

Research Article

Inhibition of Pro-Inflammatory Cytokine TNF- α by *Azadirachta indica* in LPS Stimulated Human THP-1 Cells and Evaluation of its Effect on Experimental Model of Asthma in Mice

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Abstract

Asthma is a chronic inflammatory disorder characterized by airway inflammation and airway hyper-responsiveness to inhaled stimuli. The production and release of proinflammatory cytokines (TNF- α , IL-1 β and IL-6) from monocytes/macrophages play critical role in the pathogenesis of chronic inflammatory diseases and immune response. *Azadirachta indica* is a well-known medicinal plant in the Indian traditional system with immunomodulatory action. In present study, the anti-inflammatory activity of herbal immune modulator *Azadirachta indica* was studied by evaluating the inhibition of LPS stimulated TNF- α secretion using human Monocytic THP-1 cells. Differentiation of the human monocytic cell THP-1 into macrophage-like cells was induced by exposure of the cells to Phorbol Myristate Acetate. When cells were pre-incubated with Methanolic extract of *Azadirachta indica* and then stimulated with LPS, secretion of TNF- α was significantly decreased compared to control. Further to *in vitro* study, trypsin and egg albumin induced chronic model of asthma was used to evaluate the anti-asthmatic action of *Azadirachta indica* in experimental induced asthma in mice. Various parameters such as pO₂, serum bicarbonate level, tidal volume, respiratory rate, eosinophil count in bronchoalveolar lavage fluid etc. were measured after the treatment with Methanolic extract of *Azadirachta indica* compared to control. The results of this study were in line with expected responses observed with standard anti-asthmatic agents. Thus the present study has shown that the *Azadirachta indica* inhibits the production and release of proinflammatory cytokines, TNF- α from monocytes/macrophages which play critical role in the pathogenesis of chronic inflammatory diseases and thereby suggesting its possible use in inflammatory diseases such as Asthma.

ABBREVIATIONS

ANOVA: Analysis of Variance; BAL: Broncho Alveolar Lavage; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; CO₂: Carbon Dioxide; ELISA: Enzyme Linked Immunosorbent Assay; GINA: Global Initiative for Asthma; HCL: Hydrochloric Acid; HRP: Horseradish Peroxidase; IAEC: Institutional Animal Ethics Committee; IL: Interleukins; LPS: Lipopolysaccharide; MEAI: Methanolic Extract of *Azadirachta indica*; NaOH: Sodium Hydroxide; NC: Negative Control; NCCS: National Centre of Cell Sciences; NF-kB: Nuclear Factor kB; O.D: Optical Density; PC: Positive Control; PMA: Phorbol-12-Myristate-13-Acetate; RPMI: Roswell Park Memorial Institute; SEM: Standard Error of Mean; TNF- α : Tumor Necrotic Factor α .

INTRODUCTION

Asthma is a chronic inflammatory disorder characterized by airway inflammation and airway hyper-responsiveness to inhaled stimuli. It is a very common disease with immense social

impact which afflicts 300 million individuals worldwide [1]. The physiologic and clinical features of asthma derive from an interaction among the resident and infiltrating inflammatory cells in the airway surface epithelium, inflammatory mediators, and cytokines [2]. Cytokines are biologically highly potent peptides endogenously synthesized upon stimulation and they direct and modify the inflammatory response in asthma and likely determine its severity. Key cytokines include IL-1 β and TNF- α , which amplify the inflammatory response [1].

TNF- α is the most widely studied cytokine of the TNF super family. It mediates numbers of immune-inflammatory reactions ranging from activation and differentiation of immune-inflammatory cells to expression of various adhesion molecules on various cell types and finally involved in expression of major histocompatibility complex [3].

The THP-1 cell line, a human monocytic leukaemia cells, has been widely used in *in vitro* mechanistic studies evaluating anti-inflammatory activities [4,5]. Activated macrophages cause

many of their effects via the secretion of soluble inflammatory mediators. LPS derived from gram-negative bacteria is considered to be the most potent activator of the macrophage secretory response. TNF- α is one of the earliest major proinflammatory mediators secreted by macrophages when stimulated with LPS *in vivo* and *in vitro* [6]. TNF- α has been implicated in the pathogenesis of several inflammatory diseases, including asthma [7] and its production has been suggested as a possible target for therapy in many diseases.

