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Ameliorative effects of *Artemisia pallens* in a murine model of ovalbumin-induced allergic asthma via modulation of biochemical perturbations



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ABSTRACT

Introduction: Asthma is a chronic, heterogeneous airway disorder characterized by airway inflammatory and remodeling. *Artemisia pallens* has been reported to possess antioxidant, anti-inflammatory and Anti-allergic potential.

Objective: To evaluate the anti-asthmatic effects of methanolic extract of *Artemisia pallens* (APME) against ovalbumin (OVA)-induced airway hyperresponsiveness (AHR) in rats.

Materials and method: AHR was induced in male Sprague-Dawley rats (180–200 g) by intraperitoneal (i.p.) injection of OVA and boosted with an identical OVA solution (s.c.) on day 7. Rats were either treated orally with vehicle (10 mg/kg), montelukast (10 mg/kg) or APME (100, 200 and 400 mg/kg) for next 28 days. At the end treatments, various biochemical, molecular (RT-PCR and ELISA analysis) and histological parameters were evaluated.

Results: APME (200 and 400 mg/kg) significantly attenuated ($p < 0.05$) OVA-induced alteration in lung functions measured by Whole-body plethysmography. Increased Bronchoalveolar Lavage (BAL) fluid differential cell count, as well as total protein and albumin in BAL fluid and lungs, was significantly decreased ($p < 0.05$) by APME. It also significantly attenuated ($p < 0.05$) elevated lung oxido-nitrosative stress, myeloperoxidase, and serum IgE levels. OVA-induced down-regulation in lung Nrf2 and upregulation in TNF- α , IL-1 β , IL-4, IL-6, TGF- β mRNA expression was significantly attenuated ($p < 0.05$) by APME (200 and 400 mg/kg) treatment. Histopathological analysis of lung tissue showed that APME treatment reduced OVA-induced inflammatory influx and fibrosis.

Conclusion: *Artemisia pallens* simultaneously orchestrate plethora of mechanisms viz. modulations of IgE, TGF- β , TNF- α , IL's and Nrf-2 levels to exhibit its anti-asthmatic potential in OVA-induced AHR in rats.

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1. Introduction

Asthma is a heterogeneous disease characterized by chronic airway inflammation. It is a chronic respiratory condition affecting 1% to 18% of the population in different countries [1].

According to the Global Burden of Disease Study (GBD), the total number of people suffering from asthma in the world may be as high as 334 million [2]. Allergens, exercise, cold air, upper respiratory tract infection are the possible precipitating factors for asthma [3]. Symptoms which define asthma including

Abbreviations: AHR, Airway Hyperresponsiveness; BPM, Blood Pressure per Minute; BALF, Bronchoalveolar Lavage Fluid; DBP, Diastolic Blood Pressure; EIP, End Inspiratory Pause; ELISA, Enzyme-Linked Immunosorbent Assay; Penh, Enhanced Pause; EVE, expired Volume; f, Frequency of breathing; GSH, Glutathione; IL's, Interleukin's; MDA, Malondialdehyde; MABP, Mean Arterial Blood Pressure; APME, Montelukast; MTL, Methanolic extract of *Artemisia pallens*; MPO, Myeloperoxidase; NO, Nitric Oxide; Nrf2, Nuclear factor E2-related factor 2; OVA, Ovalbumin; PEF, Peak expiratory flow; PIF, Peak inspiratory flow; ROS, Reactive Oxygen Species; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; SOD, Superoxide Dismutase; SBP, Systolic Blood Pressure; TV, Tidal Volume; TGF- β , Transforming Growth Factor- β ; TNF- α , Tumor Necrosis Factor- α .

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wheezing, coughing lead to an impediment of normal activities and narrowing of the airway reduces the efficiency of movement of air to and from the alveoli [4]. Airway hyperresponsiveness (AHR) and airway inflammation, usually accompany asthma, but these are not necessary or sufficient to make the diagnosis [1].

It has been reported that inflammatory cells played a vital role in induction and maintenance of pathogenesis of asthma [5]. Inflammatory infiltration at the site of allergic infection enhances the inflammatory responses that are resulting into tissue damage, and dysfunctioning could be due to the liberation of mediators such as platelet-activating factor, leukotrienes, prostaglandins, histamine, cytokines, chemokines, and cationic proteins [6,7]. It has been reported that serotonin is an important mediator of asthma which releases from mast cells and platelets in plasma resulted in a contraction of airway smooth muscles [8]. Furthermore, T helper cell induced type 2 immune responses in mast cell are also leading to the production of cytokines, interleukin (IL)-4, IL-5, IL-9 and IL-13, eosinophil inflammation and immunoglobulin E [9,10]. This type of immune response triggers due to airborne particles which could be allergic (e.g., dust mites, cockroach residue, furred animals, moulds, pollens) and non-allergic (e.g., infections, tobacco smoke, cold air, exercise) results in AHR. Severe, long-standing asthma changes the architecture of the airway which includes smooth muscle hypertrophy, and broncho-fibrosis can lead to an irreversible decrement in pulmonary function.

Animal models play an important role in the development of therapeutic moieties in allergens induced AHR and chronic airway inflammation [11]. Ovalbumin (OVA)-induced asthma model mimics clinicopathological features of asthma including the airway edema, goblet cell hyperplasia, increased mucus production, persistent bronchospasm along with inflammatory influx which may result in anaphylactic shock [7]. In OVA-induced asthma, aluminum hydroxide is used as adjuvant and OVA as an allergen. Aluminum hydroxide when exposed to the presence of antigen, it promotes the development of Th2 phenotype resulted in asthma [12].

Effective treatment of allergic asthma includes β_2 -agonists, anticholinergics, oral corticosteroids, immunomodulators, anti-leukotrienes or leukotriene modifiers and mast cell stabilizers [13]. Montelukast, a cysteinyl leukotriene receptor (CysLTR)1 antagonist which is widely used for the treatment of allergic asthma via modulation of Th2 cytokines and growth factors [14]. However, these treatment regimens provide mitigation in only to a small number of patients and also associated with side effect thus limit their implication in the treatment of asthma [15]. Literature cited with evidence suggests that, the drugs from herbal origin play an important role in inhibition of development of asthma [13].

Artemisia pallens Walls. ex DC (Davana) (Asteraceae) found abundantly in the southern part of India including Karnataka, Tamil Nadu, Andhra Pradesh and Maharashtra [16,17]. It is an aromatic herb which has been used by tribal people for various ailments. It possesses an array of pharmacological properties including anthelmintic, tonic, antioxidant, analgesic, antipyretic, antifungal, antibacterial, antimicrobial, antidiabetic, and anti-inflammatory activities [18,19]. The researcher also reported its hepatoprotective and nephroprotective potentials against acetaminophen-induced toxicity [17]. It has been reported that *Artemisia pallens* contain sesquiterpene lactone as an active constituent [19]. Moreover, previously sesquiterpene lactones have been found to be effective against asthma in laboratory animals [20]. Hence, the aim of present study was to evaluate the effect of *Artemisia pallens* against OVA-induced asthma by assessing various behavioral, biochemical, molecular and histological parameters in laboratory animals.

2. Material and methods

2.1. Preparation of methanolic extract of *Artemisia pallens* (APME)

The APME was prepared by macerating air dried and course powdered plant of *Artemisia pallens* with methanol according to previously reported methods [16,17].

