

Anti-inflammatory potential of turmeric, amla, and black pepper mixture against sepsis-induced acute lung injury in rats

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Abstract Acute lung injury (ALI), is a severe inflammatory lung disease. We tested the prophylactic effect of a functional food mix comprising three anti-inflammatory plant products: turmeric, amla, and black pepper (TAB) against lipopolysaccharide (LPS)-induced ALI in rats. Two-month-old male Wistar rats were randomly divided into three groups: control (C), LPS (5 mg/kg), and LPS with TAB (TAB). After 6 h of LPS injection, the rats were sacrificed by cervical decapitation to collect the lung tissue. Results showed that TAB partially ameliorated LPS-induced increase in circulating inflammatory cytokines (TNF α and IL6) and significantly prevented lung histopathological changes. TAB also suppressed LPS-activated ER stress markers (GRP78, pIRE1, and CHOP) and apoptotic markers (caspase-3 and -12) in the lung. The anti-inflammatory effects of the TAB support its potential use as an adjuvant to mitigate ALI. Importantly, TAB's ingredients have been used for centuries as part of the diet with limited or no toxic effects.

Keywords Inflammation · Cytokine storm · Immunomodulation · Herbal formulation · Functional food · Lipopolysaccharide

Abbreviations

TAB Functional food consisting of turmeric, amla, and black pepper
ALI Acute lung injury

ARDS Acute respiratory distress syndrome
TNF- α Tumor necrosis factor-alpha
IL Interleukin
MCP Monocyte chemoattractant protein
NF- κ B Nuclear factor kappa B
LPS Gram-negative bacterial cell wall lipopolysaccharide
ER Endoplasmic reticulum

Introduction

Acute lung injury (ALI), and its severe condition, acute respiratory distress syndrome (ARDS) are serious inflammatory lung diseases affecting around 1 million people annually resulting in high morbidity and mortality (Rubinfeld and Herridge 2007; Yuan et al. 2018). The most common cause of ALI is sepsis, due to microbial infection including current SARS-CoV-2 (Leist et al. 2020; Habashi et al. 2021). Increased vascular permeability, leukocyte recruitment, and overproduction of cytokines causing alveolar and interstitial pulmonary edema, alveolar collapse, and hypoxemia are the characteristics of ALI (Tomashefski 2000). Despite much research, no effective therapies are available for ALI / ARDS in clinical practice. As of now, corticosteroid therapy in ALI is beneficial only in the early phase of lung inflammation but with several adverse effects like hyperglycaemia, hypokalaemia, dyslipidaemia, hypertension, peptic ulcers, immunosuppression, neuropsychiatric disturbances, osteoporosis, myopathy, etc. (Schacke et al. 2002). Scientific evidence indicates the role of an overactive inflammatory response in the pathogenesis of early steps of ALI / ARDS. The host's uncontrolled inflammatory response, including leukocyte recruitment and cytokine storm, could impair the host pulmonary epithelial or endothelial layer (Karbian et al.

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2020; Liu et al. 2020). Hence, a strong anti-inflammatory agent or formulation could alleviate this pathology.

Turmeric (*Curcuma longa*), Amla (*Emblica Officinalis Gaertn.*), and Black pepper (*Piper nigrum* L.) are popular culinary plants and are also used for several centuries in the Indian Ayurvedic medicine system for curing numerous inflammatory disorders. Turmeric is a food component used as a spice in many parts of the world. Curcumin, responsible for the vibrant yellow color is the major active principle of turmeric has been attributed to numerous pharmacological activities of which the best-explored is its anti-inflammatory effect (Jurenka 2009). Curcumin was shown to modulate the inflammatory response by suppressing the production of tumor necrosis factor-alpha (TNF- α), IL-1, -2, -6, -8, and -12 (Abe et al. 1999). Black pepper has been reported to have anti-inflammatory potential is commonly used in food preparation and traditional medicine in several countries (Tasleem et al. 2014; Pei et al. 2020). Further, black pepper was shown to increase curcumin's bioavailability by 20 folds (Shoba et al. 1998; Patil et al. 2016). Studies have also shown that amla extract has strong anti-inflammatory effects and inhibits microbial infection-induced expression of the neutrophil chemokines interleukin (IL)-8, GRO-alpha, GRO-gamma, and pro-inflammatory cytokine, IL-6 in the bronchial epithelial cells (Nicolis et al. 2008). In addition to the immunomodulatory effect of amla (Singh et al. 2013), the synergistic effects of turmeric, amla, and black pepper are well reported in the scientific literature (Rawal et al. 2014; Pitchaiah et al. 2017; Abdul Manap et al. 2019; Chaitanya et al. 2020). Furthermore, our earlier studies suggested the prophylactic efficacy of these three components individually against diabetic complications (Suryanarayana et al. 2004; Muthenna et al. 2009; Puppala et al. 2012; Rao et al. 2012) which motivated us to investigate the combined effect of turmeric, amla, and black pepper (TAB) against sepsis-induced ALI using a rat model.