Azadirachta indica, belonging to the family of the Meliaceae and is native to India and Burma. In India Neem is famous with many names like 'Divine Tree', "Heal All", "Nature's Drugstore", "Village Dispensary", "Panacea of all Disease" etc. Each part of the neem tree has some medicinal property. The extensive literature survey for various traditional and scientific documentation indicate that the leaves of *Azadirachta indica* (Meliaceae) have shown immunomodulator characteristics [8]. It is also scientifically documented for its analgesic [9], hyperglycaemic [10], anti-ulcer [11], Antifertility [12], antimalarial [13], antibacterial [14], antioxidant [15], and hepato-protective [16] activity.

Animal models of asthma have been extensively used to examine mechanisms of disease, the activity of a variety of genes and cellular pathways, and to predict the safety of new drugs or chemicals before being used in clinical studies [17]. Asthma involves complex physiological process and as animals are biologically similar to human, animal usage is well established [18].

We undertook a study to evaluate the anti-inflammatory potential of Methanolic extract of *Azadirachta indica* (MEAI) in human monocytic THP-1 cells using a PMA induced cell differentiation model, followed by evaluation of its effect on trypsin and egg albumin induced experimental model of asthma in mice.

MATERIALS AND METHODS

In vitro cell line assay to measure anti-inflammatory action

Propagation of human acute monocytic leukemia cell line (THP-1): Human acute Monocytic leukemia cell line (THP-1) was procured from NCCS, Pune, India. The cell line was cultured in RPMI 1640 (Himedia) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Himedia), 2mM glutamine (Himedia), 1mM sodium pyruvate, 2g/l sodium bicarbonate, 0.05mM mercaptoethanol, 1% 100U/ml penicillin (Himedia) and 1% 100U/ml streptomycin (Himedia) in 25cm² culture flask (Falcon, USA) and incubated at 37°C in CO₂ (5%) incubator.

THP-1 cells were treated with PMA for 24 hrs to convert it to THP-1 macrophage attached cells. After incubation, nonattached cells were removed by aspiration, and the adherent cells were washed with culture medium three times. After attaining 80% confluency, 1x10⁶ of cells/ml were seeded in each well of the plates with complete medium and incubated at 37°C under CO₂ (5%) incubator. The growth medium was changed every 2 days. THP-1 derived macrophage cells were cultured until complete confluency had reached.

Assessment of viability of THP-1 cells: Viability of THP-1 cells was assessed by trypan blue assay. Trypan blue was used to stain the cells after end of each treatment. Cells were aliquot in 0.5ml microfuge tube and equal amount of trypan blue was added to it. Slides were prepared and observed under microscope. Number of stained (dead) vs number of unstained (live) was counted manually.

MEAI and LPS treatments to THP-1 macrophage cells: The THP-1 derived macrophages were separately pre-challenged with different concentration of MEAI. Cells treated with Diclofenac 250µg/ml were used as positive control and cells without treatment were used as negative control. Cells were further incubated with 100ng/ml LPS for 12 hrs in fresh culture medium for stimulation.

5.1.4. Detection of secreted TNF- α in culture supernatants: TNF- α production was chosen as the outcome variable for studying the anti-inflammatory effect. Human TNF- α was quantified by an ELISA using TNF- α kit (GenXbio) according to the manufacturer's instructions. 96 well ELISA plate pre-coated with TNF- α monoclonal body was used. Briefly, Cell culture media centrifuged at 1500rpm for 10 mins at 4°C. Supernatants were collected carefully. PBS was used to dilute the cell suspension to cell concentration of approximately 1 million/ml. Cells degraded through repeated freeze-thaw cycles to release interior components. Cells were centrifuged at 2000-3000rpm for approximately 20 mins at 4°C. Supernatants were collected carefully. 40µl diluted sample, 10µl of TNF- α antibody and 50µl of streptavidin-HRP were added in sample wells and incubated at 37°C for 60 mins. Plates were washed with washing buffer 5 times and 50µl of chromogen reagent A and B were added to each well and incubated for 10 mins at 37°C for color development. 50µl of stop solution was added to each well to terminate the reaction and read at 450nm. TNF- α concentration was calculated by TNF- α standard curve. The sensitivity for TNF- α detection is 1.52ng/L and assay range is 3.1 to 899ng/L.

Calculation: Each experiment was conducted with three independent cell cultures and was triplicated for each treatment. According to standard concentrations and corresponding absorbance (OD) values, linear regression equation of the standard curve was derived. Using the standard curve, the concentration of TNF- α was calculated based on the OD of the sample. Data for supernatant cytokines were expressed as percentage of inhibition relative to the control.