2.2. Animals

Adult male Sprague-Dawley rats (180–200 g) were obtained from the National Institute of Biosciences, Pune (India). They were maintained at $24 \pm 1^\circ\text{C}$, with a relative humidity of 45–55% and 12:12 h dark/light cycle. The animals had free access to standard pellet chow (Pranav Agro-industries Ltd., Sangli, India) and water throughout the experimental protocol. All experiments were carried out between 09:00 and 17:00 h. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune and performed in accordance with the guidelines on animal experimentation recommended by Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

2.3. Chemicals

Ovalbumin (OVA, egg albumin grade II), montelukast, and aluminum hydroxide was purchased from Sigma Chemical Co. (St Louis, MO, USA). Sulphanilamides, naphthylamine diamine HCl, and phosphoric acid were obtained from Loba Chemie Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and purchased from S.D. Fine Chemicals, Mumbai, India.

APME was freshly prepared daily as 1% aqueous solution of DMSO in three different dosages (100, 200 and 400 mg/kg) and administered to rats orally [17]. Montelukast was freshly prepared daily as 1% aqueous solution of distilled water and administered to rats orally at a dose of 10 mg/kg [21].

2.4. Drug treatment and OVA challenge

Sensitization procedures and aerosol challenge were performed according to reported procedure [5]. Briefly, animals were actively sensitized by an intraperitoneal (i. p.) injection of 0.66 ml of a suspension of 1 mg OVA plus 300 mg of aluminum hydroxide ($\text{Al}(\text{OH})_3$) in 0.9% (w/v) NaCl. It was considered as Day 0 of sensitization. Seven days after sensitization, the animals were boosted subcutaneously (s.c.) with an identical injection of aluminum-precipitated OVA. The aluminum precipitate acts as an adjuvant to promote IgE production [12]. A positive control montelukast (10 mg/kg) and APME (100, 200 and 400 mg/kg) treatments were given for next 28 day. On day 28, OVA-aerosol challenge was accomplished by placing the rats individually in a polyurethane chamber connected to the nebulizer, which generated an aerosol mist pumped into the exposure chamber by the airflow supplied by a small animal ventilator set at 60 strokes per minute, with an output volume of 10 ml. All rats were exposed to 1% OVA aerosol for 15 min 24 h after OVA challenge. The various parameters were evaluated.

2.5. Determination of cellular count

On day 28, blood (3 ml) was collected in a heparinized tube from each animal under light ether anesthesia and used for leukocyte count. Each sample was centrifuged at 500g for 10 min at 4°C ; the cells in the pellet were washed in 0.5 ml saline, and total cells were counted using automated cell counter (KX –21, Sysmex,

India). In order to perform differential analyses, aliquots of the cells were placed onto slides and then stained with Field's stain to identify eosinophils, lymphocytes, macrophages or neutrophils using standard morphologic determinants.

2.6. Lung function measurements

Whole-body flow-through plethysmography (EMKA Technologies, France) was used to monitor the respiratory dynamics of an unrestrained rat quantitatively on day 0, 7, 14, 21 and 28. Recorded variables included Peak inspiratory flow (PIF), Peak expiratory flow (PEF), tidal volume (TV), expired volume (EV), the frequency of breathing (f), enhanced pause (P_{enh}) value. Briefly, the system was calibrated by injecting a known volume (5 ml) into the plethysmographic chamber by use of a glass syringe. Rats were allowed to acclimate to the chamber for 15 min before recordings were made. Respiratory activity was recorded for 10 min, to establish baseline values for the variables described above, including airway obstruction parameters [22].

2.7. Measurement of peripheral blood oxygen content

To assess peripheral blood oxygen content *in-vivo*, rats were monitored for the percentage of hemoglobin saturated with oxygen (Pulse Ox). On day 14 and 28 the rats were anesthetized with ether, and a peripheral pulse Ox sensor (ChoiceMMed, V1.0CF3, MD300CF3, China) was attached to the tail. Pulse Ox readings were taken as the animal regained consciousness [23].

2.8. Invasive measurement of hemodynamic changes

Hemodynamic changes such as HR (Heart Rate), SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure) and MABP (Mean Arterial Blood Pressure) were measured by using a polyethylene cannula (PE 50) filled with heparinized saline (100 IU/ml) inserted into the right carotid artery. The cannula was connected to a transducer, and the signal was amplified by bioamplifier and an acquisition data system (AD Instruments Pvt. Ltd. with software LabChart 7.3; AD Instrument Pvt. Ltd) [24].

2.9. Bronchoalveolar lavage (BAL) and determination of BAL markers

After determination of lung function test ice-cold PBS (0.5 ml) was instilled into the lungs, bronchoalveolar lavage fluid (BALF) was obtained by three aspirations (total volume 1.5 ml) via tracheal cannulation [5]. Each BALF was centrifuged, and the supernatant collected and stored at -70°C . Total inflammatory cell numbers were assessed by counting cells in at least five squares of a hemocytometer, after exclusion of dead cells stained with trypan blue. Cell pellets were suspended in 0.5 ml PBS, and 100 μl of each solution was spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quik[®] Stain reagents (Polysciences Asia Pacific, Inc. Taiwan) according to the manufacturer's instructions.

2.10. Lung homogenate preparation

At the end of the study, all animals were sacrificed after BAL collection. Blood specimens were collected from the retro-orbital plexus and serum was obtained by centrifugation $8350 \times g$ for 10 min at 4°C . Lung artery was perfuse with PBS to remove blood cells. Tissue homogenates were prepared with 0.1 M Tris-HCl buffer (pH 7.4), and supernatant of homogenates was employed to estimate albumin, total protein, and nitric oxide.

2.11. IgE level measurement in serum of OVA-induced hyperresponsive rats

Total IgE titer in serum was evaluated using rat IgE Enzyme-Linked Immunosorbent Assay (ELISA), Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as per manufacturer's instructions. Serum samples were at 1:20 dilution, HRP labeled rat anti-mouse IgE and TMB substrate were used. Results were evaluated by the P/N value. The test was done in duplicate to avoid False-negative, and false-positive results and the average value were taken for the final calculation.

2.12. Biochemical estimations and antioxidant parameters

The activity of albumin in serum, BAL and lungs were measured using commercially available reagent kits (Pathozone Diagnostics, India) and ultraviolet-visible spectrophotometer (JASCO-V-530, JASCO Corp., Tokyo, Japan). Lung of individual rats was isolated and washed with ice-cold saline. Tissue homogenates were prepared with 0.1 M Tris-HCl buffer (pH 7.4). The supernatant obtained was used to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA). The level of SOD, GSH, and MDA in lung homogenate was determined according to earlier reported methods [25–27].

2.13. Reverse transcriptase PCR

The levels of mRNA were analyzed in lung tissue using a reverse transcription (RT)-PCR approach as described previously [28,29]. Briefly, single-stranded cDNA was synthesized from 5 μg of total cellular RNA using reverse transcriptase (MP Biomedicals India Private Limited, India) as described previously [28,30]. The primer sequences for TNF- α , IL-1 β , IL-4, IL-6, Nrf2, TGF- β , and β -actin are presented in Supplementary Table 1. Amplification of β -actin served as a control for sample loading and integrity. PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The size of amplicons was confirmed using a 100-bp ladder as a standard size marker. The amplicons were visualized, and images were captured using a gel documentation system (Alpha Innotech Inc., San Leandro, CA, USA). Gene expression was assessed by generating densitometry data for band intensities in different sets of experiments, by analyzing the gel images on the Image J program (Version 1.33, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) semi-quantitatively. The band intensities were compared with constitutively expressed β -actin. The intensity of mRNAs was standardized against that of the β -actin mRNA from each sample, and the results were expressed as PCR-product/ β -actin mRNA ratio.