Materials and methods

Chemicals and reagents

Lipopolysaccharides from *Escherichia coli* O55:B5 (LPS), horseradish peroxidase (HRP) conjugated anti-rabbit, and anti-mouse antibodies, immunoblot chemicals, and protease inhibitor cocktail were purchased from Sigma Chemicals (MO, USA). Primary antibodies for NF-kB, pNF-kB, PI3K, AKT, pAKT, ATG5, LC3, BAX, caspase 3, and caspase 12 were purchased from Cell Signaling Technology, (Massachusetts, USA). TNF α , pERK1, pJNK1, pPI3K, GRP78, pIRE1, CHOP, Beclin1, P62, Bcl2, and β -actin antibodies were purchased from Thermo Scientific (Massachusetts, USA).

Animal experiment

Two-month-old Wistar rats were received from the animal facility of ICMR- National Institute of Nutrition and adapted for a week. Rats were fed with an AIN93 pellet diet and drinking water ad libitum. The animal room was maintained at standard conditions of 22 ± 2 °C temperature, 50–60% of humidity, and 12:12 h dark: light cycle. All the procedures of animal work were approved and accorded by the Institutional Animal Ethical Committee. Rats were randomly divided into the following three groups: control (C), LPS, and LPS with a functional food mixture of turmeric, amla, and black pepper (TAB). The Group-TAB was pre-treated with the TAB in the diet for a week before LPS administration, while Groups -C and -LPS received a regular chow diet.

Functional food mixture of turmeric, amla and black pepper (TAB)

Fine quality dried turmeric root/rhizome and black pepper (dried fruits) were purchased from a local market and grounded to a fine powder. Good quality fresh amla was collected in the winter season from a local market and washed under tap water, air-dried to remove water, seeds were removed, pericarp was cut into small pieces and air-dried at room temperature under shade. The dried amla pieces were grounded into a fine powder and stored in an airtight glass container. One gram of amla powder, 0.25 gram of turmeric powder, and 0.5 gram of black pepper were added to 100 grams of the chow diet. These specified individual doses were decided based on the active principle content (curcumin in turmeric, β -glucogallin in amla, and piperine in black pepper) required for optimal efficacy, back-calculated based on our earlier studies on diabetic complications (Suryanarayana, Kumar et al. 2004, Suryanarayana, Saraswat et al. 2005, Mrudula, Suryanarayana et al. 2007, Suryanarayana, Satyanarayana et al. 2007, Saraswat, Muthenna et al. 2008, Muthenna, Suryanarayana et al. 2009, Puppala, Ponder et al. 2012, Rao, Muthenna et al. 2012).

LPS was administered on the eighth day to all rats except Group-C at a dose of 5 mg/kg body weight, dissolved in saline via intraperitoneal route. The LPS dose used in the study was based on our pilot study conducted according to the earlier reported studies (Ayaz et al. 2017; Deng et al. 2017). After 30, 60, and 120 min of LPS injection blood sample was collected from the retro-orbital plexus for analysis of inflammatory cytokines. Rats were sacrificed by cervical decapitation after 6 h of LPS injection and a part of lung tissue was fixed in formalin solution and the remaining was snap-frozen for further analysis.

Analysis of circulatory cytokines by ELISA

Plasma concentrations of cytokines IL-6, IL-10 and TNF- α were measured by the Solid Phase Sandwich ELISA method (R&D Systems, MN, USA) by following the manufacturer's instructions. Briefly, 100 μ L of plasma sample was mixed with 100 μ L of the Detection Antibody, diluted in 1% BSA in PBS, pH 7.2 and incubated for 2 h in dark at room temperature. After washing with 0.05% Tween-20 in PBS, pH 7.2, added 100 μ L of the working dilution of Streptavidin-HRP conjugate, and incubated for 20 min at room temperature. Following a wash, added 100 μ L of Substrate Solution (1:1 mixture of H₂O₂ and Tetramethylbenzidine) and incubated at room temperature for 20 min. 50 μ L of 2 N H₂SO₄ was added to each well to stop the reaction and gently tapped the plate to ensure proper mixing. Absorbance at 450 nm was measured immediately, using a microplate reader. A standard curve in the range of 62.5 to 4000 pg/mL was constructed by reducing the data generating a four-parameter logistic (4-PL) curve-fit.

Immunoblotting

Lung tissue was homogenised in 20 mM Tris lysis buffer (100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μ g/mL each of aprotinin, leupeptin, and pepstatin; pH7.5) and protein concentration was measured by the Lowry method. An equal amount of protein from each of the three experimental groups was subjected to SDS PAGE and the resolved bands were transferred to nitrocellulose membrane to probe with primary antibodies each at a specific dilution. Then incubated with respective secondary antibody conjugated with HRP enzyme. Finally, the bands are visualised after developing with enhanced chemiluminescence detection reagent (Bio-Rad Laboratories, California, USA) and images were captured by an Image Analyzer (G-Box iChemi XR; Syngene, Maryland, USA). Images were quantitated and analyzed using ImageJ software (NIH, Bethesda, USA).

Immunohistochemistry

The lung tissue was perforated with and fixed in formalin solution and embedded in paraffin. The lung Sect. (4 μ m thickness) were stained with hematoxylin and eosin (H&E) and were visualized in a microscope (Leica Microsystems, Germany) at 10X and 40X magnifications.