In vivo study to evaluate the effect MEAI on Trypsin and egg albumin induced asthma in mice

Animals: Healthy mice of either sex, weighing 25-30gms were used for the animal experiment. The animals were housed at 25°C; 12 hours light-dark cycles, in poly propylene cages with free access to food and water ad libitum during the course of experiment. The protocol was approved by IAEC (K.B. Institute of Pharmaceutical Education and Research) under the CPCSEA guideline before carried out the project.

Study design: A combination of trypsin and egg albumin was used to induce asthmatic status in mice [19]. The animals were divided into 4 groups (n=6). All animals (except group I) were exposed to trypsin aerosol (1mg/ml, 1ml/min) once daily

for 5 min, followed by a rest of 2 hrs and then exposed to egg albumin aerosol (1% m/V, 1ml/min) for 3 min. This procedure was repeated for 10 days and later egg albumin aerosol was discontinued whereas trypsin exposure was continued until the 21st day. The animals of group I were exposed to aerosol of saline for 5 minutes daily. Group II animals were exposed to trypsin and egg albumin, but did not receive any drug treatment and they served as asthmatic control. After animals developed asthma, animals of group-III received Dexamethasone (5mg/kg, *per oral*), animals of group-IV received MEAI (400mg/kg, *per oral*) treatment from day 22 to day 35. On day 35, after 2 hrs of the last dose of treatment, only egg albumin challenge was given.

On day 1 (baseline) before any exposure, on day 21 (after trypsin exposure) and on day 35 (after treatment) the following parameters were measured for each animal. pO₂, Tidal volume, Respiratory rate, serum bicarbonate. On day 35, in addition to above parameters, Eosinophil count in BAL fluid and histopathology of lung tissue was done.

Measurement of pO₂: The measurement of arterial O₂ tension (pO₂) was done with the help of pulse oxymeter [20,21].

Measurement of serum bicarbonate level: About 1 to 2ml of blood was collected from each animal under anesthesia after 1 hr of the exposure to egg albumin. The serum was separated from blood. For bicarbonate level measurement, 10ml of 1.0gm/dl saline was pipetted out in 100ml beaker. To this, 0.1ml of the serum and 2 drops of phenol red indicator was added and mixed well. In above mixture, 0.01 N NaOH was added drop wise till the end point was achieved (7.35 pH or color changed from yellow to pink). The volume of NaOH required was noted down and considered as control reading (X ml). In another set, 9.0ml of 1gm/dl saline was pipetted out in another 100ml beaker, 0.01 N 1ml HCL was added and mixed well. The above procedure was repeated and volume of NaOH required was noted (Y ml). Serum bicarbonate level (m. Eq/ ltr) was measured based on the readings [22].

Measurement of tidal volume and respiratory rate: Tidal volume and respiratory rate were measured with the help of 'Respiratory volume transducer' that was used with 'strain gage coupler' and student physiograph.

Eosinophil count in BAL fluid: On 35th day of the study, the tracheobronchial tree was lavaged with 1ml of saline 3 to 4 times. The fluid was collected and centrifuged at 2000rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 0.5ml saline. A thin film of 0.5ml suspended saline was made on a clean grease-free slide using a smooth edged spreader. The film was fixed with methyl alcohol for 3 to 5 min and dried. A few drops of Geimsa stain in phosphate buffered saline (pH 6.8) were added and kept for 15 mins. The number of each type of leukocytes was determined under the microscope at 450x magnification [23].

Histopathology of lungs: On 35th day, the animals were sacrificed and lungs were dissected out. The procedure used for histopathological study was fixation of the tissue with formalin, embedding in paraffin blocks, sectioning with microtome (0.7μ thickness) and finally stained by haematoxylin and eosin stain technique [24].

Statistical analysis: Experimental results were expressed as mean + SEM for 6 animals. The results were computed statistically. Statistical significance of the difference in parameters amongst groups was determined by one-way ANOVA followed by post hoc test. Paired t-test was also performed.

RESULTS AND DISCUSSION

PMA-induced differentiation of THP-1 cells

THP-1 cells are premonocytes, committed to the monocytic cell lineage. They grow in suspension and do not adhere to the plastic surfaces of the culture plates (Figure 1A). For the induction of terminal differentiation to macrophage-like cells, THP-1 cells were cultured in the presence of PMA for 24 hrs. After 24 hrs of culture with 50nM PMA, the cells adhered to the dish bottom and had the morphological characteristics of macrophages (Figure 1B).

Cell viability Assay

The results of the cell viability assay showed no significant decrease in viable cells after the treatment of the compound when compared to control as shown in Table 2. Thus, there were no detectable adverse effects of sample treatments on cell viability.

