pretreatment with TMC extract not only prevented the LPS-induced rise in MDA but also reduced MDA levels below the physiological baseline of the healthy control group. This drastic reduction signifies a potent antioxidant activity of TMC extract in inhibiting lipid peroxidation reactions (Martins et al., 2022). The TMC3 formula (P3) showed the highest efficacy with the lowest MDA level among all treatments, followed by TMC2 (P2) and TMC1 (P1). This difference in effectiveness suggests that the composition and ratio of active ingredients in the TMC formula are crucial in determining its antioxidant capacity. The dose combination in TMC3 (T=300, M=200, C=500 mg/kg BW) appears to provide an optimal balance, acting as a highly efficient direct radical scavenger that mitigates early oxidative damage before it cascades into systemic lipid peroxidation.

LPS is an endotoxin found in the outer membrane of Gram-negative bacteria. When injected into mice, it binds specifically to Toll-like Receptor 4 (TLR4) expressed on the surface of immune cells (such as macrophages and neutrophils). The binding of LPS to TLR4 triggers an intracellular signaling cascade, primarily activating the NF-kB (Nuclear Factor kappa B) pathway (Mahdavi-Roshan et al., 2022). Once activated, NF-kB translocates to the nucleus and upregulates the expression of pro-inflammatory enzymes, such as iNOS (Inducible Nitric Oxide Synthase) and COX-2 (Cyclooxygenase-2). These activated enzymes, along with the mitochondrial stress caused by LPS, lead to a massive burst of ROS (such as superoxide radicals O_2^- and hydrogen peroxide H_2O_2 and Reactive Nitrogen Species (RNS). This overwhelms the animal's natural antioxidant defenses (such as SOD, Catalase, and Glutathione). The excess ROS violently attacks the polyunsaturated fatty acids (PUFAs) present in the cell membranes and organelle membranes (such as the mitochondrial membrane). This chemical degradation process is known as lipid peroxidation. As the PUFAs in the cell membrane break down, they decompose into various cytotoxic compounds. Malondialdehyde (MDA) is one of the primary, stable end-products of this lipid peroxidation process. When your TMC extract reduces MDA expression, it proves that the extract is successfully scavenging ROS or inhibiting the TLR4/NF-kB pathway, thereby protecting the cell membranes from degradation (Yekti et al., 2018; Martins et al., 2022).

SOD Expression in Liver Cells

SOD enzyme activity (Figure 3b) in the normal group reached 12.51%, increased to 25.23% in the LPS group with a significant difference, while the TMC group (18.18%) showed no significant difference from LPS. Therapy administration in the P1, P2, and P3 groups (13.84%, 5.74%, and 12.74%, respectively, as shown in Figure 3a) demonstrated significant differences from the LPS group, with a significant reduction approaching normal levels. The decrease in SOD activity in the LPS group indicates enzyme exhaustion due to excessive oxidative stress, while extract therapy stimulates endogenous SOD synthesis through flavonoid and

polyphenol compounds. The strongest effect was observed in P2 (5.74%), indicating the optimal dose of this plant combination for lung antioxidant protection.

LPS induction significantly increased SOD activity from 12.51% (healthy control) to 25.23% (LPS-Induced), reflecting a compensatory response to excessive ROS production during acute lung injury (Liu et al., 2025). This elevation indicates enzymatic exhaustion rather than effective antioxidant defense. Treatment with TMC extract alone (K+) reduced SOD to 18.18%. In the TMC + LPS groups, SOD levels decreased to 13.84% (P1), 5.74% (P2), and 12.74% (P3), approaching the normal physiological range. These findings suggest that TMC extract alleviates oxidative stress by reducing ROS burden, thereby normalizing SOD activity (Varra et al., 2025). Although P2 exhibited the most pronounced reduction (5.74%), this drop below baseline raises concern for over-inhibition of endogenous antioxidant defenses. Excessive suppression of SOD may compromise immune function (Xu et al., 2022). Conversely, P1 and P3 achieved balanced restoration without overshooting, offering optimal therapeutic benefit while preserving natural immunity.

LPS binds to the TLR4/CD14/MD2 receptor complex to activate the downstream inflammatory cascade, triggering a massive production of ROS via the activation of NADPH oxidase (NOX2) in phagocytic cells. This excessive accumulation of ROS inactivates SOD through post-translational modifications, specifically the nitration of tyrosine residues at the enzyme's active site by peroxynitrite (ONOO-) (Zhao et al., 2023). Concurrently, LPS suppresses the Nrf2-Keap1 pathway, which regulates the gene expression of endogenous antioxidant enzymes, including SOD (Ngo and Duennwald., 2022).