Statistical analysis

Results are expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using the one-way ANOVA by GraphPad Prism 8 scientific software (GraphPad Software, California, USA). Tukey's multiple

comparisons test was used for post hoc analysis. $p < 0.05$ was considered as statistically significant.

Results and discussion

TAB ameliorated LPS-induced inflammatory markers

We observed no changes in the food intake and body weights among the experimental groups (Fig. 1A, B).

A profound increase in cytokines (cytokine storm) causing severe inflammation and lung damage is manifested in sepsis-induced ALI and the same is displayed in LPS injected rats. The LPS after binding to TLR4 induces the events, which converge at the activation of nuclear factor kappa light-chain-enhancer of activated B cell (NF- κ B) inducing the gene expression of pro-inflammatory mediators (Fig. 7) (Akira and Takeda 2004). We observed increased phospho-NF- κ B expression in the lungs of LPS injected rats while TAB pre-treatment significantly prevented LPS induced NF- κ B activation (Fig. 1F, G). TNF α , an inflammatory cytokine secreted by macrophages during acute inflammation, triggers several cell-signalling events. We have observed higher circulating TNF α by 30 min of LPS injection and its levels peaked by 60 min and later diminished by 120 min but still higher than control. Pre-treatment with TAB could partially ameliorate the rise in TNF α levels as seen in Fig. 1C. Immunoblotting of TNF α at the tissue level showed higher protein levels in the lungs of LPS injected rats while TAB intervention significantly prevented this rise of TNF α levels (Fig. 1F, G). Another important inflammatory cytokine, interleukin 6 (IL6) responsible for stimulating acute phase inflammatory response was increased in circulation by 60 min and a further increase till 120 min of LPS injection. However, TAB could completely ameliorate the rise of IL6 levels at 60 min and partially at 120 min of LPS injection (Fig. 1D). Interleukin 10 (IL-10), a potent anti-inflammatory cytokine that plays a vital role in avoiding damage to the host and preserving tissue homeostasis was found increased by 30 min of LPS injection and gradually decreased but greater than control even at 120 min. TAB intervened rats showed moderate levels of circulatory IL10 throughout the observed period (Fig. 1E).

TAB prevented LPS-induced lung histopathological changes

Inflammatory infiltrates, alveolar membrane thickening and severe alveolar space destruction were observed in LPS injected group compared to the control group (Fig. 2). However, pre-treatment with the TAB significantly prevented the severity of ALI compared with the rats that received LPS alone (Fig. 2).

