



Ziziphus mauritiana Lam attenuates inflammation via downregulating NFκB pathway in LPS-stimulated RAW 264.7 macrophages & OVA-induced airway inflammation in mice models

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ABSTRACT

Ethnopharmacological relevance: *Ziziphus mauritiana* Lam leaves were utilized in treating asthma, diabetes, inflammation, and hepatic diseases in Indian traditional medicine. The leaves were used as an edible vegetables in rural parts of India.

Aim of the study: The aim is to prove the anti-inflammatory activity of *Ziziphus mauritiana* Lam leaves against LPS-stimulated RAW 264.7 macrophages and OVA-induced airway inflammation in mice through its attenuation mechanism in the NFκB signalling pathway.

Materials and methods: Terpenoids present in MEZ were quantified using U(H)PLC analysis. MEZ at 50 and 100 µg/mL were tested against LPS stimulated RAW 264.7 macrophages. The concentration of NO, ROS, and cytokines was quantified from the cell culture supernatants. OVA-induced asthma in mice was adopted for screening airway inflammation. MEZ at 250 and 500 mg/kg was tested for airway hyperresponsiveness, leukocyte counting, pro-inflammatory cytokines (IL-4, IL-5, IL-13 and TNF-α), lung histopathology, and various inflammatory gene expressions in lungs for NFκB signalling pathway in asthma.

Results: Terpenoids like betulin, betulinic acid, oleanolic acid, and ursolic acid were quantified from U(H)PLC analysis. MEZ at higher doses reduced the NO, ROS, and pro-inflammatory cytokines in LPS stimulated RAW 264.7 macrophages. MEZ at 500 mg/kg significantly reduced AHR and also decreased total and differential leukocytes. MEZ also reduced the expressions of ICAM, VCAM, and Muc5C genes. Histopathological analysis revealed MEZ significantly reduced the leukocyte infiltration and mucus hypersecretion in the lungs. MEZ suppressed lung inflammation by inhibition of p65 mediated IκB-α translocation in the NFκB signalling pathway. **Conclusion:** From these findings, MEZ significantly reduced airway inflammation by inhibiting NFκB mediated inflammatory pathway. Hence, this study proved that *Ziziphus mauritiana* Lam has anti-asthmatic potential in Indian traditional medicine.

1. Introduction

Asthma is a chronic airway disease characterized by airway hyper-responsiveness, bronchoconstriction, and inflammation. Breathlessness, wheezing, and chronic cough are the main symptoms of asthma, and the severity may vary with individuals (Cukic et al., 2012). Asthma is predominantly estimated to affect >334 million people (4.9%) of the

world's population. People with asthma-like symptoms among all ages estimated to be is more than 635 million. The gender distribution of asthma depends on age and sex; the prevalence rate of males and females are 4.6% and 5.1%, respectively (Ahmed et al., 2015). The diverse interactions between genetic factors and environmental agents are the predominant cause of asthma. Aeroallergens include pollens, dust mites, and animal dander; they often have proteolytic properties that cause allergic asthma (Jenerowicz et al., 2012). Drugs from natural origin play

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Abbreviations

AHR	Airway hyperresponsiveness	iNOS	Inducible nitric oxide synthase
BAL	Bronchoalveolar lavage	LPS	Lipopolysaccharide
DCFH ₂ -DA	Dichloro-dihydro fluorescein diacetate	MEZ	Methanol extract of <i>Ziziphus mauritiana</i> Lam leaves
DMEM	Dulbecco's Modified Eagle Medium	Muc5C	Mucin-5 subtype C
FBS	Fetal bovine serum	NFκB	Nuclear Factor Kappa light chain enhancer of B cells
U(H)PLC	Ultra-High-Performance Liquid Chromatography	NO	Nitric oxide
IAEC	Institutional Animal Ethics Committee	PVDF	Polyvinylidene difluoride
ICAM	Intercellular adhesion molecule	ROS	reactive oxygen species
IFN	Interferon	RP	Reverse phase
IgE	Immunoglobulin E	RT-PCR	Reverse transcription-polymerase chain reaction
IL	Interleukin	TLR	Toll Like Receptor
IκB-α	Light polypeptide gene enhancer in B-cells	TNF-α	Tumor necrosis factor
		VCAM	Vascular cell adhesion protein

a significant role in the public health care system of the nation. (Zhang et al., 2010). An increase in population, increased demand for drugs, increase in the cost of treatment, side effects of drugs, and resistance to drugs are the primary reasons that lead mankind towards the use of plant-based herbal medicines for a variety of human disorders (Boston et al., 2019). Focus has shifted towards the phytomedicines and isolation of natural products to treat asthma. Plant-based drugs are currently tested for efficacy in clinical trials claimed no side effects.

The genus *Ziziphus* comprises more than 170 species widely spread throughout the subtropical regions of Asia and Africa. *Z. mauritiana* Lam. and *Z. jujuba* Mill. are the two main cultivated Indian jujube varieties (Alsayari and Wahab, 2021). *Ziziphus mauritiana* (Rhamnaceae), commonly known as Indian ber enticed with high nutritional values used as edible vegetable in rural parts of India and Indonesia. In Indian traditional medicine, the leaves of this plant are used to treat digestive, hepatic cardiovascular, and urinary disorders. It is also used to treat asthma, fever, obesity, diabetes, eczema, diarrhoea and insomnia (Ramar et al., 2020). Previous studies have shown that *Ziziphus mauritiana* is enriched with terpenoids, flavonoids, phenolic acids, α-tocopherol, β-carotene, and polysaccharides and possess significant anti-inflammatory activities (Kumar et al., 2017). It is already reported that *Ziziphus jujube* was used to treat asthma (Ninave and Patil, 2019). There is no scientific evidence for *Ziziphus mauritiana* Lam on its anti-asthmatic activity. Hence, the present study was carried out to screen the anti-asthmatic potential of *Ziziphus mauritiana* Lam leaves through *in-vitro* and *in-vivo* models to prove its traditional claim.

2. Materials and methods

2.1. Materials

RAW 264.7 macrophage cell lines were obtained from NCCS in Pune, India. Gibco, Thermo Fisher, USA, provided culture media and buffers. MP Biomedicals, USA provided betulinic acid, ursolic acid, and oleanolic acid (purity 97 percent). Alfa Aesar (Haverhill, MA) provided methacholine bromide (L06848) and Sigma Aldrich provided ovalbumin (A5503) (St. Louis, MO). Raybiotech, USA, provided the ELISA kits. Santa Cruz Biotechnology, USA provided antibodies like IκB-α (847), NFκB-p65 (372), and NFκB-p50 (7178). Eurofins Genomics India provided primers. All the reagents and chemicals used were of analytical grade.

2.2. Collection of plant and preliminary phytochemical analysis

We collected the leaves of *Ziziphus mauritiana* Lam from wild sources of Kolli Hills of Western Ghats in Tamil Nadu, India (GPS coordinates Latitude: 11.248514; Longitude: 78.338707) during December 2018 and identified them from the Botanical Survey of India Coimbatore. A

voucher specimen was preserved in our institution library for further references (AU/BIT/Ph/18/012). The leaves were shade dried coarsely powdered and extracted with methanol using a reflux bath for 48h (Ramar et al., 2020) and concentrated, yielding semi-solid residue of 10.25% w/v. MEZ was tested for preliminary phytochemical analysis (Verma and Singh, 2020).