2.14. Lung tissue histopathology

After BAL fluid had been obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h for histopathological studies. It was processed for 12 h using isopropyl alcohol, xylene, and paraffin embedded for light microscopic study (Nikon E200, Japan). Paraffin-embedded tissue sections cut at 5 μm thickness were prepared and stained after deparaffination using hematoxylin and eosin stain (H & E) to verify morphological assessment and Masson's trichrome (MT) stain for the severity of interstitial fibrosis [31]. Photomicrographs were captured at a magnification of $40\times$ and $100\times$.

2.15. Statistical analysis

Data were expressed as mean $\pm$ standard error mean (SEM). Data analysis was performed using GraphPad Prism 5.0 software

(GraphPad, San Diego, USA). Statistical comparisons were made between drug-treated groups and AHR control animals. Data were analyzed using one-way ANOVA, Tukey's multiple range test was applied for post hoc analysis. A value of $p < 0.05$ was considered to be statistically significant. The size of the airway tissue was determined using Image J 1.37.

3. Results

3.1. Effect of APME treatment on OVA-induced alteration in body weight, relative lung weight, hemodynamic parameters and percent oxygen saturation of rats

OVA-induced AHR is associated with reduced body weight and increased lung weight. Also, administration of OVA resulted in decreased fraction of oxygen-saturated hemoglobin which was determined by evaluating percent oxygen saturation. In the present investigation, there was significantly decreased ($p < 0.05$) in body weight, heart rate and percent oxygen saturation in OVA control group whereas significant increased ($p < 0.05$) in relative lung weight, SBP, DBP and MABP in OVA control group as compared to the normal group. Treatment with MTL (10 mg/kg) significantly increased ($p < 0.05$) body weight, heart rate and percent oxygen saturation whereas significantly decreased ($p < 0.05$) relative lung weight, SBP, DBP and MABP as compared to OVA control rats. Administration of APME (200 and 400 mg/kg) showed significantly increased ($p < 0.05$) in body weight, heart rate, and percent oxygen saturation and a significant reduction ($p < 0.05$) in the relative lung weight, SBP, DBP and MABP as compared to OVA control rats. (Table 1)

3.2. Effect of APME treatment on OVA-induced alteration in lung function test of rats

Assessment of pulmonary function test (PFT) by non-invasive method was used to determine the severity of lung injury. There was significant decreased ($p < 0.05$) in the PEF, Tidal volume, and expiratory time whereas significant increased ($p < 0.05$) in the PIF, frequency of breathing and enhanced pause of OVA control group as compared to the normal group. Treatment with MTL (10 mg/kg) significantly increased ($p < 0.05$) in PEF, Tidal volume, and expiratory time whereas PIF, the frequency of breathing and enhanced pause were decreased significantly ($p < 0.05$) in MTL (10 mg/kg) treatment group as compared to OVA control rats. Treatment with APME (200 and 400 mg/kg) also showed significant restoration ($p < 0.05$) in lung function test when compared with OVA control rats. (Table 2)

3.3. Effect of APME treatment on OVA-induced alteration in hematological parameters of rats

Evaluation of hematological parameters provides the insight to the disease state. Hence in the present investigation when compared with normal group, Hemoglobin (Hb), red blood cells (RBC), Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), Red blood cell distribution width (RDW), White blood cells (WBCs) and lymphocyte count were decreased significantly ($p < 0.05$) and Mean corpuscular hemoglobin concentration (MCHC), as well as neutrophils count, were increased significantly ($p < 0.05$) in OVA control rats. Administration of MTL (10 mg/kg) significantly increased ($p < 0.05$) RBC, MCV, MCH, RDW, WBC and lymphocyte count as compared to OVA control rats. Whereas, MCHC and neutrophils count was decreased significantly ($p < 0.05$) in MTL (10 mg/kg) treatment as compared to OVA control rats. When compared with OVA control rats, APME (200 and 400 mg/kg) also showed significant inhibition ($p < 0.05$) in OVA-induced alterations in hematological parameters level as compared to OVA control rats. (Table 3)

3.4. Effect of APME treatment on OVA-induced alteration in BALF differential cell count of rats

OVA control group showed significantly increased ($p < 0.05$) in the BALF total and differential cell count as compared to the normal group. However, treatment of MTL (10 mg/kg) showed a significant reduction ($p < 0.05$) in the OVA-induced elevated BALF total and differential cell count as compared to OVA control group. When compared with OVA control group, BALF total and differential cell count were significantly decreased ($p < 0.05$) in APME (200 and 400 mg/kg) treatment. (Table 3)

3.5. Effect of APME treatment on OVA-induced alteration in the levels of total protein and albumin in serum, BAL fluid and lungs of rats

The elevated levels of protein and albumin in the serum are considered as the hallmark of tissue damage. Hence in the present investigation, we have evaluated the effect of APME administration on the level of total protein and albumin in serum, BAL fluid and lungs. There was significantly increased ($p < 0.05$) in the levels of total protein and albumin in serum, BAL fluid as well as lungs of the OVA-control rats as compared to the normal group. OVA-induced increased in total protein and albumin levels in serum, BAL fluid and lungs were significantly decreased ($p < 0.05$) by MTL (10 mg/kg) treatment as compared to OVA control rats. When compared with OVA control rats, APME (200 and 400 mg/kg) treatment

Table 1

Effect of APME on OVA-induced alteration in body weight, relative lungs weight, hemodynamic parameters and percent oxygen saturation of rats.

Parameter	Normal	AHR Control	MTL (10)	APME (100)	APME (200)	APME (400)
Body weight (gm)	240.23 ± 6.55	167.11 ± 5.78 [#]	242.51 ± 7.95 ^{*S}	163.62 ± 6.63	194.62 ± 4.62 ^{*S}	214.77 ± 6.95 ^{*S}
Relative lungs weight	0.68 ± 0.20	1.81 ± 0.12 [#]	0.89 ± 0.17 ^{*S}	1.74 ± 0.34	1.45 ± 0.21 ^{*S}	1.24 ± 0.48 ^{*S}
SBP (mmHg)	95.63 ± 10.23	154.22 ± 8.65 [#]	128.30 ± 5.96 ^{*S}	150.23 ± 11.75	148.62 ± 7.95	117.02 ± 11.02 ^{*S}
DBP (mmHg)	75.62 ± 8.62	104.32 ± 7.02 [#]	92.56 ± 8.88 ^{*S}	102.32 ± 11.52	100.45 ± 7.95	85.45 ± 5.62 ^{*S}
MABP (mmHg)	77.54 ± 9.86	123.51 ± 5.18 [#]	98.75 ± 9.02 ^{*S}	114.05 ± 7.64	108.22 ± 5.98	95.62 ± 5.65 ^{*S}
HR (BPM)	380.21 ± 5.47	278.65 ± 7.96 [#]	345.23 ± 7.65 ^{*S}	287.62 ± 9.02	290.45 ± 6.78	329.65 ± 10.45 ^{*S}
Pulse Ox (% O ₂ saturation)	95.65 ± 3.45	85.12 ± 2.62 [#]	89.65 ± 3.22 ^{*S}	86.50 ± 4.65	88.12 ± 3.02 ^{*S}	92.56 ± 7.45 ^{*S}

Data are expressed as mean ± S.E.M. (n = 6) and one way ANOVA followed by Tukey's multiple range test. ^{*} $p < 0.05$ as compared to AHR control group, ^S $p < 0.05$ as compared to one another group and [#] $p < 0.05$ as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract. SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure and MABP: Mean Arterial Blood Pressure.

Table 2
Effect of APME on OVA-induced alteration in lung functions of rats.