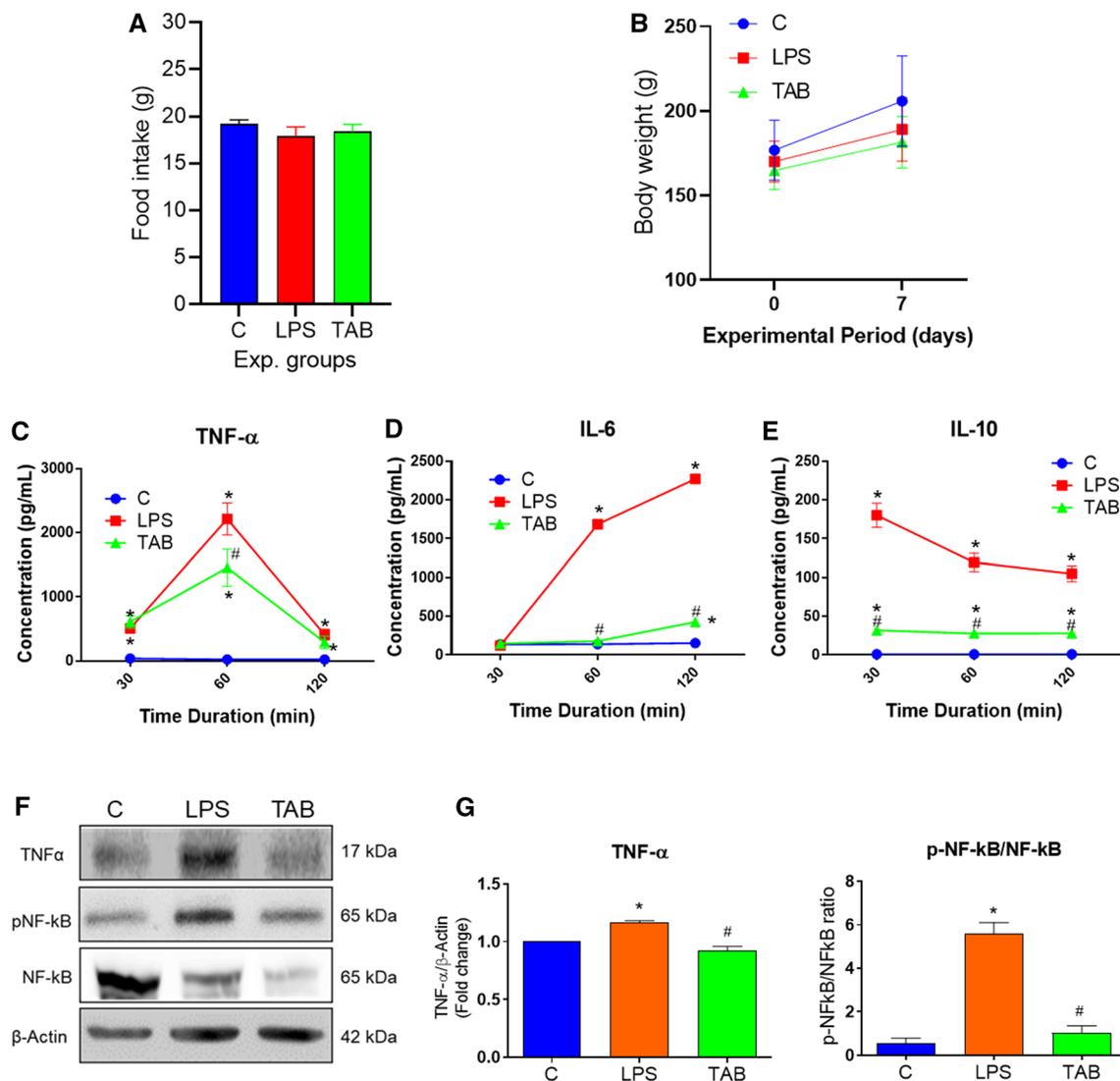


Fig. 1 Pre-treatment with TAB prevented LPS-induced inflammation. **A** Average food intake/day. **B** Bodyweight of rats. **C** Plasma TNF α levels. **D** Plasma IL6 levels. **E** Plasma IL10 levels. **F** Representative immunoblots of lung tissue inflammatory markers. **G** Quantitative data of immunoblots. Data are presented as the mean \pm SEM.

* $p < 0.05$ when compared with the control group, # $p < 0.05$ when compared with the LPS group. C=control group, LPS=lipopolysaccharide injected group, TAB=functional food mixture (turmeric + amla + black pepper) pre-treated group

TAB ameliorated LPS-induced MAPK (ERK/JNK) and PI3K/Akt pathway activation

There are several signalling pathways associated with the inflammation, however, MAPKs and PI3K/Akt are considered as two key signalling cascades affecting the translocation of NF- κ B during inflammation (Andy SN 2017) (Fig. 7).

MAPKs are well-known signalling adaptors involved in inflammatory responses. To determine the outcome of TAB pre-treatment on LPS-induced MAPKs, the levels of extracellular signal-regulated protein kinase (ERK1/2),

and c-Jun N-terminal kinase (JNK) were assessed by immunoblot. We observed increased levels of pERK1/2 and JNK1 protein expression in the lungs of the LPS group that were prevented by TAB (Fig. 3).

Phosphoinositide 3-kinases (PI3Ks) regulate inflammatory response by modulating the activation and spreading of neutrophils and macrophages (Hawkins and Stephens 2015). Hence, we next investigated the status of PI3K and Akt protein expression. We observed increased phosphorylation of PI3K and Akt proteins in the LPS group that were prevented by TAB (Fig. 3).

Fig. 2 Representative histology of LPS-induced lung injury in rats. (Hematoxylin and eosin staining; magnification, x10 and x40; scale bar = 100 μm for x40 and 200 μm for x10). LPS = lipopolysaccharide group; TAB = functional food mixture (turmeric + amla + black pepper) pre-treated group. Black filled arrows are indicating decreased alveolar spaces, red arrows are indicating thickened alveolar membrane, black open arrows are indicating macrophage infiltration near alveolar space