2.3. U(H)PLC quantification of terpenoids

Standard terpenoids like betulin, betulinic acid, oleanolic acid, and ursolic acid were weighed, dissolved in methanol, and filtered to produce a final concentration of 50 µg/mL. A gradient U(H)PLC (Nexera, Shimadzu, Japan) equipped with a PDA detector and C₁₈ column was used with 50 µL injection volume. The mobile phase used was solvent A: methanol and solvent B: 0.1% (v/v) formic acid in water. We detected the gradient conditions with the zero-time 15% solvent A increased to 75% in 30min with a 0.2 mL/min flow rate, and compounds at 280 nm (Wang et al., 2019).

2.4. LPS-stimulated RAW 264.7 macrophages

2.4.1. Cell culture

RAW 264.7 macrophages were seeded at 10⁵ cells/well in 96-well using DMEM medium supplemented with 10% FBS, penicillin (100U/mL), and streptomycin (100 mg/mL) with humidified 5% CO₂ atmosphere. The medium was changed every two days. Cells were trypsinized to attain confluence.

2.4.2. Cell viability assay

Different concentrations of MEZ (25, 50, 100, 200 µg/mL) were mixed with RAW 264.7 macrophages for 24h. Untreated cells were used as control. MTT dye reduction assay was performed to determine the percentage of cell viability upon treatment (Ogbole et al., 2017).

2.4.3. NO assay and intracellular ROS measurement

RAW 264.7 macrophages were stimulated with 1 µg/mL of LPS with and without MEZ (50 and 100 µg/mL) and incubated at 37 °C for 24h. Equal volumes of cell culture supernatants and Griess reagent were mixed, incubated at 37 °C for 15min (Park et al., 2017). Similarly, the cell culture supernatants were mixed with 10 µM of DCFH₂-DA, incubated at 37 °C for 30min with and without MEZ (50 and 100 µg/mL) (Park et al., 2019). The absorbance of the mixture was measured at 540 nm using a multimode microplate reader (EnSpire, PerkinElmer, Singapore).

2.4.4. Pro-inflammatory cytokine quantification

We used a Multi-analyte ELISArray kit (Qiagen, USA) to detect various pro-inflammatory cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12,

IL-17A, IFN γ , TNF α , GM-CSF) in culture supernatants as mentioned in the manufacturer's instructions.

2.4.5. Western blot analysis

Quantified total protein samples (30mg/lane) were resolved by 12% (W/V) SDS-PAGE and transferred on the PVDF membrane. Subsequently, it was probed with specific primary antibodies for I κ B- α , NF κ B-p65, and NF κ B-p50, followed by incubation with a specific secondary antibody conjugated with horseradish peroxidase. Densitometry analysis of the bands was performed using ImageJ software (Bio Rad, USA) (Ren et al., 2020).

2.5. OVA-induced asthma in mice

2.5.1. Animals

Female Balb/c mice (6–8 weeks old) were obtained from Sri Venkateswara Enterprises, Bangalore, India. The mice were acclimatized in 12h light/dark cycle and fed with a standard diet and water *ad libitum*. The experiment was approved by the institutional animal ethical committee of Anna University, Tiruchirappalli (AUROT/IAEC/NOV, 2018/0025 Dt. 21.11.2018).

2.5.2. OVA-sensitization and treatment

Mice were divided into five groups (6 mice/group). Group-1 received PBS and served as standard control. Group-2 received OVA sensitization

and challenge. Group-3 served as positive control and received 3 mg/kg of dexamethasone, p.o. Group-4 and 5 received various doses of MEZ (250 and 500 mg/kg, p.o. twice daily) from days 18–23. All the treatments were done by oral gavage. Mice from groups 2 to 5 were received 20 μ g OVA adsorbed on 200 μ g alum in PBS intraperitoneally on days 0 and 14 (Kim et al., 2019). Mice were then exposed to 1% OVA in PBS from day 21–23, mentioned as per the protocol in Fig. 2.

2.5.3. Assessment of airway hyperresponsiveness (AHR)

On day 24, AHR was recorded from individual mice placed into an acrylic chamber (Mouse Whole body plethysmograph, Emka, France). After baseline recording (PBS exposure), mice were exposed to increasing concentrations of methacholine in PBS (0, 3.125, 6.25, 12.5, and 25.0 mg/mL). The fold increase in Penh over baseline was calculated and compared among experimental groups (McAlexander et al., 2015) using IOX2 software (Emka, France).

2.5.4. Bronchoalveolar lavage collection and leukocyte counting

On day 25, the mice were euthanized to collect bronchoalveolar lavage. Briefly, mice were euthanized, and tracheotomy was performed. An ice-cold PBS (0.3 mL X 5 times) was infused into the lungs *via* a catheter and aspirated gently to collect the BAL. Total cells were counted from 100 μ L of BAL by the trypan blue dye exclusion method. For differential cell counts, BAL was centrifuged (200g, 4 $^{\circ}$ C, 10min), and cells were fixed in slides using cytospin. The slides were dried, stained with