Inhibition of TNF-α by MEAI

The production and release of proinflammatory cytokines (TNF-α, IL-1β and IL-6) from monocytes/macrophages play critical role in the pathogenesis of chronic inflammatory diseases [25]. They are endogenously synthesized upon stimulation and are involved in numerous cellular processes, such as inflammation, immunological responses and many others.

Table 1: Effect of various concentration of MEAI on TNF-α secretion in LPS- stimulated THP-1 cells.

Sample	TNF-α level (ng/L)*
MEAI 50μg	148.5 ± 0.8660
MEAI 100μg	142.5 ± 8.8081
MEAI 250μg	126 ± 9.1150
MEAI 750μg	159 ± 8.8881
Diclofenac 250μg/ml (Positive control)	52.5 ± 2.0207
Negative control	202.5 ± 17.7693

*Each value represents the mean ± SEM, N = 3

LPS = Lipopolysaccharide; MEAI = Methanolic extract of *Azadirachta indica*; TNF-α = Tumor necrotic factor-α

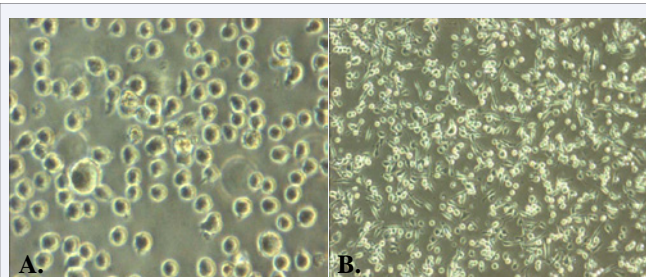


Figure 1 Induction of differentiation in THP-1 cells by PMA. THP-1 cells were incubated for 24 h without (A) or with (B) PMA (50nM).

Table 2: Effect of various concentration of MEAI on cell viability.

Sample	% cell viability*
MEAI 50 μ g	96.31 \pm 0.1493
MEAI 100 μ g	97.55 \pm 0.3546
MEAI 250 μ g	97.29 \pm 0.1162
MEAI 750 μ g	94.37 \pm 0.3724
Diclofenac 250 μ g/ml (Positive control)	94.19 \pm 0.4895
Negative control	99.88 \pm 0.0624

*Each value represents the mean \pm SEM, N = 3
MEAI = Methanolic extract of *Azadirachta indica*

The results for TNF- α release from THP-1 cells pretreated with various sample have shown in Figure 2A. At 50 μ g, 100 μ g, 250 μ g and 750 μ g, MEAI inhibited the release of TNF- α by 26.66, 29.62, 37.77 and 21.48% respectively. TNF- α secretion was maximum inhibited at concentration 250 μ g (37.77% inhibition) when compared to control (Figure 2B). This results indicates MEAI exhibit anti-inflammatory effects by potent suppression of TNF- α release which support earlier research about the anti-inflammatory potential of *Azadirachta indica* [26].

After studying the role of transcription factors involved in immune system (in context to their inflammatory response such as asthma) via *in vitro* cell line assay, an *in vivo* study was conducted to establish the efficacy of MEAI in experimental model of asthma in mice. The findings of trypsin and egg albumin induced experimental mice model of asthma measuring tidal volume, serum pO₂ level, respiratory rate, serum bicarbonate level, eosinophil count as well as observations from histopathological studies were in line with expected responses observed with standard anti-asthmatic agents.

Tidal volume is the volume of air inspired or expired per breath. As asthma is an obstructive disease, there is a difficulty in expiration resulting in reduction of the volume of air expired. In addition, there is shallow and rapid breathing thus decreasing the tidal volume and simultaneously increasing the respiratory rate. The lungs do not provide adequate respiratory exchange due to constricted air flow volume and the levels of oxygen in the blood begin to fall. In the present study, a lower tidal volume was observed in asthmatic group compared to normal control group after trypsin exposure from baseline. There was a significant (p<0.05) increase in tidal volume in the animals subjected to dexamethasone and MEAI (Figure 3). Similarly, significant (p<0.05) higher respiratory rate was observed in asthmatic group compared to normal control group after trypsin exposure