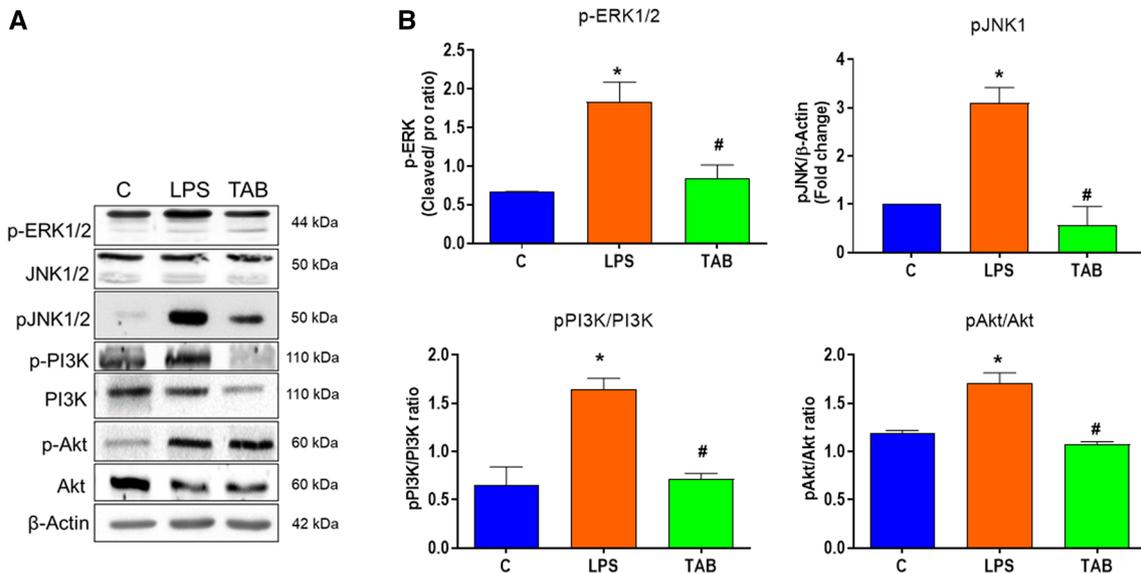
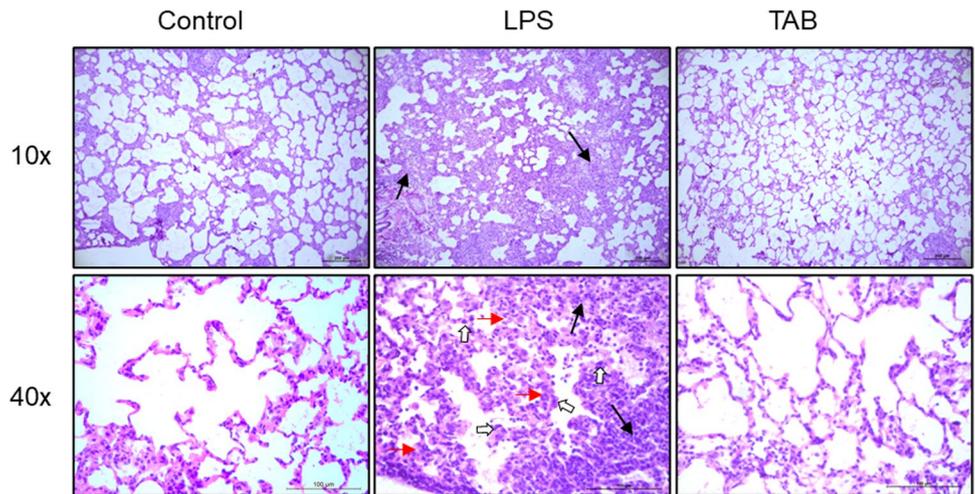


Fig. 3 TAB prevented LPS-induced MAPK and PI3K/Akt excessive activation. **A** Representative immunoblot images of MAPK and PI3K/Akt pathway proteins. **B** Quantification data of immunoblots. Data are presented as the mean ± SEM. **p* < 0.05 when compared with the control group, #*p* < 0.05 when compared with the LPS group. C = control group, LPS = lipopolysaccharide injected group, TAB = functional food mixture (turmeric + amla + black pepper) pre-treated group

pared with the control group, #*p* < 0.05 when compared with the LPS group. C = control group, LPS = lipopolysaccharide injected group, TAB = functional food mixture (turmeric + amla + black pepper) pre-treated group

TAB ameliorated LPS-induced ER stress

ER stress response plays a vital role in many infectious conditions including LPS induced sepsis (Zeng et al. 2017) and hence, we next investigated the status of ER stress markers in the rat lung. As shown in Fig. 4, we observed increased protein expression of ER stress markers like GRP78, pIRE1 and CHOP in the LPS group. However, TAB pre-treatment prevented LPS-induced ER stress.

ER stress is a protective response not only to restore protein homeostasis but also to restore cellular homeostasis by modulating several cellular signalling pathways by

activating the unfolded protein response. ER stress recruits TRAF2 to the ER membrane to initiate inflammatory responses via the NF-κB pathway and is a major contributor to several inflammatory diseases (Keestra-Gounder et al. 2016; Li et al. 2020a). The ER stress inhibitor 4-PBA was shown to prevent LPS induced inflammation through modulating ER stress and autophagy in ALI models (Zeng et al. 2017; Wang et al. 2020). In the current study, the TAB could alleviate ER stress in LPS injected rats (Fig. 4). The tannoids of amla, curcumin and piperine present in the TAB were earlier shown to combat ER stress individually by various mechanisms in varied disease models