Administering TMC extract as a preventive measure holds promise for establishing early defense mechanisms by acting as a bifunctional inducer. Theoretically, electrophilic compounds within TMC, such as curcumin and rosmarinic acid, can modify specific cysteine residues (like Cys151) on Keap1, thereby releasing Nrf2 to translocate into the nucleus. In this study, the expression of this parameter surged dramatically in the LPS-challenged group due to severe oxidative stress. Interestingly, the TMC-treated group exhibited a moderate position. Although the reduction was not statistically significant compared to the LPS group, the downward trend suggests a partial recovery effect. This suggests that while the natural enzymatic shield of the Keap1-Nrf2 pathway was not fully activated at this early stage, the TMC formulation still played a crucial role in mitigating the severity of oxidative damage (Hassanzadeh-Taheri et al., 2021; Li et al., 2026).

When inflammation peaks, the phytochemical compounds in TMC immediately switch roles to act as direct radical scavengers. Through this dual action, the extract independently clears out ROS accumulation while preventing the body's natural antioxidant system from overworking to the point of exhaustion (Ngo & Duennwald, 2022). Because the cellular oxidative stress burden is alleviated early on, the lung cells no longer need to trigger a late-stage emergency response to boost NRF2 or produce SOD on a massive scale. This breathing room allows the tissue's redox status to stabilize back toward homeostasis, proving that the internal defense system is experiencing a distinct antioxidant-sparing effect provided by the TMC formulation

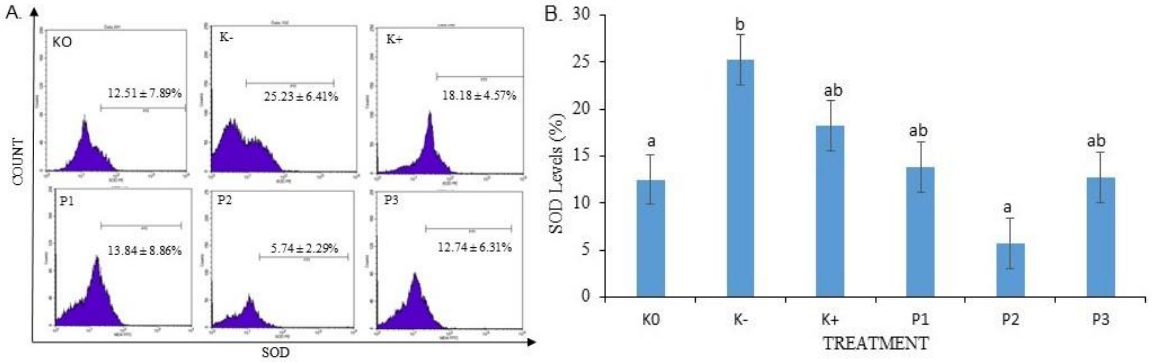

Figure 3. Administration of TMC in LPS-induced mice reduces SOD expression. The comparison showed among untreated [K(0)], LPS-induced [K(-)], LPS + TMC1 [P1], LPS + TMC2 [P2], and LPS + TMC3 [P3]. (A) Flow cytometry results, and (B) Results of the one-way ANOVA statistical test presented as mean ± standard deviation. Different letters indicate significant differences at $p \leq 0.05$ using the Tukey's HSD post-hoc test.

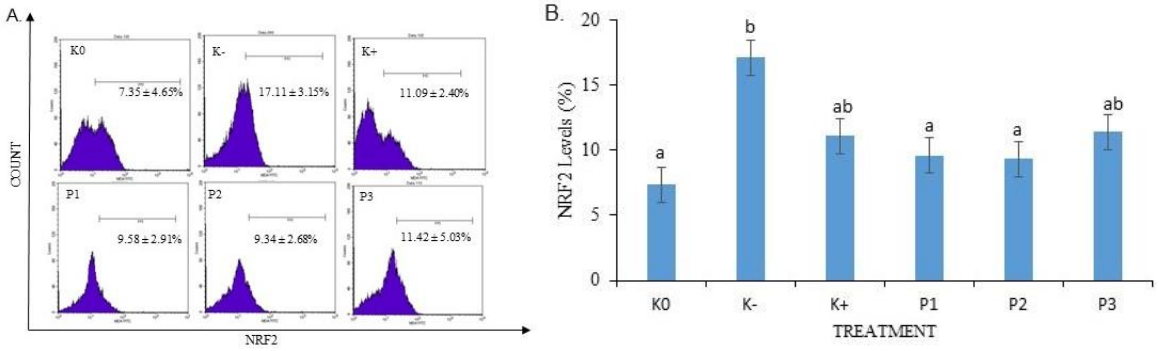

Figure 4. Administration of TMC in LPS-induced mice reduces NRF2 expression. The comparison showed among untreated [K(0)], LPS-induced [K(-)], LPS + TMC1 [P1], LPS + TMC2 [P2], and LPS + TMC3 [P3]. (A) Flow cytometry results, and (B) Results of the one-way ANOVA statistical test presented as mean ± standard deviation. Different letters indicate significant differences at $p \leq 0.05$ using the Tukey's HSD post-hoc test.