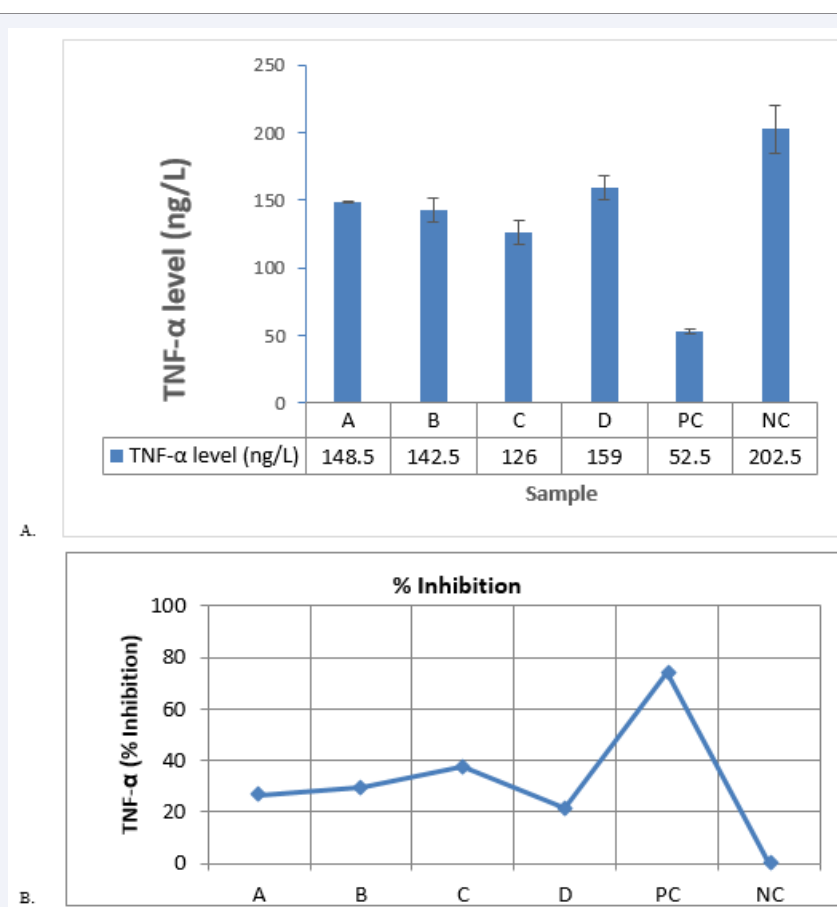


Figure 2 A. Effect of various concentrations of MEAI on TNF- α level. A = 50 μ g, B = 100 μ g, C = 250 μ g, D = 750 μ g, PC = Positive Control, NC = Negative Control Each value represents the mean + SEM, N = 3

B. Percentage inhibition of TNF- α by various concentration of MEAI concentrations. A = 50 μ g, B = 100 μ g, C = 250 μ g, D = 750 μ g, PC = Positive Control, NC = Negative Control

from baseline. Significantly ($p < 0.05$) lower respiratory rate was observed in dexamethasone and MEAI treated groups compared to asthmatic control group (Figure 4).

Further, in patients with asthma, analysis of blood gases reveals a severe hypoxemia with arterial oxygen (pO_2) lower than 60mmHg [27]. In the present study, the sensitized animal when challenged with trypsin and egg albumin, showed a significant ($p < 0.001$) lower serum pO_2 level compared to normal control animals similar to that of observed in patients suffering from asthma. pO_2 level was significantly higher ($p < 0.001$) in the animals subjected to dexamethasone and MEAI compared to asthmatic control group (Figure 5) indicating improvement in condition.

Additionally, among patients with asthma, as severity of airflow obstruction increases, pCO_2 first normalizes and subsequently increases. Increased pCO_2 level in serum will eventually result in increased bicarbonate levels because carbon dioxide in blood is transported as bicarbonates [28]. In present study, challenging animals with trypsin and egg albumin showed a significant higher serum bicarbonate level ($p < 0.05$) in asthmatic control group as compared to normal control group. Serum bicarbonate level was significantly lower ($p < 0.05$) in the animals subjected to Dexamethasone and MEAI (Figure 6).