Parameter	Normal	AHR Control	MTL (10)	APME (100)	APME (200)	APME (400)
AUC [PIF (m/s)]	355.78 ± 45.85	712.45 ± 47.62 [#]	414.32 ± 21.54 ^{*,§}	700.52 ± 52.45	514.62 ± 22.32 ^{*,§}	487.65 ± 25.62 ^{*,§}
AUC [PEF (m/s)]	545.62 ± 23.57	300.21 ± 22.78 [#]	421.45 ± 31.62 ^{*,§}	321.78 ± 18.65	358.52 ± 45.62	450.27 ± 22.84 ^{*,§}
AUC [TV (m)]	32.54 ± 1.85	17.95 ± 4.84 [#]	25.65 ± 3.22 ^{*,§}	17.65 ± 2.85	21.54 ± 2.22 ^{*,§}	22.45 ± 3.26 ^{*,§}
AUC [EV (m)]	30.45 ± 1.84	19.69 ± 2.44 [#]	24.25 ± 3.11 ^{*,§}	20.14 ± 2.96	22.32 ± 2.01 ^{*,§}	22.47 ± 2.62 ^{*,§}
AUC [f(bpm)]	5487 ± 300.21	8875 ± 324.62 [#]	6524 ± 235.78 ^{*,§}	8274 ± 451.21	7295 ± 284.66 ^{*,§}	6887 ± 247.62 ^{*,§}
AUC (P _{enh})	25.24 ± 3.11	38.25 ± 4.54 [#]	28.45 ± 2.02	37.45 ± 3.24	34.52 ± 2.07 ^{*,§}	31.02 ± 2.77 ^{*,§}

Data are expressed as mean ± S.E.M. (n = 6) and one way ANOVA followed by Tukey's multiple range test. **p* < 0.05 as compared to AHR control group, [§]*p* < 0.05 as compared to one another group and [#]*p* < 0.05 as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract.

Table 3
Effect of APME on OVA-induced alteration in hematological parameters and differential cell counts in BAL fluid of rats.

Parameter	Normal	AR Control	MTL (10)	APME (100)	APME (200)	APME (400)
Hemoglobin (g/dl)	18.20 ± 0.22	12.42 ± 0.21 [#]	13.21 ± 0.24	14.32 ± 2.44	15.12 ± 2.77	17.32 ± 3.02 ^{*,§}
RBC (X 10 ⁶ /mm ³)	8.62 ± 0.22	8.11 ± 0.21 [#]	8.42 ± 0.32 ^{*,§}	8.20 ± 0.11	8.36 ± 0.41 ^{*,§}	8.59 ± 0.32 ^{*,§}
PCV (%)	52.32 ± 2.11	40.22 ± 2.55 [#]	42.51 ± 2.55	39.65 ± 4.32	44.62 ± 2.65	45.22 ± 3.05 ^{*,§}
MCV (fL)	62.32 ± 2.55	51.32 ± 2.45 [#]	57.62 ± 2.33 ^{*,§}	50.24 ± 3.62	54.21 ± 4.03 ^{*,§}	56.65 ± 2.78 ^{*,§}
MCH (pg)	20.15 ± 0.12	16.32 ± 0.32 [#]	19.22 ± 2.03 ^{*,§}	17.20 ± 2.45	18.45 ± 1.01	18.96 ± 0.45 ^{*,§}
MCHC (g%)	32.45 ± 1.32	56.26 ± 2.45 [#]	40.32 ± 2.32 ^{*,§}	52.45 ± 3.22	48.65 ± 1.22 ^{*,§}	37.62 ± 3.88 ^{*,§}
RDW (%)	14.21 ± 0.33	11.42 ± 0.45 [#]	13.26 ± 0.78 ^{*,§}	10.45 ± 0.24	12.45 ± 0.24 ^{*,§}	13.15 ± 0.78 ^{*,§}
WBC (X 10 ³ /mm ³)	14.02 ± 1.22	8.65 ± 0.98 [#]	14.75 ± 2.62 ^{*,§}	9.65 ± 1.44	12.14 ± 2.01 ^{*,§}	13.45 ± 2.01 ^{*,§}
N (%)	35.62 ± 2.12	54.23 ± 2.01 [#]	40.32 ± 2.55 ^{*,§}	51.24 ± 3.44	45.52 ± 2.77 ^{*,§}	41.72 ± 2.99 ^{*,§}
L (%)	59.86 ± 2.77	42.32 ± 3.04 [#]	52.62 ± 4.32 ^{*,§}	40.32 ± 1.87	45.62 ± 2.87	49.62 ± 3.44 ^{*,§}
Total cell count (X 10 ³)	128.65 ± 14.32	756.95 ± 108.65 [#]	234.62 ± 32.45 ^{*,§}	698.62 ± 235.62	514.32 ± 21.32 ^{*,§}	425.62 ± 32.89 ^{*,§}
N (X 10 ³)	0.78 ± 0.21	12.32 ± 2.65 [#]	2.32 ± 0.56 ^{*,§}	10.21 ± 3.66	6.95 ± 2.67 ^{*,§}	4.66 ± 1.78 ^{*,§}
L (X 10 ³)	7.23 ± 2.32	157.62 ± 23.44 [#]	23.55 ± 2.78 ^{*,§}	147.62 ± 30.45	95.62 ± 11.78 ^{*,§}	56.95 ± 12.77 ^{*,§}
E (X 10 ³)	24.62 ± 3.78	547.62 ± 27.62 [#]	105.32 ± 28.65 ^{*,§}	476.65 ± 56.96	300.62 ± 23.62 ^{*,§}	208.65 ± 26.32 ^{*,§}
M (X 10 ³)	105.32 ± 23.63	300.45 ± 12.85 [#]	201.32 ± 22.63 ^{*,§}	312.62 ± 21.45	297.65 ± 20.54	245.62 ± 22.58 ^{*,§}

Data are expressed as mean ± S.E.M. (n = 6) and one way ANOVA followed by Tukey's multiple range test. **p* < 0.05 as compared to AHR control group, [§]*p* < 0.05 as compared to one another group and [#]*p* < 0.05 as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract. N: Neutrophils, L: Lymphocytes, E: Eosinophils and M: Monocytes.

Table 4
Effect of APME on OVA-induced alteration in total protein and albumin in serum, BAL fluid and lungs of rats.

Parameter	Normal	AHR Control	MTL (10)	APME (100)	APME (200)	APME (400)
Lungs total protein (gm/dl)	0.42 ± 0.21	1.98 ± 0.43 [#]	0.72 ± 0.14 ^{*,§}	1.87 ± 0.48	1.02 ± 0.21 ^{*,§}	0.85 ± 0.35 ^{*,§}
BAL total protein (gm/dl)	1.55 ± 0.32	3.25 ± 0.21 [#]	1.85 ± 0.45 ^{*,§}	3.21 ± 0.51	2.55 ± 1.02 ^{*,§}	1.92 ± 0.32 ^{*,§}
Serum albumin (gm/dl)	0.52 ± 0.08	1.21 ± 0.04 [#]	0.78 ± 0.06 ^{*,§}	1.32 ± 0.10	1.07 ± 0.06	0.82 ± 0.09 ^{*,§}
BAL albumin (gm/dl)	0.52 ± 0.07	3.02 ± 0.21 [#]	0.81 ± 0.07 ^{*,§}	2.98 ± 0.24	2.12 ± 0.88 ^{*,§}	1.44 ± 0.38 ^{*,§}
Lungs albumin (gm/dl)	0.42 ± 0.09	4.65 ± 0.88 [#]	0.89 ± 0.22	3.98 ± 1.54	3.12 ± 0.87 ^{*,§}	2.44 ± 0.87 ^{*,§}

Data are expressed as mean ± S.E.M. (n = 6) and one way ANOVA followed by Tukey's multiple range test. **p* < 0.05 as compared to AHR control group, [§]*p* < 0.05 as compared to one another group and [#]*p* < 0.05 as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract.