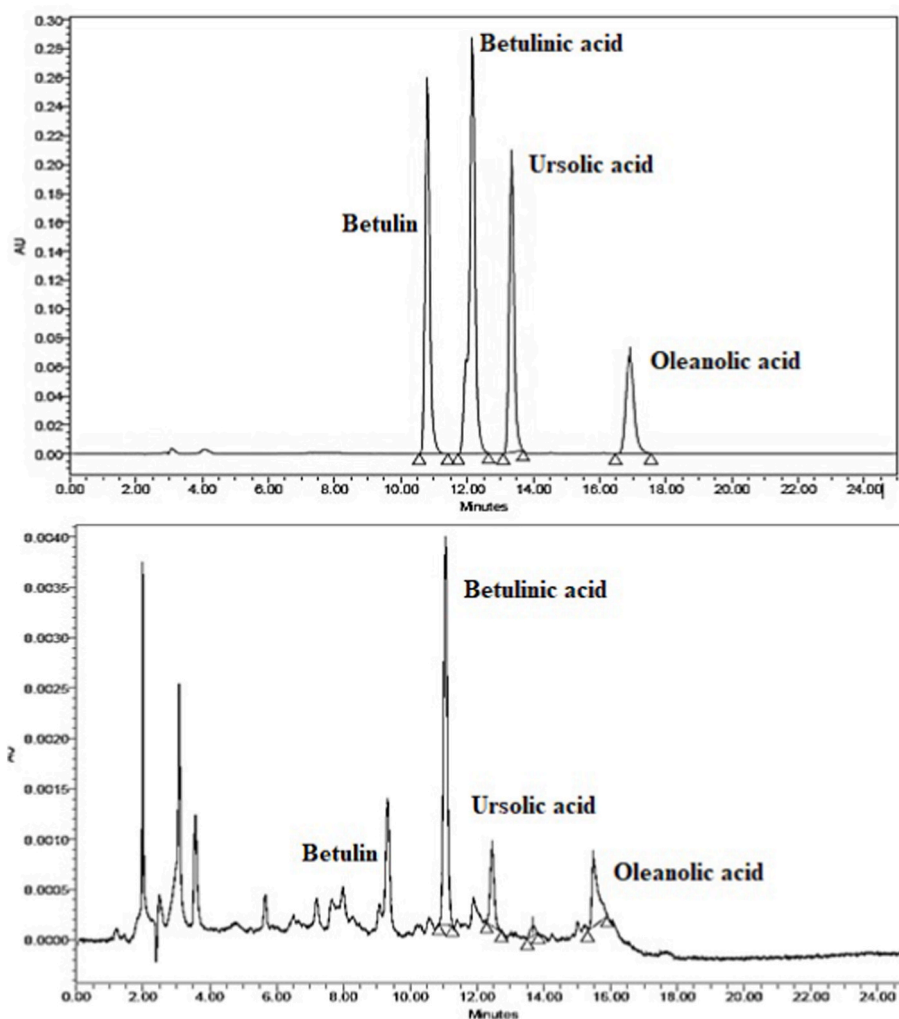


Fig. 1. U(H)PLC analysis of terpenoids and MEZ; A) HPLC chromatogram of standard terpenoids like betulin, betulinic acid, oleanolic acid, and ursolic acid; B) Chromatogram for MEZ.

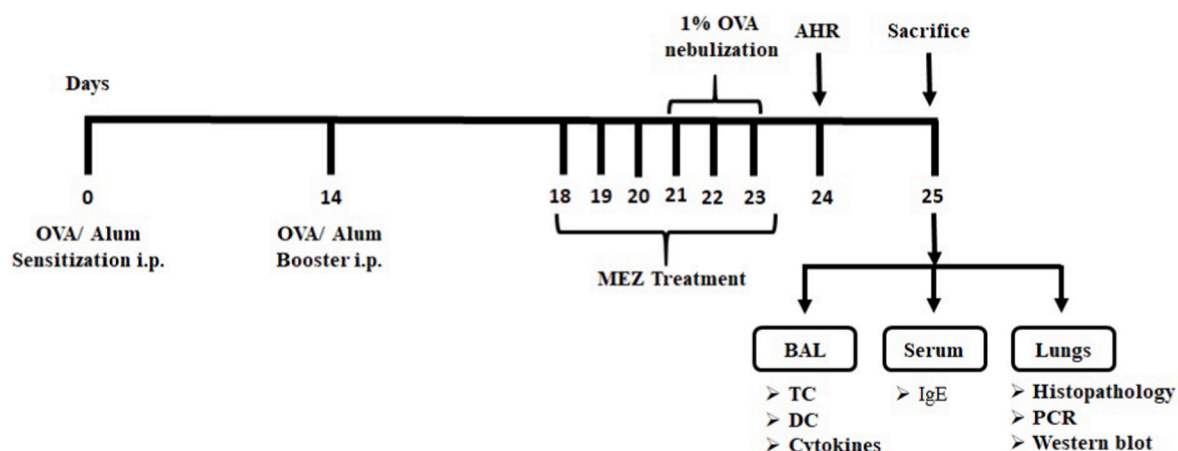


Fig. 2. Protocol for the induction of asthma in mice.

the modified Wright - Giemsa stain, and counted manually for differential leukocytes (Zhou et al., 2018). We preserved the lungs for histopathology, RT-PCR, and Western blot analysis.

2.5.5. Measurement of cytokines in BAL and serum IgE

The concentration of pro-inflammatory cytokines like TNF- α , IL-1 β , IL-6 and IL-17 were measured in BAL supernatant and total, and OVA-specific serum IgE levels from serum were determined using commercially available ELISA kits (Raybiotech, USA).

2.5.6. Lung histopathology

The lung samples were stored in 10% neutral buffered formalin. These samples were processed to make 4 μ m sections, stained with hematoxylin & eosin (H&E) and Periodic acid - Schiff (PAS).

2.5.7. Total mRNA preparation and RT-PCR analysis

Total mRNA was extracted from lung samples using trizol reagent (DSS Takara, India). The primers for the biomarkers are shown in Table 2. RT-PCR (C1000 Touch Thermal Cycler, Bio-Rad, USA) was performed from cDNA using amplification with β -actin as an internal control. The products were resolved through a 2% agarose gel, stained with ethidium bromide, and detected UV light. The density of each band was measured using ImageJ software (Bio-Rad, USA).

2.5.8. Western blot analysis

Lung tissue proteins were quantified and separated on 10% SDS polyacrylamide gels. The antibodies used and protocols followed were mentioned in section 2.4.5.

2.5.9. Statistical analysis

All grouped data was examined using the Statistical Package for Graph Pad Prism Software Version 5.0. All of the tests were repeated three times, and the results are shown as Mean Standard Error Mean (SEM). To examine statistical significance, we used analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Table 1
Quantification of terpenoids from MEZ using U(H)PLC analysis.

S. No	RT of STD	RT of MEZ	Analyte	Regression value	R ² value	LOQ (μ g/mL)	Wavelength (nm)
1	10.68	11.07	Betulin	$y = 1.837x - 0.159$	0.9987	466.82	280
2	12.07	12.79	Betulonic acid	$y = 4.909x - 0.202$	0.9967	265.92	280
3	13.69	13.78	Ursolic acid	$y = 5.592x - 0.631$	0.9906	112.76	280
4	16.73	14.76	Oleanolic acid	$y = 2.690x - 0.326$	0.9947	227.33	280

Each value represents the mean $\pm$ SD (n = 3).

Table 2
Primers used in RT-PCR analysis.

Targets	Sequences
VCAM-1	F-5'-CAAGGGTGACCAGCTCATGAA-3' R-5'-TGTGACGCCACCTGAGATCC-3'
ICAM-1	F-5'-CATCGGGGTGGTGAAGTCTGT-3' R-5'-TGTGGGGGAAGTGTGGTC-3'
Muc5ac	F-5'-GAGTGACATTGCAGGAAGCA-3' R-5'-CAGAGGACAGGAAGGTGAGC-3'
β -actin	F-5'-TCATGAAGTGTGACGTTGACATCCGT-3' R-5'-CCTAGAAGCATTTGCGGTGCACGATG-3'

VCAM-1 - Vascular cell adhesion molecule-1; ICAM-1 - Intercellular adhesion molecule-1.

Muc5ac - Mucin 5AC; F- forward; R-reverse.

3. Results

3.1. Quantification of terpenoids using U(H)PLC

The U(H)PLC chromatograms of standards and MEZ are shown in Fig. 1A and B respectively. The terpenoids identified in MEZ are betulin (1.41 μ g/mL) followed by betulonic acid (6.02 μ g/mL), oleanolic acid (2.36 μ g/mL), and ursolic acid (0.85 μ g/mL) (Table 1). The remaining unidentified peaks were not quantified.

3.2. Effect of MEZ on cell viability

The percentage viability of RAW 264.7 macrophages after MEZ treatment at 25, 50, 100, and 200 μ g/mL concentration is $110.04 \pm 5.63\%$, $105.20 \pm 5.35\%$, $102.25 \pm 4.11\%$, and $89.03 \pm 4.68\%$, respectively (Fig. 3).