Eosinophils are bone marrow derived granulocytes that play a central role in asthma. Increased numbers of eosinophils exist in the airways of most persons who have asthma [29]. An

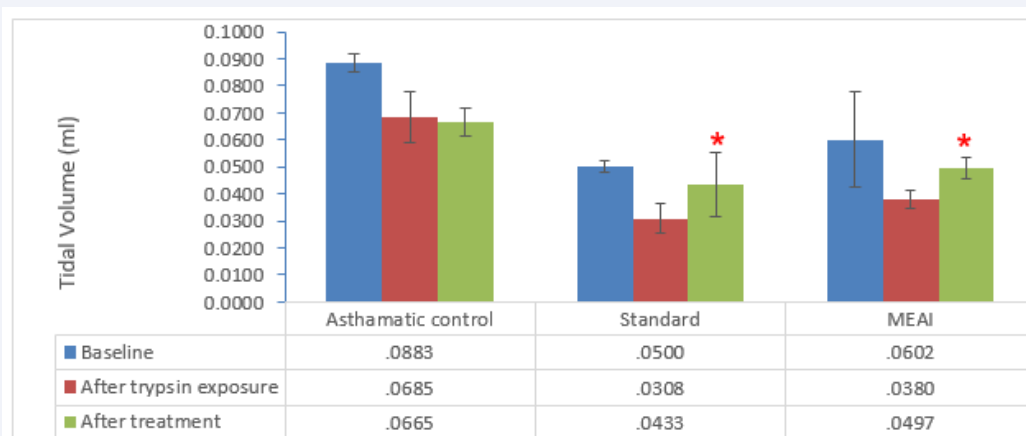


Figure 3 Effect of Dexamethasone and MEAI on tidal volume
Standard = dexamethasone, MEAI = methanolic extract of *Azadirachta indica*
*Significant difference from 21st day ($p < 0.05$) (paired t-test)

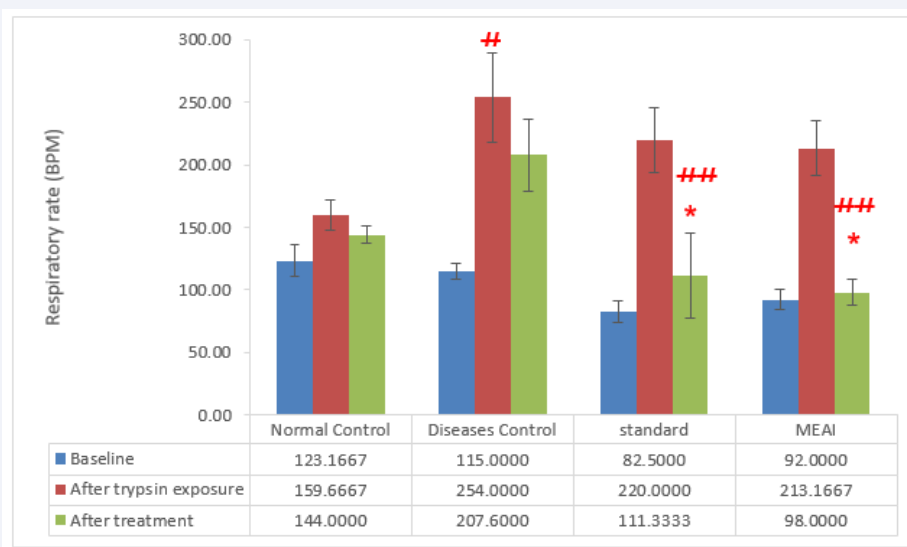


Figure 4 Effect of Dexamethasone and MEAI on Respiratory rate
Standard = Dexamethasone, MEAI = Methanolic extract of *Azadirachta indica*
#Significant difference from baseline ($p < 0.05$) (paired t-test)
*Significant difference from 21st day ($p < 0.05$) (paired t-test)
##Significant difference from asthmatic control group ($p < 0.05$) (One way ANOVA followed by post hoc test)



Figure 5 Effect of Dexamethasone and MEAI on pO₂ level
 Standard = Dexamethasone, MEAI = Methanolic extract of *Azadirachta indica*
 *Significant difference from normal control group (p<0.001)
 #Significant difference from asthmatic control group (p<0.001)
 (One way ANOVA followed by post hoc test)