showed significant inhibition (*p* < 0.05) in the OVA-induced increased total protein and albumin levels in serum, BAL fluid and lungs. (Table 4)

3.6. Effect of APME treatment on OVA-induced alteration in the levels of lung SOD, GSH, MDA, NO, MPO and serum IgE of rats

An elevated level of oxido-nitrosative stress reflected the increased reactive oxygen species (ROS) production after an injury to lung, and it was measured by an alteration in the levels of endogenous antioxidants includes SOD and GSH. There was a significant decreased (*p* < 0.05) in lung SOD and GSH level in OVA control group as compared normal rats whereas OVA control group showed significantly increased (*p* < 0.05) in the lung MDA, NO, MPO and serum IgE levels as compared to the normal group. MTL (10 mg/kg) treatment significantly increased (*p* < 0.05) SOD and GSH levels, whereas there was a significant decreased (*p* < 0.05) lung MDA, NO and serum IgE levels as compared to OVA control rats. Administration of APME (200 and 400 mg/kg) also showed significantly inhibited (*p* < 0.05) OVA-induced elevated lung oxido-

nitrosative stress as well as lung MDA and serum IgE levels as compared to OVA control rats. (Fig. 1)

3.7. Effect of APME treatment on OVA-induced alteration in lung nrf2, TNF-α, IL-1β, IL-4, IL-6, and TGF-β mRNA expressions in rats

Increased levels of pro-inflammatory mediators (such as TNF-α, IL-1's and TGF-β) in immune inflammatory response plays various important roles in AHR. In the present investigation, there was significant up-regulation (*p* < 0.05) in the lung NF-α, IL-1β, IL-4, IL-6, and TGF-β mRNA expressions of OVA control group as compared to the normal group. OVA control group showed significant down-regulation (*p* < 0.05) in Nrf2 mRNA expression as compared to the normal group. Treatment with MTL (10 mg/kg) significantly inhibited (*p* < 0.05) OVA-induced alterations in lung Nrf2, TNF-α, IL-1β, IL-4, IL-6, and TGF-β mRNA expressions as compared to OVA control group. APME (200 and 400 mg/kg) treatment showed significant down-regulation (*p* < 0.05) in lung TNF-α, IL-1β, IL-4, IL-6, and TGF-β mRNA expressions and upregulation in Nrf2 mRNA expression as compared to OVA control rats. (Fig. 2)

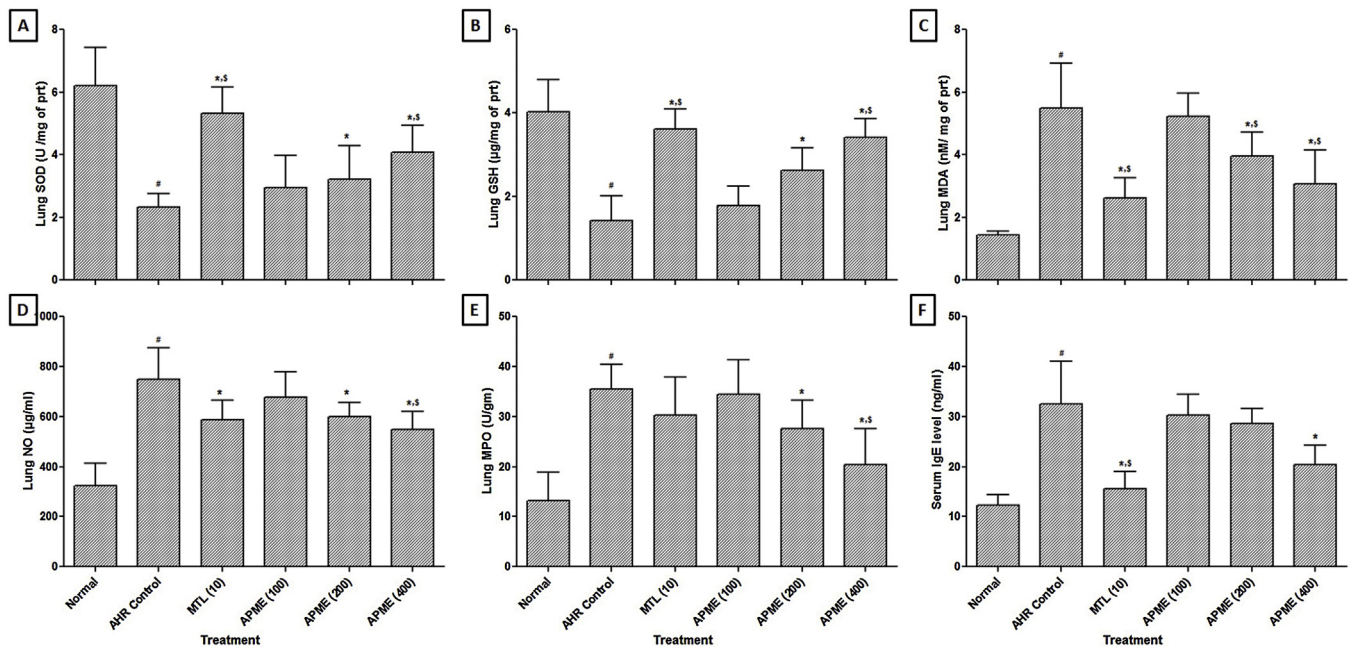


Fig. 1. Effect of APME on OVA-induced alteration in lung SOD (A), GSH (B), MDA (C), NO (D), MPO (E) and serum IgE (F) of rats. Data are expressed as mean $\pm$ S.E.M. (n = 4) and one way ANOVA followed by Tukey's multiple range test. * $p < 0.05$ as compared to AHR control group, $^{\#}p < 0.05$ as compared to one another group and $^{\#}p < 0.05$ as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract.