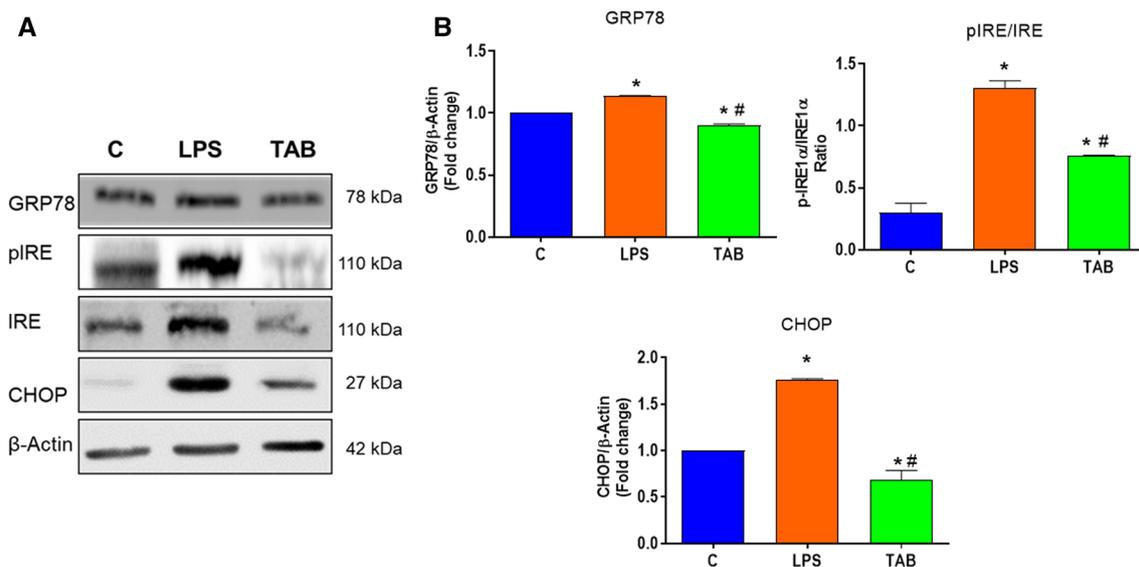


Fig. 4 TAB prevented LPS-induced ER stress. **A** Representative immunoblot images of ER stress markers. **B** Quantification data of immunoblots. Data are presented as the mean \pm SEM. * p < 0.05 when compared with the control group, # p < 0.05 when compared with the

LPS group. C=control group, LPS=lipopolysaccharide injected group, TAB=functional food mixture (turmeric + amla + black pepper) pre-treated group

(Guo et al. 2018; Dhivya Bharathi et al. 2019; Shakeri et al. 2019).

TAB ameliorated LPS-induced changes in autophagy-lysosomal system

To further understand the possible mechanisms of TAB interference in reducing inflammation, we analysed the level of Beclin1, ATG5, LC3-II and p62 proteins to inspect the possible alterations in the autophagy-lysosome system (Fig. 5). LPS-group showed increased protein expression of Beclin1, ATG5, LC3II and P62 while TAB- group showed a beneficial effect in preventing these alterations (Fig. 5).

TAB ameliorated LPS-induced apoptotic markers

Along with the inflammation lung-cell apoptosis is another key pathologic feature of ALI and modulation of apoptosis was shown to prevent LPS induced ALI (Ju et al. 2018; Xie et al. 2018). Hence, we next investigated the status of apoptotic protein expression in the lungs of experimental rats. As shown in Fig. 6, the LPS- group showed increased protein levels of Bax that plays a crucial role in the mitochondrial apoptotic process. Bax/Bcl2 ratio estimated by quantitative immunoblotting was greater in LPS-group when compared with the control group but with no statistical significance. Caspase-3 protein another marker of apoptosis was elevated in LPS-group but prevented in TAB-group. Cleaved caspase 12, a marker of ER stress-induced apoptosis was elevated in LPS-group which is partly prevented in TAB-group.

Turmeric offers several biological activities most of which are attributed to the presence of curcumin. Curcumin exerts strong anti-oxidant and anti-inflammatory activities and more than 100 clinical trials were conducted on curcumin in various diseases including autoimmune disorders (Naksuriya et al. 2014). Curcumin was shown to regulate the inflammatory response by down-regulating the activity of cyclooxygenase-2 (COX-2), lipoxygenase, and inducible nitric oxide synthase (iNOS) enzymes. It also inhibits the production of the inflammatory cytokines TNF- α , IL-1, -2, -6, -8, and -12, and down-regulates mitogen-activated JNK (Abe et al. 1999; Jagetia and Aggarwal 2007; Goel et al. 2008). Curcumin dose-dependently inhibited LPS-induced NF- κ B with an IC₅₀ of 18 μ M, interestingly turmeric extract showed an IC₅₀ of 15 μ M (Edwards et al. 2020). In another study, curcumin-free turmeric was also shown to have anti-inflammatory activity (Aggarwal et al. 2013).

Amla has been used in treating several disorders especially inflammatory diseases such as pneumonia, hepatitis, and even cancer, and is well-known for a wide range of biological activities (Wang et al. 2017). Phytochemical studies on amla showed that it is rich in tannins, polyphenols, flavonoids, gallic acid, vitamin C, and emblicol (Sarin et al. 2014). The immunomodulatory effects of Amla were mainly due to the down-regulation of pro-inflammatory genes, COX-2, iNOS, IL-16, IL-6, and TNF- α (Chatterjee et al. 2011; Sripanidkulchai and Junlatat 2014). Amla also alleviated LPS induced inflammation in macrophages by decreasing the release of pro-inflammatory mediators (Li et al. 2020). Chang et al. reported that beta-glucogallin