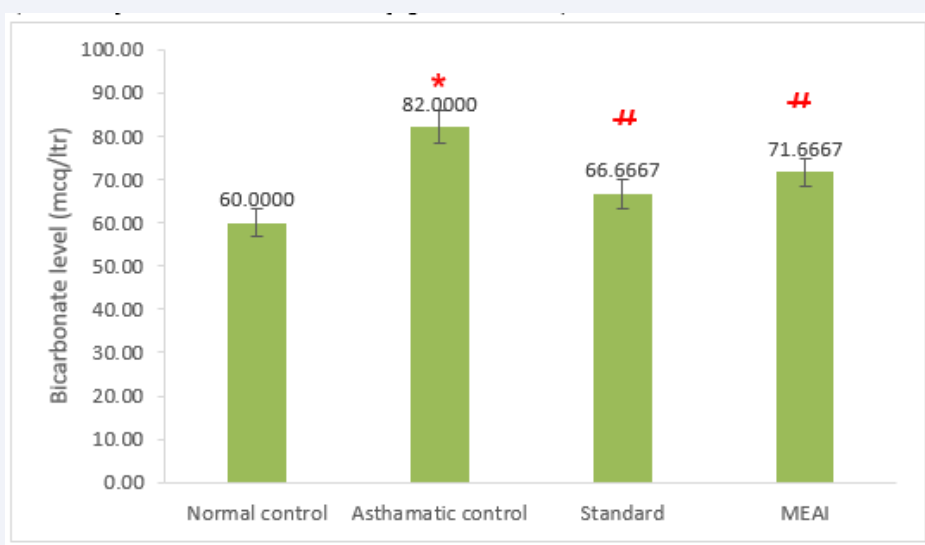


Figure 6 Effect of Dexamethasone and MEAI on serum bicarbonate level
 Standard = Dexamethasone, MEAI = Methanolic extract of *Azadirachta indica*
 *Significant difference from normal control group (p<0.05)
 #Significant difference from asthmatic control group (p<0.05)
 (One way ANOVA followed by post hoc test)

allergic reaction in the airways, caused by natural exposure to allergens, has been shown to lead to an increase in eosinophils in bronchoalveolar lavage [30]. Increases in eosinophils often correlate with greater asthma severity. In present study, challenging animals with trypsin and egg albumin showed a significant higher eosinophil count (p<0.05) in asthmatic control group as compared to normal control group. Eosinophil count was significantly lower (p<0.05) in the animals subjected to Dexamethasone and MEAI (Figure 7).

In asthma, the major physiological event leading to clinical symptoms is airway narrowing. Bronchial smooth muscle contraction (bronchoconstriction) occurs to narrow the airways in response to exposure to a variety of stimuli including allergens or irritants [31] which also cause decrease in the lumen size of the bronchiole. In present study, histopathological studies revealed an intact bronchial structure in normal control group (Figure 8A), whereas trypsin and egg albumin-sensitized animals

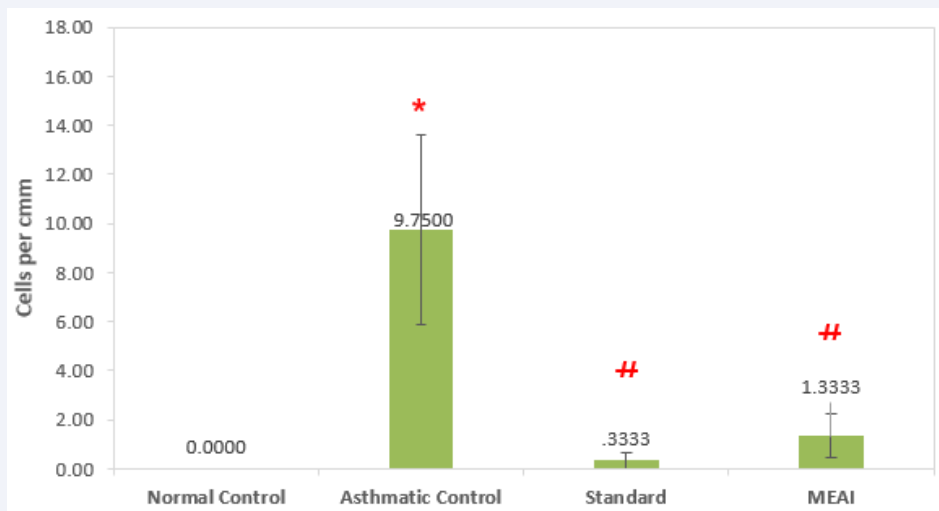


Figure 7 Effect of Dexamethasone and MEAI on eosinophil count
 Standard = Dexamethasone, MEAI = Methanolic extract of *Azadirachta indica*
 *Significant difference from normal control group ($p < 0.05$)
 #Significant difference from asthmatic control group ($p < 0.05$)
 (One way ANOVA followed by post hoc test)