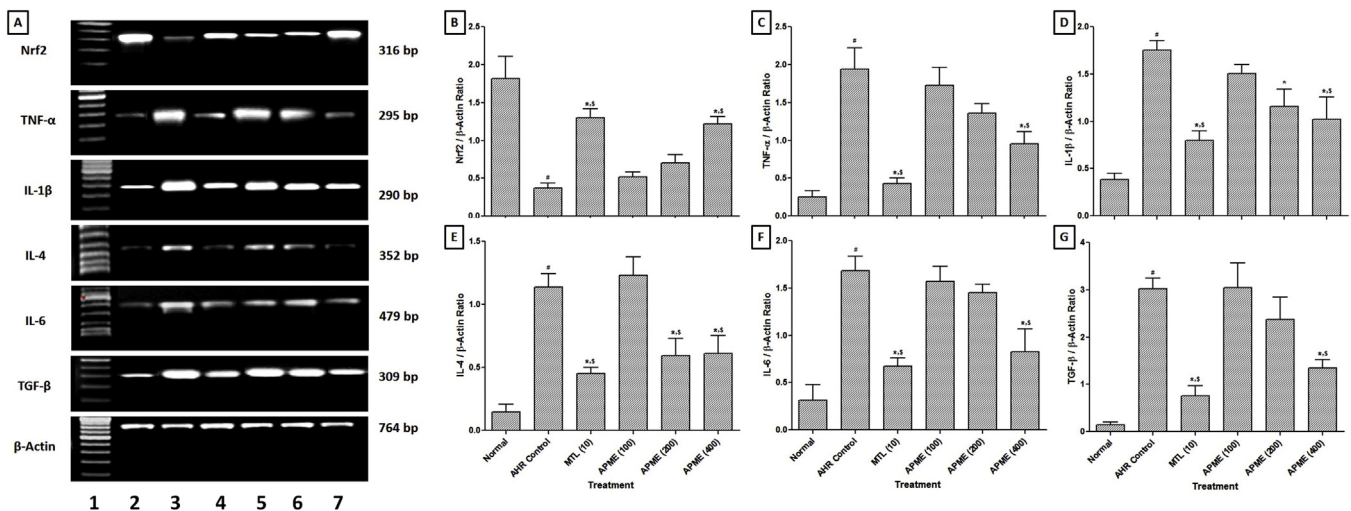


Fig. 2. Effect of APME on OVA-induced alteration in lung Nrf2, TNF- α , IL-1 β , IL-4, IL-6 and TGF- β mRNA expression of rats (A), quantitative representation of mRNA expression of Nrf2 (B), TNF- α (C), IL-1 β (D), IL-4 (E), IL-6 (F) and TGF- β (G). Data are expressed as mean $\pm$ S.E.M. (n = 4) and one way ANOVA followed by Tukey's multiple range test. * $p < 0.05$ as compared to AHR control group, $^{\#}p < 0.05$ as compared to one another group and $^{\#}p < 0.05$ as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract, Lane 1: Ladder 1000 bp; Lane 2: mRNA expression of normal group; Lane 3: mRNA expression of AHR control group; Lane 4: mRNA expression of MTL (10 mg/kg, p.o.); Lane 5: mRNA expression of APME (100 mg/kg, p.o.) treated group; Lane 6: mRNA expression of APME (100 mg/kg, p.o.) treated group and Lane 7: mRNA expression of APME (100 mg/kg, p.o.) treated group.

3.8. Effect of APME treatment on OVA-induced pathological alteration in rats lungs

Lung tissue from normal group revealed normal morphologies with thin-lined alveolar septa with well-organized alveolar space (Figs. 3 A and 4 A). Administration of OVA-induced severe inflammatory changes in lungs (grade 4), accompanied by distorted goblet cell hyperplasia (grade 3). Severe alveolar injury with thickened intra-alveolar septa collapsed alveolar spaces, huge inflammatory exudates, edema (grade 3) along with alveolar fibrosis, and interstitial fibrosis (grade 4) was observed in histology

of lung tissue from OVA control rats (Figs. 3B and 4B). Moreover, airway perimeter was increased significantly ($p < 0.05$) in OVA control group as compared to the normal group. Treatment with montelukast markedly reduced pulmonary inflammation (grade 1), alveolar fibrosis (grade 1) and distorted goblet cell hyperplasia (grade 2). It showed a significant decreased ($p < 0.05$) in the airway perimeter as compared to OVA control rats (Figs. 3C and 4C). The figure depicted the morphology of lung tissue from APME (100 mg/kg) treated rats. It evident in severe inflammatory infiltrations (grade 3), distorted goblet cell hyperplasia (grade 3), thickened intra-alveolar septa, edema (grade 3) and alveolar fibrosis (grade 3)

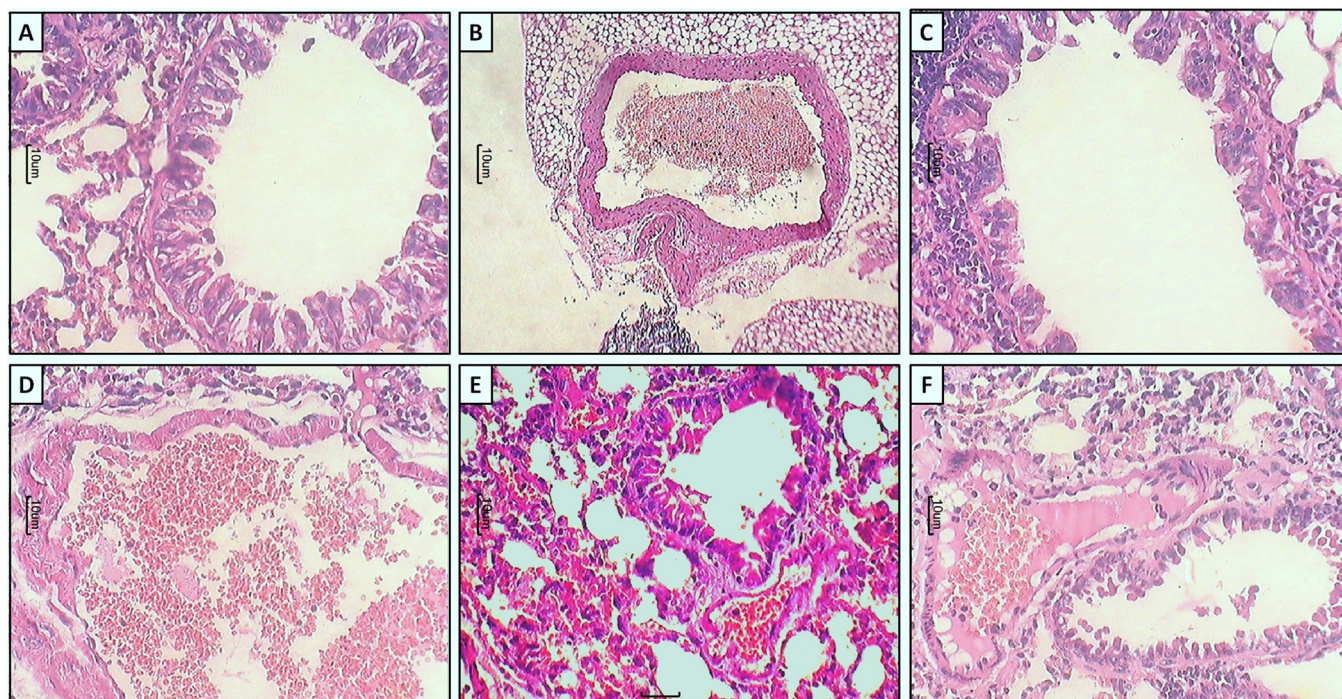


Fig. 3. Effect of APME on OVA-induced alteration in lung histology of rats.

Photomicrograph of sections of lungs of normal (A), AHR control (B), Montelukast (10 mg/kg) treated (C), and APME (100 mg/kg) treated (D), APME (200 mg/kg) treated (E) and APME (400 mg/kg) treated (F) rats. Lung H&E staining at 40 $\times$.