NRF2 Activation in Liver Cells

NRF2 expression levels (Figure 4b) in the normal group reached 7.35%; the TMC group was 11.09% (no significant difference from LPS at 17.11%), while the LPS group showed a significant difference from the normal group, indicating that TMC pretreatment did not fully prevent NRF2 oxidative stress pathway activation due to LPS. In the therapy groups, NRF2 expression significantly decreased to 9.58% (P1), 9.34% (P2), and 11.42% (P3, no significant difference from LPS), demonstrating recovery approaching normal levels, especially at higher doses.

The Nrf2 pathway is the body's primary defense system against oxidative stress. NRF2 functions as a key transcriptional regulator that activates the expression of various antioxidant genes, including the SOD enzyme, which serves as the first line of defense in neutralizing superoxide radicals (Ngo & Duennwald, 2022). Under basal conditions, NRF2 is kept inactive in the cytoplasm by binding to a repressive protein Keap1, which constantly targets NRF2 for proteosomal degradation to ensure its levels remain low when the cell is not under stress.

In this study, LPS induction significantly increased NRF2 expression from 7.35% (healthy control, K0) to 17.11% (LPS induced, K-). Physiologically, NRF2 activation serves as a protective mechanism against oxidative stress by upregulating antioxidant enzymes such as SOD, thereby preventing ROS-induced tissue damage (Ngo & Duennwald, 2022). The sudden burst of ROS caused by LPS oxidizes specific cysteine residues on Keap1, prompting NRF2 to decouple from Keap1, translocate into the nucleus, and bind to the Antioxidant Response Element (ARE). This binding triggers the mass transcription of crucial endogenous antioxidant enzymes, such as SOD, HO-1, and GSH-Px. However, as LPS-induced acute inflammation intensifies via the NF-kB pathway, negative cross-talk occurs where active NF-kB competes with NRF2 for necessary transcription co-activators. Consequently, the endogenous NRF2 defense system becomes overwhelmed and exhausted, leading to unchecked lipid peroxidation, hence the high MDA levels observed in the LPS group. Conversely, preventive intervention with the TMC extract combination significantly alleviated this oxidative stress.

Treatment with TMC extract (K+) reduced NRF2 expression to 11.09%. In the TMC + LPS groups (P1–P3), NRF2 levels decreased to 9.58% (P1), 9.34% (P2), and 11.42% (P3), approaching the basal level of healthy controls. This dose-dependent normalization confirms that TMC extract effectively modulates the NRF2 antioxidant pathway. By reducing excessive NRF2 activation induced by LPS, TMC enhances endogenous antioxidant capacity without causing sustained overactivation, thereby restoring redox balance in a physiologically appropriate manner (Hou et al., 2024).

This distinct pattern of reduced MDA accompanied by decreased NRF2 expression and low SOD activity strengthens the hypothesis that TMC extract mitigates LPS-induced oxidative stress through an antioxidant sparing effect, without necessarily relying on the upregulation of the endogenous NRF2–SOD pathway (Yang et al., 2019). Phytochemicals within the TMC extract, such as rosmarinic acid, curcumin, polyphenols, flavonoids, and terpenoids present across the *Tithonia, Moringa, and Curcuma* blend, are

demonstrated to act as direct exogenous radical scavengers. This mechanism allows for the direct neutralization of ROS at the onset of exposure without heavily engaging the host's internal antioxidant system (Sikder et al., 2025).

Because the free radicals are intercepted and quenched upstream by the TMC bioactive compounds, the intracellular oxidative stress burden is significantly lowered. This preserved cellular homeostasis leaves the cysteine residues on Keap1 unmodified, keeping NRF2 tethered in the cytoplasm and reducing the cellular requirement to initiate NRF2 nuclear translocation or synthesize compensatory enzymes like SOD. Ultimately, this condition aids in restoring tissue redox status toward homeostasis, successfully preventing lipid peroxidation while preventing the exhaustion of the host's natural antioxidant defenses.