3.3. MEZ inhibits NO and ROS productions in RAW 264.7 cells

LPS stimulation increased the nitrite levels to $40.50 \pm 2.51 \mu$ M ($p < 0.001$) in RAW 264.7 macrophages compared to control (1.88 ± 0.75

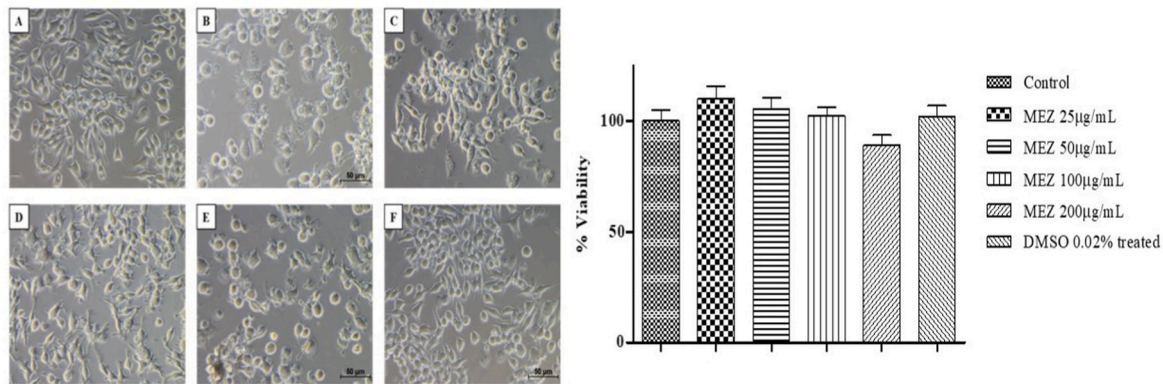


Fig. 3. Effect of MEZ on RAW 264.7 murine macrophages was determined by MTT assay after 24 h incubation. Control, DMSO 0.02% treated (negative control cells). A) Cell viability; B) Percentage viability. Each value represents mean $\pm$ SEM (n = 3).

µM). Pre-treatment with MEZ at 100 µg/mL (12.86 ± 0.98 µM) significantly reduced the nitrite levels (Fig. 4A). Similarly, ROS levels were increased in LPS stimulated RAW 264.7 macrophages (3.00 ± 0.03 folds of control) which were reduced with MEZ treatment (50 and 100 µg/mL) 2.04 ± 0.09 and 1.60 ± 0.33 folds of control, respectively (Fig. 4B).

3.4. MEZ decreases cytokine production

LPS stimulation significantly ($p < 0.001$) increased the cytokine levels except for IL-10 and IL-4, which were reduced significantly ($p < 0.001$). Pre-treatment with MEZ produced a dose-dependent reduction in cytokines production (Fig. 5). Pre-treatment with MEZ at 100 µg/mL produced significant ($p < 0.001$) reduction in the cytokines. Similarly, pre-treatment with MEZ at 100 µg/mL significantly ($p < 0.001$) increased IL-4 and IL-10 levels.

3.5. MEZ downregulating NFκB pathway in LPS stimulated RAW 264.7 macrophages

We used a Western blot experiment to look at the amount of NFκB protein expression in RAW 264.7 macrophages to see if the prior inhibitory effect was linked to the regulation of NFκB expression. LPS (1 µg/mL) increased the expression of NFκB protein (p50, p65 and IKK) as compared to the control group. The expression of NFκB was considerably reduced by pre-treatment with MEZ (100 µg/mL) (Fig. 6). It is beneficial. MEZ pre-treatment, on the other hand, hinders the translocation of NFκB and inhibits p65 mediated phosphorylation by blocking the IκB-α.

3.6. MEZ reduced AHR in OVA-induced asthma mice

OVA-induced mice produced a 3-fold increase in Penh value than the normal control (Fig. 7). MEZ treatment (250 and 500 mg/kg) produced significant protection ($p < 0.001$) against methacholine challenge.

3.7. MEZ reduced leukocyte counting in OVA-induced asthma mice

The total cell count was increased significantly ($p < 0.001$) in the BAL of OVA-induced mice, which is mainly ($p < 0.01$) reduced MEZ at 500 mg/kg treatment (Fig. 8). Similarly, the eosinophil levels, which are increased in the OVA-induced mice, are reduced by the MEZ treatment.

3.8. Effects of MEZ on pro-inflammatory cytokines

The pro-inflammatory cytokines like IL-4, IL-5; IL-13 and TNF-α were significantly ($p < 0.001$) increased in the BAL supernatant of OVA-induced/exposed mice (Fig. 9). Treatment with MEZ at 500 mg/kg significantly reduced the inflammatory cytokines (IL-5 and TNF-α ($p < 0.01$), IL-4 and IL-13 ($p < 0.05$)) levels in OVA-induced asthma mice.

3.9. Effects of MEZ on total and OVA-specific IgE levels

Total and OVA-specific IgE levels were significantly increased ($p < 0.001$) in OVA-induced mice (Fig. 10). Treatment with MEZ at 500 mg/kg significantly ($p < 0.001$) reduced the total and OVA-specific IgE levels.

3.10. Reduction in pathological changes in lungs

We presented the protective effect of MEZ on airway inflammation in

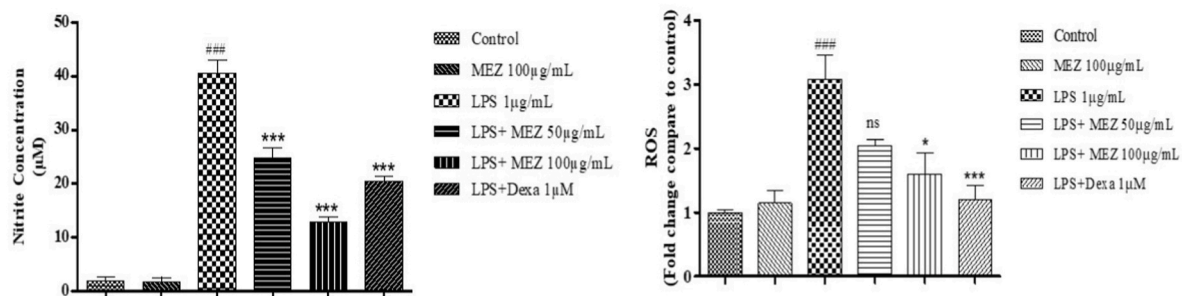


Fig. 4. Effect of MEZ on production of A) Nitric oxide; B) ROS in LPS stimulated RAW 264.7 macrophages. The control group received only media. The LPS group received only Lipopolysaccharide 1 µg/mL. Values are expressed as mean $\pm$ SEM (n = 3). ### $p < 0.001$ statistically different from control vs LPS groups. *** $p < 0.001$, ** $p < 0.01$ statistically different from LPS vs MEZ (50 and 100 µg/ml treatment) groups.