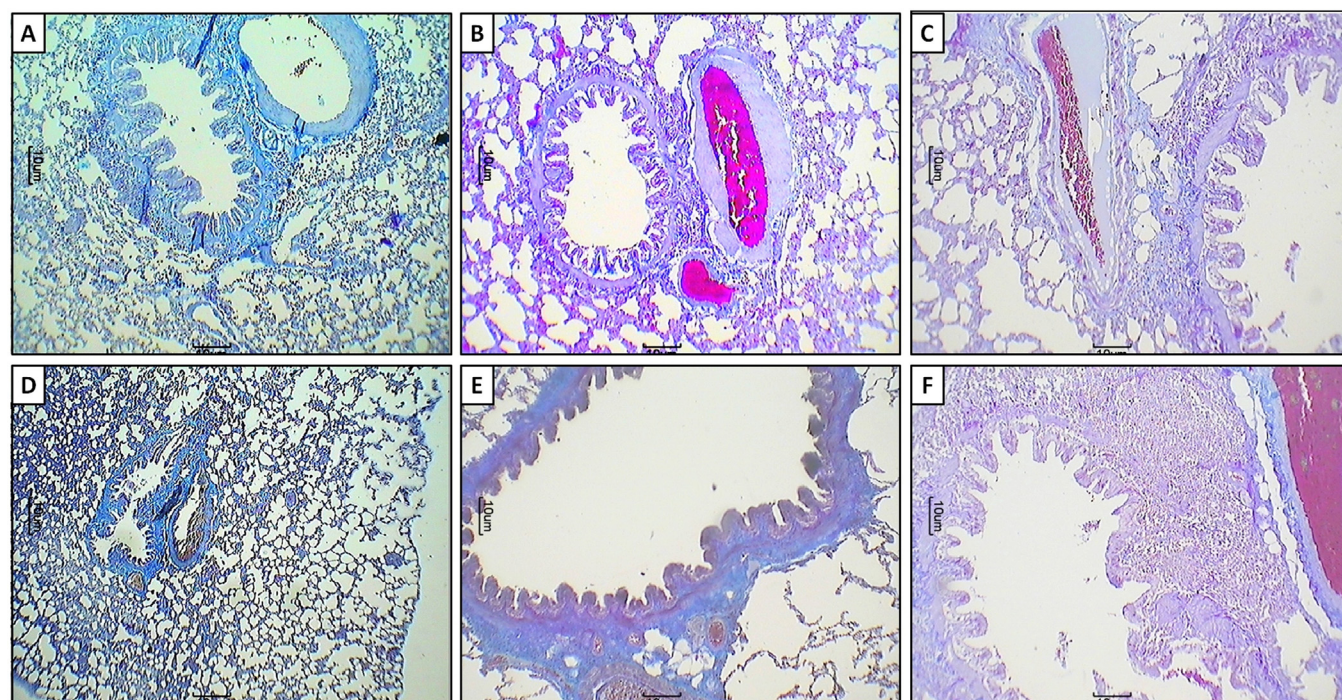


Fig. 4. Effect of APME on OVA-induced alteration in lung histology of rats.

Photomicrograph of sections of lungs of normal (A), AHR control (B), Montelukast (10 mg/kg) treated (C), and APME (100 mg/kg) treated (D), APME (200 mg/kg) treated (E) and APME (400 mg/kg) treated (F) rats. Lung MT staining at 40 $\times$.

(Figs. 3D and 4D). Lung tissue from APME (200 and 400 mg/kg) treated rats showed mild infiltration of inflammatory cells (grade 2) whereas goblet cell hyperplasia (grade 2), vascular congestion and alveolar fibrosis (grade 2) were markedly reduced (Figs. 3E–F and 4E–F). When compared with OVA control group, airway perimeter was significantly decreased ($p < 0.05$) in the APME (200 and 400 mg/kg) treated group. (Supplementary Table 2)

4. Discussion

Asthma is a chronic, complex, immune-inflammatory disorder and thus various animal models played a vital role in the understanding the mechanism as well as evaluation of various therapeutic moieties against asthma. It has been well documented that allergen-induced asthma is associated with chronic

inflammation and remodeling of airways. Thus, an array of agents such as corticosteroid, dexamethasone, leukotriene modifiers and mast cell stabilizers has employed to suppress inflammation in this allergen-induced asthma [32]. These agents have an ability to inhibit goblet cells hyperplasia and elevated serum IgE levels in an OVA model of AHR [33].

Earlier studies have suggested that inflammatory insult to the lungs resulted in an alteration in respiratory patterns. Therefore, determination of pulmonary function test (PFT) are used to diagnosis of the nature and severity of lung injury. The advantage of physiologic markers over the biochemical and pathologic biomarkers of lung injury is its non-invasiveness. Moreover, one can easily determine the nature of pulmonary damage via collective evaluation of several PFTs [31]. Amongst various PFTs, inspiratory time (Ti), expiratory time (Te), relaxation time (RT), end inspiratory pause (EIP) and enhanced pause (P_{enh}) which are flow-derived parameters that have also been used as indices of acute response to intoxicants [34]. The results of the present investigation are in accordance with the findings of the previous study where OVA-induced rats showed altered PFTs [35]. However, administration of APME showed significant restoration of these flow-derived parameters.

Clinically, determination of oxygen saturation of the arterial hemoglobin is an important measure for the patients with potential respiratory distress [36]. Breathing pattern analysis along with pulmonary mechanics is an important hallmark for the lung gas exchange properties. Moreover, the rate of gas transport across alveolar-capillary membrane reflected its integrity. Thus, the percentage of hemoglobin saturated with oxygen (Pulse Ox) is an indirect measure to determine the peripheral blood oxygen content *in vivo* [31]. In the present investigation, OVA exposure rats showed a reduction in pulse Ox readings as compared to the normal group. Administration of APME showed to reduce the severity of OVA-induced AHR as there was a significant restoration in the percentage of hemoglobin saturated with oxygen.

Literature cited with evidence suggests that macrophages are the vital cell that plays an important role in innate immune responses in the lung [37]. These cells are mainly responsible for encountering the allergen, destruction of substance that interfere with the normal gas exchange as well other threats to homeostasis [38]. These cells have a dual activity which is depending upon the signals which they received. Different macrophage phenotype has distinct functions which may be immunogenic or tolerogenic, destroying or repairing tissue and thus serves as pro- or anti-inflammatory [39]. When macrophages exposed to the elevated IL-4 response, it serves as an important mediator in tissue repair responses [40]. Consistent with previous findings, results of present investigation also showed alterations in hematological and BAL fluid differential cell counts of OVA-induced rats as compared to normal rats whereas administration of APME significantly inhibited it.

Furthermore, Th2 cytokines cause activation of eosinophils that induces the release of pro-inflammatory mediators and ROS [41]. It has been reported that increased ROS production leads to an oxidative influx in the airway and lung tissue resulting in progression of inflammation in the asthmatic airway [42]. Researchers have documented that increased production of ROS damages cell membrane phospholipids that results in impairment of membrane function leading to cell and tissue injury [43,44]. This structural destruction of unsaturated fatty acids in lipid membrane reflected as lipid peroxidation which indicates elevated levels of MDA that in turn associated with decreased antioxidant defense enzymes [45,46]. Therefore, an array of studies carried out over past decade showed that inhibition of overproduction of ROS is beneficial in the treatment of lung inflammatory diseases. In the present investigation also treatment with APME showed

significant inhibition in MDA levels that may inhibit ROS production to exert its anti-inflammatory potential.

For normal lung homeostasis, it is essential to have a balance between oxidants and antioxidants. Pulmonary insults via elevated levels of oxidants resulted in induction of these endogenous antioxidants that protects the lungs via detoxification of deleterious oxygen radicals. These endogenous antioxidants include SOD, catalase, and glutamate which serves as rate-limiting enzyme in glutathione (GSH) synthesis [47,48]. Furthermore, extracellular glutathione peroxidase also responsible for the inhibition of oxidative stress in the extracellular matrix and alveolar lining fluid [49,50]. Clinically it has been shown that activity of SOD significantly decreased in the epithelial mucosa of patients with eosinophilic sinusitis [51]. Additionally, decreased level of GSH is associated with increased allergic asthmatic responses via development of T cells from Th1 to Th2 type [52]. Several lines of studies suggested that Nuclear factor erythroid-2-related factor 2 (NRF2) plays an essential role in the lungs through the activation of antioxidant response element-regulated antioxidant [53,54]. Antioxidant responsive element (ARE) directed activation of Nrf2 signaling serves as a cellular antioxidant defense mechanism in patients with acute lung injury/acute respiratory distress syndrome [55]. In the current study, OVA-induced rats showed down-regulated expression of Nrf2 along with SOD and GSH. Treatment with APME showed significant inhibition in these OVA-induced alterations reflecting its antioxidant potential.