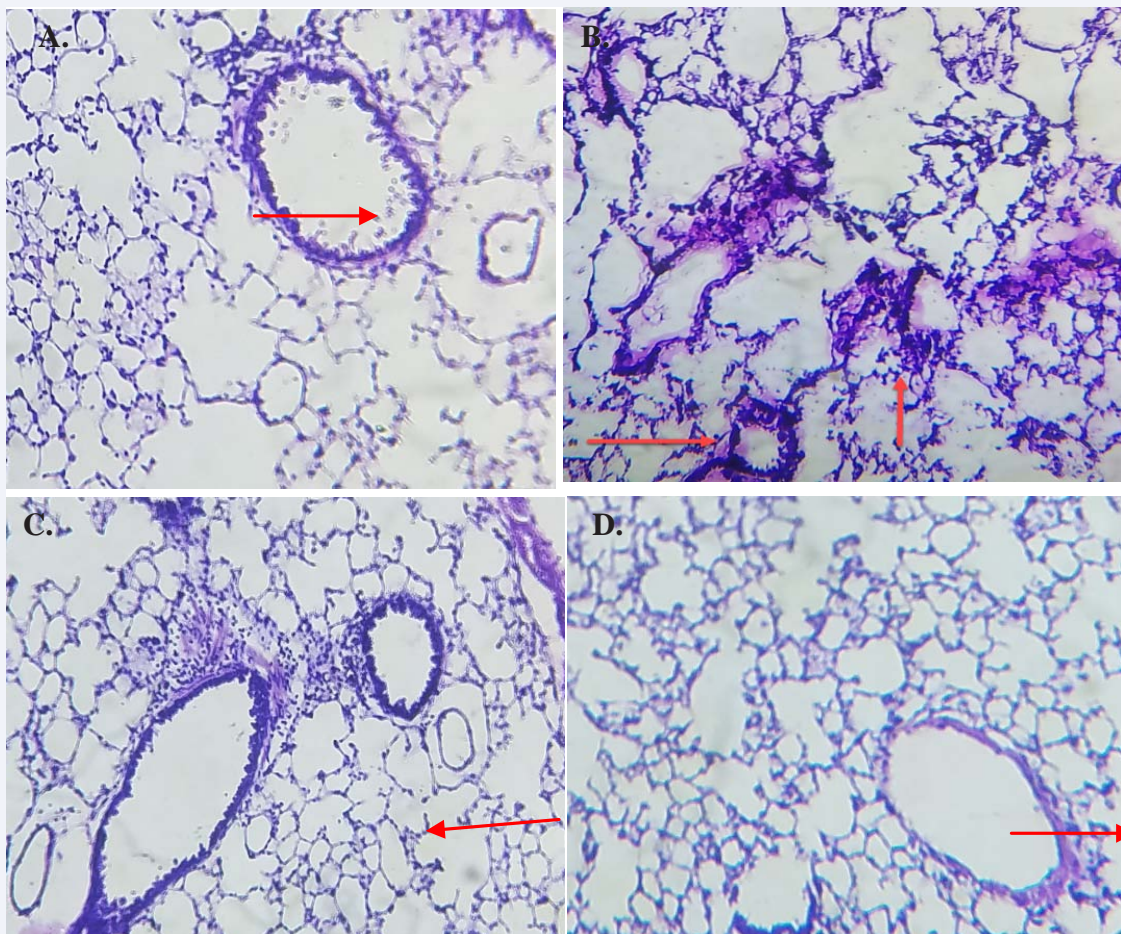


Figure 8 Photographs of tissue sections of lung isolated from mice (100x)
 A = Normal control group, B = Asthmatic control group, C = Dexamethasone treated group, D = Methanolic extract of *Azadirachta indica* treated group

showed Small (constricted) bronchus with inflammation (Figure 8B). In dexamethasone and MEAI treated animals, it showed near normal bronchus and alveoli with minimal inflammation with near normal cellular architecture (Figure 8C,D).

Results of this *in vivo* study suggest beneficial effect of MEAI in trypsin and egg albumin induced experimental model of asthma in mice. Considering that the levels of TNF- α is increased in inflammatory diseases like Asthma; in this study, a method to investigate immuno-modulating effects of *Azadirachta indica* was developed based on the THP-1 cells. Results of *in vitro* cell line further explains the suppressive effect of MEAI on proinflammatory cytokine release such as TNF- α , which could be the probable mechanism by which these plants exhibit anti-inflammatory activity.

CONCLUSION

The results obtained in the present study have shown that MEAI inhibits TNF- α secretion in LPS stimulated in human monocytic THP-1 cells. Hence, the probable mechanism, by which this immunomodulatory herbal plant extracts possess anti-inflammatory activity, might be attributed to their potent suppressive effect on proinflammatory cytokine release such as TNF- α and thereby NF- κ B which is involved in immune system and thereby suggesting its possible use in inflammatory diseases such as Asthma. However, further detailed studies are required to establish its clinical relevance/therapeutic potential.

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