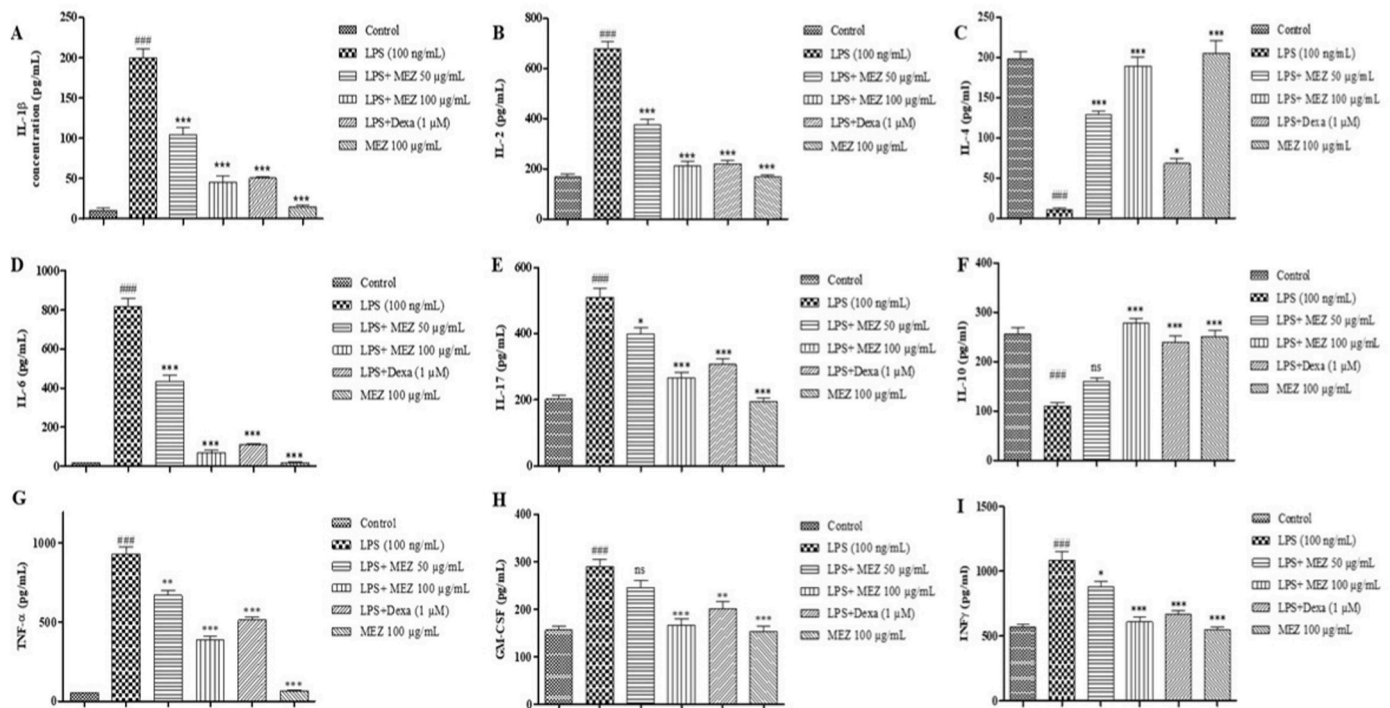


Fig. 5. Effects of MEZ on inflammatory and pro-inflammatory cytokine levels induced by LPS stimulated RAW 264.7 cells. A) IL-1 β ; B) IL-2; C) IL-4; D) IL-6; E) IL-17; F) IL-10; G) TNF- α ; H) GM-CSF; I) INF γ . Values are presented as Mean $\pm$ SEM of three separate experiments. ### $p < 0.001$ Control vs LPS groups. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ for LPS vs MEZ (50 and 100 μ g/ml treatment) and *** $p < 0.001$ for LPS vs dexamethasone treated groups.

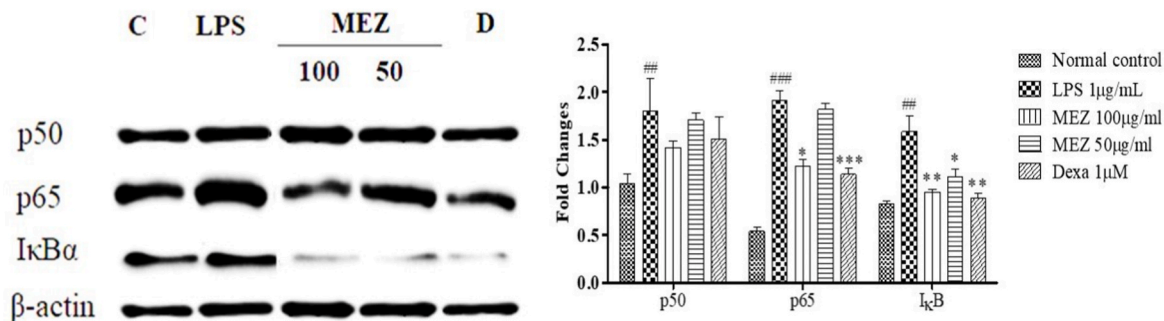


Fig. 6. A) MEZ inhibits the expression of various NF κ B proteins in LPS stimulated RAW 264.7 macrophages; B) Densitometric analysis of NF κ B proteins in LPS stimulated RAW 264.7 macrophages compared to β -actin. Values are presented as Mean $\pm$ SEM of three separate experiments. ### $p < 0.001$ Control vs LPS groups. $p < 0.05$ LPS vs MEZ 50 and 100 μ g/ml treatment. *** $p < 0.001$ for LPS vs dexamethasone treated groups.

Fig. 11. OVA induction has damaged the typical lung architecture in mice. It showed inflammatory changes in the lungs, which include thickening of the airway epithelium infiltration of the inflammatory cells into the peribronchiolar region of the lungs. MEZ treatment markedly reduced the thickness of the airway epithelium and reduced inflammatory cells recruitment into the peribronchiolar area (H&E staining). The OVA induction also increased goblet cell hyperplasia and mucus production (PAS staining). Mice treated with MEZ showed it decreased goblet cell hyperplasia and mucus production.

3.11. MEZ treatment decreases the inflammatory gene expressions

The provocative gene expressions of, VCAM, and Muc5C were increased in OVA-induced mice (Fig. 12). MEZ 500 mg/kg treatment reduced the mRNA expression of VCAM, and Muc5C genes in OVA-induced/exposed mice.

3.12. MEZ downregulating NF κ B signalling pathway

The effect of MEZ on NF κ B activation in the lung tissue of mice with OVA-induced asthma was investigated using western blotting to look at the phosphorylation levels of p50, p65, and I κ B α proteins. The researchers discovered that after being exposed to OVA, the levels of p50, p65, and I κ B α proteins in lung tissues were higher in OVA-exposed mice than in control mice (Fig. 13). The levels of p65 and I κ B α in lung tissues were drastically reduced after pre-treatment with MEZ 500 mg/kg. This finding implies that via changing the phosphorylation of the p65 protein, MEZ treatment lowers phosphorylation levels of I κ B α in lung tissue to reduce airway inflammation.