In the past, researchers have underlined the correlation between the nitric oxide and allergen-induced inflammatory responses [35,56]. This reactive nitrogen species have an ability to interact with intracellular GSH and deplete its cellular concentration leads to elevated oxido-nitrosative stress [31,57]. Additionally, epithelial cells iNOS has an ability to elevate NO level that promotes asthmatic inflammation via Th1 cells down-regulation and elevates Th2 cytokines (such as IL-4, IL-6, and IL-13) release [56]. In the current study, OVA-induced rats associated with elevated NO levels whereas APME treatment significantly decreased these OVA-induced NO influx in rats. The results of the present investigation are in line with our previous study where we reported that administration of APME caused significant inhibition in toxicant-induced elevated NO levels [17].

TNF- α is an important pro-inflammatory mediator in immune inflammatory response which plays various important roles in AHR such as airway smooth muscle activation, activation of airways eosinophils, airways neutrophil infiltration, changes in vascular permeability [58]. It has been clinically also proven that asthmatic patient has associated with elevated airways TNF- α expression [58]. Moreover, BAL fluid from asthmatics patient is also reported to have elevated IL-1 β level which is responsible for contraction of airway smooth muscle [59]. In agreement with other literature, this work showed that AHR control rats showed significant upregulation in TNF- α and IL-1 β mRNA expressions. However, APME treatment significantly inhibits this pro-inflammatory mediator which might be due to its anti-inflammatory potential.

Furthermore, it has been reported that an array of Th2 cytokines, such as IL-4, IL-6, and IL-13 have plays an important role in asthma. The elevated levels of IL-4 and IL-6 have been reported in BAL fluid of asthmatic patients [60]. These Th2 cytokines have an important role in various functions of allergic response including eosinophil recruitment, airway hyperresponsiveness, and mucus secretion. IL-4 has also been shown to have a vital role in the release of mast cell as well as secretion of histamine and leukotrienes [60]. A compelling body of evidence supports that IL-4 promotes IgE production by normal PBMCs [61,62]. Moreover, this elevated level of IgE causes mucus hypersecretion and goblet cell hyperplasia. In the present investigation, gene expression

profiling of lung tissue from AHR control group showed significant increases in the expression of these Th2 cytokines along with elevated IgE levels. Results of the present investigation reveal that administration of APME inhibit recruitment of inflammatory cells and thus decreased the levels of Th2 cytokines and in turn IgE.

Allergic inflammation in the lung is a unique histological characteristic of OVA-induced allergic asthma model. The histopathological investigation of lung tissue from OVA exposed rats demonstrating significant goblet cell hyperplasia, collagen deposition, neutrophil infiltration and tissue destruction. We have also confirmed the pathological findings made by [63]. Administration of APME inhibited histopathological aberration in lung tissue induced by OVA which might be due to a reduction in IgE levels.

Evidence from the previous studies suggested that TGF- β , a potent profibrotic growth factor released from inflammatory cells of lung causes increased collagen deposition, cell proliferation and mucus hypersecretion that leads to lung remodeling [64]. The presence of collagen in the extracellular matrix of connective tissues serve as a major source of fibrous and structural protein [65]. In the past, studies have reported a direct correlation between TNF- α with the enhanced expressions of TGF- β [31,66]. Interestingly, in the present study, we found a high intensity of collagen deposition in the alveolar space of AHR control rats as compared to normal rats. Results of our investigation showed that APME treatment significantly inhibited OVA-induced up-regulated TGF- β expression which in turn reduced the level of collagen deposition.

Of late, much attention has been laid clinically on the role of herbal medicine in the treatment of asthma. Treatment with *Ginkgo leaf*, *Solanum xanthocarpum*, *Solanum trilobatum*, *Boswellia serrata* and *Tylophora indica* provide the symptomatic relief and showed significant improvement in lung function tests in asthmatic subjects [67–70]. Moreover, it has been reported that a five-herb herbal formula containing *Astragalus mongholius* Bunge, *Cordyceps sinensis* Sacc, *Radix stemonae*, *Bulbus fritillariae cirrhosae*, and *Radix scutellariae* showed a beneficial effect in the asthmatic patient via improvement in pulmonary functions (forced expiratory volume and Forced vital capacity) [7,71].

Based on the findings of present investigation we have further carried out the isolation, identification, and characterization of the phytoconstituent present in the APME. The preliminary pharmacological study carried out in our laboratory revealed that the phytoconstituent artemisinin (3R,5aS,6R,8aS,9R,12S,12aR)-3,6,9-trimethyloctahydro-3,12-epoxy[1,2]dioxepino[4,3-i] isochromen-10(3H)-one) showed potent antiasthmatic activity against OVA-induced AHR in laboratory rats. However, further study is in progress for the elucidation of the actual mechanism of action of artemisinin isolated from APME at the molecular level.

Conclusion

Taken together, the results of present investigation suggests that administration of APME in OVA-induced AHR rats exerted its anti-asthmatic potential via a plethora of mechanisms including inhibition of oxido-nitrosative stress, IgE, TGF- β , TNF- α , IL's along with an increase in Nrf-2 level in rats. This finding may open novel landscapes in therapeutic option for the phytoconstituent like artemisinin with the antioxidant potential to treat allergen-induced asthma.

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None.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biopha.2017.08.017>.

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Supplementary Files

Supplementary Table 1: Primer sequences for Nrf2, TNF- α , IL-1 β , IL-4, IL-6, TGF- β and β -actin

Sr. No.	Gene	Sequence		Size(bp)
		Forward primer	Reverse primer	
1.	Nrf2	CCTCACCTCTGCTGCCAGT	GGGAGGAATTCTCCGGTCTC	316
2.	TNF- α	AAGCCTGTAGCCCATGTTGT	CAGATAGATGGGCTCATACC	295
3.	IL-1 β	TGATGTTCCCATTAGACAGC	GAGGTGCTGATGTACCAGTT	290
4.	IL-4	ACCTTGCTGTCACCCTGTTCTGC	GTTGTGAGCGTGGACTCATTCACG	352
5.	IL-6	TAGCCGCCCCACACAGACAG	GGCTGGCATTGTGGTTGGG	479
6.	TGF- β	GTTCTTCAATACGTCAGACATTCG	CATTATCTTTGCTGTCACAAGAGC	309
7.	β -actin	GTCACCCACACTGTGCCCATCT	ACAGAGTACTTGCGCTCAGGAG	764

Supplementary Table 2: Effect of APME on OVA-induced pathological alteration in rat lungs

Parameter	Goblet cell hyperplasia	Inflammation	Eosinophils	Infiltration of leukocytes	Edema	Hemorrhage	Alveolar fibrosis	Airway perimeter (μm^2)
Normal	+	+	-	+	-	-	+	5.66 $\pm$ 1.02
AR Control	+++	++++	+++	++++	+++	++	++++	67.62 $\pm$ 5.96 [#]
MTL (10)	++	+	-	++	+	-	+	10.32 $\pm$ 2.01 ^{*,§}
APME (100)	+++	+++	++++	++++	++++	+++	+++	60.33 $\pm$ 4.02
APME (200)	+++	++	++++	+++	+++	+	++	45.23 $\pm$ 3.99 ^{*,§}
APME (400)	++	+	++	+	++	+	+	36.95 $\pm$ 2.84 ^{*,§}

Data are expressed as mean $\pm$ S.E.M. (n=6) and one way ANOVA followed by Tukey's multiple range test. * $p < 0.05$ as compared to AHR control group, § $p < 0.05$ as compared to one another group and # $p < 0.05$ as compared to normal group. AHR: Airway Hyperresponsiveness, MTL: montelukast, APME: *Artemisia pallens* methanolic extract.

Note:

-: no abnormality detected,

+: damage/active changes up to < 25%,

++: damage/active changes up to < 50%,

+++ : damage/active changes up to < 75%,

++++: damage/active changes up to > 75%