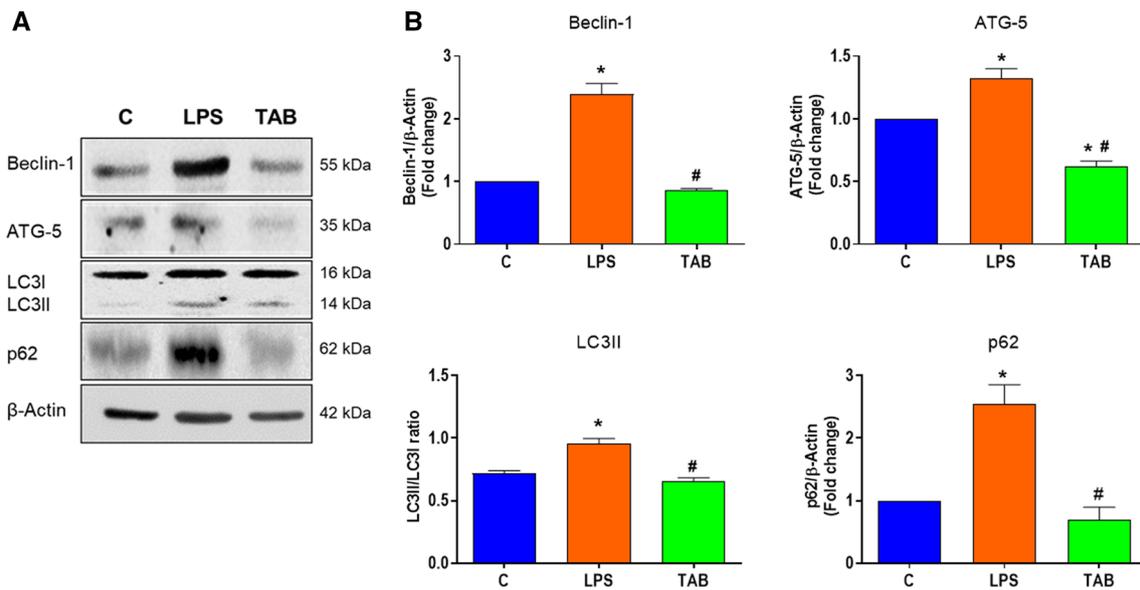


Fig. 5 TAB prevented LPS-induced alterations in autophagy- lysosomal system. **A** Representative immunoblot images of autophagy proteins. **B** Quantification data of immunoblots. Data are presented as the mean \pm SEM. * $p < 0.05$ when compared with the control group,

$p < 0.05$ when compared with the LPS group. C = control group, LPS = lipopolysaccharide injected group, TAB = functional food mixture (turmeric + amla + black pepper) pre-treated group

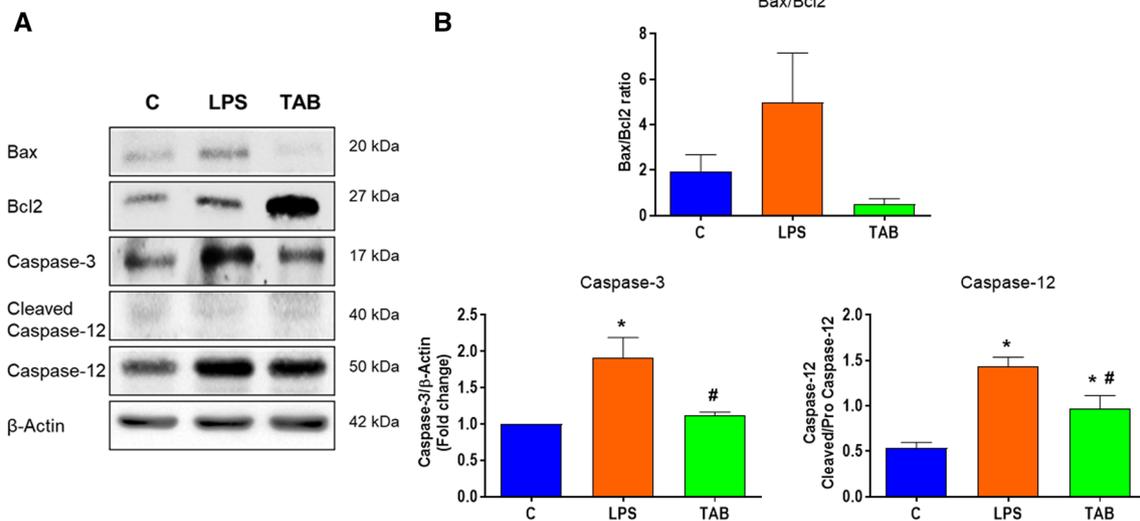


Fig. 6 TAB prevented LPS-induced alterations in apoptotic markers. **A** Representative immunoblot images of apoptotic and anti-apoptotic proteins. **B** Quantification data of immunoblots. Data are presented as the mean \pm SEM. * $p < 0.05$ when compared with the control group,

$p < 0.05$ when compared with the LPS group. C = control group, LPS = lipopolysaccharide injected group, TAB = functional food mixture (turmeric + amla + black pepper) pre-treated group

isolated from amla could inhibit LPS-induced oxidative stress by preventing activation of JNK and p38 in murine macrophages (Chang et al. 2013).