4. Discussion

The inflammation in the airways results from a series of complex interactions between the immune system and the lung's resident cells. The main features of asthma are airway hyper-responsiveness,

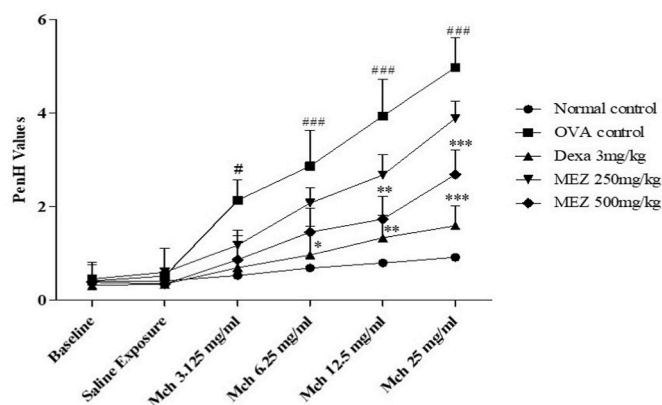


Fig. 7. Effect of MEZ on airway hyperresponsiveness in OVA-induced asthma mice. Mice were nebulized with PBS and graded doses of methacholine to record enhanced pause (PenH). Groups include - Normal control (PBS only); OVA control, OVA-sensitized/challenged mice; Dex, dexamethasone (3 mg/kg) + OVA sensitized/challenged mice; MEZ (250 and 500 mg/kg) + OVA sensitized/challenged mice. Values are expressed as mean $\pm$ SEM. ### p < 0.001 normal control vs OVA control; *** p < 0.001 OVA control vs treatment group; ** p < 0.01 OVA control vs treatment group.

bronchoconstriction, chronic inflammation with increased mucus production leading to narrowing of the airways, and airway remodelling (Kitoko et al., 2018). Plants enriched with terpenoids exhibited significant anti-inflammatory properties (Dhami et al., 2019). MEZ is enriched with terpenoids like betulinic acid, oleanolic acid, and ursolic acid. Macrophages play an essential role against various infections by acting through host defence mechanisms. Activation of macrophages worsens the pathogenic inflammatory conditions by inducing multiple inflammatory cytokines. LPS derived from the outer membrane of the gram-negative bacteria elicit inflammation in the effector cells by stimulating pro-inflammatory cytokines. LPS exposed to the RAW264.7 macrophages were extensively studied for the *in-vitro* models of inflammation (Maruthamuthu et al., 2020). NO plays a vital role in the early onset of the progression of inflammation. Among the inflammatory mediators, NO produced by iNOS plays a crucial role in initiating an immune response from the activation of macrophages. NO is one of the

significant contributors to the inflammatory response (Joo et al., 2014). When stimulated with LPS, Macrophages produce a large amount of NO, which causes inflammatory destruction in the target organs/tissues during infection. ROS are highly reactive radicals, including superoxide, hydroxyl radicals, and hydrogen peroxide, resulting in the mitochondrial respiratory chain reactions. This accumulated ROS plays a vital role in the pathogenesis of many chronic disorders by acting through the redox mechanism in the NF κ B pathway. Pro-inflammatory mediators like interleukins and tumor necrosis factors play an essential role in acute and chronic inflammatory processes. These cytokines stimulate other pro-inflammatory mediators to worsen the inflammation further. For example, interleukin IL-10 inhibits other cytokines like IL-1 β , IL-6, IL-10, IL-12, IL-18, GM-CSF, and TNF- α in the inflammatory process (Alkahtani et al., 2020). From this study, MEZ exerts anti-inflammatory activity by inhibiting ROS, NO, and cytokine produced by RAW 264.7 macrophages. LPS treatment with RAW 264.7 macrophages phosphorylates the I κ B and initiates its release from the cytosol to the nucleus. However, MEZ pre-treatment inhibits the translocation of p65mediated phosphorylation and prevents the translocation of NF κ B by blocking the IKK.

Mice models of allergic asthma had drawn much attention towards the effector phase of the disease progression. Mice induced with inhaled allergens has been used to investigate the novel targets for treating asthma (Kim et al., 2020). AHR is one of the critical pathophysiological features of asthma. It correlates airway resistance with the airway physiology of the lungs of the mice. PenH is a baseless value but is not a mathematical model representation; it is a representation of a breathing pattern. PenH is used to measure bronchial hyper-responsiveness (Lundblad, 2012). Methacholine, a muscarinic receptor agonist, provokes bronchoconstriction on airway smooth muscles, detecting AHR for fewer side effects (De Boer et al., 2017). In this study, MEZ treatment showed protection against airway hyperresponsiveness by lowering PenH values in asthmatic mice. Eosinophils are the predominant inflammatory cells in asthmatic lung tissues. Eosinophil releases eosinophil cationic protein and eosinophil peroxidase, facilitating cytotoxic molecules into the cell (Carr et al., 2016). Hence, its accumulation in lung tissue would cause severe asthmatic allergy and inflammatory response. Macrophages are the minor inflammatory cells recruited into the lungs because of the innate immune response, which engulfs invading pathogens or allergens to maintain homeostasis (Langhans,

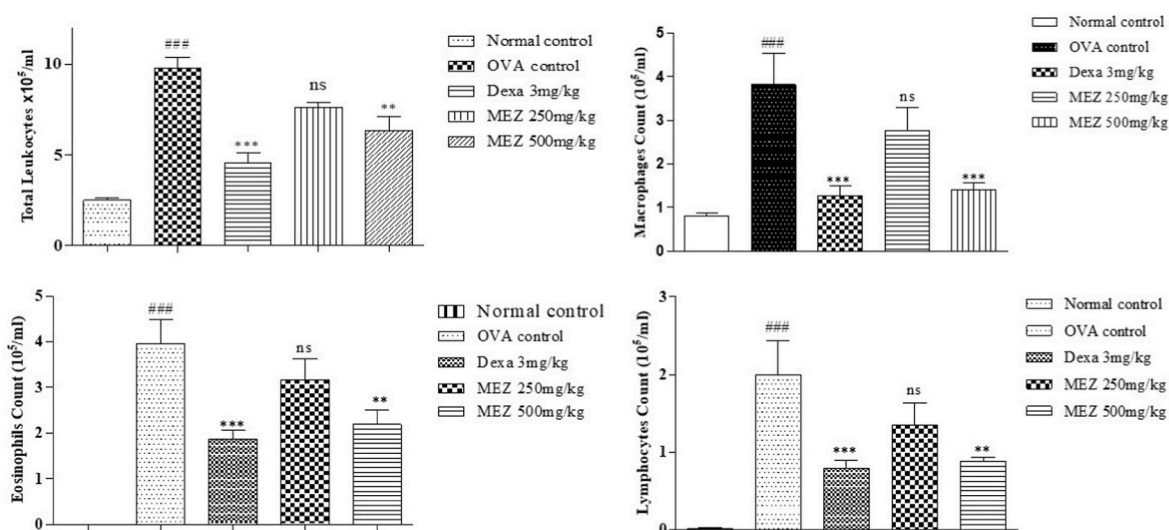


Fig. 8. Effect of MEZ on A) Total cells; B) Eosinophils; C) Macrophages; D) Lymphocytes counts in OVA induced asthma mice. Total cell counts were employed from trypan blue dye exclusion method. Differential cell counts like eosinophils, macrophages and lymphocytes were counted manually for 100 cells. Values are expressed as mean $\pm$ SEM. Groups include - Normal control (PBS only); OVA control, OVA-sensitized/challenged mice; Dex, dexamethasone (3 mg/kg) + OVA sensitized/challenged mice; MEZ (250 and 500 mg/kg) + OVA sensitized/challenged mice. ### p < 0.001 normal control Vs OVA control; *** p < 0.001- OVA control vs treatment group; ** p < 0.01- OVA control vs treatment group.