This study provides significant scientific and societal contributions by effectively bridging traditional ethnomedicine with modern molecular pharmacology. Scientifically, this research identifies the preventive TMC formulation (specifically P2) as an optimized, superior respiratory-protective agent capable of fully absorbing the cellular oxidative burden before tissue degradation occurs. From a societal and healthcare perspective, given the high mortality rates and exorbitant critical care costs associated with acute lung injury triggered by bacterial infections or sepsis, this study offers a safe, non-toxic, and highly affordable preventive supplement alternative. By validating this polyherbal blend, it elevates the economic and utilitarian value of highly abundant regional agricultural assets.

Overall, these findings strengthen the hypothesis that TMC extract possesses the ability to mitigate LPS-induced oxidative stress without necessarily relying on the regulation of the NRF2–SOD pathway. TMC extract demonstrates significant potential in maintaining the balance of the body's natural antioxidant system (Yang et al., 2019). The pattern of reduced MDA accompanied by decreased NRF2 expression and SOD activity in the P2 treatment group suggests the occurrence of an antioxidant sparing effect.

Phytochemicals within TMC extract, such as rosmarinic acid and curcumin, are hypothesized to act as direct radical scavengers. This mechanism allows for the direct neutralization of ROS without heavily engaging the endogenous antioxidant system (Sikder et al., 2025). Consequently, the oxidative stress burden on cells is significantly lowered, thereby reducing the need to activate the NRF2 signaling pathway or increase the production of compensatory enzymes like SOD. This condition ultimately aids in restoring tissue redox status toward homeostasis (balance).

Beyond its biomolecular implications, this study provides a profound interdisciplinary contribution that directly bridges United Nations Sustainable Development Goal (SDG) 3: Good Health and Well-Being and SDG 15: Life on Land. Within the framework of SDG 3, the established efficacy of the TMC polyherbal formulation in mitigating lipopolysaccharide-induced acute lung injury directly addresses Target 3.4 (reducing mortality from inflammatory and non-communicable diseases) and Target 3.b (supporting the research and development of accessible, innovative medicines). By offering a scientifically validated and economically viable preventive strategy against severe respiratory pathologies, this research outlines a practical approach to alleviating the socio-economic burdens placed on intensive healthcare systems.

Concurrently, the research actively promotes SDG 15 (Targets 15.4 and 15.5) through the targeted bioprospecting of local flora (*Tithonia diversifolia, Moringa oleifera*, and *Curcuma longa*). Elevating the pharmacological and economic status of these abundant botanical resources transforms common terrestrial vegetation into high-utility medical assets. This rigorous scientific validation provides a tangible, market-driven incentive for local communities to actively conserve terrestrial ecosystems, halt regional biodiversity loss, prevent land degradation, and adopt sustainable agroforestry and herbal farming practices, ultimately demonstrating how the preservation of terrestrial biodiversity can be strategically leveraged to safeguard human health

CONCLUSION

A combination of *Tithonia diversifolia, Moringa oleifera*, and *Curcuma longa* extracts (TMC) exerts a potent protective effect against lipopolysaccharide (LPS)-induced acute lung injury (ALI) in BALB/c mice. Intraperitoneal administration of LPS triggered histopathological alterations characterized by alveolar septal thickening and inflammatory cell infiltration, concurrently with marked oxidative stress as evidenced by elevated MDA, SOD, and NRF2 levels. Notably, administration of the P2 formulation effectively preserved lung

histological architecture and restored MDA and NRF2 levels to normal physiological baselines through an antioxidant-sparing mechanism. Further research is needed to fully elucidate the underlying mechanisms, quantifying specific pro-inflammatory cytokines (such as TNF-α, IL-1β, and IL-6). Furthermore, characterizing the long-term pharmacokinetic profile and chronic toxicity of this specific ratio constitutes a crucial step before transitioning from acute animal models to clinical applications.

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AUTHOR CONTRIBUTION STATEMENT

SNC conceived and designed the study, conducted the data analysis, and was responsible for the conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing of the original draft, writing, reviewing and editing, visualization, supervision, and project administration. BNAF, RHB, and NSYS contributed equally to the conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing of the original draft, writing, reviewing and editing, and visualization. NDL, MD, and APWM participated in the methodology, software development, writing, reviewing and editing, supervision, and project administration. HT contributed to the validation of the data, writing, reviewing, and project administration. MR was responsible for the overall conceptualization, methodology, validation, investigation, writing, reviewing and editing, supervision, and project administration. All authors have read and approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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