Black pepper is used in traditional food formulations, perfumery, alternative medicine, and cosmetics in many Asian countries. The active phenolic component of pepper, piperine, is shown to have beneficial health effects. Piperine

was shown to inhibit the translocation of activator protein 1 (AP-1) in IL1 β -treated cells thereby modulating inflammation (Bang et al. 2009). Piperine was reported to exert anti-inflammatory activity by decreasing the expression of ICAM-1 on the macrophage surface thereby inhibiting macrophage polarization (Gholijani et al. 2021). Piperine could suppress the pro-inflammatory factors IL-1 β and TNF- α , and

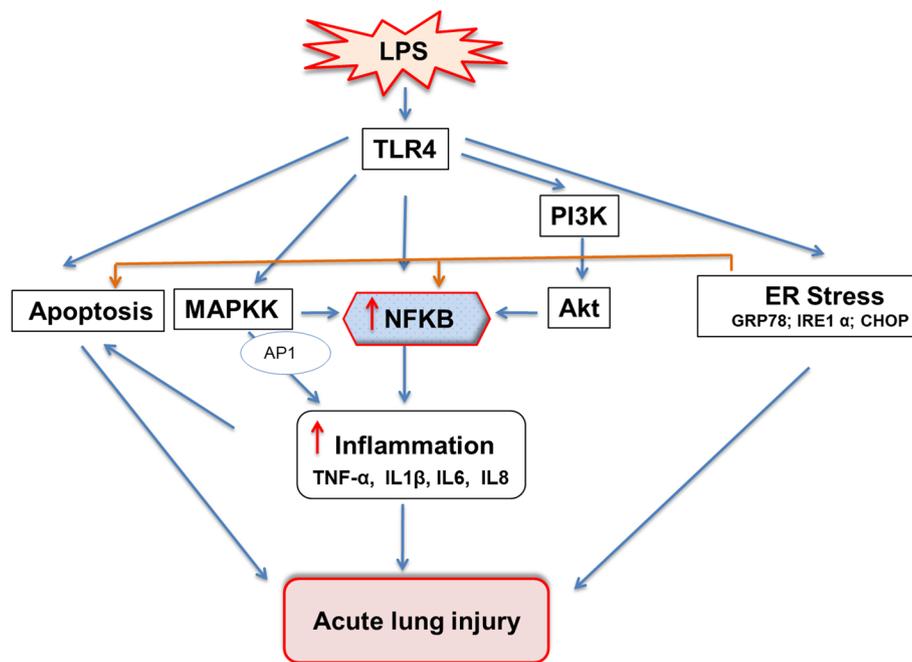


Fig. 7 Schematic diagram showing the key mediators of LPS induced acute lung injury. LPS activates TLR4 on the cell membrane that in turn triggers several cell-signalling molecules. TLR4 initiates translocation of the pro-inflammatory transcription factor NF- κ B into the nucleus from the cytoplasm to induce gene transcription. TLR4 also activates PI3K/Akt signalling to stabilise NF- κ B. MAPKs activated by TLR4 also induces pro-inflammatory cytokine synthesis via AP1. TLR4 induces apoptosis in multiple ways including ROS mediated, ER stress mediated and cytokine-mediated apoptosis. ER

stress further increases the translocation of NF- κ B for the induction of pro-inflammatory cytokine gene expression. Severe inflammation in the lung caused by excessive proinflammatory cytokines, ER stress, oxidative stress, and apoptosis collectively disturbs organ homeostasis and finally leads to acute lung injury. Pre-treatment with functional food mixture TAB could modulate the activities of several key mediators of LPS-induced ALI in rats. LPS- lipopolysaccharide, TLR4- Toll-like receptor 4, NF- κ B- nuclear factor kappa light-chain-enhancer of activated B cell, AP1- Activator protein 1

enhance the anti-inflammatory effects of IL-10 and TGF- β 1 (Yu et al. 2021). Though curcumin has several health benefits, one of the drawbacks is its poor bioavailability. Interestingly, piperine was found to increase the bioavailability of curcumin by 2000% in both humans and animals with no adverse effects (Shoba et al. 1998). Hence, the issue of curcumin's poor bioavailability could be resolved by the addition of pepper, consequently enhancing the efficacy of the formulation by synergism.

Conclusion

As summarised in Fig. 7, sepsis induces severe inflammation in which NF- κ B activation is crucial and multifactorial. Further, the uncontrolled inflammation finally damages the lung tissue causing ALI. The results showed that pre-treatment with TAB constituting turmeric, amla, and black pepper powder at specified doses prevented LPS-induced NF- κ B activation and its downstream pathologies in rat lungs. TAB has ameliorated LPS-induced ER stress response, MAPK signalling, autophagy, and cell

death processes. The anti-inflammatory effect of the TAB is well supported by these results and hence, can be used at least as an adjuvant for ALI and other inflammatory conditions. The formulation can be easily prepared as the ingredients are economical and readily available. Further, people regularly consume these constituents for centuries as part of the diet without any signs of toxicity.

Authors' contributions MN: Performed the experiments, data collection, and analysis. KKK, KPR, MS, and URA: data collection and analysis. GBR: Project administration, manuscript review, and funding acquisition. SSR: Study conception and design, supervision and manuscript writing.

Data availability Authors confirm that all relevant data are included in the article. Raw data are available with the corresponding author on request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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Ethical approval Approved by Institutional Animal Ethics Committee.

Consent to participate Not applicable.

Consent for publication Not applicable.

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