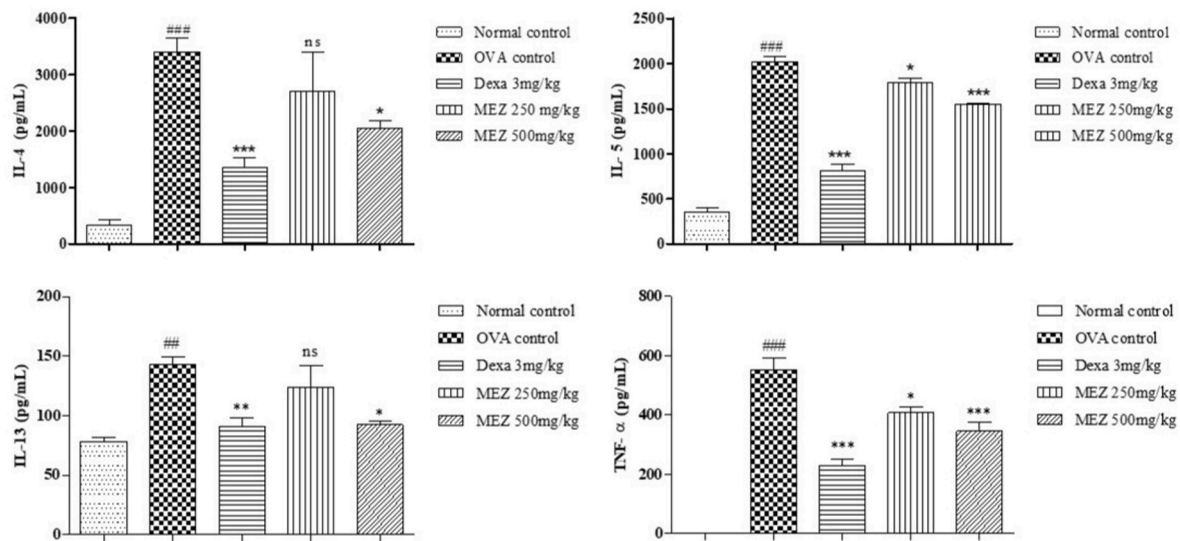


Fig. 9. Effects of MEZ on inflammatory cytokines A) IL-3; B) IL-5; C) IL-13 D) TNF- α in BALF samples on OVA-induced asthma mice. Groups include - Normal control (PBS only); OVA control, OVA-sensitized/challenged mice; Dex, dexamethasone (3 mg/kg) + OVA sensitized/challenged mice; MEZ (250 and 500 mg/kg) + OVA sensitized/challenged mice. Values are expressed as mean $\pm$ SEM. $^{###}p < 0.001$ normal control vs OVA control; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ OVA control vs treatment groups.

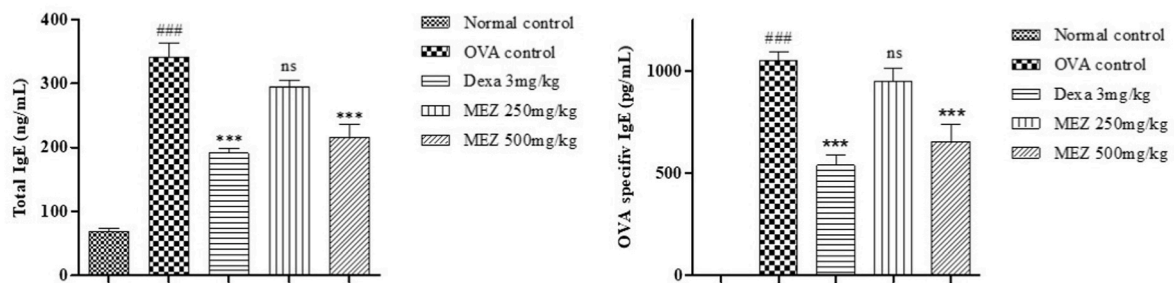


Fig. 10. Effect of MEZ on a) Total IgE; b) OVA specific IgE levels in serum samples of OVA induced asthma mice. Values are expressed as mean $\pm$ SEM (n = 5). $^{###}p < 0.001$ normal control vs OVA control; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ OVA control vs treatment groups.

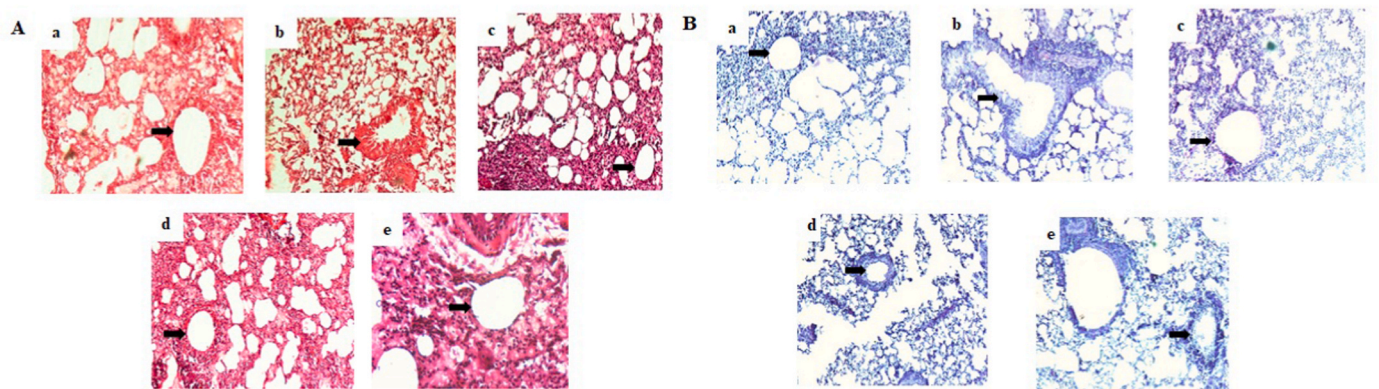


Fig. 11. Effect of MEZ on histopathological changes in the lung tissues. A) H&E Staining; B) PAS Staining. Staining with H&E showed increased inflammation and cellular infiltration in OVA mice which was reduced in MEZ treatment. Similarly PAS staining showed increased mucus secretion in OVA mice which was reduced in MEZ treatment. a) Normal control; b) OVA control; c) Dexamethasone 3 mg/kg treated mice; d) MEZ 250 mg/kg treated; e) MEZ 500 mg/kg treated. Magnification 20X, (scale bar = 100 μ m).

2018). These macrophages are classified into M1 and M2 as per their activation process. The LPS, TNF α , and IFN γ activates M1 macrophages while, IL-4, IL-13, and IL-33. Activates M2 macrophages, which in turn release excessive IL-4 and IL-13 in response to the airway inflammation (Draijer and Peters-Golden, 2017). The IL-4 induces isotype switching in

B cells from IgG to IgE, which binds to mast cells to release leukotrienes and histamine. IL-13 promotes IgE isotype switching in B cells mucus secretion in the airway mucosa (Tedesco et al., 2018). In the present study, mice treated with MEZ 500 mg/kg significantly decreased the elevated cytokines, eosinophils and macrophages.

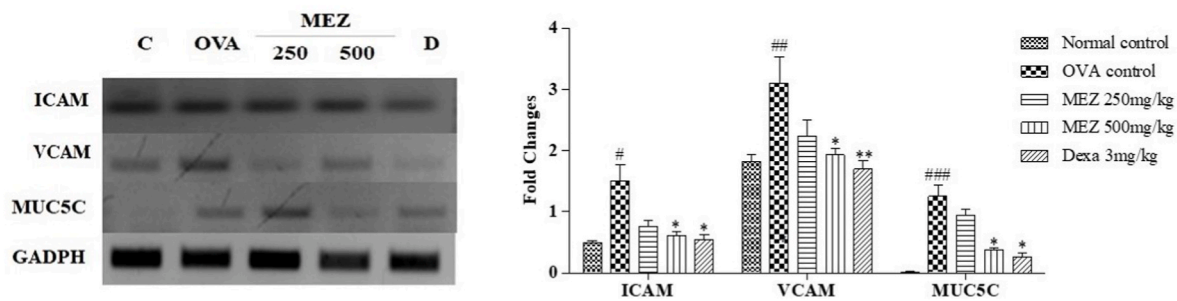


Fig. 12. MEZ inhibits the expression of various inflammatory genes like ICAM, VCAM, and Muc5C A) Gene expressions; B) Desiometric analysis in the lung of OVA-induced asthma mice. ### $p < 0.001$, ## $p < 0.01$ and * $p < 0.05$ normal control vs OVA control; ** $p < 0.01$ and * $p < 0.05$ OVA control vs treatment groups.

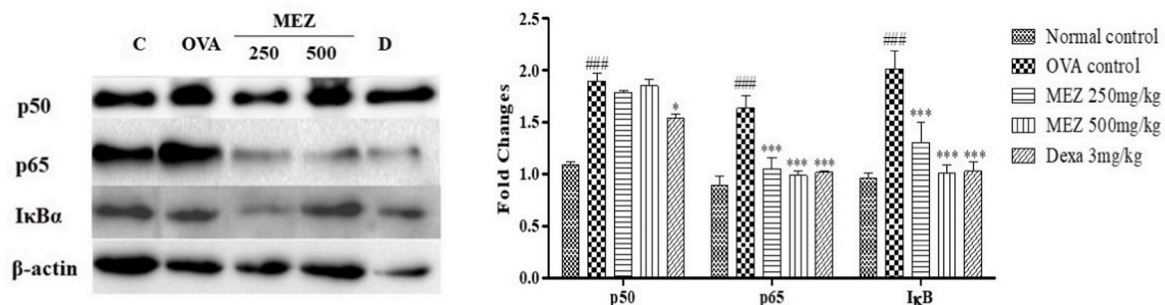


Fig. 13. MEZ inhibits the expression of various NFκB proteins in the lungs induced by OVA challenge. a) Expressions of p50, p65 and IκBα proteins in the lung tissues; b) Densitometric analysis of NFκB proteins in the lung tissues compared to β-actin. ### $p < 0.001$ normal control vs OVA control; *** $p < 0.001$ OVA vs MEZ (250 and 500 mg/kg treatment groups. *** $p < 0.001$, * $p < 0.05$ OVA vs dexamethasone 3 mg/kg treated group.

Th₂ mediated an increase in serum IgE levels confirmed type 1 hypersensitivity reactions. IgE binds with the mast cells, and basophils initiate an NFκB mediated signalling cascade, leads to activate pro-inflammatory and inflammatory cytokines (Oettgen and Burton, 2015). These cytokines activate CD40 ligands and induce the secretion of cytokines IL-4, IL-5, and IL-13, which are responsible for IgE production, eosinophil accumulation, and mucus hypersecretion (Turner et al., 2014). TNF-α is believed to amplify inflammatory reaction as a pro-inflammatory cytokine. Transcription factors and DNA binding proteins regulate the expression of inflammatory cytokines. The migration of eosinophils in asthmatic airways is a multi-step process initiated by the production of IL-5 and IL-13 and coordinated by specific chemokines such as exotoxin in combination with adhesion molecules such as ICAM-1 and VCAM-1 (Johansson, 2017). IL-5 is very specific to eosinophils and is the major hemeprotein responsible for eosinophil growth, differentiation, mobilization, recruitment, activation, and survival. According to growing evidence, IL-5 and IL-13 have a significant role in goblet cell hyperplasia and mucin Muc5C gene and protein production in mice (Esnault and Kelly, 2016). Treatment with MEZ reduced the inflammatory cytokines and IgE levels in mice.

TLR4, as a natural immune recognition receptor, identifies the receptor's signal cascade, which activates NFκB dependent pathways, resulting in the production of numerous inflammatory genes. These genes that were expressed reactivated NFκB expression, resulting positive feedback regulation (Sangaran et al., 2021). NFκB is normally inactive because it binds to its inhibitor IκB. When the epithelium is stimulated and injured (as in allergy disorders), IκBα is separated from NFκB due to the activation of specific proteins, resulting in the activation of NFκB, which then leads to the production of a large number of inflammatory factor genes (Lim et al., 2021). It is reported that OVA induction activates the NFκB mediated airway inflammation in mice (Herath et al., 2020). NFκB plays a vital role in modulating immune responses and inflammatory cytokines like IL-4, IL-5 and IL-13 (Piao et al., 2019). Our results showed MEZ inhibits the translocation of p65

from the cytoplasm to the nucleus and dephosphorylates IκBα in OVA-induced asthma mice. These observations indicate that the modulation of the p65 signalling pathway, involving IκBα and NF-κB, may be one of the molecular bases for the beneficial effects of MEZ on allergic airway disease.

5. Conclusion

This study conducted to prove the traditional claim of *Ziziphus mauritiana* Lam leaves for the treatment of asthma. We quantified the terpenoids present in the leaves using U(H)PLC method. Furthermore, studies were conducted in RAW 264.7 macrophages and OVA-induced asthma in mouse models, respectively. The results confirmed MEZ exhibits significant anti-asthmatic activity by downregulating p65 mediated inhibition of IKK-α in the NFκB signalling pathway. Therefore, *Ziziphus mauritiana* Lam may be considered as a therapeutic alternative for treating asthma. Future studies may be conducted to describe the detailed mechanism of action in which MEZ attenuates airway inflammation in asthmatics.

CCRediT authorship contribution statement

Mohan Kumar Ramar: Conceptualization, Methodology, Investigation, and, Writing – original draft, revision and finalization). **Linda Jeeva Kumari Henry:** Investigation, Formal analysis, Cell line work, and, Writing – review & editing, writing (review). **Shiyamsundar Ramachandran:** Investigation, Formal analysis, western blot and PCR. **Kumarappan Chidambaram:** Formal analysis, HPLC analysis. **Ruckmani Kandasamy:** Conceptualization, Supervision, Project administration, Funding acquisition, and, Writing – review & editing. All authors have approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further reading

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